NEUROREPAIR STRATEGIES TO INDUCE ANGIOGENESIS, NEUROGENESIS AND SYNAPTIC PLASTICITY

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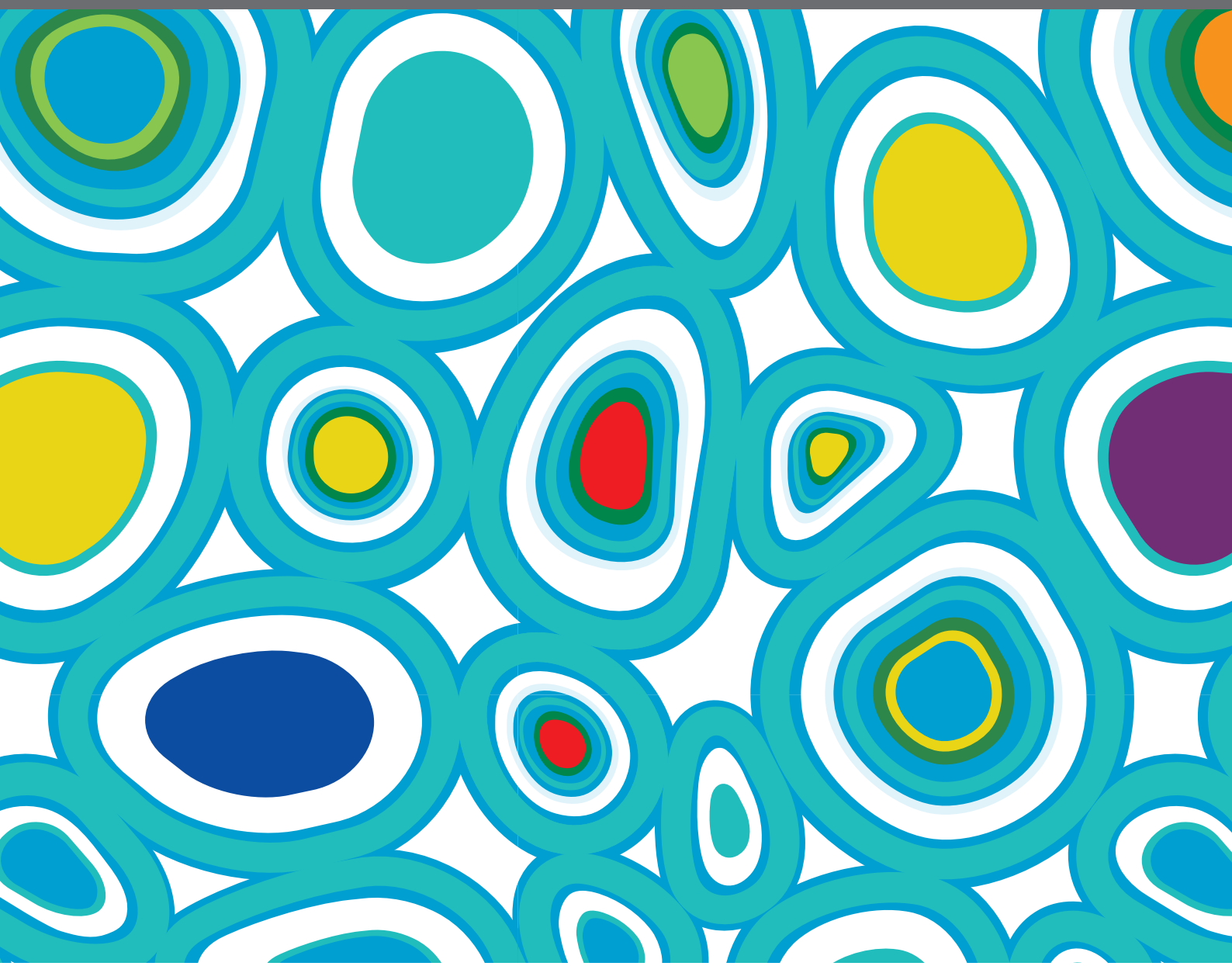

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NEUROREPAIR STRATEGIES TO INDUCE ANGIOGENESIS, NEUROGENESIS AND SYNAPTIC PLASTICITY

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EDITORIAL
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Editorial: Neurorepair Strategies to Induce Angiogenesis, Neurogenesis and Synaptic Plasticity

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Editorial on the Research Topic

Neurorepair Strategies to Induce Angiogenesis, Neurogenesis and Synaptic Plasticity

Brain damage as a result of ischemic stroke and trauma, following injury, or in neurodegenerative disease generates severe consequences leading to impairments in motor and cognitive functions. Although neuroplasticity persists in the adult human brain, the reorganization and self-repair are limited and recovery from most diseases is far from certain. Recent discoveries point to repair strategies of the adult injured or diseased human brain that require approaches targeting neuron replacement, angiogenesis, gliogenesis, and enhancing synaptic plasticity. To achieve an efficient regenerative response, the orchestration of scar formation, cleaning debris of damaged cells and integrating newborn neurons into existing circuitries is of importance. Knowledge of the various steps and the underlying neurochemical processes supporting cell genesis and integration, provides valuable insights of signaling pathway management that might lead to new therapeutic strategies in tissue repair.

For this special issue, we collected review articles and original research reports summarizing and discussing repair strategies for the rodent and human brain. The studies specifically focus on glial cells in the repair process, and examine the new exciting role of astrocytes (Chiareli et al.) and their reprograming potential following spinal cord injury (Puls et al.) or cortical brain damage (Ribeiro et al.). Thereby, two articles highlight the reprogramming capacity of reactive astrocytes *in vitro* that involves Galectin-3 triggering of Notch1 signaling in response to damage-facilitating nuclear translocation of Notch intracellular domain (Ribeiro et al.). Excitingly, Puls et al. reveal an *in vivo* astrocyte-to-neuron conversion, whereby reactive astrocytes covert into functional neurons *via* the NeuroD1 pathway that may improve neurorepair processes following spinal cord injury.

Besides astrocytes, gliogenesis of microglia and oligodendrocyte precursor cells (OPCs) in the adult brain promote repair processes and enhance signaling pathways that in turn contribute to neuroplasticity. Specifically, Neuron-Glia (NG) 2-positive OPCs constitute the main proliferating cell population outside the neurogenic niches, and display an endogenous source with multipotent potential. Importantly, demyelinating diseases require oligodendrocytes; and the review by Reyes-Haro et al. reveals OPCs express $GABA_A$ receptors, communicate with neurons through GABAergic signaling, which might contribute to remyelination as repair strategy. In addition, the transcriptome profile of corpus callosum shows the activation of oligodendrogenesis alongside an angiogenic response in cerebral hypoperfused mice highlighting the role of glial cells as therapeutic target in vascular cognitive impairment and dementia (Takase et al.).

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Microglia take part in neuroplasticity of the healthy rodent hippocampus and may be a potential therapeutic model in the maintenance of mood. In pathology, dysfunctional microglia contribute to late-stage development of Alzheimer's Disease (AD) inducing cell death and the release of brain-derived neurotrophic factor (Turkin et al.). Apolipoprotein E (APOE) is an important factor in the brain vasculature and also associated with an increased risk to develop AD by affecting cerebral vascular integrity, brain metabolism, synaptic plasticity, and neuroinflammation. In this issue, two studies present their findings and discuss the autocrine functions of *apoE* genotype in the modulation of basal phenotypic state of brain endothelial cells, the role of APOE4 in increased inflammation (Marottoli et al.), and in promoting tonic-clonic seizures (Lamoureux et al.).

The review by Cuartero et al. ask whether "Post-stroke Neurogenesis [is] Friend or Foe?" and provides an overview of the role of newborn neurons in the neurogenic niches subventricular zone and subgranular zone after ischemic stroke. The authors conclude that neurogenesis in the dentate gyrus may adopt a "maladaptive plasticity response" that contributes to "the development of post-stroke cognitive impairment and dementia." Last but not least, Park and Hayakawa discuss the role of cell-free extracellular mitochondria in non-cell-autonomous signaling in central nervous system pathophysiology. Neurorepair strategies also include the use of (external) bioscaffolds and the creation of cell platforms providing the basis for tissue engineering (Zamproni et al.).

In conclusion, the present collection of articles reflects the complexity of the central nervous system and thus the varies repair strategies and mechanisms involved in the neurorepair process. Collectively, our studies provide an overview and explore different strategic approaches including gliogenesis, angiogenesis, neurogenesis, and targeting synaptic plasticity following distinct types of brain damage. Our approach in summarizing the specific findings in this edition is to encourage new young scientists to discover novel pathways, to inspire researchers to go new innovative ways—facilitating neurorepair process and leading to the development of novel solutions that improve the quality of life of patients following injury and in neurological disease.

AUTHOR CONTRIBUTIONS

FK, FS, and MP have made a substantial, direct and intellectual contribution to this editorial, and all authors have approved it for publication.

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Regeneration of Functional Neurons After Spinal Cord Injury via *in situ* NeuroD1-Mediated Astrocyte-to-Neuron Conversion

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Spinal cord injury (SCI) often leads to impaired motor and sensory functions, partially because the injury-induced neuronal loss cannot be easily replenished through endogenous mechanisms. *In vivo* neuronal reprogramming has emerged as a novel technology to regenerate neurons from endogenous glial cells by forced expression of neurogenic transcription factors. We have previously demonstrated successful astrocyte-to-neuron conversion in mouse brains with injury or Alzheimer's disease by overexpressing a single neural transcription factor NeuroD1. Here we demonstrate regeneration of spinal cord neurons from reactive astrocytes after SCI through AAV NeuroD1-based gene therapy. We find that NeuroD1 converts reactive astrocytes into neurons in the dorsal horn of stab-injured spinal cord with high efficiency (∼95%). Interestingly, NeuroD1-converted neurons in the dorsal horn mostly acquire glutamatergic neuronal subtype, expressing spinal cord-specific markers such as Tlx3 but not brain-specific markers such as Tbr1, suggesting that the astrocytic lineage and local microenvironment affect the cell fate after conversion. Electrophysiological recordings show that the NeuroD1-converted neurons can functionally mature and integrate into local spinal cord circuitry by displaying repetitive action potentials and spontaneous synaptic responses. We further show that NeuroD1-mediated neuronal conversion can occur in the contusive SCI model with a long delay after injury, allowing future studies to further evaluate this *in vivo* reprogramming technology for functional recovery after SCI. In conclusion, this study may suggest a paradigm shift from classical axonal regeneration to neuronal regeneration for spinal cord repair, using *in vivo* astrocyte-to-neuron conversion technology to regenerate functional new neurons in the gray matter.

Keywords: spinal cord, NeuroD1, astrocyte, neuronal conversion, *in vivo* reprogramming

INTRODUCTION

Spinal cord injury (SCI) is a devastating central nervous system (CNS) disorder and often leads to loss of motor and sensory functions below the injury site, even paralysis depending on the severity of the injury (Adams and Hicks, 2005). The pathophysiological process after SCI is rather complex, resulting in neuronal loss, neuroinflammation, demyelination, and Wallerian degeneration of the axons (Norenberg et al., 2004). Reactive astrogliosis is common to CNS injury, and particularly severe after SCI. Resident astrocytes react to injury-induced cytokines and dramatically upregulate the expression of a number of proteins such as the astrocytic marker GFAP and the neural progenitor markers Nestin and Vimentin (Sofroniew, 2009). These reactive astrocytes also become proliferative and hypertrophic in cell morphology. In the acute phase of SCI, reactive astrocytes play important roles in repairing the blood-spinal cord barrier and restricting the size of the primary injury (Okada et al., 2006; Herrmann et al., 2008). However, in the sub-acute or chronic phase, reactive astrocytes constitute the major component of the glial scar, a dense tissue structure that is inhibitory to axonal regeneration (Silver and Miller, 2004). Therefore, for decades, substantial effort has been made to overcome the glial scar and promote regrowth of severed axons through the injury site (Filous and Schwab, 2017). On the other hand, the spinal neurons that are lost during and after the injury need to be replaced in order to rebuild the local neuronal circuits. In this regard, stem cell transplantation has been reported to achieve certain success (Tuszynski et al., 2014; Lu et al., 2017), however the identity of transplanted cells and restoration of functional circuitry in the injury site still require further investigation (Goldman, 2016).

In vivo neuronal reprogramming has recently emerged as a novel technology to regenerate functional new neurons from endogenous glial cells by overexpressing neurogenic transcription factors in the CNS (Grande et al., 2013; Niu et al., 2013; Guo et al., 2014; Liu et al., 2015, 2020; Gascon et al., 2016; Li and Chen, 2016; Barker et al., 2018; Lei et al., 2019; Chen et al., 2020; Wu et al., 2020). In the injured spinal cord, a combination of growth factor treatment and forced expression of the neurogenic transcription factor *Neurogenin 2* (*Ngn2*) has been reported to stimulate neurogenesis from neural progenitors (Ohori et al., 2006). However, these newly generated neurons suffer from poor long-term survival. More recently, the transcription factor *Sox2* has been shown to reprogram astrocytes into proliferating neuroblasts, which require further treatment with a histone deacetylase inhibitor, valproic acid (VPA), to differentiate into mature neurons (Su et al., 2014). With additional treatment of neurotrophic factors, *Sox2*-converted neurons can express several neuronal subtype markers, but predominately VGluT, a marker for glutamatergic neurons (Wang et al., 2016).

Our group has previously demonstrated that the neurogenic transcription factor *NeuroD1* can reprogram reactive astrocytes into functional neurons in the stab-injured brain and in a mouse model for Alzheimer's disease (Guo et al., 2014). Following studies demonstrated that NeuroD1-mediated *in vivo* astrocyte-to-neuron conversion can reverse glial scar back to neural tissue (Zhang et al., 2020) and repair the damaged motor cortex after ischemic stroke (Chen et al., 2020). More recently, by combining NeuroD1 and Dlx2 together, we have demonstrated that astrocytes in the striatum of R6/2 mouse model for Huntington's disease can be converted into GABAergic neurons (Wu et al., 2020). The major goal of the current study is to determine whether *NeuroD1* can reprogram reactive astrocytes into functional neurons in the injured spinal cord. Using adeno-associated virus (AAV) for gene delivery and a *Cre-Flex* system with *GFAP* promoter to target reactive astrocytes specifically, our results indicate that NeuroD1 can mediate direct astrocyte-to-neuron conversion with high efficiency in both stab and contusive SCI models. The NeuroD1-converted neurons preferentially acquire glutamatergic phenotype in the dorsal horn and express neuronal subtype markers specific to the spinal cord such as Tlx3. Patch clamp recordings further demonstrate that the NeuroD1-converted neurons can functionally mature and integrate into spinal cord circuitry. Interestingly, combining NeuroD1 together with Dlx2 also generates more GABAergic neurons in the spinal cord, similar to that discovered in the brain. Together, our results indicate that NeuroD1-mediated neuronal conversion opens an avenue to treat SCI with internal glial cells through AAV-based gene therapy that may regenerate a diversity of neuronal subtypes for functional repair.

MATERIALS AND METHODS

Animal Use

GAD-GFP mice (Tg[Gad1-EGFP]94Agmo/J) and wild-type C57BL/6 mice were purchased from the Jackson Laboratory and bred in house. Mice of 2–4 months old (both male and female) were used. Mice were housed in a 12 h light/dark cycle and supplied with sufficient food and water. All animal use and studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University. All procedures were carried out in accordance with the approved protocols and guidelines of National Institute of Health (NIH).

Retrovirus and AAV Production

Retroviral vectors expressing *GFP* and *NeuroD1-GFP* under the *CAG* promoter (*pCAG*) were previously described (Guo et al., 2014). Retrovirus packaging, purification and titering were performed as previously described (Guo et al., 2014). The viral titers were determined by serial dilution to be $\sim 1 \times 10^7$ genome copy (GC)/ml.

For AAV-mediated gene expression, the *Cre-Flex* system was applied to target transgene expression specifically to reactive astrocytes using the *human GFAP* (*hGFAP*) promoter. To generate *pAAV-hGFAP::Cre* vector, the *hGFAP* promoter was first amplified from *pDRIVE-hGFAP* plasmid (InvivoGen) by PCR and inserted into *pAAV-MCS* (Cell Biolab) between the MluI and SacII sites to replace the *CMV* promoter. The *Cre* gene coding fragment was then similarly subcloned from *phGFAP-Cre* (Addgene plasmid #40591) and inserted into *pAAV MCS*

between the EcoRI and SalI sites. To construct *pAAV-FLEX* vectors expressing transgenes, the coding sequences of *NeuroD1*, *mCherry* or *GFP* were amplified by PCR from the corresponding retroviral constructs. The *NeuroD1* fragment was fused with either *P2A-mCherry* or *P2A-GFP* and subcloned into the *pAAV-FLEX-GFP* vector (Addgene plasmid #28304) between the KpnI and XhoI sites. All plasmid constructs were confirmed by sequencing. The AAV-CamKII-GFP plasmid was purchased from Addgene (#64545). For AAV production, HEK 293T cells were transfected with the *pAAV* expression vectors, *pAAV9-RC* vector (Cell Biolab), and *pHelper* vector (Cell Biolab) to generate AAV particles carrying our transgenes. Three days after transfection, the cells were scraped in their medium and centrifuged. The supernatant was then discarded and the cell pellet was frozen and thawed four times, resuspended in a discontinuous iodixanol gradient, and centrifuged at 54,000 rpm for 2 h. Finally, the virus-containing layer was extracted, and the viruses were concentrated using Millipore Amicon Ultra Centrifugal Filters. The viral titers were determined using the QuickTiter™ AAV Quantitation Kit (Cell Biolabs) and then diluted to a final concentration of 1×10^{10} GC/ml for injection (except for our GFAP-Cre virus, diluted to a final concentration of 1×10^{8} GC/ml).

