Astrocytic LRRK2 Controls Synaptic Connectivity through ERM Phosphorylation.

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Abstract

Astrocytes tightly control neuronal connectivity and function in the brain through direct contact with synapses1-5. These glial cells become reactive during disease pathogenesis6-8 including Parkinson’s disease (PD)9-14. However, it remains unknown if astrocyte dysfunction is an initiating factor of PD pathogenesis and whether astrocytes can be targeted to stop or reverse the synaptic dysfunction seen in PD. Using in vitro and in vivo methods, we found that the PD-linked gene Lrrk2 controls astrocyte morphology via regulating the phosphorylation of ERM proteins (Ezrin, Radixin, and Moesin), a structural component of the perisynaptic astrocyte processes. ERM phosphorylation is robustly elevated both in mice and humans carrying the LRRK2 G2019S Parkinsonism mutation. Importantly, the reduction of the ERM phosphorylation, specifically in the LRRK2 G2019S in adult astrocytes, is sufficient to restore excitatory synapse number and function deficits in the LRRK2 G2019S knock-in mouse cortex. These results show a role for Lrrk2 in controlling astrocyte morphogenesis and synaptogenic function and reveal that early astrocyte dysfunction in PD could be causal to disruptions in cortical excitatory synaptic connectivity. The astrocytic dysfunction can be corrected by dampening ERM phosphorylation, pinpointing astrocytes as critical cellular targets for PD therapeutics.
Astrocytes instruct the formation and function of synaptic circuits\textsuperscript{3,12,15}. Most
synapses in the central nervous system are in direct contact with perisynaptic astrocyte
processes\textsuperscript{16-19}. This structural association is essential for the regulation of synaptic
function; therefore, the perisynaptic astrocyte process and the pre- and postsynaptic
neuronal compartments were coined the term “the tripartite synapse”\textsuperscript{18,20}. At the tripartite
synapse, astrocytes clear excess neurotransmitters, maintain ion homeostasis, and
secrete factors regulating synapse development and function\textsuperscript{21-25}. To accomplish these
crucial roles at the synapse, astrocytes gain a highly elaborate morphology and form a
network coupled through gap junctions to continuously tile the entire brain
parenchyma\textsuperscript{2,19,26}. Previous studies found that astrocyte morphogenesis, tiling, and
neuronal synaptogenesis are interdependent processes controlled by bidirectional
signaling via secreted factors and cell adhesion molecules\textsuperscript{27-32}.

Astrocytes become reactive in numerous neurological disorders, including
Parkinson’s Disease (PD)\textsuperscript{33,34}. PD is a progressive neurodegenerative motor disorder
affecting over 8 million people worldwide\textsuperscript{35-37}. PD is commonly diagnosed at the onset of
motor symptoms, including tremors, bradykinesia, and rigidity. By the time these motor
symptoms are apparent, the PD patients have already lost many of their dopaminergic
neurons in their substantia nigra pars compacta (SNPc)\textsuperscript{38,39}. However, decades before
the onset of motor dysfunction, individuals with PD often display non-motor symptoms,
such as cognitive, mood, and sleep disorders\textsuperscript{40-43}. These clinical observations suggest
that during the early phases of PD, synaptic circuit dysfunction exists across multiple brain
regions, including the cerebral cortex\textsuperscript{44,45}. Recent studies suggested that astrocytes
contribute to neurodegeneration in PD by producing neurotoxic factors, increasing glutamate-mediated excitotoxicity, disrupting K+ buffering and Ca2+ homeostasis10,46,47. However, it remains unknown if astrocyte dysfunction is an initiating factor or a reaction to neuronal pathology in PD.

**Astrocytic LRRK2 controls astrocyte morphology through ERM phosphorylation.**

Even though most PD cases are idiopathic, ~15% are caused by mutations in Parkinsonism genes48. The PD-linked genes identified to date point to dysfunction in fundamental cellular processes such as lysosome and mitochondria function, vesicular trafficking, and secretion49-51. However, how the dysfunction of these genes results in impaired synaptic circuits and dopaminergic neuron death is unknown. Moreover, many cell types in the brain and body express these PD-linked genes52-54. Thus, the cellular origins of PD may not be restricted to neurons.

Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene cause familial PD55,56. In particular, the LRRK2 G2019S is the most common mutation that causes familial PD43,49,57. It is largely accepted that LRRK2 G2019S is a gain-of-function mutation that increases LRRK2’s kinase activity58-63. PD patients with the LRRK2 G2019S mutation exhibit symptoms and disease progression that resembles idiopathic PD, such as late-onset disease, responsiveness to L-DOPA treatment, and the presence of Lewy body and Tau-tangle pathology64,65. PD patients with the LRRK2 G2019S mutation also have non-motor symptoms, such as depression, hallucinations, and sleep and cognitive disorders43,57,66. Several rodent models with LRRK2 G2019S mutation have been generated; however, these models do not show motor dysfunction or dopaminergic
neuron loss\textsuperscript{67-69}. However, non-motor symptoms such as cognitive dysfunction is common in PD, indicating impairments in numerous brain regions, including the cerebellar cortex\textsuperscript{70,71}. Previous human and rodent studies showed that LRRK2 is highly expressed in the cortex and LRRK2 G2019S alters cortical and cortico-striatal circuits and causes cognitive problems such as impaired goal-directed actions, reflecting critical non-motor symptoms of PD\textsuperscript{72-81}.

Because astrocytes play essential roles in cortical circuit formation and function\textsuperscript{5,82}, and cortical connectivity is altered in PD, we tested whether mouse and rat cortical astrocytes express Lrrk2. Using a knockout (KO)-verified LRRK2 antibody\textsuperscript{83,84}, we visualized the expression of Lrrk2 protein in cortical mouse astrocytes by immunostaining coronal brain sections from wild-type (WT) or LRRK2 G2019S knock-in (\textit{ki/ki}) mice\textsuperscript{76,85}. These mice also contained the Aldh1l1-eGFP transgene\textsuperscript{28}, labeling all astrocytes with GFP. Lrrk2 staining was evident in both WT and LRRK2 G2019S mouse astrocytes, localizing throughout astrocytic processes (Extended Data Fig. 1a-b). Similarly, cortical astrocytes from WT or LRRK2 G2019S BAC transgenic rats also express Lrrk2 \textit{in vitro}, which we verified by immunoblotting (Extended Data Fig. 1c-d; Extended Data Fig. 1e-f).

To investigate the function of LRRK2 in astrocyte morphogenesis, we first utilized a primary rat cortical astrocyte and neuron co-culture system to examine how the loss of Lrrk2 affects astrocyte morphology. To do so, we knocked down Lrrk2 with a short hairpin RNA (shRNA) that targets both the rat and mouse \textit{Lrrk2} (Extended Data Fig. 1g-h). In this neuron-astrocyte co-culture assay, we isolated WT (unmanipulated) cortical neurons and astrocytes from neonatal rats and cultured them separately for 9 days \textit{in vitro} (DIV). Astrocytes were then transfected with GFP-tagged shRNA against \textit{Lrrk2} (\textit{shLrrk2}) or a
scrambled control shRNA (shControl). Two days post-transfection, astrocytes were plated onto DIV11 WT neurons (Fig. 1a). As previously shown\textsuperscript{28,86}, astrocytes transfected with shControl acquired a complex morphology in response to 48 hours of neuronal contact (Fig. 1b). On the contrary, Lrrk2 knockdown stunted astrocyte morphological complexity (Fig. 1b). To avoid possible off-target effects from shRNA, we also isolated cortical astrocytes from LRRK2 KO rats\textsuperscript{87-90}. We examined their morphological complexity in response to contact with WT neurons. Like shLrrk2 astrocytes, LRRK2 KO astrocytes had significantly diminished arborization compared to WT rat astrocytes, indicating that Lrk2 is required for neuron-induced astrocyte morphogenesis \textit{in vitro} (Fig. 1b).

To study how LRRK2 G2019S affects astrocyte morphological complexity, we isolated cortical astrocytes from WT or LRRK2 G2019S BAC-transgenic rats\textsuperscript{87-89} and examined their morphological complexity in response to contact with WT neurons \textit{in vitro}. We found that LRRK2 G2019S rat astrocytes showed a reduction in astrocyte complexity compared to WT astrocytes (Fig. 1c-d). These results suggest that both loss and gain of LRRK2 kinase function impair astrocyte morphogenesis \textit{in vitro}.

To determine if neuronal LRRK2 G2019S mutation could impact astrocyte morphogenesis in a cell non-autonomous manner, we cocultured WT or LRRK2 G2019S astrocytes with LRRK2 G2019S neurons. When cultured on top of LRRK2 G2019S neurons, the WT astrocytes also displayed reduced arborization, indicating that LRRK2 G2019S mutation bi-directionally impairs astrocyte-neuron signaling required for astrocyte morphogenesis (Fig. 1c-d). These results show that LRRK2 G2019S impairs astrocyte morphology in both a cell-autonomous and non-cell autonomous manner,
illustrating important roles for astrocytic and neuronal Lrrk2 function in establishing proper astrocyte-neuron contacts.

Lrrk2 is a large multidomain protein containing ROC (Ras of complex), COR (C-terminal of ROC), catalytic kinase, and 4 protein-protein interactions domains (armadillo, ankyrin, leucine-rich repeats, WD40 domains)\textsuperscript{64,91,92}. The PD-linked mutation, LRRK2 G2019S, is in the kinase domain and is known to increase LRRK2 kinase activity \textit{in vitro} and \textit{in vivo}\textsuperscript{58-60,62}. Previous studies identified numerous intracellular proteins (Rab8A, Rab10, endophilin A1, auxillin, ribosomal protein s15, N-ethylmaleimide sensitive fusion protein, synaptojanin, and P62/SQSTM1) as LRRK2 kinase substrates\textsuperscript{62,93-99}. LRRK2 plays critical roles in ciliogenesis, lysosome function, immune system, and synaptic vesicle trafficking by phosphorylation of its substrates\textsuperscript{100-115}.

ERM proteins (Ezrin, Radixin, and Moesin) are structural components of perisynaptic astrocyte processes (PAPs)\textsuperscript{16,17}. LRRK2 phosphorylates a conserved threonine residue present in all ERM proteins\textsuperscript{60,116,117}. When phosphorylated, ERM proteins change conformation and link the F-actin cytoskeleton to the cell membrane\textsuperscript{118,119}. This switch is thought to play an important role in organizing cell morphology by allowing process outgrowth\textsuperscript{119,120}. Among the ERM proteins, ezrin is specifically and highly enriched in astrocytes (Extended Data Fig. 1i-j) and is known to preferentially localize at the perisynaptic astrocyte processes\textsuperscript{16}. Genetic ablation or down regulation of ezrin has been shown to reduce astrocyte morphological complexity \textit{in vivo}\textsuperscript{121-123}. Given these potential functional links between LRRK2 and ERM proteins, we wondered whether Lrrk2 controls astrocyte morphology via ERM phosphorylation (phospho-ERM).
To determine if LRRK2 function in astrocytes is required for ERM phosphorylation, we analyzed the levels of phospho-ERM in WT rat astrocytes treated with two different doses of MLi-2, a highly specific LRRK2 kinase inhibitor and compared phospho-ERM levels to the vehicle (DMSO) treated astrocyte cultures using immunoblotting. The well-established LRRK2 phosphorylation substrate Rab10 served as a control for this assay. As expected, MLi-2 treatment inhibited Rab10 phosphorylation (Fig. 1e). We found that, compared to astrocytes treated with DMSO, both 30 and 100 nM MLi-2 treatment decreased phospho-ERM levels in WT rat astrocytes by ~75%, indicating that LRRK2 kinase function is required for ERM phosphorylation in astrocytes (Fig. 1f). Next, we compared the levels of ERM phosphorylation in astrocytes isolated from WT or LRRK2 G2019S rats and found an increase (increased by ~100%) in ERM phosphorylation in LRRK2 G2019S astrocytes (Fig. 1g-h). These data reveal an important role for LRRK2 in mediating ERM phosphorylation in astrocytes in vitro. These findings also suggest that LRRK2 controls astrocyte morphology by regulating ERM phosphorylation levels.

**ERM phosphorylation state underlies Lrrk2-mediated astrocyte morphological complexity.**

Because both loss of Lrrk2 function (shRNA and LRRK2 KO) and gain of Lrrk2 kinase function (LRRK2 G2019S) perturbed astrocyte morphogenesis, we hypothesized that a balance between phosphorylated and unphosphorylated ERM protein levels may be crucial for regulating astrocyte morphological complexity. To test this hypothesis, we manipulated the balance between phosphorylated and unphosphorylated ERM in shLrrk2-transfected or LRRK2 G2019S astrocytes using two EZRIN mutants with
phosphorylation-mimicking or -deficient properties. Switching the amino acid threonine (T) at location 567 with aspartate (D) results in a conformation of EZRIN that mimics a constitutively phosphorylated state\(^{124,125}\). On the other hand, mutating the same residue T567 to alanine (A) results in a version of the protein that cannot be phosphorylated: phospho-dead EZRIN\(^{119,120}\). Phospho-dead EZRIN binds to the plasma membrane like WT EZRIN but is poorly associated with the actin cytoskeleton\(^{119,120,126,127}\).

We first tested if overexpressing phospho-mimetic EZRIN in sh\textit{Lrrk2}-transfected astrocytes would rescue their morphology. Astrocytes transfected with shControl, and WT EZRIN or phospho-mimetic EZRIN served as controls. In these experiments, only astrocytes were manipulated and plated onto WT rat neurons. WT or phospho-mimetic EZRIN transfection did not change the morphological complexity of shControl astrocytes (Fig. 1i). However, overexpression of phospho-mimetic EZRIN, but not WT EZRIN, fully rescued the complexity of sh\textit{Lrrk2} astrocytes (Fig. 1j).

We next tested if increasing unphosphorylated EZRIN would rescue the morphogenesis of LRRK2 G2019S astrocytes. To do so, we isolated cortical astrocytes from WT or LRRK2 G2019S rats, and over-expressed WT or phospho-dead EZRIN, and plated transfected astrocytes on WT cortical neurons. We found that overexpression of WT EZRIN did not change the morphology of WT or LRRK2 G2019S astrocytes. However, overexpression of the phospho-dead EZRIN reduced the morphological complexity of WT astrocytes (Fig. 1k). Conversely, overexpression of the phospho-dead EZRIN fully rescued the morphological complexity of LRRK2 G2019S astrocytes \textit{in vitro} (Fig. 1l). These results show that LRRK2 mediates astrocyte morphological complexity by regulating ERM phosphorylation and strongly suggest that the balance between
phosphorylated and unphosphorylated ERM proteins is needed for neuronal contact-induced astrocyte morphogenesis.