Laminectomy, Injury, and Stereotaxic Viral Injection

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (80–120 mg/kg ketamine; 10–16 mg/kg xylazine). A laminectomy was then performed at the T11–T12 vertebrae to expose dorsal surface of the spinal cord, and a stab or contusion injury was performed. The stab injury was conducted with a 31-gauge needle at the center of the exposed surface, 0.4 mm lateral to the central artery with a depth of 0.4 mm, while the contusion injury was generated with a force of 45 kdyn on an Infinite Horizon Impactor (IH-0400, Precision Systems and Instrumentation) directly at the center of the exposed surface. For conversion after stab injury, 1.0 μL of AAV (1×10^{10} GC/ml) was injected using a 50 μl Hamilton syringe with a 34-gauge injection needle at a rate of 0.05 μL/min at the same coordinates immediately after the stab injury, while retrovirus (1×10^{7} GC/ml) was injected at 4 days post stab injury. For conversion after contusive injury, AAV (1×10^{10} GC/ml) was injected at 10 days or 16 weeks following contusion injury and at 1 mm away from the injury site with a depth of 0.4–0.8 mm. The viral injection needle was kept in place for 3 min after injection to prevent drawing out the virus during withdrawal. The surgical area was then treated with antibiotic ointment and the skin was clipped for a week to allow the skin to re-suture. The mice were kept on a heating pad and treated with Carprofen for pain relieve via subcutaneous injection (5 mg/kg) on the day of surgery and drinking water (10 mg/kg) for 3 days after surgery and closely monitored for 1 week to ensure full recovery of health.

Electrophysiology

Mice were sacrificed at defined time points by anesthetization with 2.5% Avertin and decapitation. The spinal cord segment was then removed from the spine into cutting solution (125 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, and 10 mM glucose adjusted to pH 7.4 and 295 mOsm/L and bubbled for 1 h with 95% O_2/5% CO_2) cooled on ice, where it was encased in an agarose matrix (Sigma) and cut into 300 μm thickness slices using a VT3000 vibratome (Leica). Slices were then incubated for 1 h in holding solution (92 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 15 mM glucose, 12 mM N-Acetyl-L-cysteine, 5 mM Sodium ascorbate, 2 mM Thiourea, 3 mM Sodium pyruvate, 2 mM $MgSO_4$, and 2 mM $CaCl_2$, adjusted to pH 7.4 and 295 mOsm/L and bubbled continuously with 95% O_2/5% CO_2) at room temperature before patch-clamp recording in standard ACSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose adjusted to pH 7.4 and 295 mOsm/L and bubbled for 1 h with 95% O_2/5% CO_2). Both native and converted cells were recorded by whole-cell recording using standard inner solution (135 mM K-gluconate, 10 mM KCl, 5 mM Na-phosphocreatine, 10 mM HEPES, 2 mM EGTA, 4 mM MgATP, and 0.5 mM Na_2GTP, adjusted to pH 7.4 and 295 mOsm/L) with the membrane potential held at −70 mV. Typical values for the pipette and total series resistances were 2–10 and 20–60 MΩ, respectively. Data were collected using the pClamp 9 software (Molecular Devices) by sampling at 10 kHz and filtering at 1 kHz. Data were then analyzed and plotted with the Clampfit 9.0 software (Molecular Devices).

Immunohistochemistry, Immunocytochemistry, and Microscopy

After perfusion, the target region of the spinal cord (∼0.5 cm in length) was surgically dissected, fixed in 4% paraformaldehyde (PFA) in PBS for 1 day, dehydrated in 30% sucrose solution for 1 day, and sectioned into 30 μm coronal or horizontal slices using a Leica CM1950 cryostat. The slices were collected serially in 24-well plates so that distance from the injury site could later be ascertained. The samples were then stored at 4°C in 0.02% sodium azide (NaN_3) in PBS to prevent bacterial degradation. Spinal cord slices were chosen for immunohistochemistry based on infection of the dorsal horn by inspecting the reporter protein (mCherry or GFP) in the storage solution under a fluorescent microscope. For the stab injury experiments, care was taken to select coronal slices at least 100 μm from the injury site to ensure tissue integrity. On the first day of staining, samples were washed in PBS three times for 5 min per wash, permeablized with 2% Triton X-100 in PBS for 20 min, and blocked using a 5% normal donkey serum (NDS) and 0.1% Triton-X in PBS for 2 h to reduce non-specific binding of the antibodies. The samples were then incubated with primary antibodies diluted in the same blocking buffer at 4°C for two nights to allow thorough penetration of the antibodies. On the third day, the samples were recovered to room temperature, washed in PBS three times for 5 min per wash, and incubated with secondary antibodies diluted in blocking buffer for 1 h. Finally, the samples were washed in PBS three more times for 10 min per wash and mounted on glass slides with coverslips using anti-fading mounting solution (Invitrogen). The immunostained samples were examined and imaged using Olympus FV1200 and Zeiss LSM 800 laser confocal microscopes.

Z-stacks were collected for the *in vivo* images for the whole thickness of the samples and maximum intensity and z-stack projections were used for image preparation and analysis.

Quantification and Data Analysis

As a result of our carefully selected injection coordinates described above, infected cells were mostly found in the dorsal horn of the spinal cord, Rexed laminae 1-6 (Rexed, 1954). For most of the quantification, including cell conversion and NeuN acquisition, cells were counted if they appeared in any part of this region. For quantification based on cell subtype (**Figures 3**, **4**), cells were only counted if they appeared in Rexed laminae 1-3, centered about the substantia gelatinosa, a region dominated by small, excitatory interneurons and easily demarcated due to its high cell density (Santos et al., 2007). This region was chosen for its ease of demarcation and so that a consistent local population of neuronal subtypes could be expected from sample to sample. Quantification was performed on collected images using the z-stacked images as a guide and the layered stacks to check the vertical dimension. Strict background cutoffs for positive signals were calculated for each channel as three times the average background intensity for the relevant tissue and antibody. Cells were binned by presence (i.e., above the background cutoff) or absence (i.e., below the background cutoff) for each marker in question, using the viral fluorophore (mCherry or GFP) to identify infected cells and DAPI to confirm each cell for counting. To estimate the total number of converted neurons per infection for our contusion experiments, we multiplied the average number of $NeuN^+$ neurons among the infected cells per horizontal section, calculated from one dorsal, one central, and one ventral section, by the total number of horizontal sections per sample. While this might over estimate the number of cells, it gives a rough estimate of the number of converted neurons in the infected areas. All quantification was performed on three biological replicates per data point and is reported as the means and standard deviations of the three replicates.

RESULTS

NeuroD1 Reprograms Reactive Astrocytes Into Neurons in the Injured Spinal Cord

We previously demonstrated that expressing NeuroD1 in reactive astrocytes after brain injury can directly convert astrocytes into neurons (Guo et al., 2014; Chen et al., 2020; Zhang et al., 2020). In this study, we investigated whether such *in vivo* direct conversion technology can regenerate functional new neurons in injured spinal cord. To target the dividing reactive astrocytes after injury, we employed retroviruses that mainly express ectopic genes in dividing cells but not in neurons, which cannot divide. We injected NeuroD1-expressing retroviruses at 4 days post-stab injury (dpi), when many dividing reactive astrocytes have been detected (Chen et al., 2008; Hong et al., 2014), and analyzed samples at 1, 3, and 6 weeks post-injection (wpi) (**Figure 1A**). In this study, we chose the spinal cord dorsal horn as our major region of interest because it is composed of both excitatory and inhibitory neurons and is critical to afferent sensory information processing (**Figure 1B**). We are currently investigating motor neuron regeneration in the spinal cord ventral horn in a separate study. We first explored the cell types infected by our control CAG::GFP retroviruses. At 1 wpi, we found that the control GFP retroviruses infected a mixture of glial cell types including reactive astrocytes ($GFAP^+$ and some $GFAP^+/Olig2^+$), oligodendrocyte progenitor cells (OPCs) ($Olig2^+$), and microglia ($Iba1^+$) (**Figures 1C,D**), but not $NeuN^+$ neurons (**Figure 1D**). In contrast, cells infected by the CAG::NeuroD1-GFP retrovirus showed an increasing number of $NeuN^+$ cells with neuronal morphology over time (**Figure 1E**), and quantitatively reached 93.5% at 6 wpi (**Figure 1F**), indicating a successful glia-to-neuron conversion in the injured spinal cord.

While retrovirus has the advantage to target only dividing glial cells for conversion, avoiding non-dividing neurons, such advantage also limits its capability to convert non-dividing glial cells into neurons. In order to move our *in vivo* reprogramming technology toward clinical settings in the future, we adopted an AAV gene delivery system in which the transgene expression is controlled by an astrocyte-specific *GFAP* promoter to target both dividing and non-dividing glial cells (**Figure 2A**). Specifically, we used a *Cre-Flex* gene expression system, which contains two AAV vectors, with one encoding *GFAP-Cre* and the other encoding the transgene in reverse form flanked by double LoxP sites (FLEX vector) (Atasoy et al., 2008; Liu et al., 2015; Chen et al., 2020). Thus, when the two AAVs are co-injected into the spinal cord, Cre recombinase will be expressed in the infected reactive astrocytes and turn on the transgene expression in FLEX vector by flipping the transgene sequence into the correct form for transcription (**Figure 2B**). We first confirmed the specificity of the *Cre-Flex* AAV system in the spinal cord by co-injecting AAV GFAP::Cre and AAV FLEX-CAG::mCherry (or::GFP) into the stab-injured dorsal horn. The control virus infected cells were mostly $GFAP^+$, $NeuN^-$ astrocytes at 4 wpi (**Figure 2C**). Next, we co-injected AAV GFAP::Cre with AAV FLEX-CAG::NeuroD1-P2A-mCherry into the stab-injured dorsal horn. In contrast to the control AAV, the NeuroD1-mCherry infected cells were mostly $NeuN^+/GFAP^-$ neurons with clear neuronal morphology at 4 wpi (**Figure 2D**). NeuroD1 overexpression in the infected cells was confirmed by immunostaining (**Supplementary Figure 1**). Interestingly, besides $NeuN^+/GFAP^-$ converted neurons, we also observed many NeuroD1-AAV-infected cells at 2 wpi with co-immunostaining of both $GFAP^+$ and $NeuN^+$ (**Figure 2E**), suggesting a potential intermediate stage during astrocyte-to-neuron conversion. We termed these $GFAP^+/NeuN^+$ cells induced by NeuroD1 expression in astrocytes as "AtN transitional cells." We did not observe any such transitional cells in the control mCherry-infected spinal cord after injury, suggesting that AtN conversion does not happen following neural injury but can be induced by ectopic expression of transcription factors such as NeuroD1. Quantitative analysis revealed that the control AAV-infected cells were mostly $GFAP^+$ astrocytes by 8 wpi (**Figure 2F**, left red bar), whereas NeuroD1 AAV-infected cells showed a progressive increase in the percentage of neurons ($NeuN^+/GFAP^-$, blue bar in **Figure 2F**) from 2 to 8 wpi, reaching ∼95% at 8 wpi (**Figure 2F**, right blue bar). Note that at 2 wpi, over 60%

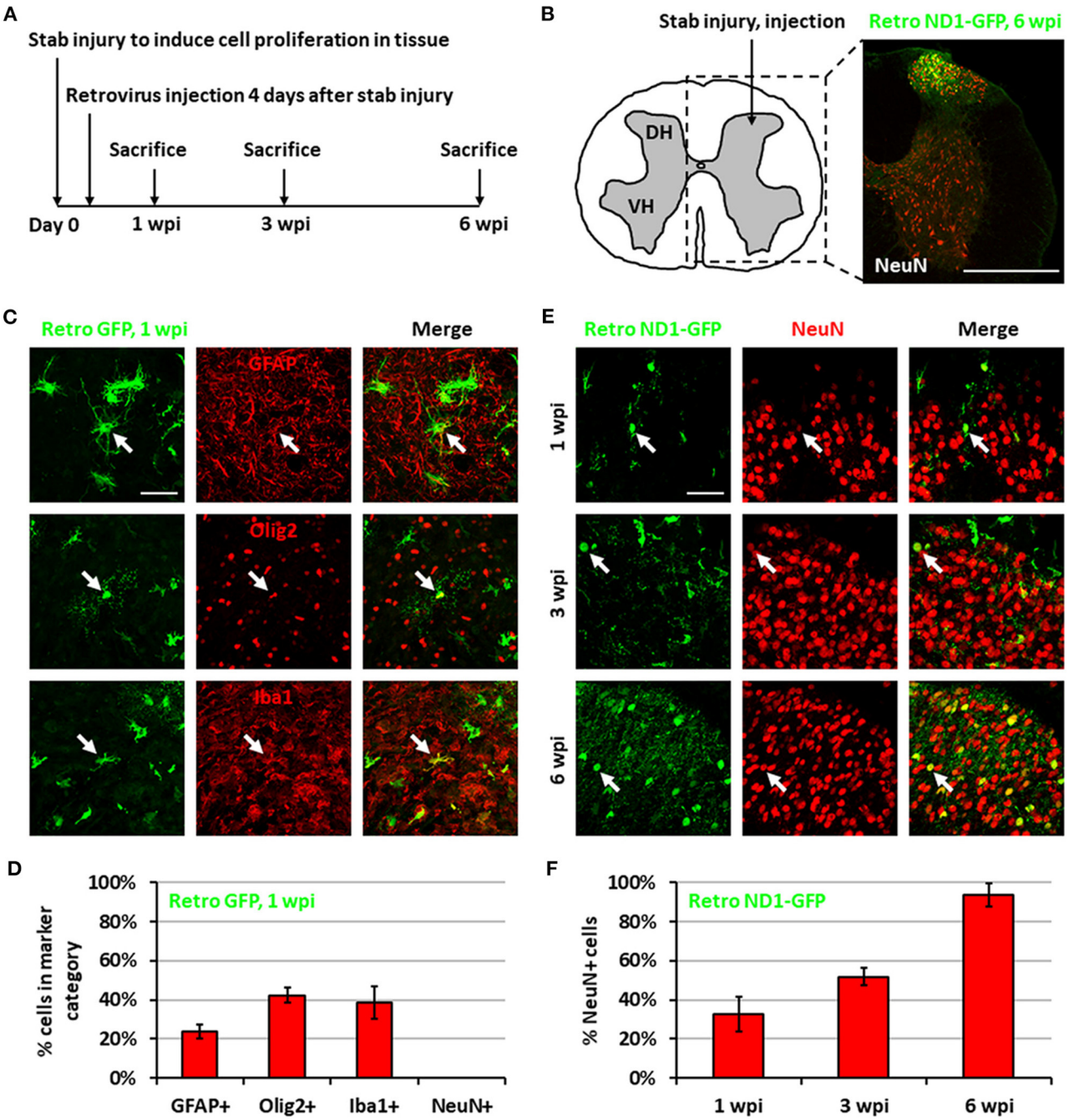

FIGURE 1 | Neuronal conversion induced by NeuroD1-expressing retroviruses after stab injury in the spinal cord dorsal horn. **(A)** Experiment paradigm. **(B)** Schematic illustration of dorsal horn injury and viral injection, together with an actual viral infection image. Coordinates are 0.4 mm lateral of the central artery and 0.4 mm below the tissue surface. Stab injury was performed with a 32-guage needle followed by stereotaxic injection at the injury site. Scale bar, 500 μm. **(C)** Three main types of proliferating glial cells at 1 wpi after injection of control retrovirus expressing GFP alone: astrocytes (GFAP), OPCs (Olig2), and microglia (Iba1). Scale bar, 50 μm. **(D)** Quantification based on staining for control retrovirus GFP at 1 wpi. Bars show the mean and standard deviation of three replicates. **(E)** NeuroD1-GFP-infected cells in the dorsal horn at 1, 3, and 6 wpi after retroviruses expressing NeuroD1-GFP. NeuroD1-GFP-infected cells gradually acquired more and more NeuN signal. Arrows show example $NeuN^+$ cells. Scale bar, 500 μm. **(F)** Quantification based on staining for retroviruses expressing NeuroD1-GFP. Bars show the mean and standard deviation of three replicates.

of NeuroD1-infected cells were $GFAP^+/NeuN^+$ transitional cells (green bar in **Figure 2F**), which gradually decreased at 4 wpi and 8 wpi together with a decrease of $GFAP^+$ astrocytes (red bar in **Figure 2F**) among the NeuroD1-infected cell population. Further analysis showed that neither transitional cells nor converted neurons exhibited significant cell death

suggesting that apoptosis does not play a significant role during the NeuroD1-mediated cell conversion process (**Supplementary Figure 2**). Comparing to Ngn2-mediated or Ascl1-mediated AtN conversion (Gascon et al., 2016), less apoptosis was detected during NeuroD1-mediated conversion process, which may suggest that different transcription factors act through different signaling and metabolic pathways to carry out cell conversion.

NeuroD1 Converts Dorsal Spinal Astrocytes Into Tlx3$^+$ Glutamatergic Neurons

After demonstrating astrocyte-to-neuron conversion in the spinal cord, we next investigated which subtypes of neurons were generated through NeuroD1-mediated conversion. The dorsal horn of the spinal cord contains two main neuronal subtypes: glutamatergic and GABAergic neurons (Abraira and Ginty, 2013). During spinal cord development, two transcription factors, Tlx3 and Pax2, appear to play critical roles in determining cell fate specification in the dorsal horn (Cheng et al., 2005; Huang et al., 2008). Interestingly, by examining AAV NeuroD1-GFP-infected cells in the dorsal horn at 8 wpi, we found that the majority of NeuroD1-converted neurons were Tlx3$^+$ (62.6 $\pm$ 3.3%), suggesting a majority glutamatergic neuronal subtype (**Figure 3A**). In contrast, only a small percentage of NeuroD1-converted neurons in the dorsal horn were Pax2$^+$ (8.8 $\pm$ 1.3%), suggesting a minority GABAergic neuronal subtype (**Figure 3A**). Because AAV might infect a small proportion of neurons (**Figure 2F**, control), we further examined retrovirus NeuroD1-GFP-infected cells in the dorsal horn at 6 wpi and found that, similar to our AAV experiments, the retrovirus NeuroD1-converted neurons were mainly Tlx3$^+$ (50.3 $\pm$ 17.0%), with a minority being Pax2$^+$ (16.4 $\pm$ 4.3%) (**Figure 3B**; quantified in **Figure 3C**). These results suggest that the majority of NeuroD1-converted neurons in the dorsal horn of spinal cord are Tlx3$^+$ neurons, with a small proportion being Pax2$^+$ neurons.

We further confirmed the neuronal subtypes after NeuroD1 conversion using AAV CaMKII-GFP to identify glutamatergic neurons and GAD-GFP transgenic mice to identify GABAergic neurons. When co-injecting AAV GFAP::Cre and Flex-NeuroD1-mCherry together with AAV CaMKII::GFP (Dittgen et al., 2004), we observed 89.5 $\pm$ 5.2% ($n = 3$) of GFP$^+$ cells co-expressing Tlx3$^+$, confirming that these Tlx3$^+$ neurons are indeed glutamatergic (**Figure 3D**). Many NeuroD1-mCherry converted neurons were also colocalizing with CaMKII-GFP (**Figure 3D**), suggesting that they were glutamatergic neurons. When injecting AAV GFAP::Cre and Flex-NeuroD1-mCherry in GAD-GFP mice (Ma et al., 2006), in which GABAergic neurons are genetically labeled with GFP, we did not observe GAD-GFP co-expression with Tlx3$^+$ ($n = 3$), as expected. Indeed, in the dorsal horn of spinal cord in uninjured and untreated mice, we observed that the CaMKII-GFP co-stained consistently with endogenous Tlx3$^+$ while GAD-GFP co-stained with Pax2$^+$ (**Supplementary Figure 3**). Therefore, the majority of NeuroD1-converted neurons in the dorsal horn of the spinal cord are glutamatergic neurons, consistent with our findings in the mouse cortex (Chen et al., 2020).

Dlx2 Combined With NeuroD1 Increases the Conversion of Dorsal Spinal Astrocytes Into Pax2$^+$ GABAergic Neurons

Having generated a majority of glutamatergic neurons using NeuroD1 alone in the spinal cord, we further investigated whether it's possible to increase the proportion of GABAergic neurons by combining NeuroD1 with other transcription factors. In a separate study on Huntington's disease, we have discovered that combining NeuroD1 and Dlx2 together can successfully convert striatal astrocytes into GABAergic neurons (Wu et al., 2020). Dlx2 is a transcription factor that has been reported to play a significant role in GABAergic neuron specification and maturation during brain development (Anderson et al., 1999; McKinsey et al., 2013; Victor et al., 2014; Yang et al., 2017; Pla et al., 2018). Therefore, we injected a 1:1 ratio of AAV5 FLEX-NeuroD1-mCherry and AAV5 FLEX-Dlx2-mCherry in combination with AAV5-GFAP-Cre (**Figure 4**; $n = 3$). We first confirmed the co-expression of NeuroD1 and Dlx2 with immunostaining after viral infection (**Figure 4A**). Immunostaining experiments demonstrated that many NeuroD1+Dlx2-converted neurons were Tlx3$^+$ or Pax2$^+$ neurons (**Figure 4B**). Quantitative analysis revealed that 32.5 $\pm$ 2.1% of NeuroD1+Dlx2-converted neurons were Pax2$^+$ neurons (**Figure 4C**), a 5-fold increase compared to that generated by NeuroD1 alone (6.3%; $p = 0.05$, Kruskal-Wallis H-test). The percentage of Tlx3$^+$ neurons generated by NeuroD1 + Dlx2 was 56.2 $\pm$ 3.4% (**Figure 4C**). Importantly, the GABAergic identity of our NeuroD1+Dlx2-converted neurons was further confirmed in GAD-GFP mice, where we observed co-localization of NeuroD1+Dlx2-converted neurons were both Pax2$^+$ and GAD-GFP$^+$ (**Figure 4D**; $n = 3$; 4 wpi). These results suggest that the ratio of newly converted Tlx3$^+$ vs. Pax2$^+$ neurons in the dorsal horn of spinal cord can be determined through the combinations of NeuroD1 and Dlx2 transcription factors.

NeuroD1-Converted Neurons Express Region-Specific Neuronal Subtype Markers

While NeuroD1-converted neurons appear to be mainly glutamatergic neurons in both the mouse cortex and spinal cord, we further investigated whether they are the same type of glutamatergic neurons or not. For this purpose, we injected the same AAV GFAP::Cre and AAV FLEX-NeuroD1-mCherry into the mouse M1 motor cortex and the spinal cord, and then performed a serial immunostaining using both cortical neuronal markers (FoxG1 and Tbr1) and spinal neuronal markers (Tlx3 and Pax2) at 4 wpi (**Figure 5**). The majority of NeuroD1-infected cells were converted into neurons in both the brain and the spinal cord at 4 wpi (**Figures 5A,C**). Strikingly, when we compared the neuronal subtypes resulting from NeuroD1-mediated conversion in the brain vs. the spinal cord side-by-side, a distinct pattern emerged: the converted neurons in the mouse cortex acquired cortical neuron markers such as FoxG1

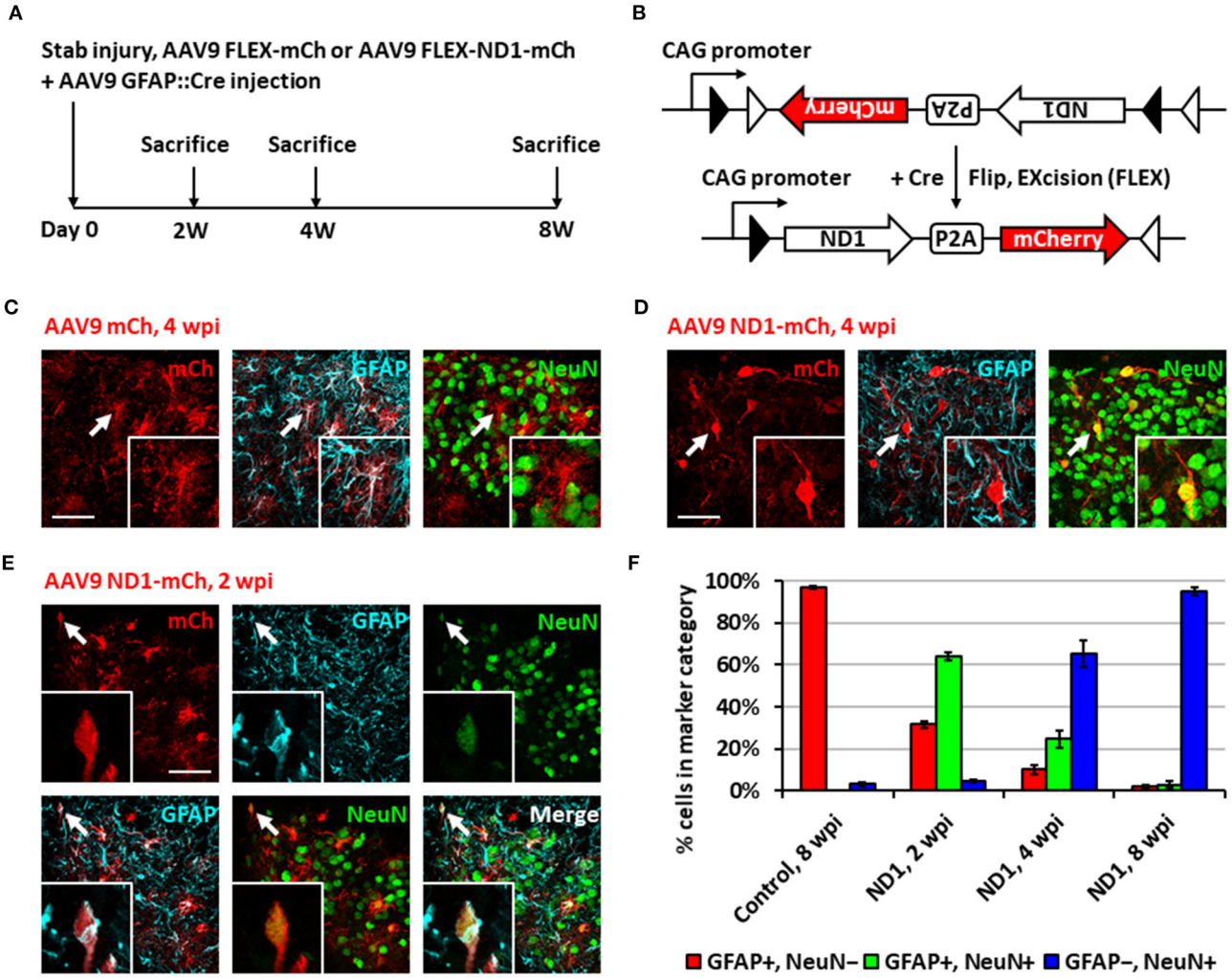

FIGURE 2 | Neuronal conversion using AAV9 NeuroD1 viruses after stab injury in the spinal cord dorsal horn. **(A)** Experiment paradigm. **(B)** AAV9 GFAP::Cre and AAV9 FLEX-NeuroD1-mCherry system (abbreviated elsewhere as AAV9 ND1-mCh). GFAP promoter restricts infected cells to astrocytes. Control virus replaces the NeuroD1 transgene with an additional mCherry. **(C)** Infected astrocytes in the dorsal horn at 4 wpi after control AAV9 mCherry injection. Arrows and inset show an example of mCherry-infected GFAP$^+$ astrocyte. Scale bar, 50 μm. **(D)** AAV9 NeuroD1-mCherry-infected cells became converted neurons in the dorsal horn at 4 wpi after viral injection. Arrows and inset show an example of NeuroD1-mCherry-labeled NeuN$^+$ cell. Scale bar, 50 μm. **(E)** A transitional cell (arrows and inset) captured during astrocyte-to-neuron conversion process that shows both GFAP$^+$ and NeuN$^+$ signals at early time point of 2 weeks after NeuroD1-mCherry infection. Scale bar, 50 μm. **(F)** Quantification data based on GFAP and NeuN staining for control or NeuroD1-infected cells at different time points (2, 4, and 8 wpi). Bars show the mean and standard deviation of three replicates. Infected cells at 2 wpi are mostly transitional, staining positive for both NeuN and GFAP. By 4 wpi, NeuroD1-infected cells are mostly converted neurons, staining positive for only NeuN.

(66.1 ± 14.3%) and Tbr1 (17.1 ± 1.9%), but not spinal neuron markers such as Tlx3 (0%) or Pax2 (0%) (**Figures 5A,B**); in contrast, the converted neurons in the spinal cord acquired spinal neuron markers Tlx3 (46.4 ± 2.2%) and Pax2 (4.2 ± 0.3%), but not cortical neuron markers FoxG1 (0%) or Tbr1 (0.6 ± 0.5%) (**Figures 5C,D**). Morphologically, the converted neurons in the brain resembled cortical pyramidal neurons with larger cell bodies (**Figure 5A**), while those in the spinal cord resembled dorsal horn interneurons with smaller cell bodies (**Figure 5C**). The relative lower percentage of Tbr1$^+$ cells among the converted neurons in the cortex suggest that the newly converted neurons may not be mature enough at 4 wpi and may take longer time to fully acquire their neuronal identity. These distinct differences in the neuronal identity after conversion by the same transcription factor in the brain vs. the spinal cord suggest that the glial cell lineage, here cortical lineage vs. spinal lineage, as well as the local environment may exert an important influence on the resulting subtypes of converted neurons.

NeuroD1-Converted Neurons Are Physiologically Functional

To test the functionality and circuit-integration of NeuroD1-converted neurons, we performed patch-clamp electrophysiological recordings of native and converted

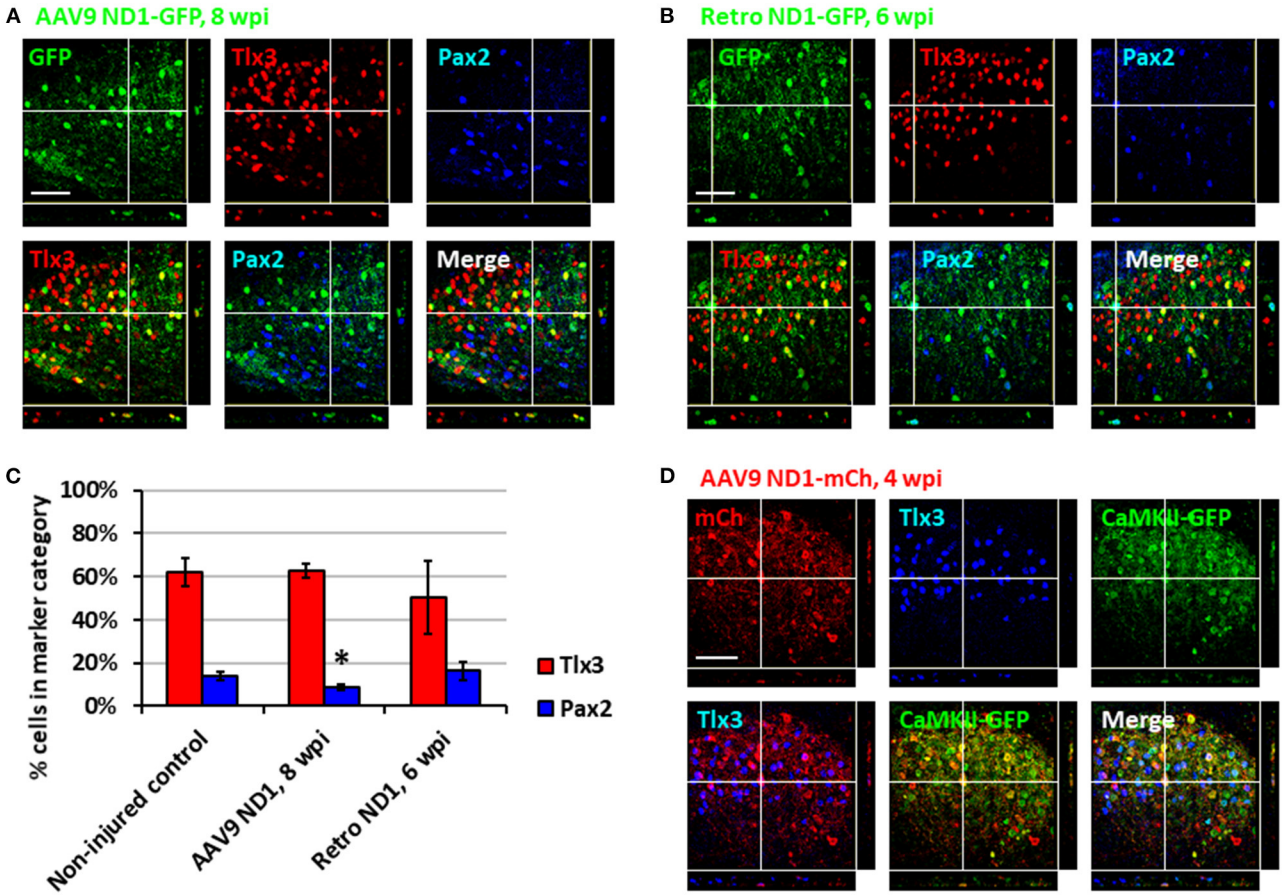

FIGURE 3 | Subtypes of NeuroD1-converted neurons in the spinal cord dorsal horn. **(A)** Tlx3 (glutamatergic) and Pax2 (GABAergic) subtype staining for converted neurons in the dorsal horn at 8 wpi after AAV9 NeuroD1-GFP injection. Z-projection targets an example of Tlx3$^+$ neuron. Scale bar, 50 μm. **(B)** Tlx3 and Pax2 subtype staining for converted neurons in the dorsal horn 6 wpi after retrovirus ND1-GFP injection. Z-projection targets an example of Pax2$^+$ neuron. Scale bar, 50 μm. **(C)** Quantification based on subtype staining after AAV9 ND1-GFP (8 wpi) or retrovirus ND1-GFP (6 wpi) conversion. Control data is based on NeuN$^+$ cells in uninjured, untreated tissue. Bars show the mean and standard deviation of three replicates. Kruskal-Wallis *H*-tests show no significant difference in % of Tlx3$^+$ or Pax2$^+$ neurons between the non-injured control and the retrovirus ND1-GFP groups ($p > 0.05$). Kruskal-Wallis *H*-tests show no significant difference in % of Tlx3$^+$ neurons ($p > 0.05$) but a significant difference in % of Pax2$^+$ neurons between the non-injured control and the AAV9 ND1-GFP groups (*$p < 0.05$). **(D)** AAV9 ND1-mCherry and CaMK2-GFP co-injection showed strong (89.5 ± 5.2%; $n = 3$) co-labeling of CaMK2 for converted Tlx3$^+$ neurons at 4 wpi. Z-projection targets an example of Tlx3$^+$, CaMK2$^+$ neuron. Scale bar, 50 μm.

neurons on spinal cord slices from mice sacrificed at 8–10 wpi (**Figure 6A**). The converted neurons could generate repetitive action potentials (**Figure 6B**) and displayed large Na^+ and K^+ currents (**Figure 6C**). Moreover, we detected robust spontaneous EPSCs from the NeuroD1-converted neurons (**Figure 6D**). Quantitatively, we found that the NeuroD1-converted neurons showed no significant difference in Na^+ currents (**Figure 6E**) and spontaneous EPSCs from their neighboring native neurons (**Figure 6F**). Immunostaining with a series of synaptic markers including SV2 and VGlut1/VGlut2 further confirmed that the NeuroD1-converted neurons were surrounded by numerous synaptic puncta with many of them directly innervating the neuronal soma and dendrites (**Figures 6G,H**, cyan and yellow dots). Finally, cFos, an immediate early gene that is typically activated by neuronal activity during functional tasks, was clearly detected in some of the NeuroD1-converted neurons, indicating that they were functionally active in the local spinal cord circuits (**Figure 6I**). Altogether, our results demonstrate that NeuroD1 can reprogram reactive astrocytes into functional neurons in the dorsal horn of the injured spinal cord.