To determine if LRRK2 G2019S mutation impacts ERM phosphorylation in astrocytes in vivo, we examined the levels of phospho-ERM in coronal brain sections from 21-day-old (P21) and 12-week-old WT or LRRK2 G2019S<sup>ki/ki</sup> mice in which astrocytes were labeled via the Aldh1L1-eGFP transgene. When compared to WTs, we observed an increase in phospho-ERM staining in both P21 and 12-week-old LRRK2 G2019S<sup>ki/ki</sup> mice. This increase was not uniform but rather localized exclusively to some of the astrocytes within the layers L1 and L2/3 of the anterior cingulate cortex (ACC) and the primary motor cortex (MOp) (Fig. 2a-c; Extended Data Fig. 2a-b). In P21 LRRK2 G2019S<sup>ki/ki</sup> mice, the phospho-ERM integrated densities in the ACC Layer 1 and Layer 2/3 and the MOp Layer 1 and Layer 2/3 were increased by ~412% and ~158% compared to the WTs, respectively (Fig. 2d). In 12-week-old (P84) LRRK2 G2019S<sup>ki/ki</sup> mice, the phospho-ERM integrated densities in the ACC Layer 1 and Layer 2/3 and in the MOp Layer 1 and Layer 2/3 were increased by ~700% and ~398% compared to the WTs, respectively (Extended Data Fig. 2c). However, the phospho-ERM integrated density in neighboring brain regions such as the primary somatosensory cortex (SSp) and dorsal medial striatum (DMS) was not different between LRRK2 G2019S<sup>ki/ki</sup> or WTs (Extended Data Fig. 2h). These findings show that LRRK2 G2019S mutation induces aberrant ERM phosphorylation in astrocytes. Intriguingly, this increase in ERM phosphorylation is not uniform but rather localized to ACC and MOp, which are cortical regions that are impacted in PD patients<sup>128-130</sup>.

To determine the in vivo functions of astrocytic Lrrk2, we examined astrocyte morphological complexity in Layer 1 and Layer 2/3 of ACC and MOp of LRRK2 G2019S<sup>ki/ki</sup>
mice. To do so, we used postnatal astrocyte labeling by electroporation (PALE) combined with the PiggyBac transposon system to introduce a membrane-targeted fluorescent label (mCherry-CAAX) into a sparse population of cortical astrocytes in WT and LRRK2 G2019S<sup>ki/ki</sup> mice at P0 (Extended Data Fig. 2k). At P21, following the period of astrocyte growth and extensive elaboration, the territory volumes for LRRK2 G2019S astrocytes were significantly smaller (reduced by ~34%) than WT astrocytes (Fig. 2f). In addition, compared to WT astrocytes, the branching complexity of LRRK2 G2019S astrocytes was reduced (Fig. 2g). The reduction in astrocyte size and complexity in LRRK2 G2019S<sup>ki/ki</sup> astrocytes did not result in an increase in the numbers of astrocytes in these brain regions (Extended Data Fig. 3a-e). The number of Aldh1L1-EGFP/Sox9 double-positive astrocytes in these brain regions was similar in WT and LRRK2 G2019S<sup>ki/ki</sup> mice (Extended Data Fig. 3c). However, there were patches within ACC and MOp parenchyma in LRRK2 G2019S<sup>ki/ki</sup> mice in which the EGFP-positive astrocyte processes had noticeably reduced. These changes in astrocytes were not due to a typical reactive gliosis phenotype because we did not detect any increase in the levels of GFAP, which differentially labels reactive astrocytes in the cortical grey matter (Extended Data Fig. 3f).

Taken together, these results reveal that LRRK2 G2019S mutation reduces astrocyte morphological complexity without affecting astrocyte numbers or reactivity in vivo.

To determine if LRRK2 G2019S-mediated astrocyte morphological deficits are due to the phospho-ERM upregulation in astrocytes, we tested whether phospho-dead EZRIN can rescue the morphological complexity of LRRK2 G2019S astrocytes in vivo. To do so, we performed PALE to introduce mCherry-CAAX and phospho-dead EZRIN into astrocytes of WT or LRRK2 G2019S<sup>ki/ki</sup> mice. Like our in vitro results, the overexpression
of phospho-dead EZRIN in WT astrocytes is sufficient to decrease the territory volume and morphological complexity (Fig. 2e-g). However, the overexpression of phospho-dead EZRIN restored LRRK2 G2019S astrocyte territory volume and complexity (Fig. 2f-g). These results indicate that balancing phosphorylated and unphosphorylated ERM levels is crucial for regulating astrocyte morphology, and overexpression of phospho-dead EZRIN can rescue morphological deficits of LRRK2 G2019S astrocytes in vivo.

To further test this hypothesis, we studied if knocking down LRRK2 in WT astrocytes affects astrocyte morphological complexity in vivo. We introduced shControl or shLrrk2 into a sparse population of cortical astrocytes in WT mice by using PALE. We found that, like LRRK2 G2019S astrocytes, shLrrk2 astrocytes had significantly smaller territory volumes (reduced by ~36%) and reduced morphological complexity when compared to shControl astrocytes (Fig. 2h-j). To determine whether phospho-mimetic EZRIN can restore astrocyte morphology in LRRK2 knockdown astrocytes in vivo, we performed PALE to introduce either shLrrk2 or shControl together with phospho-mimetic EZRIN overexpression vector into astrocytes of the ACC and MOp. We found that overexpression of phospho-mimetic EZRIN in shControl astrocytes did not change astrocyte territory volume (Fig. 2h-i). However, expression of phospho-mimetic EZRIN in shLrrk2-containing astrocytes restored astrocyte territory volume and morphological complexity up to the WT levels (Fig. 2j). Taken together, these findings revealed that the balance between phosphorylated and unphosphorylated ERM is crucial for LRRK2-mediated astrocyte morphological complexity in vivo. Disrupting the balance of ERM phosphorylation status in astrocytes compromises astrocyte morphological complexity,
and modulation of this balance can be used to restore astrocyte morphology in the LRRK2 G2019S genotype.

ERM phosphorylation is upregulated in the frontal cortices of PD patients carrying LRRK2 G2019S mutation.

To determine if phospho-ERM abundance is altered in PD patients carrying LRRK2 G2019S mutation, we stained frontal cortices from 3 patients who carry this mutation and compared them to age and sex-matched donors (Supplemental Table 1). We observed that, compared to sex and age-matched human control subjects, the overall levels of phosphorylated ERM were robustly increased by 7-fold in the frontal cortex of PD patients carrying the LRRK2 G2019S mutation (Fig. 2m). The elevated signal of phospho-ERM is often surrounded by GFAP positive astrocytes and astrocyte processes. The astrocytic organization, determined by GFAP staining, appeared to be disrupted in PD patients carrying the LRRK2 G2019S mutation (Fig. 2l). We observed increased GFAP levels in grey matter astrocytes (increased by ~42%) of LRRK2 G2019S mutation carriers, suggesting the presence of reactive gliosis in PD patients carrying LRRK2 G2019S mutation (Fig. 2n). These data indicate that ERM phosphorylation is dysregulated in PD patients carrying the LRRK2 G2019S mutation. Together, our data show that phospho-ERM levels are elevated in the cortices of PD patients with LRRK2 G2019S mutation. The increase in phospho-ERM is concurrent with reactive gliosis and astrocyte morphology changes. Distinct from the P21 and 3-month-old LRRK2 G2019S^ki/ki^ mice where phospho-ERM was restricted to astrocytes, in the human patient brains (~80 years old), we also saw phospho-ERM accumulation outside the GFAP-labelled astrocytes. This observation
suggests a role for aberrant ERM phosphorylation in multiple cell types in later stages of PD caused by the LRRK2 G2019S mutation.

**LRRK2 G2019S mutation disrupts excitatory and inhibitory synapse numbers and function in the cortex.**

Astrocyte morphological complexity is a crucial indicator of proper astrocyte-synapse interactions that control synapse numbers\(^2,27,131-133\). LRRK2 G2019S\(^{ki/ki}\) mice revealed no dopaminergic neuron loss or motor dysfunction\(^{68,69}\), however, synapse dysfunction at cortico-striatal circuits was observed in adult mice. For example, LRRK2 G2019S alters presynaptic glutamatergic transmission and disrupts glutamate receptor trafficking at striatal postsynaptic terminals\(^{72,76,85,134-136}\). However, whether LRRK2 G2019S affects cortical connectivity is unknown. Given the critical role of LRRK2 in synapses and PD-related cortical circuits\(^{137-141}\), we next investigated the role of LRRK2 G2019S mutation in synaptic connectivity in the ACC and MO\(\text{P}\) (Fig. 3a). To quantify the density of structural cortical synapses, we used the Vesicular Glutamate Transporter 1 (VGlut1) and the postsynaptic density protein 95 (PSD95) or the Vesicular GABA Transporter (VGAT), and gephyrin (GEPHYRIN), which mark the respective pre- and post-synaptic compartments of intracortical excitatory and inhibitory synapses\(^{142-146}\) (Fig. 3b). Structural synapses were identified as the apposition and co-localization of pre and postsynaptic markers, which are in two distinct neuronal compartments (i.e., axons and dendrites) and would only appear to co-localize at synapses due to their close proximity\(^{147}\).

Synapse densities were quantified in the Layers 1 and 2/3 of the ACC and MO\(\text{P}\) of 12-week-old LRRK2 G2019S\(^{ki/ki}\) and WT mice. There was a significant decrease (~30%) in the VGlut1/PSD95-positive excitatory synapse densities in the ACC of the LRRK2
G2019S<sup>ki/ki</sup> mice compared to WT controls, whereas the VGAT/Gephyrin-positive inhibitory synapse numbers in the ACC were not different between genotypes (Fig. 3c-d; Extended Data Fig. 4a-b). On the other hand, we found no changes in the densities of VGlut1/PSD95-positive excitatory synapses in the Layers 1 and 2/3 of the MOp in LRRK2 G2019S<sup>ki/ki</sup> mice compared to WT controls (Extended Data Fig. 4c-d). However, compared to the 12-week-old WT controls, the density of VGAT/Gephyrin-positive inhibitory synapses in Layer 1 and in Layer 2/3 of MOp of LRRK2 G2019S<sup>ki/ki</sup> mice were increased by ~45% and ~35%, respectively (Fig. 3e-f). These data indicate that LRRK2 G2019S mutation differentially impacts the excitatory and inhibitory synapse densities in the ACC and the MOp.

We next tested the effects of altered synapse density in the ACC and the MOp of LRRK2 G2019S mice on synaptic function using electrophysiology. To do so, we recorded miniature excitatory postsynaptic currents (mEPSCs) in the ACC and miniature inhibitory postsynaptic currents (mIPSCs) in the MOp layer 2/3 pyramidal neurons from acute brain slices of 12-week-old LRRK2 G2019S<sup>ki/ki</sup> and WT mice. In agreement with a reduction in excitatory synapse density in the ACC, LRRK2 G2019S<sup>ki/ki</sup> neurons displayed reduced mEPSC frequency by ~65% and a corresponding right shift in the cumulative distributions of mEPSC inter-event intervals (Fig. 3g-h) when compared to WT neurons. There were no significant changes in the mean mEPSC amplitude or the cumulative probability between genotypes (Fig. 3i). mEPSC frequency changes correlate with changes in synapse numbers or the probability of presynaptic glutamate release<sup>148</sup>, whereas the mEPSC amplitude is an indicator of the synaptic strength reflecting the number of AMPARs at the synapse<sup>149</sup>. Therefore, together with our anatomical data,
these physiological analyses reveal that LRRK2 G2019S reduced the number of intracortical excitatory connections made onto the Layer 2/3 pyramidal neurons in the ACC without a major change in the synaptic strength.

In the MOp motor cortex, on the other hand, we found a robust increase (~73%) in mIPSC frequency when we recorded from the Layer 2/3 pyramidal neurons of the LRRK2 G2019S<sup>ki/ki</sup> mice compared with WT, with no significant change in the amplitude (Fig. 3j-l). The increased mIPSC frequency aligns with our findings that inhibitory synapse numbers are elevated in the MOp of the LRRK2 G2019S mice. This increase could be a result of enhanced inhibitory synaptogenesis or a local increase in the number of inhibitory interneurons in this brain region.

Altogether, these electrophysiological and neuroanatomical analyses show that LRRK2 G2019S reduces the density and the function of excitatory synaptic inputs made onto the ACC Layer 2/3 but increases the density and the function of inhibitory synaptic inputs made onto the MOp Layer 2/3. These data are in accordance with our finding that LRRK2 G2019S decreases excitatory synapse numbers in the ACC but increases inhibitory synapse numbers in the MOp. Furthermore, these results suggest that LRRK2 kinase function is critical to control proper excitatory and inhibitory synapse development and function in the cortex.

**Phospho-dead EZRIN rescues excitatory synapse density in the ACC of adult LRRK2 G2019S<sup>ki/ki</sup> mice.**

Because in LRRK2 G2019S<sup>ki/ki</sup> mice, all cell types, including astrocytes and neurons, express the mutant LRRK2, the cellular origin of the synaptic phenotypes is unclear. Moreover, if these synaptic phenotypes can be rescued by manipulating
astrocytes in the adult brain is unknown. Given the robust effects of phospho-dead EZRIN on rescuing LRRK2 G2019S astrocyte morphology, we sought to test whether manipulating the balance of ERM phosphorylation in adult LRRK2 G2019S astrocytes can reverse the synaptic phenotypes we observed in the ACC or MOp of the LRRK2 G2019S<sup>ki/ki</sup> mice.

To do so, 9-week-old WT and LRRK2 G2019S<sup>ki/ki</sup> mice were retro-orbitally injected with AAVs to express Hemagglutinin (HA)-tagged WT EZRIN or phospho-dead EZRIN under the control of the astrocyte-specific GfaABC1D promoter (Extended Data Fig. 5a). The injected mouse brains were harvested at 12 weeks (3 weeks after injection) for synapse quantification, and electrophysiology (Extended Data Fig. 5a). The widespread viral expression of EZRIN proteins in ACC astrocytes was verified by staining with anti-HA tag antibody (Extended Data Fig. 5b). Overexpression of WT EZRIN in WT mouse brains did not cause a noticeable increase in phospho-ERM levels in the cortex. However, WT EZRIN overexpression was accompanied by intense phospho-ERM elevation in the LRRK2 G2019S<sup>ki/ki</sup> cortices. Whereas overexpression of the phospho-dead EZRIN eliminated the phospho-ERM elevation in the LRRK2 G2019S<sup>ki/ki</sup> cortex (Extended Data Fig. 5b). This observation indicates that overexpression of phospho-dead EZRIN suppresses the elevation of ERM phosphorylation in the LRRK2 G2019S<sup>ki/ki</sup> cortices.

Overexpression of WT EZRIN was not sufficient to rescue the excitatory synapse density decline in layer 1 and layer 2/3 ACC of the LRRK2 G2019S<sup>ki/ki</sup> mice. However, overexpression of phospho-dead EZRIN reduced excitatory synapse density in the WT background by around 30% (Fig. 4a-b). Strikingly, astrocytic overexpression of phospho-dead EZRIN in the LRRK2 G2019S<sup>ki/ki</sup> fully rescued the excitatory synapse numbers up
to WT levels in ACC layer 1 and layer 2/3 (Fig. 4a-b). These data show that modulating
the phosphorylation status of astrocytic ERMs through overexpression of the phospho-
dead EZRIN in astrocytes is sufficient to generate synapse number deficits in WT mice,
whereas in LRRK2 G2019S\(^{ki/ki}\) mice, the same manipulation rescues synapse deficits.
These data strongly suggest that the reduction we observed in synapse numbers in the
ACC of the LRRK2 G2019S\(^{ki/ki}\) mice is largely due to an astrocytic dysfunction caused by
the imbalance of ERM phosphorylation. These results also indicate that astrocytes are
crucial contributors to excitatory synaptic dysfunction in the LRRK2 G2019S\(^{ki/ki}\) mouse
cortex.