NeuroD1-Mediated Cell Conversion in the Contusive SCI Model

To move closer toward clinical situations, we evaluated NeuroD1-mediated neuronal conversion in the contusive SCI model. Compared with stab injury, contusive injury creates a much more severe injury environment, which could affect the efficiency of neuronal conversion and the survival of converted neurons. We therefore performed two experiments to test our AAV GFAP::Cre and Flex-NeuroD1-GFP system after contusive SCI: one short-delay injection to test our treatment as a response to acute injury (**Figure 7**) and one long-delay injection to

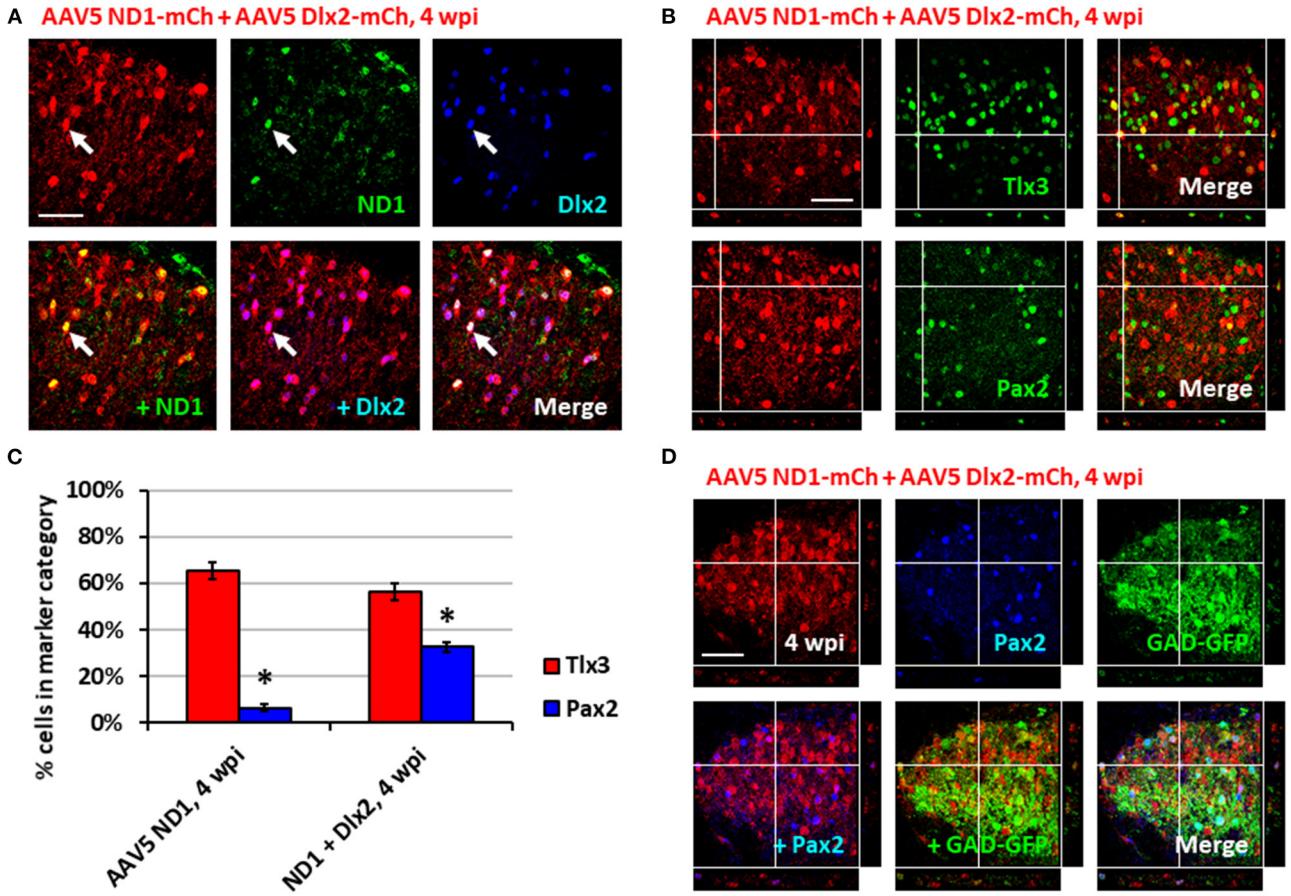

FIGURE 4 | Combining Dlx2 together with NeuroD1 generated more GABAergic neurons in the spinal cord dorsal horn. **(A)** Immunostaining confirmed the overexpression of both NeuroD1 and Dlx2 at 4 wpi following AAV5 NeuroD1-mCherry + AAV5 Dlx2-mCherry infection (1:1 ratio of 1×10^{10} GC/ml AAV5 NeuroD1-mCherry and 1×10^{10} GC/ml AAV5 Dlx2-mCherry mixed 9:1 with 1×10^{9} GC/ml AAV5 GFAP-Cre for a final titer of 5×10^{9} GC/ml AAV5 NeuroD1-mCherry, 5×10^{9} GC/ml Dlx2-mCherry, 1×10^{8} GC/ml AAV5 GFAP-Cre; 1 μL of virus mixture injected per mouse). Scale bar, 50 μm. **(B)** Tlx3 and Pax2 staining for converted neurons in the dorsal horn at 4 wpi after NeuroD1 and Dlx2 infection. Z-projections target example of $Tlx3^+$ or $Pax2^+$ neurons. Scale bar, 50 μm. **(C)** Quantitative comparison of Tlx3 and Pax2 subtype staining between neurons converted by NeuroD1 alone (4 wpi) or NeuroD1 + Dlx2 (4 wpi). Bars show the mean ± S.D. ($n = 3$ replicates). Kruskal-Wallis *H*-tests show significant differences in % $Tlx3^+$ and % $Pax2^+$ between the two groups ($^*p < 0.05$). **(D)** Co-immunostaining showing co-localization of NeuroD1+Dlx2-converted neurons (mCherry+) together with Pax2 and GAD-GFP at 4 wpi ($n = 3$). Z-projection targets an example of converted neuron being $Pax2^+$ and GAD^+. Scale bar, 50 μm.

test our treatment as a response to chronic injury (**Figure 8**). The advantage of the short-delay experiment is to maximize infection rate by taking advantage of the post-injury proliferation of reactive astrocytes, while the advantage of the long-delay experiment is to maximize the neuronal survival after conversion by allowing injury-induced neuroinflammation to taper down and minimize the secondary effects of the contusion injury. In our short-delay experiment, viral injection was conducted at 10 days post-contusive injury and tissues were collected at 6 weeks post-viral infection (**Figure 7A**). Viral injections were performed 1 mm away from the contusion site to avoid the injury core (**Figure 7B**). The injury core is apparent after contusion and is characterized by the loss of $NeuN^+$ neuronal cell bodies (**Figure 7C**, labeled by *). Viral injection at 10 days post-contusion resulted in many GFP^+ cells surrounding the injury core in both control GFP and NeuroD1-GFP groups (**Figure 7C**), indicating good infection rate and survival of the AAV-infected cells in the contusive SCI model. On the other hand, the AAV NeuroD1-GFP infected cells showed a dramatic morphological difference from the control GFP group (**Figure 7C**). As illustrated in the enlarged images in **Figure 7C**, the GFP infected cells in the control group showed typical astrocytic morphology and colocalization with GFAP signal (magenta), but rarely showed any colocalization with the neuronal marker NeuN (red). In contrast, NeuroD1-GFP infected cells were often colocalized with NeuN but rarely colocalized with GFAP (**Figure 7C**), indicating successful neuronal conversion. Quantitatively, we counted the total number of converted neurons to be ∼2,000 cells surrounding the lesion core areas (**Figure 7D**). The efficiency of NeuroD1-mediated neuronal conversion in the short-delay experiment as measured by NeuN immunoreactivity was ∼55% (**Figure 7E**), while the remaining cells were mostly $GFAP^+$

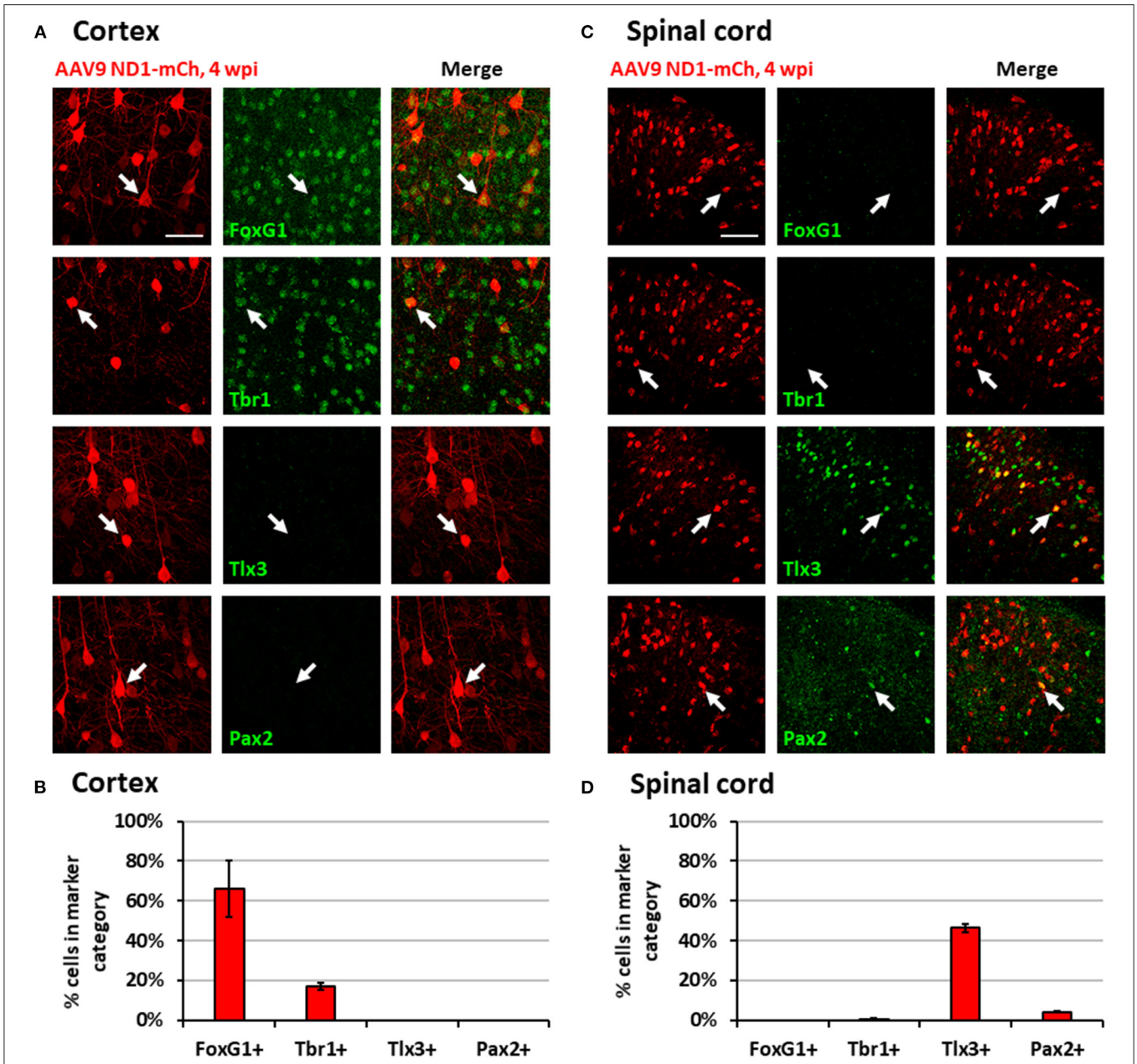

FIGURE 5 | Region-specific subtypes of NeuroD1-converted neurons in the brain vs. the spinal cord. **(A)** Subtype staining for converted neurons in the cortex 4 wpi after AAV9 NeuroD1-mCherry injection. Arrows show examples of cells positive for each subtype. Scale bar, 50 μm. **(B)** Quantification based on subtype staining in the cortex for AAV9 NeuroD1-mCherry samples (4 wpi). Bars show the mean ± S.D. ($n = 3$ replicates). **(C)** Subtype staining for converted neurons in the spinal cord dorsal horn 4 wpi after AAV9 NeuroD1-mCherry injection. Arrows show examples of cells positive for each subtype. Scale bar, 50 μm. **(D)** Quantification based on subtype staining in the spinal cord for AAV9 NeuroD1-mCherry samples (4 wpi). Bars show the mean ± S.D. ($n = 3$ replicates).

(**Figure 7F**). In contrast, the GFP-infected cells were mostly GFAP$^+$ astrocytes and rarely NeuN$^+$ neurons (only 3.9% NeuN$^+$ in GFP group) (**Figures 7E,F**).

In our long-delay experiment, viral injection was conducted at 4 months post-contusive injury, when glial scar has been well formed after contusion, and tissues were collected at 10 weeks post-viral infection (**Figure 8A**). As in the short-delay experiment, the injury core was apparent after contusion and characterized by the loss of NeuN$^+$ signal (**Figure 8B**, labeled by *). In the control AAV GFP alone group, the viral infected cells were mainly S100b$^+$ astrocytes (**Figure 8C**), but rarely showed any NeuN$^+$ signal (**Figure 8D**, top row). In contrast, the majority of NeuroD1-GFP infected cells were converted into NeuN$^+$ neurons (**Figure 8D**, bottom row; quantified in **Figure 8E**). The NeuroD1-mediated conversion efficiency reached >95% (**Figure 8E**). We observed mature

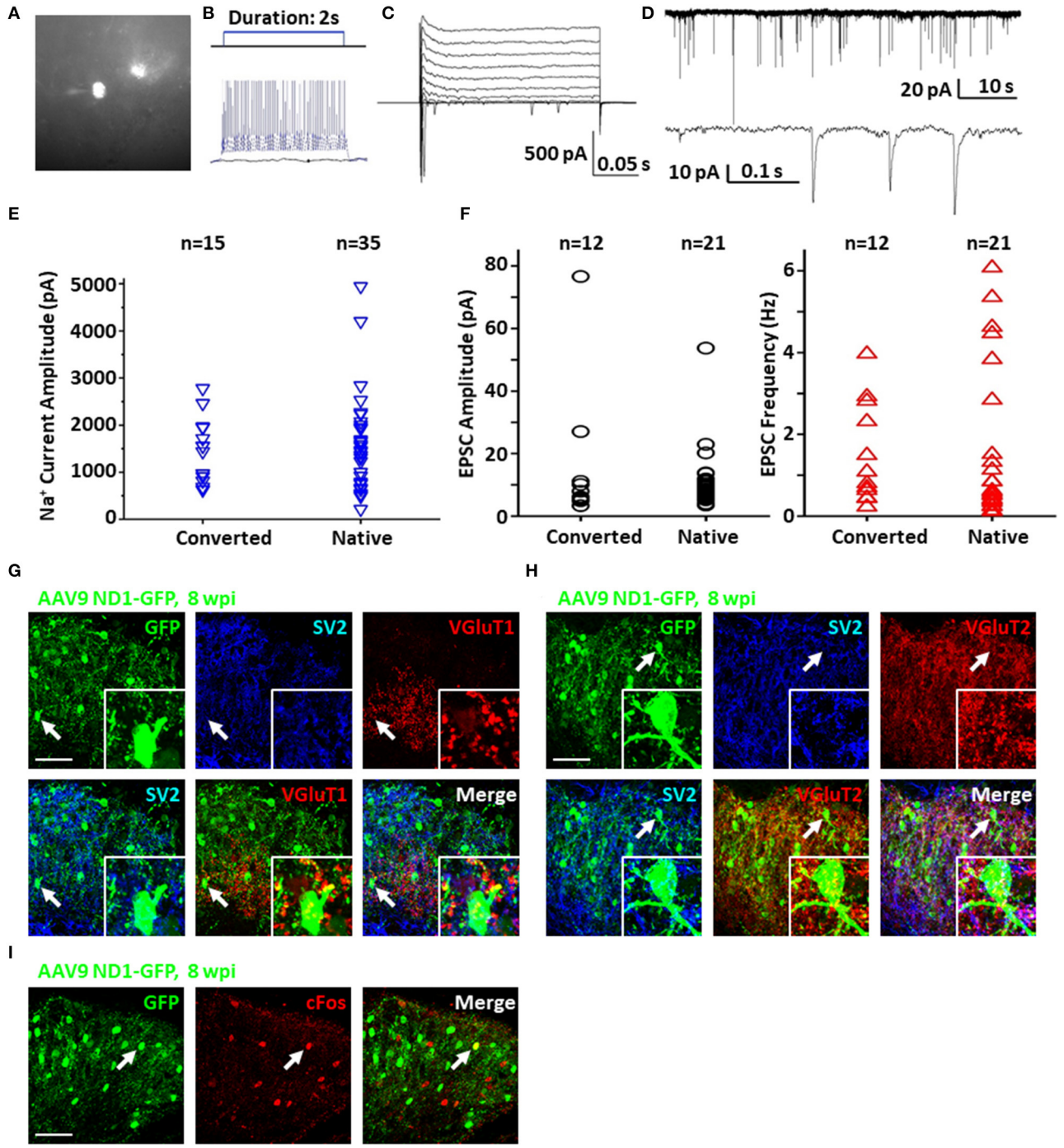

FIGURE 6 | Functionality of NeuroD1-converted neurons in the spinal cord dorsal horn. **(A)** Representative image of a NeuroD1-converted neuron for patch-clamp recording in spinal cord slices. **(B)** Sample action potentials of a converted neuron. **(C)** Sample Na^+ and K^+ currents of a converted neuron. **(D)** Sample EPSCs of a converted neuron. **(E)** Na^+ current amplitudes for converted and native neurons. Student's two-tailed *T*-test shows no significant difference between the two groups ($p > 0.5$). **(F)** EPSC amplitudes and frequencies for converted and native neurons. Student's two-tailed *T*-tests show no significant difference between the two groups ($p > 0.5$). **(G)** Synaptic SV2 and VGluT1 puncta observed on converted neurons in the dorsal horn at 8 wpi after AAV9 NeuroD1-GFP injection. Arrows and inset show puncta on the soma and processes. Scale bar, 50 μm. **(H)** Synaptic SV2 and VGluT2 puncta on converted neurons in the dorsal horn 8 wpi after AAV9 NeuroD1-GFP injection. Arrows and inset show puncta on the soma and processes. Scale bar, 50 μm. **(I)** Integration of converted neurons into local network in the dorsal horn 8 wpi after AAV9 ND1-GFP injection. Activated neurons indicated by c-Fos staining were only a small subset of all neurons. C-Fos staining was performed at 2 h after mouse exercised for 15 min on a running wheel.