We next investigated whether the same manipulation of adult astrocytes can
rescue the inhibitory synapse upregulation in the MOp. We found that neither the WT nor
the phospho-dead EZRIN overexpression in adult astrocytes reduced the density of
inhibitory synapses back to WT levels in the MOp layer 1 and layer 2/3 of the LRRK2
G2019S\(^{ki/ki}\) mice (Extended Data Fig. 5c-d). These data suggest that overexpression of
phospho-dead EZRIN in adult WT or LRRK2 G2019S\(^{ki/ki}\) astrocytes is sufficient to modify
the excitatory synapse numbers in the ACC but cannot restore or impair inhibitory
synaptic connectivity changes seen in the MOp. It is plausible that LRRK2 G2019S
impairs inhibitory synapses in the MOp by altering the number or connectivity of cortical
interneurons through a developmental mechanism. Thus, manipulation in the adult is not
sufficient to eliminate this phenotype. Future studies on the role of Lrrk2 in cortical
development are likely to be beneficial in determining its role in controlling inhibition and
how this control is disrupted in PD.
Because astrocytic overexpression of phospho-dead EZRIN impacted excitatory synapse numbers in the WT and LRRK2 G2019S<sup>ki/ki</sup> mice. Next, we performed whole cell patch clamp recordings from the ACC layer 2/3 pyramidal neurons of WT and LRRK2 G2019S<sup>ki/ki</sup> mice transduced with the same AAVs (Extended Data Fig. 5a. Overexpression of WT EZRIN in LRRK2 G2019S<sup>ki/ki</sup> astrocytes did not rescue the decreased frequency of mEPSCs in the ACC layer 2/3 neurons. However, astrocytic overexpression of the phospho-dead EZRIN significantly reduced the frequency of mEPSCs (reduced by ~54%) in the WT ACC neurons but revamped the frequency of mEPSCs in the LRRK2 G2019S<sup>ki/ki</sup> ACC neurons (Fig. 4d). Similarly, the cumulative distribution analysis of mEPSCs showed that inter-event interval durations were significantly impacted by astrocytic phospho-dead EZRIN expression in both genotypes in opposite directions (Fig. 4d). In contrast, no significant differences were observed in the mean mEPSC amplitudes across all 4 conditions using posthoc comparisons (Fig. 4e).

Together, our results reveal that astrocytic overexpression of phospho-dead EZRIN in adult LRRK2 G2019S<sup>ki/ki</sup> mice can recover the excitatory synapse density and function, whereas the same manipulation disrupts synapse numbers and function in WT ACC neurons. These findings show that the balance of ERM phosphorylation status in astrocytes is crucial for maintaining excitatory synapse density and function in the ACC. This work also reveals a causal link between the astrocytic and synaptic dysfunction induced by the LRRK2 G2019S mutation, providing a non-cell autonomous mechanism for rescuing aberrant cortical wiring in PD pathogenesis.
In summary, here we describe a previously unknown role for Lrrk2, a PD-linked gene, in controlling astrocyte morphology and astrocyte-mediated synaptogenic function. Lrrk2 is needed for establishing astrocyte morphological complexity through the phosphorylation of ERMs. LRRK2 G2019S mutation, which increases ERM phosphorylation in astrocytes, also impaired astrocyte morphogenesis in vitro and in vivo. These findings indicate that ERM phosphorylation balance is critical for the acquisition of complex astrocyte morphology. EZRIN and other ERM proteins are known to interact with cytoskeletal proteins such as F-actin and control the dynamics of F-actin polymerization in a phosphorylation-dependent manner. Future studies investigating how phosphorylated and unphosphorylated states of EZRIN control astrocyte morphogenesis through modulation of the cytoskeleton will be helpful in deciphering mechanisms of astrocyte morphogenesis.

LRRK2 G2019S\textsuperscript{ki/ki} mice do not recapitulate cardinal motor symptoms of PD because there is no detectable dopaminergic neuron loss in these mice\textsuperscript{67-69}. LRRK2 mutation carriers show non-motor symptoms in comparison to non-carriers, such as hyposmia and decreased cognitive performance\textsuperscript{150 41,151,152}. These non-motor symptoms arise long before motor dysfunction in PD, indicating that the aberrant synaptic connectivity within multiple brain circuits is present long before the death of the dopaminergic neurons\textsuperscript{40-43}. We reasoned that LRRK2 G2019S\textsuperscript{ki/ki} mice may display early circuit dysfunction akin to the non-motor symptoms of prodromal PD. In agreement with this possibility, previous studies showed that LRRK2 G2019S\textsuperscript{ki/ki} mice have deficits in attention and goal-directed learning and differential response to stress\textsuperscript{74-76,153}. Our findings showing synaptic deficits in the ACC of LRRK2 G2019S\textsuperscript{ki/ki} mice fit well with these
studies because the ACC is a brain region that controls attention, goal-directed actions, and mood\textsuperscript{154-159}.

We observed synaptic deficits in the ACC of LRRK2 G2019S\textsuperscript{ki/ki} mice concurrently with enhanced ERM phosphorylation in astrocytes. Remarkably, overexpression of the phospho-dead EZRIN in the astrocytes of 9-week-old mice can restore the excitatory synapse numbers and function in the ACC layer 2/3 neurons from 12-week-old mice. This result causally links astrocyte dysfunction to the cortical synaptic dysfunction in the LRRK2 G2019S\textsuperscript{ki/ki} mice. Moreover, our findings indicate that restoring astrocyte function, even after development is completed, can be used to treat excitatory synaptic pathology. Future work aimed at understanding the molecular underpinnings of how LRRK2 mutations alter synaptogenic functions of astrocytes may lead to effective strategies to treat PD.

How does ERM phosphorylation control both the structure and the synaptogenic functions of astrocytes? Astrocytes control synapse formation and function both through cell adhesion and secreted proteins that signal to neurons\textsuperscript{31,160}. The canonical function of ERM proteins is linking the actin cytoskeleton with the plasma membrane, thus allowing cellular process outgrowth and morphogenesis\textsuperscript{161-163}. Less is known about the roles of ERM in the secretion process. However, it is plausible that ERM phosphorylation regulates synaptogenic protein secretion from astrocytes independent of its roles in astrocyte morphology. Another alternative explanation is a role for astrocytic phospho-ERM proteins in controlling synapse elimination by astrocytes. In agreement with this possibility, a recent study found that synapses are more likely to be eliminated if phospho-ERM proteins were localized to peri-synaptic astrocyte processes in ALS patient brains.
and mouse models\textsuperscript{164}. Future studies investigating LRRK2 and ERM interactomes in astrocytes will help uncover the molecular pathways involved in LRRK2-ERM signaling at the tripartite synapse.

In summary, our findings reveal a critical function for LRRK2 in astrocyte development and how LRRK2 G2019S mutation derails important roles of astrocytes in maintaining synaptic connectivity in the developing and mature cortex. Furthermore, here we pinpoint the regulation of astrocytic ERM phosphorylation as a molecular mechanism that is impaired when LRRK2 G2019S mutation is present in astrocytes. These findings suggest that LRRK2-mediated ERM phosphorylation is an early mediator of PD pathogenesis in astrocytes and that astrocytes can be directly responsible for synaptic dysfunction seen in the early stages of PD.
METHODS

Animals:

All mice and rats were used in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Duke Division of Laboratory Animal Resources (DLAR) oversight (IACUC Protocol Numbers A147-17-06 and A117-20-05). All mice and rats were housed under typical day/night conditions of 12-hour cycles. LRRK2 G2019S\textsuperscript{ki/ki} mice (RRID: IMSR_TAC:13940) were obtained through Taconic. Aldh1l1-EGFP (RRID: MMRRC_011015-UCD) mice were obtained through MMRRC. Wild-type CD1 mice used for PALE were purchased through Charles River Laboratories (RRID: IMSR_CRL:022). LRRK2 G2019S-BAC Transgenic rats (RRID: IMSR_TAC:10681) were obtained through Taconic. LRRK2 knock-out rats (RRID: RGD_7241056) were obtained through SAGE Labs, Inc. in St. Louis, MO.

Mice and rats were used for experiments as specified in the text and figure legends. For all experiments, age, and sex-matched mice were randomly assigned to experimental groups based on genotypes. For MLi-2 treatment of astrocytes, only female rat astrocyte cultures were used for Western blot and analysis because male rat astrocyte cultures have low phospho-ERM levels \textit{in vitro}. For other experiments, mice of both sexes were included in the analysis, and we did not observe any influence or association of sex on the experimental outcomes.

Primary cortical neuron isolation and culture:

Purified (glia-free) rat cortical neurons were prepared as described previously\textsuperscript{28}. Briefly, cortices from P1 rat pups of both sexes (Sprague Dawley, Charles River Laboratories, SD-001) were micro-dissected, digested in papain (\textasciitilde 7.5 units/ml) at 33\textdegree C for 45 minutes,
triturated in low and high ovomucoid solutions, resuspended in panning buffer (DPBS (GIBCO 14287) supplemented with BSA and insulin) and passed through a 20 μm mesh filter (Elko Filtering 03-20/14). Filtered cells were incubated on negative panning dishes coated with Bandeiraea Simplicifolia Lectin 1 (x2), followed by goat anti-mouse IgG+IgM (H+L) (Jackson ImmunoResearch 115-005-044), and goat anti-rat IgG+IgM (H+L) (Jackson ImmunoResearch 112-005-044) antibodies, then incubated on positive panning dishes coated with mouse anti-L1 (ASCS4, Developmental Studies Hybridoma Bank, Univ. Iowa) to bind cortical neurons. Adherent cells were collected by forceful pipetting with a P1000 pipette. Isolated neurons were pelleted (11 minutes at 200 g) and resuspended in serum-free neuron growth media (NGM; Neurobasal, B27 supplement, 2 mM L-Glutamine, 100 U/ml Pen/Strep, 1 mM sodium pyruvate, 4.2 μg/ml Forskolin, 50 ng/mL BDNF, and 10 ng/mL CNTF). 70,000 neurons were plated onto 12 mm glass coverslips coated with 10 μg/ml poly-D-lysine (PDL, Sigma P6407) and 2 μg/ml laminin and incubated at 37°C in 10% CO₂. On day in-vitro (DIV) 2, half of the media was replaced with NGM Plus (Neurobasal Plus, B27 Plus, 100 U/mL Pen/Strep, 1 mM sodium pyruvate, 4.2 μg/ml Forskolin, 50 ng/ml, BDNF, and 10 ng/ml CNTF) and AraC (10 μM) was added to stop the growth of proliferating contaminating cells. On DIV 3, all the media was replaced with NGM Plus. In experiments involving lentivirus infection, 100 μl of supernatant containing lentivirus plus polybrene (1 μg/ml) was added to the AraC NGM mixture on DIV 2 and completely washed out on DIV 3 and replaced with NGM Plus containing 100 ng/ml BDNF. Neurons were fed on DIV 6 and DIV 9 by replacing half of the media with NGM Plus.

**Primary cortical astrocyte isolation and culture:**
Rat cortical astrocytes were prepared as described previously. P1 rat cortices from both sexes were micro-dissected, papain digested, triturated in low and high ovomucoid solutions, and resuspended in astrocyte growth media (AGM: DMEM (GIBCO 11960), 10% FBS, 10 μM hydrocortisone, 100 U/ml Pen/Strep, 2 mM L-Glutamine, 5 μg/ml Insulin, 1 mM Na Pyruvate, 5 μg/ml N-Acetyl-L-cysteine). Between 15-20 million cells were plated on 75 mm² flasks (non-ventilated cap) coated with poly-D-lysine and incubated at 37°C in 10% CO₂. On DIV 3, the removal of non-astrocyte cells was performed by forcefully shaking closed flasks by hand for 10-15 s until only an adherent monolayer of astrocytes remained. AraC was added to the media from DIV 5 to DIV 7 to eliminate contaminating fibroblasts. On DIV 7, astrocytes were trypsinized (0.05% Trypsin-EDTA) and plated into 12-well or 6-well dishes. On DIV 8, cultured rat astrocytes were transfected with shRNA and/or expression plasmids using Lipofectamine LTX with Plus Reagent (Thermo Scientific) per the manufacturer’s protocol. Briefly, 1 μg (12-well) or 2 μg (6-well) total DNA was diluted in Opti-MEM containing Plus Reagent, mixed with Opti-MEM containing LTX (1:2 DNA to LTX), and incubated for 30 minutes. The transfection solution was added to astrocyte cultures and incubated at 37°C for 3 hours. On DIV 10, astrocytes were trypsinized, resuspended in NGM plus, plated (20,000 cells per well) onto DIV 10 neurons, and co-cultured for 48 hours.

**shRNA plasmids:**

pLKO.1 Puro plasmids containing shRNA (pLKO.1-shRNA) against mouse/rat *Lrrk2* (sh*Lrrk2*: TRCN0000322193; GGCCGAGTTGTGGATCATATT), was obtained from the RNAi Consortium (TRC) via Dharmacon. A scrambled shRNA sequence was generated (GTTGCTGAATGGCGGATCTAT) and cloned into the pLKO.1
TRC cloning vector\textsuperscript{165} according to Addgene protocols (https://www.addgene.org/protocols/plko/). To generate pLKO.1 shRNA plasmids that express EGFP (pLKO.1-shRNA-EGFP), CAG-EGFP was removed from pLenLox-shNL1-CAG-EGFP\textsuperscript{166} and inserted between Kpn1 and SpeI sites in pLKO.1 Puro, replacing the puromycin resistance gene. pLKO.1 shRNA mCherry plasmids were generated by replacing EGFP with mCherry between KpnI and Nhel sites.

**PiggyBac plasmids:**

pPB-CAG-EGFP and pGLAST-PBase were a gift from Dr. Joseph Loturco\textsuperscript{167}. To generate pPB-CAG-mCherry-CAAX, mCherry-CAAX was inserted between Xmal and NotI restrictions sites to replace EGFP. To insert the hU6 promoter and shRNA in pPB-CAG-mCherry-CAAX, a DNA fragment containing hU6 and shRNA was amplified from pLKO.1-shRNA using Phusion High-Fidelity DNA Polymerase (NEB) with primers that introduced SpeI restriction sites (Forward Primer: GGACTAGTCAGGCCGAAGGAATAGAAG; Reverse Primer: GGACTAGTGCCAAAGTGTCTCTGCTG). PCR products were purified, digested with SpeI, and ligated into pPBCAG-mCherry-CAAX at the SpeI restriction site. An analytical digest with EcoRI followed by sequencing was used to confirm the orientation of the inserted DNA fragment.