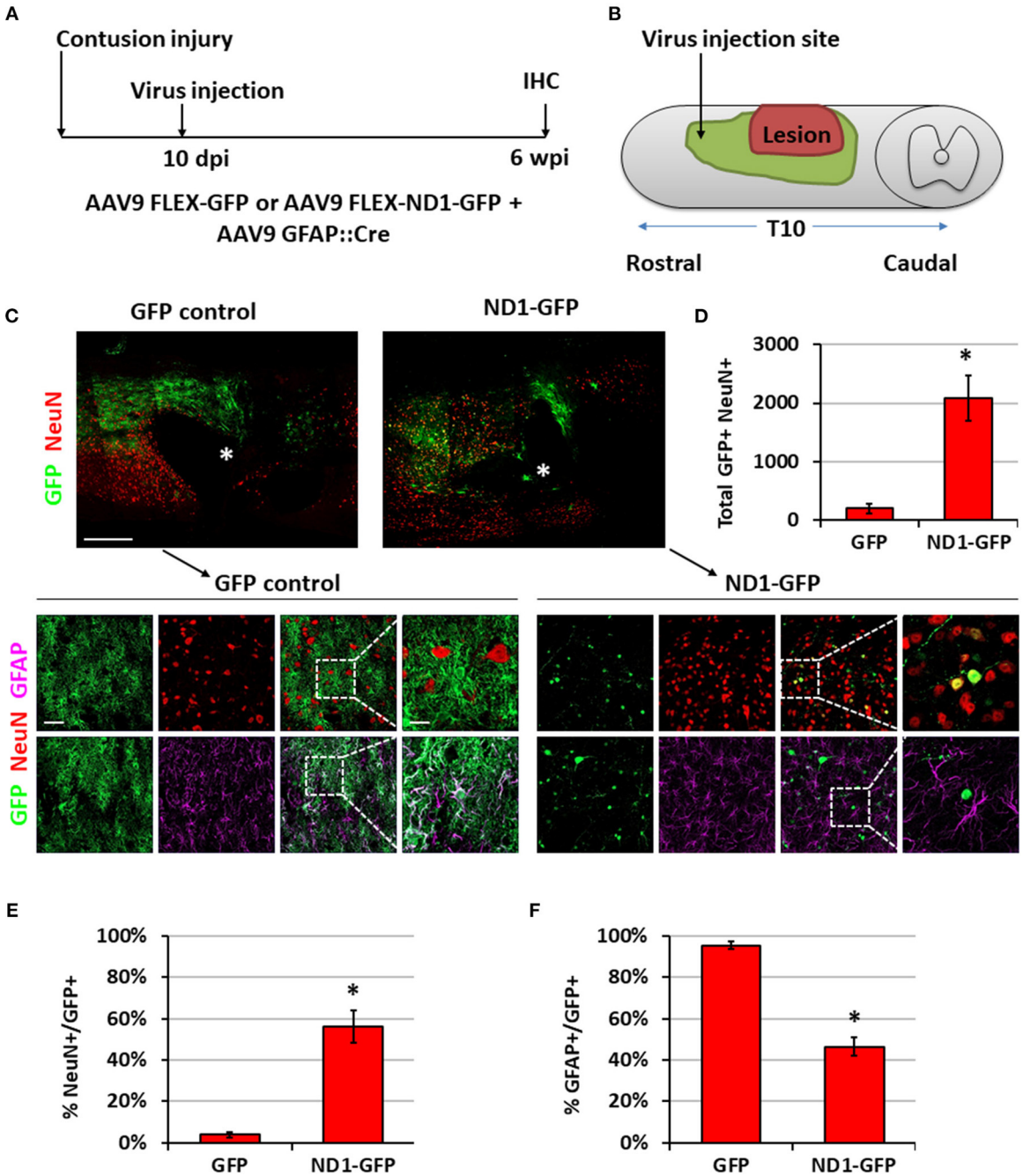

FIGURE 7 | NeuroD1 converts reactive astrocytes into neurons around the injury core with a short delay of viral injection after contusive SCI. **(A)** Experimental design showing viral injection at 10 days post a contusive SCI (30 Kdyn force). Spinal cords were analyzed at 6 weeks post viral infection. **(B)** Schematic drawing shows viral injection position: 1 mm anterior of the contusion site, 0.4 mm lateral of the central artery, and from 0.8 mm to 0.4 mm below the tissue surface. **(C)** Many infected cells survived around the injury core (indicated by *) and showing distinct cellular morphology between the two groups. Immunostaining of the neuronal markers GFAP and NeuN indicates successful neuronal conversion from reactive astrocytes by NeuroD1-GFP. Scale bars, 1 mm at overview, 50 μm at low-mag, 20 μm at high-mag. **(D)** Quantified number of converted neurons per infection (i.e., the average number of both GFP^+ and $NeuN^+$ cells per horizontal section calculated from one dorsal, one central, and one ventral section, and then multiplied by the total number of horizontal sections per sample). Kruskal-Wallis *H*-test shows a significant difference between the two groups ($n = 3$ for each group; $p = 0.05$). **(E)** Quantified $NeuN^+$ signal at 6 wpi. Kruskal-Wallis *H*-test shows a significant difference between the two groups ($n = 3$ for each group; $p = 0.05$). **(F)** GFAP staining shows a significant decrease in the number of astrocytes after NeuroD1 conversion at 6 wpi. Kruskal-Wallis *H*-test shows a significant difference between the two groups ($n = 3$ for each group; $p = 0.05$).

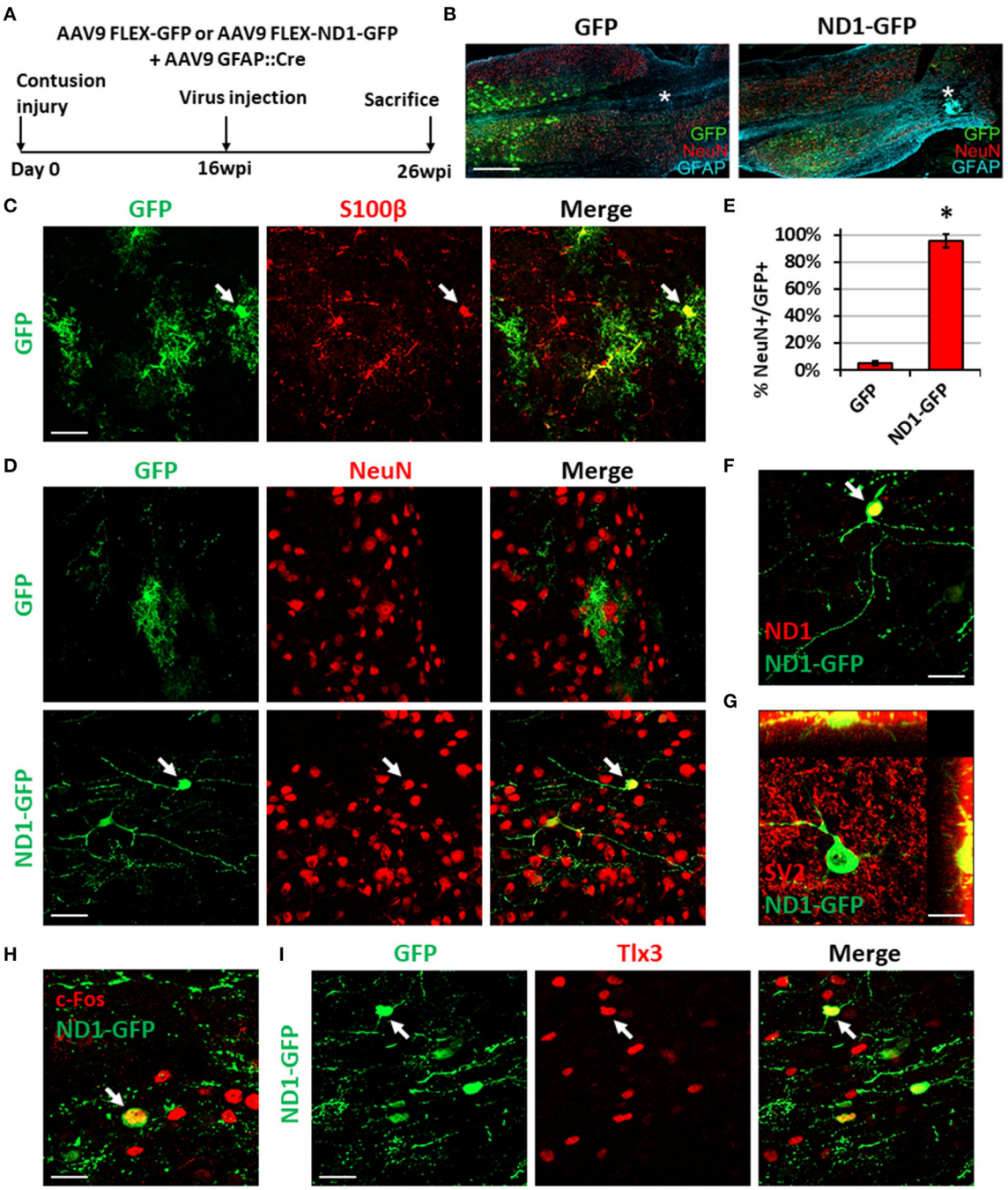

FIGURE 8 | NeuroD1-mediated neuronal conversion with a long delay of viral injection after contusive SCI. **(A)** Experimental design showing viral injection at 16 weeks after a contusive SCI (30 Kdyn force). The spinal cords were analyzed at 10 weeks post viral infection. **(B)** Many infected cells survived around the injury core (indicated by *) and showing distinct cellular morphology between the two groups. Scale bar, 1 mm. **(C)** Co-expression of the astrocyte marker S100b in control AAV GFP-infected cells. Scale bar, 50 μm. **(D)** Immunostaining of the neuronal marker NeuN indicates successful neuronal conversion from reactive astrocytes by NeuroD1-GFP with high efficiency. Scale bar, 50 μm. **(E)** Quantified neuronal conversion efficiency (95.6 ± 5.1%) at 10 wpi. Kruskal-Wallis *H*-test shows a significant difference between the two groups ($n = 4$ for the NeuroD1-GFP group and $n = 3$ for the GFP group; $p = 0.034$). **(F)** Co-expression of NeuroD1 protein in the NeuroD1-GFP-infected cells. Scale bar, 20 μm. **(G)** Co-expression of synaptic marker SV2 on the NeuroD1-GFP$^+$ cells. **(H)** Co-expression of the neuronal activity marker c-Fos in the NeuroD1-GFP$^+$ cells. Scale bar, 20 μm. **(I)** Co-expression of the glutamatergic subtype marker Tlx3 in NeuroD1-GFP$^+$ neurons in the spinal cord dorsal horn. Arrows show an example Tlx3$^+$ cell. Scale bar, 20 μm.

neuronal morphology including longer and more branching processes at this late time point and confirmed NeuroD1 overexpression in the NeuroD1-GFP infected cells (**Figure 8F**). Furthermore, NeuroD1-converted neurons at 10 wpi were surrounded by many synaptic puncta (SV2) with some of them directly innervating the soma and dendrites (**Figure 8G**, yellow dots). We also identified c-Fos$^+$ cells among NeuroD1-converted neurons (**Figure 8H**), indicating that they were able to integrate into the local spinal cord functional circuitry. Lastly, some of the NeuroD1-converted neurons in the contusive SCI model at 10 wpi showed glutamatergic subtype through expression of Tlx3 in the dorsal horn (**Figure 8I**), consistent with our stab injury model. Altogether, these results indicate that NeuroD1 overexpression can reprogram reactive astrocytes into functional neurons after contusive SCI under both acute and chronic treatment conditions, with higher conversion efficiency achieved after glial scar formation. This clinically relevant model can be used in future studies to further test functional improvement after SCI using *in vivo* cell conversion technology.

DISCUSSION

In this study, we have demonstrated in different SCI models that overexpression of NeuroD1 in the reactive astrocytes can convert them into Tlx3-positive glutamatergic neurons in the dorsal horn of injured spinal cord. Other ongoing studies are also testing different transcription factors to reprogram spinal astrocytes into GABAergic neurons or motor neurons in the ventral horn. Using AAV Cre-Flex system, we efficiently reprogrammed reactive astrocytes after stab injury in the dorsal horn of spinal cord into functional neurons that integrate into the local synaptic network. Importantly, we also observed efficient NeuroD1-mediated neuronal conversion in a contusive SCI model making this technique a potential intervention to treat SCI by regenerating functional new neurons in the gray matter. The NeuroD1-converted neurons in the dorsal horn of injured spinal cord were Tlx3$^+$ glutamatergic spinal neurons, but not Tbr1$^+$ cortical neurons, indicating regional specificity of *in vivo* reprogramming. The fact that the same neural transcription factor NeuroD1 can convert cortical astrocytes into cortical neurons and spinal astrocytes into spinal neurons may hold the key to region-specific neural repair by using internal glial cells for neuroregeneration. In contrast, transplantation of external cells does not have this advantage of intrinsic cell lineage with region-specificity.

AAV Gene Delivery System for Neuronal Conversion

We have accomplished successful NeuroD1-mediated neuronal conversion with retroviral vectors in this study and previous studies (Guo et al., 2014; Chen et al., 2020). However, since retrovirus particles are relatively large, limiting the viral titer, and since retrovirus only infects proliferating cells, the viral injection timing is confined to a narrow time window after the injury during which glial cell proliferation is increased. In contrast, because AAV infects both proliferating and quiescent cells, the AAV injection depends less on timing and can be performed in both acute and chronic injury conditions. Additionally, AAV has passed clinical trials and elicits little immune response when applied *in vivo* (Zaiss et al., 2002; High and Roncarolo, 2019). AAV particles are small and can be prepared at very high titer, therefore high dosage of conversion factors can be achieved when multiple AAV particles infect a single cell. Consistent with this, we observed a slower neuronal conversion when a lower titer of AAV-NeuroD1 was injected in the injured spinal cord (unpublished observation). Since certain serotypes of AAV can cross the blood-brain-barrier (BBB), intravenous delivery of conversion factors has made it possible to reach a broader area of the CNS (Foust et al., 2009; Chan et al., 2017). Following our earlier report of efficient conversion of reactive astrocytes into functional neurons by NeuroD1 through retroviral infection (Guo et al., 2014), intravenous injection of AAV9 expressing NeuroD1 has been reported to infect a small but significant number of resting astrocytes in the striatum and convert them into neurons (Brulet et al., 2017). On the other hand, our intracranial injection of AAV9 expressing NeuroD1 in stab injury model (Zhang et al., 2020) and ischemic injury model (Chen et al., 2020) have both resulted in high conversion efficiency, suggesting that reactive astrocytes after injury are more likely converted into neurons than the resting astrocytes. Consistent with this hypothesis, our results in this study also support that reactive astrocytes in injured spinal cord can be effectively converted into neurons with ∼95% efficiency, regardless of retrovirus or AAV delivery system. Compared to the high efficiency of astrocyte-converted neurons in the NeuroD1 group, we did observe a small percentage (typically <5%) of neurons labeled by GFP in the control group (**Figure 8E**). This might be due to the fact that AAV can infect both neurons and astrocytes efficiently. While we have used GFAP promoter to restrict the transgene expression in astrocytes, it is known that promoters usually are not 100% specific, and GFAP promoter can have low activity in neurons especially under injury condition.

The high efficiency of astrocyte-to-neuron conversion is the major reason why we target astrocytes among glial cells for *in vivo* conversion. Importantly, we have demonstrated in the mouse cortex that after astrocyte-to-neuron conversion, the remaining astrocytes can proliferate and replenish themselves (Zhang et al., 2020). Unlike killing reactive astrocytes which has been reported to make injury worse (Anderson et al., 2016), we demonstrated that converting reactive astrocytes into functional new neurons significantly ameliorated the glial scar in the brain, leading to a reversal of glial scar back to neural tissue (Zhang et al., 2020). Another practical reason for targeting astrocytes is that among glial cells including astrocytes, NG2 cells (OPCs), and microglia, AAV preferentially infect astrocytes, with much less infection rate on NG2 cells or microglia. AAV-based gene therapy so far is relatively safe and preferred choice for CNS disorders, and FDA has approved a series of clinical trials using AAV. In order to develop AAV-based gene therapy for the treatment of CNS disorders, we selected astrocytes as our preferred target for *in vivo* cell conversion. Of course, other glial cells such as NG2 cells and microglia might have their own advantage for some particular

purpose (Guo et al., 2014; Heinrich et al., 2014; Torper et al., 2015; Pereira et al., 2017; Matsuda et al., 2019).

Distinct Functions of NeuroD1 During Neuronal Conversion

Neuronal conversion can be achieved by several neurogenic transcription factors (Li and Chen, 2016). Besides NeuroD1, Sox2, Ngn2, and Ascl1 have all been reported to convert glial cells into neurons (Grande et al., 2013; Niu et al., 2013; Guo et al., 2014; Heinrich et al., 2014; Su et al., 2014; Liu et al., 2015; Gascon et al., 2016). Sox2 is expressed in neural progenitors and functions to maintain progenitor identity (Bylund et al., 2003; Graham et al., 2003; Bani-Yaghoub et al., 2006). Therefore, it is not surprising that Sox2-mediated neuronal conversion has to go through a proliferation stage as shown by incorporation of BrdU and the expression of Ki67 (Su et al., 2014). In contrast, NeuroD1 is a neuronal differentiation transcription factor that instructs terminal differentiation of neuroprogenitors into neurons during early neural development (Miyata et al., 1999; Morrow et al., 1999; Gao et al., 2009). This might partially explain why NeuroD1 can achieve high neuronal conversion efficiency (>90%) comparing to ∼6% in the case of Sox2 (Su et al., 2014). An earlier report also showed that Ngn2-expressing retrovirus was able to promote neurogenesis in the injured spinal cord, but the number of newly generated neurons greatly decreases over time even when combined with growth factor treatment (Ohori et al., 2006). When combined with Bcl2, an anti-apoptotic gene, Ngn2-mediated neuronal conversion acquires a much higher efficiency, although the authors claim that Bcl2 plays a role independent of apoptotic pathways (Gascon et al., 2016). In sharp contrast, we rarely observe apoptotic cells during and after NeuroD1-mediated conversion as determined by TUNEL assay. The difference of cell survival in converted neurons between different transcription factors may be explained by the fact that NeuroD1 is not only a conversion factor but also a survival factor. During development, NeuroD1 is required for survival of a variety of neuronal subtypes in the developing and adult CNS (Miyata et al., 1999; Morrow et al., 1999; Gao et al., 2009). This dual role of NeuroD1 during neuronal conversion and neuronal survival may explain its higher conversion efficiency over Sox2 and Ngn2. Ascl1 has also been reported to induce high efficiency of *in vivo* astrocyte-to-neuron conversion in the midbrain (Liu et al., 2015), suggesting that Ascl1 might share certain common properties with NeuroD1. Since long-term survival of converted neurons is essential to their integration into local neuronal circuitry in order to have a role in functional repair, future studies on clinical translation must pay much attention to the total number of newly generated neurons that can survive for years to have effective therapies.

Environmental Cues to Impact Neuronal Conversion in Addition to Intrinsic Factors

Transcription factor-mediated *in vivo* neuronal reprogramming illustrates intrinsic power to convert reactive astrocytes into neurons. However, environmental cues also play a role in the success of neuronal conversion (Heinrich et al., 2014) as well as the phenotype of converted neurons (Grande et al., 2013). In this study, we found that NeuroD1-converted neurons in the injured mouse cortex were $Tbr1^+$ cortical neurons, but in the injured spinal cord the NeuroD1-converted neurons were $Tlx3^+$ spinal neurons. Therefore, the local environment, together with astroglial lineage, may be essential to functional integration of converted neurons into the local neuronal circuitry as they mature. Together, a complete neuronal conversion would need both intrinsic factors (transcription factors and glial lineage factors) and extrinsic factors (local cues) to solidify the identity of converted neurons.