**Ezrin plasmids:**

pZac2.1-GfaABC1D-Lck-GCaMP6f was a gift from Dr. Baljit Khakh (Addgene plasmid #52924). pZac2.1-GfaABC1D-BiolD2-HA was generated by PCR of BiolD2 from pAAV-hSyn-BiolD2-Linker-Synapsin1a-HA\textsuperscript{168} (primers Fw: 5'-ctagcctgagaatcattttcttttttg-3', Rv: 5'-ccgggtcgactctagatgcgttagcgtaatccggtacatcg-...
3') and insertion into the EcoRI and XbaI restriction sites of pZac2.1-GfaABC1D-Lck-GCaMP6f using In-Fusion cloning (TaKaRa). pHJ421(pEGFP-Ezrin WT) and pHJ423 (pEGFP-Ezrin T567D) were a gift from Stephen Shaw (Addgene plasmid # 20680 and # 20681). pZac2.1-GfaABC1D-Ezrin WT-BiolID2-HA and pZac2.1-GfaABC1D-Ezrin T567D-BiolID2-HA were generated by PCR of Ezrin from pHJ421 or pHJ423 (primers Fw: 5'-ctagcctcgagaattcaccatgccgaaaccaatca-3', Rv: 5'-tgaacatggtgaattccgacagggcctcgaactcg-3') and insertion into the EcoRI restriction sites of pZac2.1-GfaABC1D-BiolID2, respectively. To generate pZac2.1-GfaABC1D-Ezrin T567A-BiolID2-HA plasmid, Q5® Site-Directed Mutagenesis Kit (NEB) was used with mutagenesis primers (Fwd: CAAGTACAAGCGCTGCGGCAGA; Rev: TCCCGGCCTTGCCCTCATG)

**Lentivirus production and transduction:**

Lentiviruses containing shRNA targeting vectors were produced to test the knockdown efficiency of shRNA constructs in cultured primary astrocytes or to bulk transduce neurons with shRNA and GFP. To produce lentivirus, HEK293T cells were transfected with a pLKO.1 shRNA Puro targeting plasmid (for astrocyte transduction), an envelope plasmid (VSVG), and a packaging plasmid (dR8.91) using X-tremeGENE (Roche). One day after transfection, the media was replaced with AGM (for astrocyte transduction), and media containing lentivirus was collected on days 2 and 3 post-transfection. To assess the knockdown efficiency of shRNAs in astrocytes, rat primary astrocytes at DIV 7 were plated in 6-well dishes in 2 ml of AGM. On DIV 8, 1 ml of AGM was removed, and 500 μl of fresh AGM was added along with 500 μl of lentivirus-containing media and 1 μg/ml polybrene. Cultured astrocytes were treated with puromycin (1 μg/ml) from DIV 10-15 to
select for transduced cells. Cultured astrocytes were lysed at DIV 15 for protein extraction and Western blot analysis.

**MLi-2 treatment of astrocytes:**
Cortical astrocytes were isolated from P0-P1 rat female pups as described in the “Cortical astrocyte isolation and culture” section. Cultured astrocytes were treated with 0, 30, and 100 nM LRRK2 kinase inhibitor, MLi-2 (Tocris; Catalog number: 5756), for 72 hours. Cultured astrocytes were lysed at DIV 11 for protein extraction and Western blot analysis.

**Protein extraction and Western blotting:**
Protein was extracted from cultured rat astrocytes using membrane solubilization buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.5% NP-40, and protease inhibitors). Cultured astrocytes were washed twice with ice-cold TBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ and incubated on ice in membrane solubilization buffer for 20 minutes with occasional agitation. Cell lysates were collected, vortexed briefly, and centrifuged at 4°C at high speed for 10 minutes to pellet non-solubilized material. The supernatant was collected and stored at −80°C.

Pierce BSA Protein Assay Kit (Thermo Fisher) was used to determine protein concentration, and lysates were mixed with 4x Pierce™ Reducing Sample Buffer (Thermo Scientific) and incubated at 45°C for 45 minutes to denature proteins. 7-10 μg (cultured astrocyte lysates) of protein was loaded into Bolt™ 4–12% Bus-Tris Plus gels (Thermo Scientific) and run at 150 V for 1 hour. Proteins were transferred at 100 V to PVDF membrane (Millipore) for 1.5 hours, blocked in 5% BSA in TBST (137 mM NaCl, 2.68 mM KCl, 24.7 mM Tris-Base, 0.1% (w/v) Tween 20) for 1 hour and incubated in primary
antibodies overnight at 4°C. Primary antibodies used were: anti-LRRK2 (Rabbit, 1:500; ab133474, abcam), GAPDH (mouse, 1:5000; ab8245, abcam), β-actin (mouse, 1:5000; A5441, Millipore Sigma), phospho-ERM (Rabbit, 1:500; #3141, Cell Signaling), ERM (Rabbit, 1:500; #3142, Cell Signaling), phospho-Rab10 (Rabbit, 1:500; ab230261, abcam), Rab10 (Rabbit, 1:500; ab237703, abcam). The next day, membranes were washed with TBST, incubated in HRP secondary antibodies (Thermo Fisher Scientific) for 2 hours, washed in TBST, and imaged on a Biorad Gel Doc imaging system. Protein levels were quantified using FIJI.

**Immunocytochemistry:**

Astrocyte-neuron co-cultures on glass coverslips were fixed on DIV 12 with warm 4% PFA for 7 minutes, washed 3 times with PBS, blocked in a blocking buffer containing 50% normal goat serum (NGS) and 0.4% Triton X-100 for 30 minutes, and washed in PBS. Samples were then incubated overnight at 4°C in primary antibodies diluted in blocking buffer containing 10% NGS, washed with PBS, incubated in Alexa Fluor conjugated secondary antibodies (Life Technologies) for 2 hours at room temperature, and washed again in PBS. Coverslips were mounted onto glass slides (VWR Scientific) with Vectashield mounting media containing DAPI (Vector Labs), sealed with nail polish, and imaged on an AxioImager M1 (Zeiss) fluorescence microscope. Images of healthy astrocytes with strong expression of fluorescent markers that did not overlap with other fluorescent astrocytes were acquired at 40x magnification in red, green, and/or DAPI channels using a CCD camera. Astrocyte morphological complexity was analyzed in FIJI using the Sholl analysis plugin[^169], as described previously[^28] (https://github.com/Eroglu-Lab/In-Vitro-Sholl). Statistical analyses were conducted using a custom code in R.
A mixed-effect model with multiple comparisons made using the Tukey post-test was used for Sholl analysis to account for the variability per experiment as a random effect to evaluate differences between the conditions. The exact number of independent experiments and the exact number of cells analyzed are indicated in the figure legend for each experiment. To ensure the health of astrocyte-neuron co-cultures, the peak for the number of astrocyte intersections must be greater than or equal to 25 in the control condition. We imaged non-overlapping astrocytes that contained a single nucleus (DAPI stain) and expressed consistent fluorescent markers and EZRIN constructs according to the experimental conditions.

**Postnatal astrocyte labeling by electroporation (PALE):**

Late P0/early P1 mice were sedated by hypothermia until anesthetized, and 1 μl of plasmid DNA mixed with Fast Green Dye was injected into the lateral ventricle of one hemisphere using a pulled glass pipette (Drummond). For shRNA knockdown experiments in wild-type CD1 mice, the 1 μl of DNA contained 1 μg of pGLAST-PBase and 1 μg of pPB-shRNA-mCherryCAAX was injected. To label astrocytes in WT and LRRK2 G2019S<sup>ki/ki</sup> mice, the 1 μl of DNA contained 1 μg of pGLAST-PBase and 1 μg of pPB-mCherry-CAAX was injected per mouse.

For PALE-mediated overexpression of phospho-mimetic EZRIN in shRNA knockdown experiments, 0.5 μg pGLAST-PBase, 0.5 μg pPB-shRNA-mCherryCAAX, and 1 μg pZac2.1-GfaABC1D-Ezrin T567D-BioID2-HA were injected in a total volume of 1 μl. For phospho-dead EZRIN overexpression in WT and LRRK2 G2019S<sup>ki/ki</sup> mice, 0.5 μg pGLAST-PBase, 0.5 μg of pZac2.1-gfaABC1D-mCherry-CAAX and 1 μg of pZac2.1-GfaABC1D-Ezrin T567A-BioID2-HA were injected in a total volume of 1 μl. Following DNA
injection, electrodes were oriented with the positive terminal above the frontal cortex and
the negative terminal below the chin, and 5 discrete 50 ms pulses of 100 V spaced
950 ms apart were applied. Pups were recovered on a heating pad, returned to their home
cage, and monitored until collection at P21. The number of mice used for each experiment
is indicated in the figure legends. All animals appeared healthy at the time of collection.

Brain sections were examined for the presence of electroporated cells before staining.

**Adeno-associated virus (AAV) production and administration:**

Purified AAVs were produced as described previously\(^{170}\). Briefly, HEK293T cells were
transfected with pAd-DELT A F6, serotype plasmid AAV PHP.eB, and AAV plasmid
(pZac2.1-GfaABC1D-Ezrin-BioID2-HA or pZac2.1-GfaABC1D-Ezrin T567A-BioID2-HA).
Three days after transfection, cells were collected in 15 mM NaCl, 5 mM Tris-HCl, pH 8.5,
and lysed with repeat freeze-thaw cycles followed by treatment with Benzonase (Novagen
70664) at 37°C for 30 minutes. Lysed cells were pelleted by centrifugation, and the
supernatant, containing AAVs, was applied to an Optiprep density gradient (Sigma D1556,
15%, 25%, 40%, and 60%) and centrifuged at 67,000 rpm using a Beckman Ti-70 rotor
for 1 hour. The AAV-enriched fraction was isolated from between 40% and
60% iodixanol solution and concentrated by repeated washes with sterile PBS in an
Amicon Ultra-15 filtration unit (NMWL: 100 kDa, Millipore UFC910008) to a final volume
of \(\sim 100 \mu l\) and aliquoted for storage at \(-80^\circ C\). 9-week-old WT or LRRK2 G2019S\(^{ki/ki}\) mice
placed in a stereotaxic frame were anesthetized through inhalation of 1.5% isofluorane
gas. 10 \(\mu l\) of purified AAVs having a titer of \(\sim 1 \times 10^{12} \) GC/ml was introduced into the
mouse brain intravenously by injection into the retro-orbital sinus. After 3 weeks at 12-
week-old, mice were anesthetized with 200 mg/kg Tribromoethanol (Avertin) and
transcardially perfused with TBS/Heparin and 4% paraformaldehyde (PFA) at room temperature (RT). Harvested brains were post-fixed overnight in 4% PFA, cryoprotected in 30% sucrose, and the brain blocks were prepared with O.C.T. (TissueTek) to store at −80°C. 30 µm thick brain sections were obtained through cryosectioning using a Leica CM3050S (Leica, Germany) vibratome and stored in a mixture of TBS and glycerol at −20°C for further free-float antibody staining procedures.

**Immunohistochemistry on mouse brain sections:**

Mice used for immunohistochemistry were anesthetized with 200 mg/kg Avertin and perfused with TBS/Heparin and 4% PFA. Brains were collected and post-fixed in 4% PFA overnight, cryoprotected in 30% sucrose, frozen in a solution containing 2 parts 30% sucrose and 1-part O.C.T. (TissueTek), and stored at −80°C. Floating coronal tissue sections of 30 µm, 40 µm or 100 µm thickness were collected and stored in a 1:1 mixture of TBS/glycerol at −20°C. For immunostaining, sections were washed in 1x TBS containing 0.2% Triton X-100 (TBST), blocked in 10% NGS diluted in TBST, and incubated in primary antibody for 2-3 nights at 4°C with gentle shaking. Primary antibodies used were anti-LRRK2 (Rabbit, 1:500; ab133474, Abcam), phospho-ERM (Rabbit, 1:500; #3141, Cell Signaling), Sox9 (Rabbit, 1:500; AB5535, Millipore), GFAP (Rabbit, 1:500; Z0334, Agilent DAKO), VGluT1 (Guinea pig, 1:2000; 135304, Synaptic Systems), PSD95 (Rabbit, 1:300; 51-6900, Invitrogen), VGAT (Guinea pig, 1:1000; 131004, Synaptic Systems), and GEHPYRIN (Rabbit, 1:1000; #14304S, Cell Signaling). Following the primary incubation, sections were washed in TBST, incubated in Alexa Fluor conjugated secondary antibodies diluted 1:200 (Life Technologies) for 2-3 hours at room temperature, washed with TBST, and mounted onto glass slides using a homemade mounting media.
(90% Glycerol, 20 mM Tris pH 8.0, 0.5% n-Propyl gallate) and sealed with nail polish. For DAPI staining, DAPI (1:50,000) was added to the secondary antibody solution for the final 10 minutes of incubation. Images were acquired with an Olympus FV 3000 microscope.

**Mouse astrocyte territory volume analysis:**

To assess the territory volume of individual astrocytes in the mouse cortex, 100 μm-thick floating sections containing anterior cingulate cortex (ACC) and primary motor cortex (MOp) astrocytes labeled sparsely via PALE with mCherry-CAAX were collected. High-magnification images containing an entire astrocyte (50-60 μm z-stack) were acquired with an Olympus FV 3000 microscope with the 60x objective. Criteria for data inclusion required that the entirety of the astrocyte could be captured within a single brain section and that the astrocyte was in layer 2/3 of the ACC or MOp. Astrocytes in which the entire astrocyte could not be captured within the section or was in other layers or outside of the ACC or MOp were not imaged. Imaged astrocytes were analyzed using Imaris Bitplane software as described previously. Briefly, surface reconstructions were generated, and the Imaris Xtensions “Visualize Surface Spots” and “Convex Hull” were used to create an additional surface render representing the territory of the astrocyte. The volume of each territory was recorded, and astrocyte territory sizes from biological replicates were analyzed across experimental conditions using a nested two-way ANOVA followed by Bonferroni posthoc test. For 3D Sholl analysis of individual PALE astrocytes, we first loaded images onto Imaris and then created a surface. After generating the surface of astrocytes, we created filaments using ‘Add new filament (leaf icon)’. For the quantification of complexity, we clicked on the gear tool on Imaris to display Sholl
intersections. The number of animals and cells/animals analyzed are specified in the figure legend for each experiment.

**Mouse synapse imaging and analysis:**

Staining, image acquisition, and analysis of synaptic density were performed as described previously\(^{28,171}\) with slight adjustments. Synaptic staining was performed in coronal sections (30 \(\mu m\) thick) containing the ACC and MOp from WT and LRRK2 G2019S\(^{\text{ki/ki}}\) mice. To label pre and postsynaptic proteins, the following antibody combinations were used: VGluT1 and PSD95 (excitatory, intracortical), VGAT, and GEPHYRIN (inhibitory). High magnification 60x objective plus 1.64x optical zoom z-stack images containing 15 optical sections spaced 0.34 \(\mu m\) apart were obtained using an Olympus FV 3000 inverted confocal microscope. Each z-stack was converted into 5 maximum projection images (MPI), each corresponding to a 1 \(\mu m\) section of z plane, by using FIJI. Synapses were identified by the colocalization of pre and postsynaptic puncta. The number of co-localized synaptic puncta of excitatory intracortical (VGluT1/PSD95) and inhibitory (VGAT/GEPHYRIN) synapses were obtained using the FIJI plugin Puncta Analyzer\(^{147}\). 15 MPIs were analyzed for each mouse (5 MPI/tissue section, 3 tissue sections/mouse). Between 4 and 5, age and sex-matched mice/genotype/condition were analyzed for each synaptic staining combination, as indicated in the figure legends. All animals appeared healthy at the time of collection. No data were excluded.

**Cell counting, imaging, and analysis:**

Tile scan images containing the anterior cingulated cortex (ACC) and primary motor cortex (MOp) from P21 WT and LRRK2 G2019S\(^{\text{ki/ki}}\); Aldh111-eGFP mice were acquired...
on a confocal Leica SP8 STED microscope using the galvo scanner and 20x objective.

For ALDH1L1-EGFP and SOX9 double positive cell counting in the ACC and MOp, the cells labeled with ALDH1L1-EGFP and SOX9 were counted by hand using the cell counter tool in FIJI. 2-4 sections per brain from 3 sex and age-matched mice/genotype were analyzed.

**Human brain section staining:**

Floating human frontal cortex sections of 40 μm thickness were obtained from Banner Sun Health Research Institute in Sun City, Arizona (4 control and 3 LRRK2 G2019S mutation carrier subjects). None of the control subjects had a history of dementia or neurological or psychiatric disorders at the time of death (See Supplemental Table 1). Informed and written consent was obtained from the donors. For immunostaining, sections were washed in 1x TBS containing 0.3% Triton X-100 (TBST), blocked in 3% NGS diluted in TBST, and incubated in primary antibody 2-3 nights at 4°C with shaking. Primary antibodies used were GFAP (chicken, 1:250; AB5541, Millipore Sigma) and phospho-ERM (Rabbit, 1:250; #3726, Cell Signaling). Following primary incubation, sections were washed in TBST, incubated in Alexa Fluor conjugated secondary antibodies diluted 1:200 (Life Technologies) for 2-3 hours at room temperature, washed with TBST, and mounted onto glass slides using a homemade mounting media (90% Glycerol, 20 mM Tris pH 8.0, 0.5% n-Propyl gallate) and sealed with nail polish.

**Mouse brain slice electrophysiology:**

For whole-cell patch-clamp recordings, 3-4 mice of each genotype and condition were used for miniature excitatory postsynaptic current (mEPSC) and miniature inhibitory
postsynaptic current (mIPSC) measurements. WT and LRRK2 G2019S<sup>ki/ki</sup> mice of both sexes were anesthetized with 200 mg/kg tribromoethanol (avertin) and decapitated. After decapitation, the brains were immersed in ice-cold artificial cerebrospinal fluid (aCSF, in mM): 125 NaCl, 2.5 KCl, 3 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 10 glucose, 25 NaHCO<sub>3</sub>, 1.25 NaHPO<sub>4</sub>, 0.4 L-ascorbic acid, and 2 Na-pyruvate, pH 7.3-7.4 (310 mOsmol). 350 µm thick coronal slices containing the ACC were obtained using a vibrating tissue slicer (Leica VT1200; Leica Biosystems). Slices were immediately transferred to standard aCSF (33°C, continuously bubbled with 95% O<sub>2</sub> – 5% CO<sub>2</sub>) containing the same as the low-calcium aCSF but with 1 mM MgCl<sub>2</sub> and 1-2 mM CaCl<sub>2</sub>. After 30-minute incubation at 33°C, slices were transferred to a holding chamber with the same extracellular buffer at room temperature (RT: ~25°C). Brain slices were visualized by an upright microscope (BX61WI, Olympus) through a 40x water-immersion objective equipped with infrared-differential interference contrast optics in combination with a digital camera (ODA-IR2000WCTRL). Patch-clamp recordings were performed by using an EPC 10 patch-clamp amplifier, controlled by Patchmaster Software (HEKA). Data were acquired at a sampling rate of 50 kHz and low-pass filtered at 6 kHz.

To measure mEPSCs, the internal solution contained the following (in mM): 125 K-glucuronate, 10 NaCl, 10 HEPES, 0.2 EGTA, 4.5 MgATP, 0.3 NaGTP, and 10 Na-phosphocreatine, pH adjusted to 7.2 – 7.4 with KOH and osmolality set to ~ 300 mOsmol. mEPSCs were measured in the aCSF bath solution containing 1 µM tetrodotoxin and 50 µM Picrotoxin at -70 mV in voltage-clamp mode. To measure mIPSCs, the internal solution contained the following (in mM): 77 K-glucuronate, 77 KCl, 10 HEPES, 0.2 EGTA, 4.5 MgATP, 0.3 NaGTP, and 10 Na-phosphocreatine, pH adjusted to 7.2 – 7.4 with KOH
and osmolality set to ~ 300 mOsmol. mIPSCs were measured in the aCSF bath solution containing 1 µM tetrodotoxin and 10 µM 6-cyano-7-nitroquinoxaline-2,3- dione (CNQX), and 50 µM D-2-amino-5-phosphonopentanoate (D-AP5) at -70 mV in voltage-clamp mode. mEPSCs and mIPSCs recorded at -70 mV were detected using Minhee Analysis software (https://github.com/parkgilbong/Minhee_Analysis_Pack). To analyze the frequency, events were counted over 5 minutes of recording. To obtain the average events for each cell, at least 100 non-overlapping events were detected and averaged. The peak amplitude of the average mEPSCs was measured relative to the baseline current.

Quantification and statistical analysis:

All statistical analyses were performed in GraphPad Prism 9. Exact value of n, what n represents, and specific statistical tests for each experiment are indicated in the figure legend for each experiment. All data are represented as mean ± standard error of the mean, and data points are shown where applicable. Exact P-values are listed in the figure for each experiment. Where indicated, the unpaired two-tailed t-tests were run using Welch’s correction, and a correction for multiple comparisons was applied using Hom-Sidak method with an alpha threshold of 0.05 for adjusted P-value. A Geisser-Greenhouse correction was used for both One-way and Two-way ANOVA analyses. For Sholl analysis, statistical analyses were conducted using a custom code in R (https://github.com/Eroglu-Lab/In-Vitro-Sholl). A mixed-effect model with multiple comparisons made using the Tukey post-test was used for Sholl analysis to account for the variability per experiment as a random effect to evaluate differences between the conditions. Sample sizes were determined based on previous experience for each
experiment to yield high power to detect specific effects. No statistical methods were used
to predetermine the sample size. All experimental animals that appeared healthy at the
time of tissue collection were processed for data collection.
Acknowledgments:

We thank Drs. Nicole Calakos, Katja Brose, Andrew Singleton, Dolores Irala, Francesco P. Ulloa Severino, Juan Ramirez, Justin T. Savage, Gabrielle Séjourné, and other Eroglu lab members for critical feedback about the manuscript. We thank Adam Okinaga and Donna Porter for excellent technical help. This work was supported by a Foerster Bernstein Women in STEM Postdoctoral Fellowship to SW, and a Chan Zuckerberg Initiative, Neurodegeneration Challenge Network Collaborative grant (2018-191999 and DAF2021-237435) to CE and ARLS, and by the N.I.H. grants R35-NS122140 to A.R.L.S. and R01-NS102237 to CE, and by the Michael J. Fox Foundation for Parkinson’s Research (MJFF) Astrocyte Biology in PD pilot grant (MJFF 17914 to CE and ARLS), and by the joint efforts of MJFF and the Aligning Science Across Parkinson’s (ASAP) initiative. MJFF administers the grant [ASAP-020607 to CE and SHS] on behalf of ASAP and itself Michael J Fox Foundation. Dr. Cagla Eroglu is an HHMI Investigator. We are grateful to the Banner Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona for the provision of human biological materials. The Brain and Body Donation Program has been supported by the National Institute of Neurological Disorders and Stroke (U24 NS072026 National Brain and Tissue Resource for Parkinson’s Disease and Related Disorders), the National Institute on Aging (P30AG19610 and P30AG072980, Arizona Alzheimer’s Disease Center), the Arizona Biomedical Research Commission contracts 4001, 0011, 05-901 and 1001 to the Arizona Parkinson’s Disease Consortium) and the Michael J. Fox Foundation for Parkinson’s Research.
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Author contributions

Conceptualization, SW, ARLS, and CE; Methodology, SW, DSB, CXT, TT, MPR, KD, SHS, and CE; Investigation, SW, DSB, CXT, KD; Formal analysis, SW, CXT, and MPR; Resources, KS; Writing – original draft, SW and CE; Writing – Review & Editing, SW, SHS, CXT, TT, MPR, KD, KS, ARLS, DSB, and CE; Funding Acquisition, SW, SHS, ARLS, and CE.

Declaration of interests

The authors declare no competing interests.

Data Availability

The data that support the findings of this study are available from the corresponding author (Dr. Cagla Eroglu) upon request after the publication of this manuscript. The data include experiment records, raw data, and analysis. The authors confirm that all data underlying the findings will be made fully available without restriction upon publication.

Code availability

The code used to analyze Sholl data is available on the link https://github.com/Eroglu-Lab/Sholl_MACRO.
FIGURE LEGENDS

Figure 1. Astrocytic LRRK2 controls astrocyte morphology by balancing ERM phosphorylation levels in vitro. a, Schematic of astrocyte-neuron co-culture assay. b, (Upper) Rat cortical astrocytes transfected with scrambled shRNA (shControl-GFP) or shRNA targeting LRRK2 (shLrrk2-GFP) and LRRK2 knockout (KO) rat cortical astrocytes co-cultured with wild-type cortical neurons. Scale bar, 10 μm. (Lower) Quantification of astrocyte branching complexity. n = 56 (shControl-GFP), 54 (shLrrk2-GFP), 52 (LRRK2 KO) astrocytes co-cultured with wild-type (WT) cortical neurons compiled from three independent experiments. We fitted a linear mixed model with the number of intersections as an outcome variable, condition as a predictor, and the number of cells and cell radius were entered as random effects. Within this model, shLrrk2 (beta = 5.91, t(171) = 12.59) and LRRK2 KO (beta = 5.43, t(171) = 11.28) led to a significant decrease of number of intersections compared to shControl (beta = 12.37, t(171) = 25.72). Tukey multiple comparisons test revealed a significant difference between shControl-GFP and LRRK2 KO (p < 0.001) and between shControl-GFP and shLrrk2-GFP (p < 0.001). Tukey multiple comparisons test revealed no difference between shLrrk2-GFP and LRRK2 KO (p = 0.7579). c, WT or LRRK2 G2019S rat cortical astrocytes transfected with GFP and co-cultured with WT cortical neurons or LRRK2 G2019S cortical neurons. Scale bar, 10 μm. d, Quantification of astrocyte branching complexity. n = 61 (WT), 61 (LRRK2 G2019S) astrocytes co-cultured with WT cortical neurons compiled from three independent experiments. n = 41 (WT), 40 (LRRK2 G2019S) astrocytes co-cultured with LRRK2 G2019S cortical neurons compiled from two independent experiments. We fitted a linear mixed model with number of intersections as outcome variable, condition as predictor and
number of cells and cell radius were entered as random effects. Within this model, LRRK2 G2019S (beta = 7.55, t(113) = 18.95) led to a significant decrease of number of intersections compared to WT astrocytes (beta = 10.86, t(113) = 28.47). WT (beta = 7.12, t(152) = 15.44) and LRRK2 G2019S (beta = 6.49, t(152) = 13.72) astrocytes co-cultured on LRRK2 G2019S neurons led to a significant decrease of number of intersections compared to WT astrocytes co-cultured with WT neurons (beta = 14.42, t(152) = 30.09). Tukey multiple comparisons test revealed no difference between WT and LRRK2 G2019S astrocytes co-cultured on LRRK2 G2019S neurons (p = 0.7696). Tukey multiple comparisons test revealed a significant difference between WT and LRRK2 G2019S astrocytes co-cultured on WT neurons (p = 1.99E-09).

e, WT astrocytes treated with DMSO, 30 nM MLi-2, or 100 nM MLi-2 were lysed and subjected to Western blotting. Western blotting was performed using phospho-ERM, ERM, phospho-Rab10, and Rab10 antibodies. Results are representative of 4 independent experiments. f, Densitometric analysis of phospho-ERM levels in Fig. 1e. Signals corresponding to phospho-ERM were normalized to that for ERM. Statistical significance was determined by One-way ANOVA [F (2, 9) = 354.8, p < 0.0001], Bonferroni multiple comparison revealed a significant difference between 0 nM and 30 nM MLi-2 treatments (p < 0.0001, 95% C.I. = [0.6566, 0.8600]) and between 0 nM and 100 nM MLi-2 treatments (p < 0.0001, 95% C.I. = [0.7341, 0.9375]) and no differences between 30 nM and 100 nM MLi-2 treatments (p = 0.1568, 95% C.I. = [-0.02422, 0.1792]), alpha = 0.05. g, WT or LRRK2 G2019S astrocytes were lysed and subjected to Western blotting, using phospho-ERM and ERM antibodies. Results are representative of 3 independent experiments. h, Densitometric analysis of phospho-ERM levels in Fig. 1g. Signals corresponding to phospho-ERM were normalized
to that for ERM. Statistical significance was determined by Unpaired Two-tailed t-test [t (5.944) = 5.536], p = 0.0015. i, (Upper) Representative rat cortical astrocytes transfected shControl-GFP + WT EZRIN, shControl-GFP + Phospho-mimetic EZRIN, and co-cultured with WT cortical neurons. Scale bar, 10 µm. (Lower) Quantification of astrocyte branching complexity. n = 47 (shControl-GFP + WT EZRIN O/E), 46 (shControl-GFP + Phospho-mimetic EZRIN O/E) astrocytes co-cultured with WT cortical neurons compiled from three independent experiments. We fitted a linear mixed model with number of intersections as outcome variable, condition as predictor and number of cells and cell radius were entered as random effects. Within this model, neither WT EZRIN (beta = 14.76, t(107) = 19.17) or Phospho-mimetic EZRIN (beta = 13.22, t(107) = 19.07) lead to significant changes of number of intersections of shControl-GFP astrocytes (beta = 12.75, t(107) = 19.82). Tukey multiple comparisons test revealed no difference between shControl-GFP and shControl-GFP + WT EZRIN O/E (p = 0.1110), shControl-GFP and shControl-GFP + Phospho-mimetic EZRIN O/E (p = 0.8727), and shControl-GFP + WT EZRIN O/E and shControl-GFP + Phospho-mimetic EZRIN O/E (p = 0.2969). j, (Upper) Representative rat cortical astrocytes transfected shLrrk2-GFP + WT EZRIN, shLrrk2-GFP + Phospho-mimetic EZRIN, and co-cultured with WT cortical neurons. Scale bar, 10 µm. (Lower) Quantification of astrocyte branching complexity. n = 55 (shLrrk2-GFP + WT EZRIN O/E), 47 (shLrrk2-GFP + Phospho-mimetic EZRIN O/E) astrocytes co-cultured with WT cortical neurons compiled from three independent experiments. We fitted a linear mixed model with number of intersections as outcome variable, condition as predictor and number of cells and cell radius were entered as random effects. Within this model, WT EZRIN (beta = 4.99, t(162) = 10.32) did not increase the number of intersections of shLrrk2-GFP
astrocytes but Phospho-mimetic EZRIN (beta = 15.27, t(162) = 27.93) increased number of intersections of shLRRK2-GFP astrocytes (beta = 4.80, t(162) = 10.63). Tukey multiple comparisons test revealed no difference between shLrrk2-GFP and shLrrk2-GFP + WT EZRIN O/E (p = 0.9926). Tukey multiple comparisons test revealed significant difference between shLrrk2-GFP and shLrrk2-GFP + Phospho-mimetic EZRIN O/E (p < 0.0001). K- I, (Upper) Representative WT (k) or LRRK2 G2019S (l) rat astrocytes transfected GFP + WT EZRIN, or GFP + Phospho-dead EZRIN, and co-cultured with WT cortical neurons, respectively. Scale bar, 10 μm. (Lower) Quantification of astrocyte branching complexity. n = 57 (WT + WT EZRIN O/E), 66 (WT + Phospho-dead EZRIN O/E), 57 (LRRK2 G2019S + WT EZRIN O/E), 48 (LRRK2 G2019S + Phospho-dead EZRIN O/E) astrocytes co-cultured with WT cortical neurons compiled from three independent experiments. We fitted a linear mixed model with number of intersections as outcome variable, condition as predictor and number of cells and cell radius were entered as random effects. Within this model, WT EZRIN (beta = 13.49, t(137) = 25.15) did not change the number of intersections of WT astrocytes, but Phospho-dead EZRIN (beta = 5.37, t(137) = 10.02) decreased number of intersections of WT astrocytes (beta = 10.86, t(137) = 24.82). WT EZRIN (beta = 7.52, t(209) = 15.91) did not change the number of intersections of LRRK2 G2019S astrocytes but Phospho-dead EZRIN (beta = 10.88, t(209) = 22.54) increased number of intersections of LRRK2 G2019S astrocytes (beta = 7.55, t(209) = 16.77). Tukey multiple comparisons test revealed a significant difference between LRRK2 G2019S and LRRK2 G2019S + Phospho-mimetic EZRIN O/E (p = 2.03E-06), and LRRK2 G2019S + WT EZRIN O/E and LRRK2 G2019S + Phospho-mimetic EZRIN O/E (p = 4.83E-06). Tukey multiple comparisons test revealed no difference between LRRK2 G2019S and
LRRK2 G2019S + WT EZRIN O/E (p = 0.9999), and between WT and LRRK2 G2019S + Phospho-mimetic EZRIN O/E (p = 0.9999). Data are presented as mean ± s.e.m.