Even within the spinal cord, local environment can be drastically different between the gray matter vs. the white matter, with neuronal soma confined to the gray matter and neuronal axons occupying the white matter. In our experiments using AAV, we rarely observed converted neurons in the white matter; using retrovirus, we observed some converted neurons in the white matter at early time points, but they rarely survived to 6 wpi. This has been similarly observed in cell conversion studies in the white matter (corpus callosum) of mouse brains (Liu et al., 2020). Reasons for the lack of conversion in the white matter in this study could include our targeted injection technique which delivers virus precisely into the dorsal horn of the gray matter or the lack of appropriate viral receptors in the white matter. White matter may also lack sufficient trophic factors for the survival of newly generated neurons. On the other hand, Sox2-mediated neuronal conversion can result in many newborn neurons located in the white matter of the spinal cord, particularly in p21 knockout mice (Wang et al., 2016). An interesting feature of these neurons is that they appear as clusters, which, by providing trophic factors to each other, could be the reason they survive. It is also possible that the local environment in the white matter of p21 knockout mice has been altered during Sox2-mediated neuronal conversion in combination with BDNF and noggin. Further studies will be required to determine the differential effects of not only gray matter vs. white matter but also dorsal horn vs. ventral horn on neuronal conversion.

Generation of Neuronal Subtypes via NeuroD1-Mediated Conversion

We demonstrate here that NeuroD1 converts reactive astrocytes into primarily Tlx3-positive glutamatergic neurons in the dorsal horn of the injured spinal cord. Interestingly, Sox2-converted neurons in the injured spinal cord are also mainly glutamatergic (Wang et al., 2016), raising the possibility that glutamatergic neurons might be a default subtype of converted neurons. On the other hand, when we combine NeuroD1 with Dlx2 together, the proportion of GABAergic neurons was significantly increased after conversion, suggesting that the composition of neural transcription factors play an important role in the fate determination after conversion. NeuroD1 overexpression itself has been shown to inhibit GABAergic neuronal differentiation by suppressing Ascl1 (Mash1) (Roybon et al., 2010). Our result of NeuroD1 + Dlx2 suggests that Dlx2 can at least partially

antagonize the effect of NeuroD1 and pushing the astrocyte conversion more toward GABAergic neurons.

We noted that the glutamatergic neurons in the dorsal horn of the spinal cord can be $Tlx3^+$, $BarH1^+$, or $FoxD3^+$ (Bermingham et al., 2001; Gross et al., 2002; Cheng et al., 2005; reviewed in Lu et al., 2015). Therefore, some of the non-identified neurons may include $BarH1^+$ or $FoxD3^+$ glutamatergic neurons. On the other hand, considering the newly generated neurons were converted from astrocytes, they may not be mature enough to acquire definitive neuronal identity yet, or perhaps some will not become the neurons similar to their neighbors. More studies are required to fully characterize the neuronal identity after conversion for much longer time, such as 4–6 months after viral infection.

CONCLUSION

In summary, our study demonstrates that AAV NeuroD1-based gene therapy can convert reactive astrocytes into functional new neurons with high efficiency in the injured spinal cord. The AAV-NeuroD1 converted neurons can functionally mature and integrate into local neural networks. Interestingly, NeuroD1-converted neurons in the spinal cord dorsal horn mainly acquire a $Tlx3^+$ glutamatergic neuronal subtype, while combining NeuroD1 and Dlx2 together can generate more GABAergic neurons. Our next challenge is to further identify transcription factors that can regenerate motor neurons in the ventral horn, and ultimately test the beneficial effects of this cutting-edge *in vivo* astrocyte-to-neuron conversion technology in spinal cord repair.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Penn State IACUC.

AUTHOR CONTRIBUTIONS

GC conceived the idea, supervised the entire project, discussed the results, analyzed the data, and revised and finalized the manuscript. HL helped with the supervision of the project, performed some surgery experiment, discussed and analyzed the result, and helped with the manuscript writing. BP performed the major experiments, analyzed the data, made the figures, and wrote the draft of the manuscript. YD performed the contusive SCI experiments with the help from HL. BP analyzed the figure, made the figures, and wrote part of the manuscript. All other authors helped with the experiments and analyzed the data.

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This manuscript has been released as a pre-print at bioRxiv (Puls et al., 2019).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.591883/full#supplementary-material

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MINI REVIEW
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Extracellular Mitochondria Signals in CNS Disorders

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Mitochondria actively participate in the regulation of cell respiratory mechanisms, metabolic processes, and energy homeostasis in the central nervous system (CNS). Because of the requirement of high energy, neuronal functionality and viability are largely dependent on mitochondrial functionality. In the context of CNS disorders, disruptions of metabolic homeostasis caused by mitochondrial dysfunction lead to neuronal cell death and neuroinflammation. Therefore, restoring mitochondrial function becomes a primary therapeutic target. Recently, accumulating evidence suggests that active mitochondria are secreted into the extracellular fluid and potentially act as non-cell-autonomous signals in CNS pathophysiology. In this mini-review, we overview findings that implicate the presence of cell-free extracellular mitochondria and the critical role of intercellular mitochondrial transfer in various rodent models of CNS disorders. We also discuss isolated mitochondrial allograft as a novel therapeutic intervention for CNS disorders.

Keywords: extracellular mitochondria, central nervous system, mitochondrial transfer, biomarker, neurovascular unit

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INTRODUCTION

Mitochondria are the powerhouse of cells and essential for maintaining cellular function in mammals (Devine and Kittler, 2018). The role of mitochondria is especially important in a high-metabolic-rate organ like the brain. Mitochondria produce adenosine triphosphate (ATP) (Jonckheere et al., 2012; Murphy et al., 2019), play a central role in fatty acid biosynthesis (Kastaniotis et al., 2017) and cellular calcium homeostasis (David and Barrett, 2003; Pivovarova and Andrews, 2010), and also regulate intracellular mechanisms that modulate viability, immune cell activation, and mitophagy (Tait and Green, 2012). Accumulating mitochondrial reactive oxygen species (ROS) and inflammasome along with imbalanced mitochondrial membrane permeability may cause progression of cell death and neuroinflammation (Green et al., 2011; Angelova and Abramov, 2018). Therefore, restoring mitochondrial perturbation within cells is a major therapeutic strategy in many CNS disorders including stroke (Rutkai et al., 2019), hemorrhagic stroke (Wang et al., 2018), spinal cord injury (Gollihue et al., 2018a), Alzheimer's disease (AD) (Wang et al., 2020), and Parkinson's disease (PD) (Requejo-Aguilar and Bolanos, 2016).

Recent studies in rodents and humans demonstrate that mitochondria may be secreted into the extracellular milieu and transported or exchanged between cells in the CNS (Spees et al., 2006; Islam et al., 2012; Hayakawa et al., 2016; Sinha et al., 2016; Yao et al., 2018; Gao J. et al., 2019; Gao L. et al., 2019; Liu et al., 2019; Marlein et al., 2019; Miliotis et al., 2019; Al Amir Dache et al., 2020; Eun Jung et al., 2020; Park et al., 2020b). Under pathophysiological conditions in the CNS, extracellular mitochondria and their components might contribute to signals between cells that

could evoke detrimental inflammation or promote beneficial neuroprotection. In this regard, intact and active extracellular mitochondria may provide a novel therapeutic intervention to restore the bioenergetic needs of damaged or diseased recipient cells. In this mini-review, we collected studies of endogenous mitochondrial transfer as interdependent signals between cells, discuss extracellular mitochondria and its components as a novel class of mediators and biomarkers of injury or disease, and ultimately explore the clinical relevance of mitochondrial transplantation as a therapeutic approach for CNS disorders.

MECHANISMS OF EXPERIMENTAL MITOCHONDRIAL TRANSFER

Mitochondria support energy homeostasis in cells. But emerging evidence implicates that mitochondria are surprisingly transferred or exchanged between cells. The biological process was initially discovered in an *in vitro* cell culture system wherein mitochondrial DNA (mtDNA) from human mesenchymal progenitor cells (hMSCs) was delivered to A549 ρo cells (Spees et al., 2006). Similar observations have been subsequently reported by other research groups that showed evidence of mitochondrial transfer between progenitor cells and damaged cells *via* the formation of tunneling nanotubes (TNTs) (Onfelt et al., 2006; Kesner et al., 2016). The TNT-dependent mitochondrial transfer has been suggested in human-originated endothelial progenitors (Koyanagi et al., 2005) and MSCs but not in fibroblast (Yang et al., 2016) and rescued UV stress damage in PC12 cells, cigarette smoke-induced lung damage, and acute respiratory distress syndrome in rodents (Li et al., 2014; Wang and Gerdes, 2015; Jackson et al., 2016).

Extracellular vesicles (EVs) or exosomes can also deliver intracellular mitochondria or the components to other cells. It has been shown that glioblastoma cells and astrocytes produced exosome-enclosed mtDNA, supporting the idea that mtDNA may be delivered to cells through exosomal transfer (Guescini et al., 2010). In the retina, the vesicle-mediated transfer of mitochondria allows neurons to eliminate non-functional mitochondria within astrocytes *via* transmitophagy while these molecular mechanisms are still understudied (Davis et al., 2014). On contrary, astrocyte-to-neuron mitochondrial transfer has been reported as neuroprotection mechanism that reactive astrocytes after stroke produced extracellular mitochondria through CD38-cADPR signaling and these mitochondria were able to enter damaged neurons through an integrin-src/syk pathway to rescue damaged neurons (Babenko et al., 2015; Hayakawa et al., 2016).

It is important to note that the beneficiality of mitochondrial transfer may depend on context. For instance, activated astrocytes after focal cerebral ischemia may express regenerative genes (A2), whereas neurodegenerative diseases activate proinflammatory phenotype (A1) (Zamanian et al., 2012; Khakh et al., 2017; Liddelow et al., 2017; Trias et al., 2018; Yun et al., 2018). Inflammatory microenvironment where A1 astrocytes are enriched also increases proinflammatory microglia, and the crosstalk between inflammatory glia may exacerbate neurodegeneration through intercellular crosstalk *via* disrupted and fragmented mitochondria (Joshi et al., 2019). Collectively, extracellular mitochondria produced by glia can also be deleterious by expanding neuroinflammation and rigorous assessment of the functional property may provide a glimpse into the severity of neurodegeneration.

Although the molecular mechanisms underlying mitochondrial release and internalization remain to be fully elucidated, several pathways are being actively investigated. Recent proof-of-concept studies demonstrate that CD38 signaling may regulate mitochondrial transfer (Hayakawa et al., 2016; Huang et al., 2019; Lippert and Borlongan, 2019; Sun et al., 2019). Stimulating a CD38 downstream with cADPR or amplifying CD38 expression increased functional extracellular mitochondria secreted from astrocytes (Hayakawa et al., 2016). When astrocytes had a mutation R239C in GFAP gene, astrocyte-mediated mitochondrial transfer to neurons was disrupted accompanied by decreasing CD38 expression (Gao L. et al., 2019). What remains missing from the collective literature is the mechanism of how mitochondria are able to maintain their functionality. It has been proposed that mitochondrial protein posttranslational modification may support the functionality outside cells and be a key mechanism to support mitochondrial energy production, mitochondrial membrane potential, and motility in recipient cells postmitochondrial transfer (Yuzwa et al., 2012; Wang et al., 2016; Yang and Qian, 2017; Park et al., 2020a). A proof-of-concept study has been reported. When astrocytic CD38 was stimulated by NAD+, O-GlcNAc posttranslational modification in mitochondrial proteins was amplified, and these O-GlcNAcylated mitochondria maintained mtDNA content and membrane potential in extracellular space, thus improving neuroprotective effects after mitochondrial transfer (Park et al., 2020b).

Other intracellular mechanisms have been studied. The interaction between mitochondria and the ER may facilitate mitochondrial transfer within the osteocyte dendritic network (Gao J. et al., 2019). Moreover, gap junction protein connexin 43 or mitochondrial transport coordinator Miro 1 may be involved in mitochondrial transfer mechanisms regulated by mesenchymal stem cells (Islam et al., 2012; Ahmad et al., 2014). Cellular stress or stimulus can also trigger mitochondrial secretion and transfer. It has been reported that stressed cells that had lost cytochrome *c* triggered mitochondrial transfer to prevent apoptosis in PC12 cells subjected to UV exposure (Wang and Gerdes, 2015). ROS produced during oxidative stress have been suggested to trigger the secretion of mitochondria (Islam et al., 2012; Torralba et al., 2016; Zhang et al., 2016). TNT was produced in induced pluripotent stem cells (iPSCs) by tumor necrosis factor alpha (TNF-α) and the microstructure interacted with cardiomyocytes through TNFαIP2 expression, which may promote the effective transfer of mitochondria (Zhang et al., 2016). Furthermore, when extracellular mitochondria were internalized into the cells, activation of several pathways including endocytosis (Wei et al., 2018), integrin-dependent pathways (Hayakawa et al., 2016), macro-pinocytosis (Kitani et al., 2014), TNT, or cell fusion (Spees et al., 2006; Torralba et al., 2016) have been observed. Future studies are

warranted to identify the specific internalization pathway when extracellular mitochondria are either healthy or disrupted under pathophysiological conditions (**Figure 1**).

EXTRACELLULAR MITOCHONDRIA AS A BIOMARKER FOR MITOCHONDRIAL INTEGRITY IN THE CNS

Under normal conditions, healthy mitochondria maintain their functionality through continuous fission and fusion cycles. During injury or disease, the disruption of the process to integrate damaged mitochondria into the mitochondrial homeostatic system subsequently activates the process of mitochondrial elimination *via* mitophagy, mitochondria-specific autophagy that is a subtype of autophagy regulated by autophagosomes and lysosomes (Ashrafi and Schwarz, 2013), and recovered amino acids and fatty acids following the degradation process can be recycled to generate ATP (Twig et al., 2008; Nakatogawa et al., 2009), supporting that mitophagy is involved in maintaining mitochondrial quality and metabolic status.

The similar mitochondria elimination mechanism may be present in neuron–glial network in retina (Davis et al., 2014). In this study, electron microscopy and confocal microscopy determined that retinal ganglion cells transferred mitochondria-contained vesicles to retinal astrocytes to eliminate damaged mitochondria through so called transmitophagy. In the context of PD, the mechanism of transmitophagy may be crucial to maintain mitochondrial function in dopaminergic neurons and prevent neuroinflammation mediated by disrupted mitochondrial secretion (Morales et al., 2020). In this study, Morales and colleagues observed that damaged dopaminergic neurons *via* 6-OHDA infusion generated spheroid-like structure containing mitochondria and transferred them to adjacent astrocytes for digesting damaged mitochondria through STX17/Lamp1/Lamp2/LC3+ autophagolysosomes

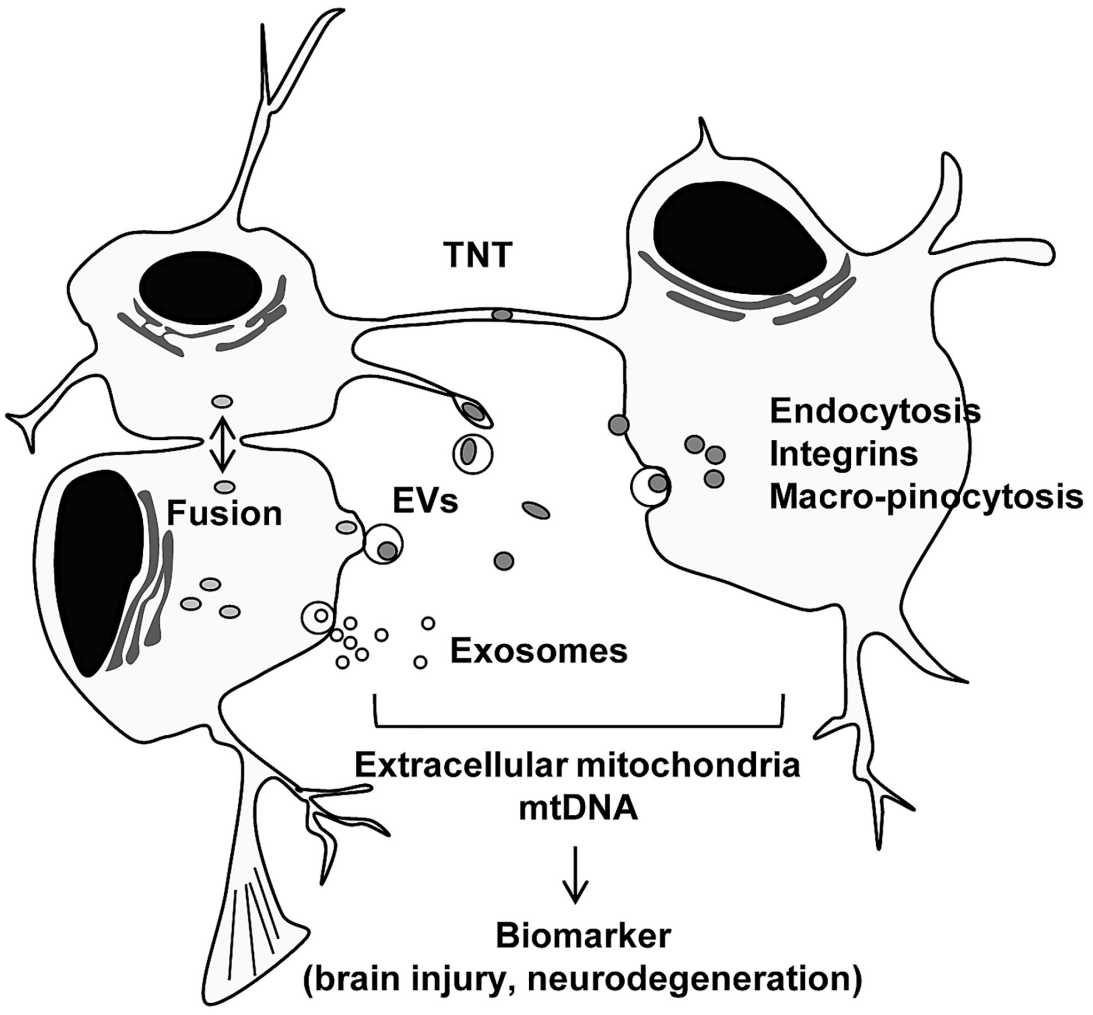

FIGURE 1 | Intercellular mitochondrial transfer. Intercellular mitochondrial transfer can be regulated by tunneling nanotubles (TNT), extracellular vesicles (EVs), exosomes, and cell fusion. Extracellular mitochondria may be incorporated into cells through canonical endocytosis pathway, integrin-mediated pathway, and macro-pinocytosis. Extracellular mitochondria and mtDNA may provide a glimpse into the status of tissue metabolism, disease severity and recovery in the CNS.