Extended Data Figure 1 (related to Figure 1). Localization and expression of LRRK2 in astrocytes. a-b, LRRK2 (red) in the primary motor cortex (MOp) of LRRK2 G2019Ski/ki; Aldh1l1-eGFP mice at P21. Scale bar, 10 µm. c, Cultured WT and LRRK2 KO rat cortical astrocytes were lysed, and cytoplasmic proteins were subjected to Western blotting, using LRRK2 and β-actin antibodies. Results are representative of 4 independent experiments. d, Densitometric analysis of LRRK2 levels in Extended Data Fig. 1c. Signals corresponding to LRRK2 were normalized to that for β-actin. Relative LRRK2 levels were then normalized to the LRRK2 signals in WT rat cortical astrocytes. Statistical significance was determined by an Unpaired Two-tailed t-test [t (6) = 13.79], p < 0.0001. e, Cultured WT and LRRK2 G2019S rat cortical astrocytes were lysed, and cytoplasmic proteins were subjected to Western blotting using LRRK2 and β-actin antibodies. Results are representative of 3 independent experiments. f, Densitometric analysis of LRRK2 levels in Extended Data Fig. 1e. Signals corresponding to LRRK2 were normalized to that for β-actin. Relative LRRK2 levels were then normalized to the LRRK2 signals in WT rat cortical astrocytes. Statistical significance was determined by an Unpaired Two-tailed t-test [t (4) = 47.96], p < 0.0001. g, Cultured WT, and shControl and shLrrk2 transfected rat cortical astrocytes were lysed, and cytoplasmic proteins were subjected to Western blotting using LRRK2 and GAPDH antibodies. Results are representative of 3 independent experiments. h, Densitometric analysis of LRRK2 levels in Extended Data Fig. 1g. Signals corresponding to LRRK2 were first normalized to that for β-actin. Relative LRRK2 levels were then normalized to the LRRK2 signals in WT rat cortical astrocytes.
Statistical significance was determined by One-way ANOVA \[F (2, 6) = 92.25, p < 0.0001\], Bonferroni multiple comparison revealed a significant difference between WT and shLrrk2 \((p < 0.0001, 95\% \text{C.I.} = [0.6743, 1.183])\) and between shLrrk2 and shControl \((p < 0.0001, 95\% \text{C.I.} = [-1.145, -0.6363])\) and no differences between WT and shControl \((p > 0.9999, 95\% \text{C.I.} = [-0.2165, 0.2923])\), alpha = 0.05. i-j, Bar plots of Ezr, Rdx, and Msn gene expression in various brain cell types in mice (yellow) or humans (red). These plots are generated using publicly available data at https://www.brainrnaseq.org/52,172.

**Figure 2.** Astrocytic LRRK2 controls astrocyte morphology by balancing ERM phosphorylation levels *in vivo*. a-c, Representative confocal images of ERM phosphorylation (purple) in the anterior cingulate cortex (ACC) and primary motor cortex (MOp) of WT or LRRK2 G2019S<sup>ki/ki</sup> Aldh1l1-eGFP transgenic mice at P21. Scale bar, 200 μm (a-b). Scale bar, 10 μm (c). d, Quantification of phospho-ERM integrated density in a-b, n = 4 (WT, 2 males and 2 females), 4 (LRRK2 G2019S<sup>ki/ki</sup>, 2 males and 2 females) mice. For phospho-ERM quantification in the ACC, nested t-test, unpaired Two-tailed t-test. \(t (16) = 15.16, p < 0.0001\). For phospho-ERM quantification in the MOp, nested t-test, unpaired Two-tailed t-test. \(t (14) = 8.963, p < 0.0001\). Grey dots are the data acquired from each image. Black dots are the averaged data acquired from each WT mouse. Blue dots are the averaged data acquired from each LRRK2 G2019S<sup>ki/ki</sup> mouse. e, Images of ACC and MOp layer 2/3 WT and LRRK2 G2019S<sup>ki/ki</sup> astrocytes at P21 expressing PB-mCherry-CAAX ± Phospho-dead EZRIN. Astrocyte territory in cyan. Scale bar, 10 μm. f, Average territory volumes of P21 astrocytes. n = 16 (WT; 6 mice), 18 (WT + Phospho-dead EZRIN O/E; 6 mice), 16 (LRRK2 G2019S; 6 mice), 17 (LRRK2 G2019S + Phospho-dead EZRIN O/E; 6 mice). Statistical significance was determined by One-way ANOVA.
Bonferroni multiple comparisons revealed a significant difference between WT and LRRK2 G2019S ($p = 0.0178, 95\% \text{C.I.} = [0.4542, 6.313]$), between WT and WT + Phospho-dead EZRIN O/E ($p = 0.0037, 95\% \text{C.I.} = [1.132, 6.991]$), and between WT + Phospho-dead EZRIN O/E and LRRK2 G2019S + Phospho-mimetic EZRIN O/E ($p = 0.0165, 95\% \text{C.I.} = [-6.346, -0.4875]$), and no differences between WT and LRRK2 G2019S + Phospho-mimetic EZRIN O/E ($p > 0.9999, 95\% \text{C.I.} = [-2.251, 3.608]$), between LRRK2 G2019S and WT + Phospho-dead EZRIN O/E ($p > 0.9999, 95\% \text{C.I.} = [7.6, 18.52]$), between LRRK2 G2019S and LRRK2 G2019S + Phospho-dead EZRIN O/E ($p < 0.0001, 95\% \text{C.I.} = [-22.19, -13.43]$), and between WT + Phospho-dead EZRIN O/E and LRRK2 G2019S + Phospho-dead EZRIN O/E ($p < 0.0001, 95\% \text{C.I.} = [-37.02, -24.72]$), and no difference between WT and LRRK2 G2019S + Phospho-dead EZRIN O/E ($p = 0.5469, 95\% \text{C.I.} = [-1.932, 8.796]$).
shControl-mCherry-CAAX or shLrrk2-mCherry-CAAX ± Phospho-mimetic EZRIN.

Astrocyte territory in cyan. Scale bar, 10 μm. i, Average territory volumes of P21 astrocytes. n = 18 (shControl; 6 mice), 18 (shControl + Phospho-mimetic EZRIN O/E; 5 mice), 22 (shLrrk2; 6 mice), 19 (shLrrk2 + Phospho-mimetic EZRIN O/E; 7 mice). Statistical significance was determined by One-way ANOVA [F (3, 20) = 5.674, p = 0.0056], Bonferroni multiple comparison revealed a significant difference between shControl and shLrrk2 (p = 0.0104, 95% C.I. = [0.5585, 5.322]), and between shLrrk2 and shLrrk2 + Phospho-mimetic EZRIN O/E (p = 0.0114, 95% C.I. = [-5.096, -0.5065]), and no differences between shControl and shControl + Phospho-mimetic EZRIN O/E (p > 0.9999, 95% C.I. = [-1.426, 3.570]), between shControl and shLrrk2 + Phospho-mimetic EZRIN O/E (p > 0.9999, 95% C.I. = [-2.156, 2.433]), and between shControl + Phospho-mimetic EZRIN O/E and shLrrk2 + Phospho-mimetic EZRIN O/E (p > 0.9999, 95% C.I. = [-3.349, 1.482]), alpha = 0.05. Data points are mouse averages.

j, Quantification of astrocyte branching complexity. n = 16 (shControl), 24 (shControl + Phospho-mimetic EZRIN O/E), 23 (shLrrk2), 18 (shLrrk2 + Phospho-mimetic EZRIN O/E). Two-way ANOVA for repeated measures. Main effects of conditions [F (2.435, 7008) = 225.0, p < 0.0001] and radius [F (88, 8633) = 325.4, p < 0.0001] and interaction [F (264, 8633) = 5.908, p < 0.0001]. Bonferroni multiple comparisons revealed a significant difference between shControl and shLrrk2 (p < 0.0001, 95% C.I. = [31.02, 40.20]), between shLrrk2 and shControl + Phospho-mimetic EZRIN O/E (p < 0.0001, 95% C.I. = [-38.59, -29.67]), between shLrrk2 and shLrrk2 + Phospho-mimetic EZRIN O/E (p < 0.0001, 95% C.I. = [-34.32, -25.69]), between shControl and shLrrk2 + Phospho-mimetic EZRIN O/E (p = 0.0139, 95% C.I. = [0.7510, 10.47]), and between shControl + Phospho-mimetic EZRIN
O/E and shLrrk2 + Phospho-mimetic EZRIN O/E ($p = 0.0188$, 95% C.I. = [0.4426, 7.813]), and no difference between shControl and shControl + Phospho-mimetic EZRIN O/E ($p > 0.9999$, 95% C.I. = [-3.083, 6.044]). Data are presented as mean ± s.e.m. k, Schematic representation of analyzed human brain regions for phospho-ERM staining, frontal cortex. l, Representative confocal images of GFAP (green) and ERM phosphorylation (purple) in the frontal cortex of human control subjects or human PD patients carrying LRRK2 G2019S mutation carriers at age >80 years old. Scale bar, 200 μm. n = 4 (Human control, 3 males and 1 female), 3 (LRRK2 G2019S mutation carriers, 2 males and 1 female) subjects. m, Quantification of phospho-ERM integrated density in l, n = 4 (Human control, 3 males and 1 female), 3 (LRRK2 G2019S mutation carriers, 2 males and 1 female) subjects, nested t-test, unpaired Two-tailed t-test. $t (4) = 3.551, p = 0.0238$. The grey dot is the data acquired from each image. The black dot is the averaged data acquired from each control subject. The blue dot is the averaged data acquired from each LRRK2 G2019S mutation carrier. n, Quantification of GFAP integrated density in l, n = 4 (Human control, 3 males and 1 female), 3 (LRRK2 G2019S mutation carriers, 2 males and 1 female) subjects, nested t-test, unpaired Two-tailed t-test. $t (18) = 3.612, p = 0.0020$. Grey dots are the data acquired from each image. Black dots are the averaged data acquired from each control subject. Blue dots are the averaged data acquired from each LRRK2 G2019S mutation carrier.

Extended Data Figure 2 (related to Figure 2). Phospho-ERM is upregulated in LRRK2 G2019S$^{ki/ki}$ astrocytes in ACC and MOp but not SSp and DMS. a-b, Representative confocal images of ERM phosphorylation (purple) in the MOp and ACC of WT or LRRK2 G2019S$^{ki/ki}$ Aldh1I1-eGFP transgenic mice at P84. Scale bar, 200 μm. n
Quantification of phospho-ERM integrated density in **a-b**, n = 4 (WT, 2 males and 2 females), 4 (LRRK2 G2019S\textsuperscript{ki/ki}, 2 males and 2 females) mice. For phospho-ERM quantification in the ACC, nested t-test, unpaired Two-tailed t-test. t (14) = 11.84, p < 0.0001. For phospho-ERM quantification in the MOp, nested t-test, unpaired Two-tailed t-test. t (14) = 6.585, p < 0.0001. Grey dots are the data acquired from each image. Black dots are the averaged data acquired from each WT mouse. Blue dots are the averaged data acquired from each LRRK2 G2019S\textsuperscript{ki/ki} mouse. **d-e**, Representative confocal images of ERM phosphorylation (purple) in the MOp and ACC of WT or LRRK2 G2019S\textsuperscript{ki/ki} Aldh111-eGFP transgenic mice at P84. Scale bar, 20 \(\mu\)m. **f-g**, Representative confocal images of ERM phosphorylation (purple) in the SSp and DMS of WT or LRRK2 G2019S\textsuperscript{ki/ki} Aldh111-eGFP transgenic mice at P21. Scale bar, 200 \(\mu\)m. **h**, Quantification of phospho-ERM integrated density in **f-g** and **i-j**, n = 4 (WT, 2 males and 2 females), 4 (LRRK2 G2019S\textsuperscript{ki/ki}, 2 males and 2 females) mice. For phospho-ERM integrated density at P21, nested t-test, unpaired Two-tailed t-test. t (22) = 0.1556, p = 0.8777. For phospho-ERM integrated density at 12-week-old, nested t-test, unpaired Two-tailed t-test. t (4) = 0.5028, p = 0.6415. Grey dots are the data acquired from each image. Black dots are the averaged data acquired from each WT mouse. Blue dots are the averaged data acquired from each LRRK2 G2019S\textsuperscript{ki/ki} mouse. **i-j**, Representative confocal images of ERM phosphorylation (purple) in the SSp and DMS of WT or LRRK2 G2019S\textsuperscript{ki/ki} Aldh111-eGFP transgenic mice at P84. Scale bar, 200 \(\mu\)m.
G2019S<sup>ki/ki</sup>, 2 males and 2 females) mice. k, Overview of postnatal astrocyte labeling by electroporation (PALE) with PiggyBac plasmids.