(Morales et al., 2020). Collectively, the transmitophagy may be a mechanism of mitochondrial quality control through intercellular mitochondrial transfer in the CNS.

Secreted mitochondria and its components in blood and cerebrospinal fluid (CSF) can be considered promising biomarkers for injury or disease. The basic hypothesis is that extracellular mitochondrial functionality reflects intracellular metabolism and can be indirectly assessed the underlying tissue metabolic integrity (Hayakawa et al., 2018; Miliotis et al., 2019). In CSF analysis in subarachnoid hemorrhage (SAH), membrane potentials of extracellular mitochondria were decreased in SAH patients, and higher JC1 ratios were associated with better clinical recovery at 3 months after SAH (Chou et al., 2017; Youn et al., 2020). Cell-free mtDNA becomes a damage-associated molecular pattern (DAMP) acting as a "danger signal" (Galluzzi et al., 2012; Thurairajah et al., 2018). Therefore, the amount of mtDNA in CSF may also implicate inflammation status. In fact, higher mtDNA contents in CSF were correlated to the progression of AD (Cervera-Carles et al., 2017) or anti-NMDA receptor encephalitis (Peng et al., 2019).

Altogether, extracellular mitochondria and mtDNA may be a biomarker for mitochondrial integrity, disease progression, or recovery in CNS pathology including neuroinflammation, hemorrhagic stroke, and neurodegenerative disease (**Figure 1**). Additional studies are needed to address whether the concept of extracellular mitochondria as a biomarker is applicable in other CNS disorders such as spinal cord injury and PD.

MITOCHONDRIAL TRANSPLANTATION AS CNS THERAPIES

In the CNS, neuron-glial-vascular interaction is essential for a homeostatic network, the so-called neurovascular unit in the CNS (Iadecola, 2004; Lo et al., 2004; Hawkins and Davis, 2005; Zacchigna et al., 2008; Zlokovic, 2008; del Zoppo, 2009) and functional impairment of mitochondria within the unit can be one of the major reasons to cause CNS disorders. McCully and colleagues have studied mitochondrial transplantation to the heart as a promising therapy without inducing detrimental immune response (McCully et al., 2016; Shin et al., 2019). These studies have supported scientists to investigate therapeutic efficacy utilizing exogenous mitochondria for CNS injury or neurodegeneration. Accumulating evidence has shown the beneficial actions of mitochondrial transplantation and the limitation in various animal models of CNS disorders (**Figure 2**).

Spinal Cord Injury

The therapeutic potential of extracellular mitochondria has been reported in a mouse model of spinal cord injury (SCI) (Gollihue et al., 2018a,b). In this study, GFP-labeled mitochondria (50–150 μg) were isolated from PC12 cells and then transplanted into the injury site after SCI onset. Following direct microinjection, macrophages, endothelial cells, and astrocytes incorporated injected mitochondria accompanied by improving acute mitochondrial bioenergetics and mitochondrial respiration (Gollihue et al., 2018a). Intriguingly, injected mitochondria were not found in neurons and did not support functional neuroprotection, implicating that mechanisms of mitochondrial internalization may be different among cell types. Ultimately, the study provides a very important aspect that mitochondrial incorporation efficiency in neurons may need to be improved to accelerate functional recovery after SCI.

Kuo and colleagues attempted to prevent axonal degeneration by mitochondrial transplantation in a rat model of sciatic nerve crush injury. In this study, BHK cell-derived mitochondria were utilized for therapeutic intervention. Dosages of mitochondria (0, 65, 130, 195, and 269 μg) were initially assessed in the cultured sciatic nerve. After the determination, extracellular mitochondria (195 μg) were injected directly into the injured nerve. Notably, mitochondria transplantation increased neuronal regeneration and decreased oxidative stress along with improving functional behaviors and physiological neuronal and muscle activities. Moreover, mitochondrial transplantation increased the expression of neurotrophic factors such as BDNF and CNTF in injured nerves along with restoring muscular integrity and increasing muscle progenitors and total muscle mass (Kuo et al., 2017).

Parkinson's Disease

Many genes involved in familial PD have been identified and known to directly link to mitochondrial dysfunction (Lill, 2016). The impairment of the respiratory chain, redox homeostasis, mitophagy, and mitochondrial biogenesis in dopaminergic neurons may aggravate PD pathogenesis (Moon and Paek, 2015). Given the fact that mitochondria-targeting antioxidants such as coenzyme Q10 and creatine monohydrate did not effectively ameliorate PD pathology (Beal et al., 2014; Kieburtz et al., 2015), healthy mitochondrial transplants to the damaged brain area can be an alternative approach to recover mitochondria-mediated redox homeostasis within neurons if these mitochondria can successfully incorporate into disrupted cells.

A study has been conducted using a mouse model of MPTP-induced PD. Fluorescent-labeled mitochondria were delivered systemically *via* tail vein injections in this model. Intriguingly, infused mitochondria (IV route, 0.5 mg/kg body weight) were ubiquitously found in CNS tissues and peripheral organs. Most importantly, mitochondrial infusion significantly improved motor functions accompanied by recovering mitochondrial complex I activity and ATP production, thus inhibiting cell death pathways in the striatum in comparison with the vehicle group (Shi et al., 2017), suggesting that intravenous infusion of mitochondria may be feasible to target not only peripheral organs but also CNS tissues.

Another study has utilized a mitochondrial modification technique with Pep-1 to improve mitochondrial delivery into CNS tissues. Interestingly, mice treated with Pep-1-conjugated mitochondria into the substantia nigra pars compacta (1.05 μg/5 μl) showed better behavioral outcomes in locomotor activity, moving distance, and moving speed compared to ones treated with unmodified control mitochondria (Chang et al., 2016). Concomitantly, mitochondrial incorporation in TH+ dopaminergic neurons was improved, suggesting that mitochondrial modification can amplify the therapeutic potential

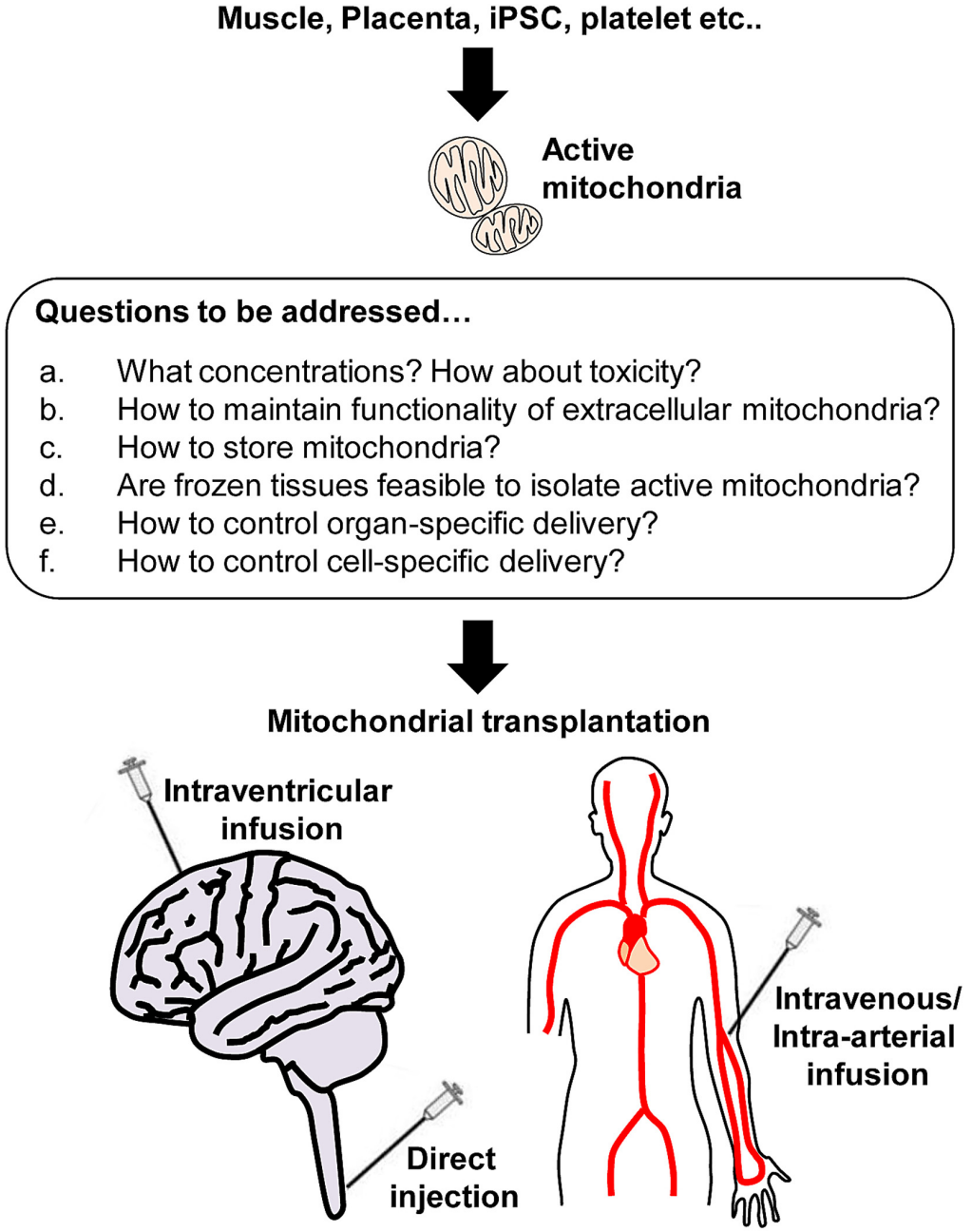

FIGURE 2 | Active mitochondria isolation from various sources and beyond. Respiratory active mitochondria are able to isolate from various sources including skeletal muscle, placenta, iPSC, and platelets. However, many questions remain to be answered regarding mitochondrial transplantation. a: It is critical to identify the optimal concentration of mitochondria that do not induce toxicity. b: Protecting extracellular mitochondria against pathological environments (high calcium, ionic imbalance, high ROS, glycation etc.) would be challenging, but it is required to avoid disrupting mitochondria while maximize the benefit induced by mitochondrial transplantation. c-d: Storing mitochondria is the most challenging issue. Instead, it might be worth seeking if frozen tissues can be sources to isolate a larger amount of active mitochondria. e-f: Finally, it is critical to investigate how to deliver mitochondria to target tissues or cells to maximize the therapeutic efficacy.

of exogenous mitochondria and the beneficiality to enhance neuronal incorporation in the CNS.

Alzheimer's Disease

Alzheimer's disease is a neurodegenerative disease along with severe memory loss with impairment of episodic memory in the initial phases (Swerdlow et al., 2014). Accumulating evidence indicates that mitochondrial impairment may contribute to the pathology of AD. Indeed, brain mitochondria in AD have shown dysfunctional respiration chain complexes, decreased ATP generation, and an excessive amount of free radicals that may lead to neurodegeneration

(Parker and Parks, 1995; Pohland et al., 2018). In addition to therapies attacking amyloid-beta, targeting mitochondrial dysfunction may indirectly ameliorate AD pathology. Nitzan and colleagues have attempted mitochondrial transplantation to restore mitochondrial function in a mouse AD model produced by Aβ 1–42 intracerebroventricular infusion (Nitzan et al., 2019). Mitochondria isolated from HeLa cells were injected through the tail vein. As a result, IV-injected mitochondria were observed in the liver, but there was no signal in the brain following IV injection. Intriguingly, mitochondrial infusion still improved cognitive functions accompanied by decreasing loss of neurons and suppressing hippocampal glial activation. Moreover, mitochondrial functional parameters in the liver and brain were also restored. Collectively, mitochondrial transplantation through IV route can be a new therapeutic strategy for AD (Nitzan et al., 2019).

Mitochondrial transplantation also improved diabetes-associated cognitive impairment (DACI) which is associated with elevated Aβ deposition (Ma et al., 2020). In this study, platelet-derived mitochondria were used as the donor source. After intracerebroventricular injection of mitochondria in db/db mice, mitochondrial internalization to hippocampal neurons was observed, and subsequently, DACI was alleviated accompanied by restored mitochondrial function as well as decreased accumulation of Aβ. Although further rigorous assessments are required, it may be promising that mitochondrial transplantation may ameliorate AD pathology *via* inhibiting Aβ generation and deposition.

Ischemic Stroke

Therapeutic interventions including recombinant tissue plasminogen activator or endovascular thrombectomy provide effective ways to achieve reperfusion for acute ischemic stroke (Vosler et al., 2009). While restoring cerebral blood flow is essential for decreasing infarction, reperfusion alone may not be sufficient to fully salvage penumbral tissue in some patients where cells are dying through active cell death pathways (Lo, 2008). Therefore, it is still important to continue the search for neuroprotective approaches that can be added to ischemia reperfusion (Vosler et al., 2009). The brain with ischemic stroke results in a lack of supplementations for glucose and oxygen and mitochondrial disruption to generate ATP, thus causing mitochondrial metabolic impairment and leading to neuronal apoptosis. Collectively, mitochondrial restoration therapy utilizing extracellular mitochondria may also be applicable in ischemic stroke (Watts et al., 2013; Baek et al., 2014; Russo et al., 2018).

Mitochondrial transplantation may indeed provide some beneficial effects to protect neurons in models of ischemic stroke. It has been shown that intact mitochondria isolated from BHK-21 cell lines successfully protected neurons against ischemic insult. In this study, mitochondria were treated directly into the striatum (75 μg) or infused through arteries (750 μg) after transient focal cerebral ischemia. Neurons and glia in the peri-infarct area appeared to incorporate mitochondria treated *via* both routes at 4 weeks after ischemic stroke. Moreover, treatment with isolated mitochondria significantly improved motor function accompanied by decreasing infarction and TUNEL-positive cells in acute stroke (Huang et al., 2016). Treatment with skeletal muscle-derived mitochondria may also provide beneficial neuroprotective effects (Zhang et al., 2019). Freshly isolated mitochondria from skeletal muscle (5×10^6/10 μl) were infused into the lateral ventricle immediately after reperfusion after focal cerebral ischemia. Consistent with prior findings, the peri-infarct neurons incorporated the treated mitochondria. Subsequently, the allograft remarkably attenuated infarct formation accompanied by restoring neurological impairments, reducing oxidative stress response and gliosis, as well as neuronal death at 28 days poststroke. Collectively, mitochondrial allograft may not only protect neurons against acute injury but also improve long-term outcomes after stroke.

Tissue source for viable mitochondrial isolation is important for practical use of mitochondria in therapy. A proof-of-concept study using placenta-derived mitochondria was performed in a mouse stroke model. From the snap-frozen placentae obtained from E17 pregnant female mice, mitochondria were isolated and evaluated the functionality. Surprisingly, flow cytometry analysis demonstrated that up to 87% of placental mitochondria were viable and maintained JC1 membrane potentials after isolation. Placental mitochondrial fractions contained ATP equivalent to mitochondrial fractions isolated from skeletal muscle and brown fat tissue. Moreover, glutathione reductase, Mn-SOD, and HSP70 were highly preserved in placental mitochondrial fractions. Then, placental-derived mitochondrial fractions (100 μg/100 μl) were infused intravenously immediately after reperfusion with full blinding and randomization. Strikingly, treatment with placental mitochondrial fractions significantly decreased infarction after focal cerebral ischemia in mice. Collectively, cryopreserved placenta can be a feasible source for viable mitochondrial isolation and transplantation with placental mitochondria may amplify beneficial effects of reperfusion in stroke (Nakamura et al., 2020).

CONCLUSION

Status of mitochondrial function is a key for recovery after CNS injury or disease (Anne Stetler et al., 2013; Madsen et al., 2017). Accumulating evidences implicate that mitochondria can be surprisingly present outside cells and transported from cell to cell. Moreover, extracellular mitochondria are found not only in rodents but also in clinical samples, and assessments of mitochondrial functionality in extracellular fluids may provide us a biomarker-like glimpse into injury or repair/regeneration. Within the emerging paradigm of targeting mitochondria after CNS injury or disease (She et al., 2017; Vekaria et al., 2017; Simon et al., 2019), viable mitochondria may be feasible to isolate and utilized for CNS therapy. Nonetheless, more rigorous studies are required to validate the reliability, safety, and efficacy of extracellular mitochondria in a wide range of applications

from diagnosis to therapeutic intervention in various CNS disorders.

AUTHOR CONTRIBUTIONS

J-HP and KH collected literature and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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APOE4 Promotes Tonic-Clonic Seizures, an Effect Modified by Familial Alzheimer's Disease Mutations

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Seizures are emerging as a common symptom in Alzheimer's disease (AD) patients, often attributed to high levels of amyloid β (Aβ). However, the extent that AD disease risk factors modulate seizure activity in aging and AD-relevant contexts is unclear. *APOE4* is the greatest genetic risk factor for AD and has been linked to seizures independent of AD and Aβ. The goal of the present study was to evaluate the role of *APOE* genotype in modulating seizures in the absence and presence of high Aβ levels *in vivo*. To achieve this goal, we utilized EFAD mice, which express human *APOE3* or *APOE4* in the absence (EFAD−) or presence (EFAD+) of familial AD mutations that result in Aβ overproduction. When quantified during cage change day, we found that unlike *APOE3*, *APOE4* is associated with tonic-clonic seizures. Interestingly, there were lower tonic-clonic seizures in E4FAD+ mice compared to E4FAD− mice. Restraint handing and auditory stimuli failed to recapitulate the tonic-clonic phenotype in EFAD mice that express *APOE4*. However, after chemical-induction with pentylenetetrazole, there was a higher incidence of tonic-clonic seizures with *APOE4* compared to *APOE3*. Interestingly, the distribution of seizures to the tonic-clonic phenotype was higher with FAD mutations. These data support that *APOE4* is associated with higher tonic-clonic seizures *in vivo*, and that FAD mutations impact tonic-clonic seizures in a paradigm dependent manner.