**Extended Data Figure 3 (related to Figure 2).** LRRK2 G2019S does not change GFAP level and ALDH1L1+/SOX9+ cell numbers in the ACC and MOp. a-b, Representative confocal images of SOX9 (purple) in the ACC and MOp of WT or LRRK2 G2019S<sup>ki/ki</sup> Aldh1l1-eGFP transgenic mice at P21. Scale bar, 200 μm. c, Quantification of ALDH1L1+/SOX9+ cell numbers in a-b, n = 3 (WT, 2 males and 1 female), 3 (LRRK2 G2019S<sup>ki/ki</sup>, 2 males and 1 female) mice, For ALDH1L1+/SOX9+ cell counting in the ACC, nested t-test, unpaired Two-tailed t-test. t (9) = 1.533, p = 0.2470. For ALDH1L1+/SOX9+ cell counting in the MOp, nested t-test, unpaired Two-tailed t-test. t (2) = 1.544, p = 0.2625. Grey dots are the data acquired from each image. Black dots are the averaged data acquired from each WT mouse. Blue dots are the averaged data acquired from each LRRK2 G2019S<sup>ki/ki</sup> mouse. d-e, Representative confocal images of GFAP (purple) in the ACC and MOp of WT or LRRK2 G2019S<sup>ki/ki</sup> Aldh1l1-eGFP transgenic mice at P21. Scale bar, 200 μm. f, Quantification of GFAP integrated density in d-e, n = 3 (WT, 2 males and 1 female), 3 (LRRK2 G2019S<sup>ki/ki</sup>, 2 males and 1 female) mice, For GFAP quantification in the ACC, nested t-test, unpaired Two-tailed t-test. t (10) = 1.524, p = 0.1586. For GFAP quantification in the MOp, nested t-test, unpaired Two-tailed t-test. t (10) = 0.5028, p = 0.6260. Grey dots are the data acquired from each image. Black dots are the averaged data acquired from each WT mouse. Blue dots are the averaged data acquired from each LRRK2 G2019S<sup>ki/ki</sup> mouse.

**Figure 3.** LRRK2 G2019S affects excitatory and inhibitory synapse number and function in the ACC and MOp. a, Schematic representation of analyzed brain regions.
(ACC and MOp) for excitatory and inhibitory synapse numbers.  

b, Schematic representations of methodologies used to quantify the VGluT1-PSD95 and VGAT-GEPHYRIN colocalized puncta. c, (Left) Representative images from the ventral ACC layer 1 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGluT1 and PSD95 antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGluT1/PSD95 co-localized puncta, normalized using the means of WT values in the ventral ACC layer 1. n = 5 (WT, 3 males and 2 females), 5 (LRRK2 G2019S<sup>ki/ki</sup>, 3 males and 2 females) mice, nested t-test, unpaired Two-tailed t-test.  

t (28) = 4.358,  

p = 0.0002.  
d, (Left) Representative images from the ventral ACC layer 2/3 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGluT1 and PSD95 antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGluT1/PSD95 co-localized puncta, normalized using the means of WT values in the ventral ACC layer 2/3. n = 5 (WT, 3 males and 2 females), 5 (LRRK2 G2019S<sup>ki/ki</sup>, 3 males and 2 females) mice, nested t-test, unpaired Two-tailed t-test.  

t (28) = 4.287,  

p = 0.0002.  
e, (Left) Representative images from MOp layer 1 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGAT and GEPHYRIN antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGAT/GEPHYRIN co-localized puncta, normalized using the means of WT values in MOp layer 1. n = 6 (WT, 3 males and 3 females), 6 (LRRK2 G2019S<sup>ki/ki</sup>, 3 males and 3 females) mice, nested t-test, unpaired Two-tailed t-test.  

t (34) = 7.164,  

p < 0.0001.  
f, (Left) Representative images from MOp layer 2/3 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGAT and GEPHYRIN antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGAT/GEPHYRIN co-localized puncta, normalized using the means of WT values in MOp layer 2/3. n = 6 (WT, 3 males and 3 females), 6 (LRRK2 G2019S<sup>ki/ki</sup>, 3 males and 3
Representative mEPSC traces from the ventral ACC layer 2/3 pyramidal neurons in acute brain slices of WT and LRRK2 G2019S\textsuperscript{ki/ki} mice. h, Cumulative frequency plots and quantification of synaptic event frequency, n = 15 (WT), 13 (LRRK2 G2019S\textsuperscript{ki/ki}) neurons from 4 mice per genotype. Kolmogorov-Smirnov test (D = 0.504, \(p < 0.001\)). The average frequency of mEPSC in WT (2.521 ± 0.3846) and LRRK2 G2019S\textsuperscript{ki/ki} (0.8691 ± 0.1309) mice. Unpaired Two-tailed t-test \([t (26) = 3.828, \(p = 0.0007\)]\). i, Cumulative frequency plots and quantification of synaptic event amplitude, n = 15 (WT), 13 (LRRK2 G2019S\textsuperscript{ki/ki}) neurons from 4 mice per genotype. Kolmogorov-Smirnov test (D = 0.194, \(p < 0.0001\)). The average amplitude of mEPSC in WT (15.53 ± 0.8105) and LRRK2 G2019S\textsuperscript{ki/ki} (13.52 ± 0.5818) mice. Unpaired t-test \([t (26) = 1.951, \(p = 0.0619\)]\). j, Representative mIPSC traces from MO\textsuperscript{p} layer 2/3 pyramidal neurons in acute brain slices of WT and LRRK2 G2019S\textsuperscript{ki/ki} mice. k, Cumulative frequency plots and quantification of synaptic event frequency, n = 12 (WT), 11 (LRRK2 G2019S\textsuperscript{ki/ki}) neurons from 4 mice per genotype. Kolmogorov-Smirnov test (D = 0.424, \(p < 0.0001\)). The average frequency of mIPSC in WT (0.7606 ± 0.06816) and LRRK2 G2019S\textsuperscript{ki/ki} (1.312 ± 0.1041) mice. Unpaired Two-tailed t-test \([t (21) = 4.503, \(p = 0.0002\)]\). l, Cumulative frequency plots and quantification of synaptic event amplitude, n = 12 (WT), 11 (LRRK2 G2019S\textsuperscript{ki/ki}) neurons from 4 mice per genotype. Kolmogorov-Smirnov test (D = 0.382, \(p < 0.0001\)). The average amplitude of mIPSC in WT (8.776 ± 0.4961) and LRRK2 G2019S\textsuperscript{ki/ki} (9.921 ± 0.5043) mice. Unpaired t-test \([t (21) = 1.617, \(p = 0.1209\)]\). Data are presented as mean ± s.e.m.

**Extended Data Figure 4 (related to Figure 3).** LRRK2 G2019S does not change excitatory synapse numbers in the primary motor cortex. a, (Left) Representative
images from the ventral ACC layer 1 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGAT and Gephyrin antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGAT/Gephyrin co-localized puncta, normalized using the means of WT values in the ventral ACC layer 1. n = 4 (WT, 2 males and 2 females), 4 (LRRK2 G2019S<sup>ki/ki</sup>, 2 males and 2 females) mice, nested t-test, unpaired Two-tailed t-test. t (22) = 0.3365, p = 0.7397. Grey dots are the data acquired from each synapse image. Black dots are the averaged data acquired from each WT mouse. Blue dots are the averaged data acquired from each LRRK2 G2019S<sup>ki/ki</sup> mouse. 

b, (Left) Representative images from the ventral ACC layer 2/3 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGAT and Gephyrin antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGAT/Gephyrin co-localized puncta, normalized using the means of WT values in the ventral ACC layer 2/3. n = 4 (WT, 2 males and 2 females), 4 (LRRK2 G2019S<sup>ki/ki</sup>, 2 males and 2 females) mice, nested t-test, unpaired Two-tailed t-test. t (21) = 0.4692, p = 0.6438. 

c, (Left) Representative images from the MO<sub>p</sub> layer 1 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGluT1 and PSD95 antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGluT1/PSD95 co-localized puncta in c, normalized using the means of WT values in the MO<sub>p</sub> layer 1. n = 5 (WT), 5 (LRRK2 G2019S<sup>ki/ki</sup>) mice, 3 males and 2 females, nested t-test, unpaired Two-tailed t-test. t (28) = 1.012, p = 0.3203. d, (Left) Representative images from the MO<sub>p</sub> layer 2/3 of WT and LRRK2 G2019S<sup>ki/ki</sup> mice that were stained with VGluT1 and PSD95 antibodies at 12-week-old. Scale bar, 10 μm. (Right) Quantification of VGluT1/PSD95 co-localized puncta in c, normalized using the means of WT values in the MO<sub>p</sub> layer 2/3. n = 5 (WT), 5 (LRRK2 G2019S<sup>ki/ki</sup>) mice, 3 males and 2 females, nested t-test, unpaired Two-tailed t-test. t (4) = 0.20465, p = 0.8479.
Figure 4. Overexpression of phospho-dead EZRIN in adult LRRK2 G2019S\textsuperscript{ki/ki} astrocytes restores excitatory synapse number and function in the ventral ACC. a, Representative images from the ventral ACC layer 1 of WT and LRRK2 G2019S\textsuperscript{ki/ki} mice injected with AAV-HA-tagged-WT EZRIN or AAV-HA-tagged-Phospho-dead EZRIN that were stained with VGluT1 and PSD95 antibodies at 12-week-old. Scale bar, 10 μm. Quantification of VGluT1/PSD95 co-localized puncta, normalized using the means of WT mice injected with AAV-HA-tagged-WT EZRIN in the ventral ACC layer 1. n = 6 (WT + WT EZRIN O/E, 3 males and 3 females), 5 (WT + Phospho-dead Ezrin O/E, 3 males and 2 females), 6 (LRRK2 G2019S\textsuperscript{ki/ki} + WT EZRIN O/E, 3 males and 3 females), 6 (LRRK2 G2019S\textsuperscript{ki/ki} + Phospho-dead EZRIN O/E, 3 males and 3 females) mice. One-way ANOVA [F (3, 19) = 5.882, p = 0.0051], Bonferroni’s multiple comparisons test revealed a significant difference between WT + WT EZRIN O/E and WT + Phospho-dead Ezrin O/E (p = 0.0493, 95% C.I. = [0.05586, 53.21]), between WT + WT EZRIN O/E and LRRK2 G2019S\textsuperscript{ki/ki} + WT EZRIN O/E (p = 0.0269, 95% C.I. = [2.396, 53.08]), and between LRRK2 G2019S\textsuperscript{ki/ki} + WT EZRIN O/E and LRRK2 G2019S\textsuperscript{ki/ki} + Phospho-dead Ezrin O/E (p = 0.049, 95% C.I. = [-50.77, -0.08163]). There were no differences between WT + WT EZRIN O/E and LRRK2 G2019S\textsuperscript{ki/ki} + Phospho-dead Ezrin O/E (p > 0.9999, 95% C.I. = [-23.03, 27.66]), between WT + Phospho-dead Ezrin O/E and LRRK2 G2019S\textsuperscript{ki/ki} + WT EZRIN O/E (p > 0.9999, 95% C.I. = [-25.48, 27.68]), and between WT + Phospho-dead Ezrin O/E and LRRK2 G2019S\textsuperscript{ki/ki} + Phospho-dead Ezrin O/E (p = 0.0863, 95% C.I. = [-50.90, 2.259]), alpha = 0.05. b, Representative images from the ventral ACC layer 2/3 of WT and LRRK2 G2019S\textsuperscript{ki/ki} mice injected with AAV-HA-tagged-WT EZRIN or AAV-HA-tagged-Phospho-dead EZRIN that were stained with VGluT1 and PSD95 antibodies at
12-week-old. Scale bar, 10 μm. Quantification of VGlut1/PSD95 co-localized puncta, normalized using the means of WT mice injected with AAV-HA-tagged-WT EZRIN in the ventral ACC layer 2/3. n = 6 (WT + WT EZRIN O/E, 3 males and 3 females), 5 (WT + Phospho-dead EZRIN O/E, 3 males and 2 females), 6 (LRRK2 G2019Ski/ki + WT EZRIN O/E, 3 males and 3 females), 6 (LRRK2 G2019Ski/ki + Phospho-dead EZRIN O/E, 3 males and 3 females) mice. One-way ANOVA [F (3, 18) = 10.45, \( p = 0.0003 \)], Bonferroni’s multiple comparisons test revealed a significant difference between WT + WT EZRIN O/E and WT + Phospho-dead Ezrin O/E (\( p = 0.0041, 95\% \) C.I. = [8.857, 55.39]), between WT + WT EZRIN O/E and LRRK2 G2019Ski/ki + WT EZRIN O/E (\( p = 0.0016, 95\% \) C.I. = [11.67, 56.22]), between WT + Phospho-dead EZRIN O/E and LRRK2 G2019Ski/ki + Phospho-dead EZRIN O/E (\( p = 0.0232, 95\% \) C.I. = [-47.19, 16.71]) and between LRRK2 G2019Ski/ki + WT EZRIN O/E and LRRK2 G2019Ski/ki + Phospho-dead Ezrin O/E (\( p = 0.0092, 95\% \) C.I. = [-47.97, -5.49]). There were no differences between WT + WT EZRIN O/E and LRRK2 G2019Ski/ki + Phospho-dead Ezrin O/E (\( p > 0.9999, 95\% \) C.I. = [-15.06, 29.49]), and between WT + Phospho-dead Ezrin O/E and LRRK2 G2019Ski/ki + WT EZRIN O/E (\( p > 0.9999, 95\% \) C.I. = [-20.45, 24.10]), alpha = 0.05. c, Representative mEPSC traces from the ventral ACC layer 2/3 pyramidal neurons in acute brain slices of WT and LRRK2 G2019Ski/ki mice injected with AAV-HA-tagged-WT EZRIN or AAV-HA-tagged-Phospho-dead EZRIN. d, Cumulative frequency plots and quantification of synaptic event frequency. n = 14 (WT + WT EZRIN O/E), 15 (WT + Phospho-dead EZRIN O/E), 11 (LRRK2 G2019Ski/ki + WT EZRIN O/E), 14 (LRRK2 G2019Ski/ki + Phospho-dead EZRIN O/E) neurons from 4 mice per condition. Kruskal-Wallis test [H (3) = 36.83, \( p < 0.0001 \)]. Average frequency of mEPSC in WT + WT EZRIN O/E (1.555 ± 0.1125), WT +
Phospho-dead EZRIN O/E (0.8416 ± 0.05145), LRRK2 G2019S^{ki/ki} + WT EZRIN O/E (0.7154 ± 0.05959), and LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (1.253 ± 0.04424) mice. The posthoc Dunn’s test with Bonferroni adjustments showed to be significant for WT + WT EZRIN O/E vs. WT + Phospho-dead EZRIN O/E (p < 0.0001), WT + WT EZRIN O/E vs. LRRK2 G2019S^{ki/ki} + WT EZRIN O/E (p < 0.0001), WT + Phospho-dead EZRIN O/E vs. LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (p = 0.0084), and LRRK2 G2019S^{ki/ki} + WT EZRIN O/E vs. LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (p = 0.0005). The posthoc Dunn’s test with Bonferroni adjustments revealed no significant difference between WT + WT EZRIN O/E and LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (p > 0.9999) and between WT + Phospho-dead EZRIN O/E and LRRK2 G2019S^{ki/ki} + WT EZRIN O/E (p > 0.9999). Cumulative frequency plots and quantification of synaptic event amplitude. n = 11 (WT + WT EZRIN O/E), 15 (WT + Phospho-dead EZRIN O/E), 11 (LRRK2 G2019S^{ki/ki} + WT EZRIN O/E), 13 (LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E) neurons from 4 mice per condition. Kruskal-Wallis test [H (3) = 7.051, p = 0.0703]. Average amplitude of mEPSC in WT + WT EZRIN O/E (4.873 ± 0.1034), WT + Phospho-dead EZRIN O/E (6.339 ± 0.4284), LRRK2 G2019S^{ki/ki} + WT EZRIN O/E (5.354 ± 0.2988), and LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (5.591 ± 0.2247) mice. The posthoc Dunn’s test with Bonferroni adjustments revealed no significant difference for WT + WT EZRIN O/E vs. WT + Phospho-dead EZRIN O/E (p = 0.0688), WT + WT EZRIN O/E vs. LRRK2 G2019S^{ki/ki} + WT EZRIN O/E (p > 0.9999), WT + Phospho-dead EZRIN O/E vs. LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (p > 0.9999), and LRRK2 G2019S^{ki/ki} + WT EZRIN O/E vs. LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (p > 0.9999), WT + WT EZRIN O/E vs. LRRK2 G2019S^{ki/ki} + Phospho-dead EZRIN O/E (p > 0.9999).
+ Phospho-dead EZRIN O/E ($p = 0.3605$), and WT + Phospho-dead EZRIN O/E vs. LRRK2 G2019S$^{ki/ki}$ + WT EZRIN O/E ($p = 0.9468$). alpha = 0.05. Data are presented as mean ± s.e.m.

Extended Data Figure 4 (related to Figure 5). Overexpression of AAV-HA-tagged-WT EZRIN and AAV-HA-tagged-Phospho-dead EZRIN in WT and LRRK2 G2019S$^{ki/ki}$ astrocytes. a, Experiment workflow of AAV-HA-tagged-WT EZRIN and AAV-HA-tagged-Phospho-dead EZRIN in WT and LRRK2 G2019S$^{ki/ki}$ astrocytes. b, Representative images from the ventral ACC of WT and LRRK2 G2019S$^{ki/ki}$ mice injected with AAV-HA-tagged-WT EZRIN or AAV-HA-tagged-Phospho-dead EZRIN that were stained with HA and phospho-ERM antibodies at 12-week-old. Scale bar, 200 μm. n = 6 (WT + WT EZRIN O/E, 3 males and 3 females), 5 (WT + Phospho-dead EZRIN O/E, 3 males and 2 females), 6 (LRRK2 G2019S$^{ki/ki}$ + WT EZRIN O/E, 3 males and 3 females), 6 (LRRK2 G2019S$^{ki/ki}$ + Phospho-dead EZRIN O/E, 3 males and 3 females) mice. c, Representative images from the MOp layer 1 of WT and LRRK2 G2019S$^{ki/ki}$ mice injected with AAV-HA-tagged-WT EZRIN or AAV-HA-tagged-Phospho-dead EZRIN that were stained with VGAT and Gephyrin antibodies at 12-week-old. Scale bar, 10 μm. Quantification of VGAT/ Gephyrin co-localized puncta, normalized using the means of WT mice injected with AAV-HA-tagged-WT EZRIN in MOp layer 1. n = 3 (WT + WT EZRIN O/E, 2 males and 1 female), 5 (WT + Phospho-dead EZRIN O/E, 3 males and 2 females), 6 (LRRK2 G2019S$^{ki/ki}$ + WT EZRIN O/E, 3 males and 3 females), 6 (LRRK2 G2019S$^{ki/ki}$ + Phospho-dead EZRIN O/E, 3 males and 3 females) mice. One-way ANOVA [F (3, 55) = 19.7, $p < 0.0001$], Bonferroni’s multiple comparisons test revealed a significant difference between WT + WT EZRIN O/E and LRRK2 G2019S$^{ki/ki}$ + WT EZRIN O/E ($p <$
0.0001, 95% C.I. = [-98.23, -32.48]), and between WT + Phospho-dead EZRIN O/E and LRRK2 G2019S^ki/ki + Phospho-dead EZRIN O/E (p < 0.0001, 95% C.I. = [-82.37, -26.61]). There were no differences between WT + WT EZRIN O/E and WT + Phospho-dead EZRIN O/E (p = 0.6323, 95% C.I. = [-53.85, 13.40]), and between LRRK2 G2019S^ki/ki + WT EZRIN O/E and LRRK2 G2019S^ki/ki + Phospho-dead EZRIN O/E (p > 0.9999, 95% C.I. = [-36.33, 17.61]), alpha = 0.05. d, Representative images from the MOp layer 2/3 of WT and LRRK2 G2019S^ki/ki mice injected with AAV-HA-tagged-WT EZRIN or AAV-HA-tagged-Phospho-dead EZRIN that were stained with VGAT1 and Gephyrin antibodies at 12-week-old. Scale bar, 10 μm. Quantification of VGAT/Gephyrin co-localized puncta, normalized using the means of WT mice injected with AAV-HA-tagged-WT EZRIN in MOp layer 2/3. n = 3 (WT + WT EZRIN O/E, 2 males and 1 female), 5 (WT + Phospho-dead EZRIN O/E, 3 males and 2 females), 6 (LRRK2 G2019S^ki/ki + WT EZRIN O/E, 3 males and 3 females), 6 (LRRK2 G2019S^ki/ki + Phospho-dead EZRIN O/E, 3 males and 3 females) mice. One-way ANOVA [F (3, 54) = 15.10, p < 0.0001], Bonferroni’s multiple comparisons test revealed a significant difference between WT + WT EZRIN O/E and LRRK2 G2019S^ki/ki + WT EZRIN O/E (p = 0.0107, 95% C.I. = [-65.46, -5.94]), and between WT + Phospho-dead EZRIN O/E and LRRK2 G2019S^ki/ki + Phospho-dead EZRIN O/E (p < 0.0001, 95% C.I. = [-78.07, -26.92]). There were no differences between WT + WT EZRIN O/E and WT + Phospho-dead EZRIN O/E (p > 0.9999, 95% C.I. = [-37.73, 23.15]), and between LRRK2 G2019S^ki/ki + WT EZRIN O/E and LRRK2 G2019S^ki/ki + Phospho-dead EZRIN O/E (p = 0.0610, 95% C.I. = [-48.84, 0.6819]), alpha = 0.05.
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Figure 1.

(a) WT rat astrocytes

(b) shControl shLRRK2 LRRK2 KO

(c) WT LRRK2 G2019S

(d) WT Astrocytes

(e) LRR2 inhibitor MLi-2 (nM)

(f) Normalized pERM / ERM

(g) WT Astrocytes

(h) Normalized pERM / ERM

(i) shControl Astrocytes

(j) shLRRK2 Astrocytes

(k) WT Astrocytes

(l) LRRK2 G2019S Astrocytes
Figure 2.

**a** Postanal day 21 (P21)

- WT ACC
- G2019S MOp
- WT ACC
- G2019S MOp

**b** Postanal day 21 (P21)

- WT ACC
- G2019S MOp
- WT ACC
- G2019S MOp

**c** Postanal day 21 (P21)

- WT ACC
- G2019S MOp
- WT ACC
- G2019S MOp

**d**

- WT
- G2019S

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**e**

- WT + Phospho-dead EZRIN O/E
- G2019S + Phospho-dead EZRIN O/E

**f**

- WT
- G2019S

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**g**

- WT
- G2019S
- G2019S + Phospho-dead EZRIN O/E
- WT + Phospho-dead EZRIN O/E

<table>
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<tr>
<th>Radius (µm)</th>
<th>Number of intersections</th>
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<tbody>
<tr>
<td>0</td>
<td>shControl</td>
</tr>
<tr>
<td>10</td>
<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
<tr>
<td>20</td>
<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
<tr>
<td>30</td>
<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
<tr>
<td>40</td>
<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
<tr>
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<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
<tr>
<td>60</td>
<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
<tr>
<td>70</td>
<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
</tbody>
</table>

**h**

- shControl + Phospho-mimetic EZRIN O/E
- shLRRK2 + Phospho-mimetic EZRIN O/E

**i**

- shControl
- shLRRK2

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<th>Territory volume (1x10^4 µm^3)</th>
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<tr>
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<td>3</td>
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<tr>
<td>4</td>
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**j**

- shControl
- shLRRK2
- shLRRK2 + Phospho-mimetic EZRIN O/E
- shControl + Phospho-mimetic EZRIN O/E

<table>
<thead>
<tr>
<th>Radius (µm)</th>
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<tr>
<td>70</td>
<td>shLRRK2 + Phospho-mimetic EZRIN O/E</td>
</tr>
</tbody>
</table>

**k**

Analyzed human brain regions for GFAP and pERM staining

- Frontal cortex
- Human LRRK2 G2019S

**l**

- Human Control
- Frontal Cortex
- Human LRRK2 G2019S

<table>
<thead>
<tr>
<th>GFAP integrated density (AU x 10^6)</th>
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<tr>
<td>0</td>
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<tr>
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<tr>
<td>4</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>8</td>
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**m**

- Control
- G2019S

<table>
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<th>pERM integrated density (AU)</th>
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<tr>
<td>2000</td>
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<tr>
<td>3000</td>
</tr>
<tr>
<td>4000</td>
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<tr>
<td>5000</td>
</tr>
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**n**

- Control
- G2019S

<table>
<thead>
<tr>
<th>GFAP integrated density (AU x 10^6)</th>
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<tbody>
<tr>
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<tr>
<td>6</td>
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</table>

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Figure 3.

(a) Analyzed brain regions for synapse number and function.

(b) Synapse quantification.

(c) ACC Layer 1
- WT • G2019S
- VGLUT1/PSD95 12-week-old
- VGLUT1/PSD95 Coloc. puncta (% of WT)
- WT G2019S
- 10 µm
- p = 0.0002

(d) ACC Layer 2/3
- WT • G2019S
- VGLUT1/PSD95 12-week-old
- VGLUT1/PSD95 Coloc. puncta (% of WT)
- WT G2019S
- 10 µm
- p = 0.0002

(e) MOp Layer 1
- WT • G2019S
- VGAT/GEPHYRIN 12-week-old
- VGAT/GEPHYRIN Coloc. puncta (% of WT)
- WT G2019S
- 10 µm
- p < 0.0001

(f) MOp Layer 2/3
- WT • G2019S
- VGAT/GEPHYRIN 12-week-old
- VGAT/GEPHYRIN Coloc. puncta (% of WT)
- WT G2019S
- 10 µm
- p < 0.0001

(g) mEPSC recordings from the ACC layer 2/3
- WT 12-week-old
- G2019S 12-week-old
- 1 s 15 pA

(h) Cumulative frequency for mEPSCs
- mEPSC Frequency (Hz)
- WT G2019S
- p = 0.0007

(i) Cumulative frequency for mEPSCs
- mEPSC Amplitude (pA)
- WT G2019S
- p = 0.06

(j) mIPSC recordings from the MOp layer 2/3
- WT 12-week-old
- G2019S 12-week-old
- 1 s 15 pA

(k) Cumulative frequency for mIPSCs
- mIPSC Frequency (Hz)
- WT G2019S
- p = 0.0002

(l) Cumulative frequency for mIPSCs
- mIPSC Amplitude (pA)
- WT G2019S
- p = 0.1209
**Figure 4.**

(a) + WT EZRIN O/E

G2019S + WT EZRIN O/E

WT + WT EZRIN O/E

WT + Phospho-dead EZRIN O/E

(b) + WT EZRIN O/E

G2019S + WT EZRIN O/E

WT + WT EZRIN O/E

WT + Phospho-dead EZRIN O/E

(c) WT + WT EZRIN O/E

WT + Phospho-dead EZRIN O/E

G2019S + WT EZRIN O/E

G2019S + Phospho-dead EZRIN O/E

(d) mEPSC Cumulative frequency

Interevent interval (sec)

(e) mEPSC Amplitude (pA)

Amplitude (pA)
Extended Data Figure 1.

(a) P21 WT Mouse cortex

(b) P21 G2019S Mouse cortex

(c) Rat astrocyte cultures

(d) Normalized LRRK2 levels in rat astrocyte cultures

(e) Rat astrocyte cultures

(f) Normalized LRRK2 levels in rat astrocyte cultures

(g) Rat astrocyte cultures

(h) Normalized LRRK2 levels in rat astrocyte cultures

(i) Ezr - Mus musculus

(j) Ezr - Homo sapiens

Rdx - Mus musculus

(q) Rdx - Homo sapiens

Man - Mus musculus

(r) Man - Homo sapiens

Fetal Astrocytes

Mature Astrocytes

Neurons

Microglia

Oligodendrocytes

Endothelial

OPC

Ezr - Homo sapiens

Rdx - Homo sapiens

Man - Homo sapiens

FPKM

FPKM

FPKM
Extended Data Figure 2. Wang et al., was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

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Extended Data Figure 3.

**a** WT ACC P21

**b** WT MOp P21

**c** WT G2019S

**d** WT ACC P21

**e** WT MOp P21

**f** WT G2019S

GFAP integrated density (AU)

ALDH1L1+/SOX9+ cell counts/mm²

G2019S ACC P21

G2019S MOp P21

GFAP MERGE

ALDH1L1-GFP

SOX9 MERGE

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Extended Data Figure 4. 

(a) ACC Layer 1

WT | G2019S

VGLUT1/PSD95 Colocalization (as a percentage of WT)

(b) ACC Layer 2/3

WT | G2019S

VGLUT1/PSD95 Colocalization (as a percentage of WT)

(c) MOp Layer 1

WT | G2019S

VGAT/Gephyrin Colocalization (as a percentage of WT)

(d) MOp Layer 2/3

WT | G2019S

VGAT/Gephyrin Colocalization (as a percentage of WT)

p-values:

- ACC Layer 1: p = 0.7397
- ACC Layer 2/3: p = 0.6438
- MOp Layer 1: p = 0.3203
- MOp Layer 2/3: p = 0.8479
Extended Data Figure 5  

**a**  
- AAV-HA-WT EZRIN  
- AAV-HA-Phospho-dead EZRIN  
  
  Retro-orbital injection  
  - WT mice  
  - LRRK2 G2019S<sup>ki/ki</sup> mice  
  
  9-week → 12-week  

- Synapse counting  
- mEPSC recording  
- Fixation, IHC, Imaging  
- Electrophysiology  

**b**  

<table>
<thead>
<tr>
<th>+ HA-tagged WT EZRIN O/E</th>
<th>+ HA-tagged Phospho-dead EZRIN O/E</th>
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<tbody>
<tr>
<td><strong>HA staining in ACC</strong></td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>G2019S</td>
</tr>
<tr>
<td></td>
<td>200 µm</td>
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<tr>
<td><strong>pERM in ACC</strong></td>
<td>WT</td>
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<tr>
<td></td>
<td>G2019S</td>
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<td></td>
<td>200 µm</td>
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</table>

**c**  

<table>
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<tr>
<th>+ WT EZRIN O/E</th>
<th>+ Phospho-dead EZRIN O/E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VGLUT1/VGAT/GEPHYRIN Coloc. puncta (%) of WT + WT EZRIN O/E</strong></td>
<td>p = 0.0107</td>
</tr>
<tr>
<td>p &gt; 0.9999</td>
<td>p = 0.0610</td>
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**d**  

<table>
<thead>
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<th>+ Phospho-dead EZRIN O/E</th>
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<tr>
<td><strong>VGLUT1/VGAT/GEPHYRIN Coloc. puncta (%) of WT + WT EZRIN O/E</strong></td>
<td>p = 0.0107</td>
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<tr>
<td>p &gt; 0.9999</td>
<td>p = 0.0010</td>
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