Keywords: Alzheimer's disease, apolipoprotein E, seizure, amyloid beta, sex

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INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia and is defined by cognitive decline, extracellular plaques containing amyloid-β (Aβ) and intraneuronal tangles of tau. In addition, accumulating evidence suggest that seizures are a common and important component of the AD phenotype (reviewed in Palop and Mucke, 2009; Friedman et al., 2012; Pandis and Scarmeas, 2012; Lam and Noebels, 2020). For example, the risk of clinical unprovoked seizures of unknown etiology is 6–10-fold higher in AD and between 1 and 60% of AD patients experience unprovoked seizures (Hauser et al., 1986; Risse et al., 1990; Romanelli et al., 1990; McAreavey et al., 1992; Mendez et al., 1994; Volicer et al., 1995; Hesdorffer et al., 1996; Amatniek et al., 2006; Lozsadi and Larner, 2006; Rao et al., 2009; Scarmeas et al., 2009; Bernardi et al., 2010; Irizarry et al., 2012; Imfeld et al., 2013; Vossel et al., 2013; Cheng et al., 2015; Sarkis et al., 2016). However, the

incidence of seizures in AD is likely to be underestimated because AD patients also experience non-convulsive seizures that are often unrecognized due to their symptomatic overlap with other behavioral changes such as memory loss, hallucinations, anxiety, and confusion. In support of this concept, subclinical epileptiform activity has been observed in up to 40% of AD patients when assessed by electroencephalogram recordings (Vossel et al., 2013, 2016; Brunetti et al., 2020; Lam et al., 2020). Recent data also suggest that seizures occur in early, preclinical stages of dementia and accelerate disease progression (Cretin et al., 2016; DiFrancesco et al., 2017; Costa et al., 2019; Keret et al., 2020). Therefore, evaluating the role of AD risk factors in modulating seizure activity is a crucial step to establish whether there is link with disease progression.

APOE genotype is the greatest genetic risk factor for sporadic AD, with *APOE4* increasing risk up to 12-fold compared to *APOE3* (reviewed in Mahley et al., 2007; Liu et al., 2013; Flowers and Rebeck, 2020). The role of *APOE* in AD is extremely complex and includes modulation of functions both independent and dependent of AD pathology (particularly Aβ). For example, *APOE4* is associated with learning and memory dysfunction during aging, independent of AD (reviewed in Tai et al., 2016), which is recapitulated in *APOE* targeted replacement mice (Grootendorst et al., 2005; Villasana et al., 2006; Bour et al., 2008; Rodriguez et al., 2013; Tai et al., 2016; Thomas et al., 2017; Zaldua et al., 2020). Critically, there is an association between *APOE* genotype and seizures (Hirsch, 2007). With *APOE4* there is higher epilepsy risk (Liang et al., 2019), particularly post trauma (Diaz-Arrastia et al., 2003; Harden, 2004), an earlier age of onset for intractable seizures (Briellmann et al., 2000; Gambardella et al., 2005; Kauffman et al., 2010) and greater memory dysfunction in patients with chronic temporal lobe epilepsy (Gouras et al., 1997; Gambardella et al., 2005; Busch et al., 2007). Unfortunately, *in vivo* research on this topic is limited, with only one report of higher seizures with *APOE4* in *APOE*-targeted replacement mice (Hunter et al., 2012). This study highlights the ability of *APOE* to modulate brain function independent of AD pathology, but there is a strong link between *APOE4* and Aβ. In humans and mice that overproduce Aβ, with *APOE4* levels of all different types of Aβ (soluble, soluble oligomeric, intraneuronal, extracellular) are higher compared to *APOE3*. Aβ itself is also linked to higher seizures as evidenced by the higher incidence of seizures found in familial AD (FAD) patients (reviewed in Palop and Mucke, 2009). FAD accounts for 5% of all cases and is caused by mutations in proteins (the amyloid precursor protein, or presenilins) that result in higher Aβ production; 40–80% of FAD patients experience seizures (Palop and Mucke, 2009). Mouse models of FAD mutations also develop epileptic spiking consistent with partial seizures (Palop et al., 2007; Minkeviciene et al., 2009; Siwek et al., 2015; Gureviciene et al., 2019), have a lower seizure threshold to pentylenetetrazole (Del Vecchio et al., 2004) and have higher audiogenic seizures (Westmark et al., 2010; Kazim et al., 2017). However, it is currently unknown whether *APOE* genotypes modulate seizures in the presence of human Aβ (i.e., *APOE*/FAD mice), and if the combination of *APOE4* and Aβ results in higher seizures compared to *APOE4* alone. Addressing these questions could provide novel insight on the contribution of *APOE* genotype to seizures in AD-relevant contexts, and therefore provide the framework for future research focused on identifying the underlying mechanisms.

The goal of the present study was to evaluate the role of *APOE* genotype in modulating seizure incidence in the absence and presence of high Aβ levels *in vivo*. To achieve this goal, we utilized EFAD mice that express *APOE3* or *APOE4* in the absence (EFAD−) or presence (EFAD+) of Aβ overproduction. We recorded total seizure incidence during cage changes, evaluated whether handling restraint or auditory cues precipitated seizures and assessed seizure threshold to pentylenetetrazole.

METHODS

Animals

All experiments procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. We used EFAD mice (Youmans et al., 2012), which were produced by crossing mice that express 5 Familial Alzheimer's disease mutations (5xFAD; APP K670N/M671L + I716V + V717I and PS1 M146L + L286V, C57BL6/B6xSJL) with *APOE*-targeted replacement mice (*APOE*-TR, C57BL6). EFAD non-carrier mice are $APOE^{+/+}$ $5xFAD^{-/-}$ (E4FAD−) and carriers are $APOE^{+/+}/5xFAD^{\pm}$ (EFAD+). All EFAD mice were at least 8 months old at the start of the study. Mice were group-housed in a 12–12-h light-dark cycle (lights off at 11 a.m. and on at 11 p.m.).

Colony Observations of Seizures

Tonic-clonic seizures are relatively easy to identify in the mouse colony as the noise during an episode is unique and loud and were recorded in EFAD mice during routine once weekly cage change by trained members of the animal husbandry staff. Cages and the corresponding mouse (tail) were marked that displayed seizure behavior during cage change at the end of the light cycle. Tonic-clonic seizures were reported if they occurred when the cage was removed from the housing rack, during cage changing in the changing station, or once the cage was placed back on the housing rack.

Acute Handling Restraint Stress-Induced Seizures

The prevalence of acute handling restraint stress-induced seizure behavior (tonic-clonic) was assessed in the same mice as those evaluated for seizures during cage change day. Mice were manually restrained for a maximum of 30 s using the scruffing technique that involves grasping the loose skin located around the dorsal aspect of the mouse's neck. If a seizure began while in restraint, the mouse was placed into an empty cage to assess the duration of tonic-clonic seizure and recovery. If a seizure did not begin while in restraint, the mouse was placed in a new cage and observed for 1 min for seizure occurrence, prior to returning to the home cage.

Audiogenic-Induced Seizures

A subset of EFAD mice were subjected to an audiogenic seizure protocol adapted from Yagi et al. (2005) to investigate seizure susceptibility triggered by sound. Briefly, mice were allowed to acclimate in the sound-attenuating cabinet (Ugo Basile) for 1 min prior to the presentation of four 11 kHz tone at an intensity of 105 dB for 20 s, with a 2 s interval. All behavioral changes were recorded using a video camera affixed to the chamber and evaluated off-line.

Pentylenetetrazole (PTZ)-Induced Seizures

A single dose (60 mg/kg, s.c.) of pentylenetetrazole (PTZ, Sigma-Aldrich™, St. Louis, United States; purity ≥ 99%) was used to evaluate seizure thresholds in the same cohort of mice exposed to the audiogenic protocol. After injection, mice were placed in acrylic boxes, and behavioral changes induced by PTZ were recorded for 30 min using a video camera above the testing arena and analyzed off-line by investigators blinded to *APOE* genotype and sex. This dose of PTZ typically induces a range of seizure-like behaviors from freezing and myoclonic twitches to tonic-clonic seizures (Brault et al., 2011; Garcia-Cabrero et al., 2013; Bezzina et al., 2015; Van Erum et al., 2020). Tonic-clonic seizures begin with freezing behavior alternating with myoclonic twitching of the forelimbs that progress to violent jumping and running. Mice with tonic-clonic seizures lasting greater than 3 min were euthanized. We also assigned a score to the types of seizures; 3 = tonic-clonic, 2 = freezing and 1 = no seizure.

Statistics

In **Figures 1** and **2** comparisons were made using Chi-squared test (GraphPad Prism version 8) to assess the incidence of seizure behaviors. Fisher's exact test was utilized when one variable equaled zero. $p < 0.05$ was considered significant. We employed sequential statistical analysis, by first focusing on our primary research question of whether *APOE* genotype modulates seizure behaviors (*APOE3* vs. *APOE4*). We then evaluated the impact of sex (male vs. female), and FAD mutations (EFAD + vs. EFAD-) on seizures within each *APOE* genotype. In **Figure 3** seizure score was evaluated via two-way ANOVA.

RESULTS

To identify how *APOE* genotype modulates behavior and brain function in aging and AD-relevant contexts, we utilize EFAD mice, which express human *APOE3* or *APOE4* in the absence (EFAD-) or presence (EFAD+) of FAD mutations that result in Aβ overproduction. Over the last few years, we started to observe seizures in mice expressing *APOE4*, especially after spending more time in the mouse colony during the dark/active cycles. Thus, the goal of this study was to systematically determine the effect of *APOE* on seizure frequency and thresholds in EFAD− and EFAD+ mice. Specifically, we utilized E3FAD−, E3FAD+, E4FAD−, and E4FAD+ mice, both male and female that were at least 8 months of age at the start of the study. The age cut-off was selected as anecdotally we had had not observed seizures in younger mice.

APOE4 but Not *APOE3* Is Associated With Tonic-Clonic Seizures During Cage Change Day

The types of seizures we observe in E4FAD− and E4FAD+ mice are tonic-clonic, which typically begins with freezing behavior, progresses to severe myoclonic twitching of the forelimbs with violent movements (uncontrolled jumping, running), followed by recumbency. Tonic-clonic seizures are readily identifiable, and after discussions with the animal husbandry staff, it became apparent that some EFAD mice had been seizing in response to the stimuli produced during routine cage change. Therefore, we sequentially evaluated the effect of *APOE* genotype, sex and FAD/Aβ on seizure frequency during weekly cage change over a 5-week period. All available mice of at least 8 months of age in the colony were utilized for this study: 23 male E3FAD−, 25 female E3FAD−, 58 male E4FAD−, 34 female E4FAD−, 28 male E3FAD+, 29 female E3FAD+, 46 male E4FAD+, 37 female E4FAD+ mice.

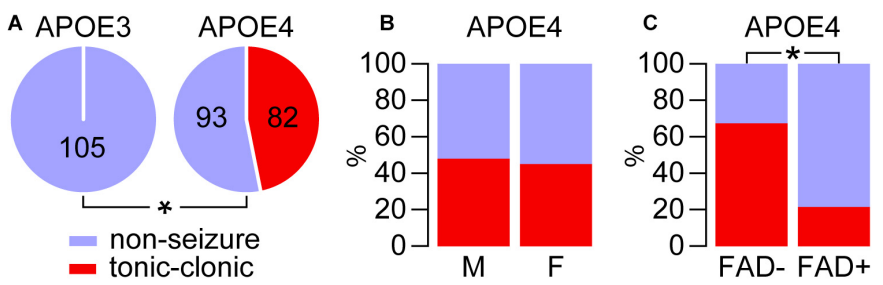

FIGURE 1 | *APOE4* but not *APOE3* is associated with tonic-clonic seizures during cage change day. The incidence of tonic-clonic seizures was evaluated during cage change day over a 5-week period in mice that express *APOE3* or *APOE4* in the absence (E3FAD– and E4FAD–) and presence (E3FAD+ and E4FAD+) of five familial AD mutations. **(A)** Tonic-clonic seizures manifested in mice that express *APOE4*, but not *APOE3* (*$p < 0.00001$, E4FAD– and E4FAD+ vs. E3FAD– and E3FAD+ mice, Fisher Exact test). **(B)** There were similar seizure frequencies in male and female *APOE4* mice ($p = 0.70$, male E4FAD– and E4FAD+ vs. female E4FAD– and E4FAD+, Chi-square test). **(C)** Interestingly, there were higher seizures in the absence of FAD mutations in *APOE4* mice (*$p < 0.0001$, E4FAD– vs. E4FAD+ mice, Chi-square test). $n = 23$ male E3FAD–, 25 female E3FAD–, 58 male E4FAD–, 34 female E4FAD–, 28 male E3FAD+, 29 female E3FAD+, 46 male E4FAD+, 37 female E4FAD+ mice.

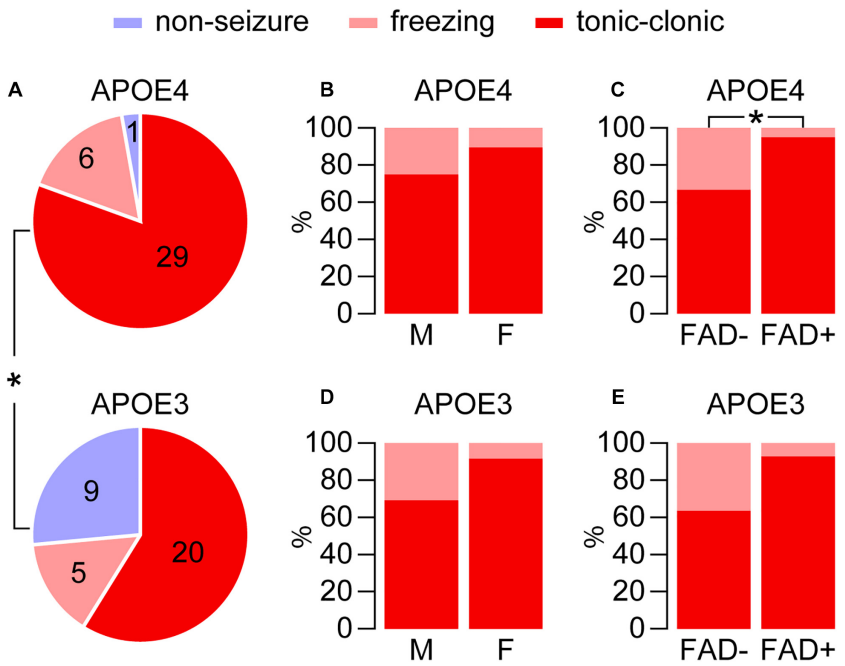

FIGURE 2 | Higher seizures with *APOE4* for pentylenetetrazole-induced seizures. Seizure frequency was evaluated in EFAD mice after treatment with pentylenetetrazole (PTZ, 60 mg/kg), which resulted in a range of seizure-like behaviors from freezing and myoclonic twitches to tonic-clonic seizures. **(A)** Seizure behaviors were modulated by *APOE* genotype and were higher with *APOE4* in EFAD mice (*$p < 0.028$, E4FAD– and E4FAD+ vs. E3FAD– and E3FAD+ mice, Chi-square test). **(B)** With *APOE4* expression, in male (12/16) and female (17/19) mice there was a similar distribution of seizures (tonic-clonic compared to total, $p = 0.26$, male E4FAD– and E4FAD + vs. female E4FAD– and E4FAD+, Chi-square test). **(C)** However, there was higher distribution of tonic-clonic seizures in E4FAD+ (19/20) compared to E4FAD– (10/15) mice (*$p = 0.028$, E4FAD– vs. E4FAD+ mice, Chi-square test). With *APOE3* expression **(D)** tonic-clonic seizure frequency was also similar for each sex ($p = 0.16$, 9/13 male E3FAD– and E3FAD+ vs. 11/12 female E3FAD– and E3FAD+, Chi-square test). **(E)** Although not statistically significant, there was trend of higher tonic-clonic seizure incidence with FAD mutations ($p = 0.07$, 7/11 E3FAD– vs. 13/14 E3FAD+ mice, Chi-square test). n = 6 male E3FAD–, 11 female E3FAD–, 8 male E4FAD–, 8 female E4FAD–, 9 male E3FAD+, 8 female E3FAD+, 9 male E4FAD+, 11 female E4FAD+.

We first assessed whether *APOE* genotype was associated with higher seizure frequency, though combining data for FAD- and FAD+ mice for each genotype (**Figure 1A**). There were tonic-clonic seizures in 46.9% of mice that express *APOE4* (82 out of 175) compared to 0% that express *APOE3* (0 out of 105). We next evaluated whether within the *APOE4* genotype group (E4FAD− and E4FAD+), biological sex affected seizure frequency. We found that both male and female *APOE4* mice showed similar frequency of seizure occurrence (**Figure 1B**): 48.1% in males (50/104) vs. 45.1% in females (32/71). Thus, unlike some other readouts found in this model (Aβ levels, neuroinflammation, cerebrovascular function) (reviewed in Tai et al., 2017; Balu et al., 2019), there is no effect of sex on seizures with *APOE4* during cage changing. We next asked whether *APOE4* alone or *APOE4* and FAD mutations/Aβ levels contribute to tonic-clonic seizures (**Figure 1C**). Our data demonstrate that E4FAD− mice (67.4%, 62/92) are more likely to experience seizure behaviors than E4FAD+ mice (24.1%, 20/93 E4FAD+). This effect was found within each sex, as both male (60.3%, 35/58 E4FAD− vs. 32.6%, 15/46 E4FAD+, $p < 0.01$, Chi-square) and female (79.4% 27/34 E4FAD− vs. 13.5%, 5/37 E4FAD+, $p < 0.0001$, Chi-square) E4FAD− mice showed higher incidence of seizures when compared to E4FAD+ mice. Collectively, these data demonstrate that *APOE4* is associated with tonic-clonic seizures compared to *APOE3*, and that FAD mutations/Aβ levels are associated with a lower number of seizures in *APOE4* mice.

Acute Handling Restraint and Auditory Stimulus Do Not Trigger Seizures in EFAD Mice

We next explored whether the effect of *APOE* genotype on tonic-clonic seizures can be elicited by acute stress alone. To this end, we conducted a standardized handling restraint test in all the mice that were evaluated for seizures during cage change day. During restraint handling, only 1.1% of mice that express *APOE4* (2 out of 175; 1 male E4FAD− and 1 female E4FAD−) showed seizures, whereas none of the *APOE3* mice (0/105) did. These data indicate that acute stress associated with handling is insufficient to induce seizures to the extent that we observed during cage change day with *APOE4* in EFAD mice.

An additional stressor that can induce seizures in mice, inducing FAD mice, is auditory stimuli, which we next evaluated in a subset of EFAD mice; 6 male E3FAD−, 11 female E3FAD−, 8 male E4FAD−, 8 female E4FAD−, 9 male E3FAD+, 8 female E3FAD+, 9 male E4FAD+, 11 female E4FAD+. We found that auditory stimulus alone is insufficient to induce seizures in any of the groups of EFAD mice tested. Together, these results suggest that acute stress due to handling and auditory stimuli do not

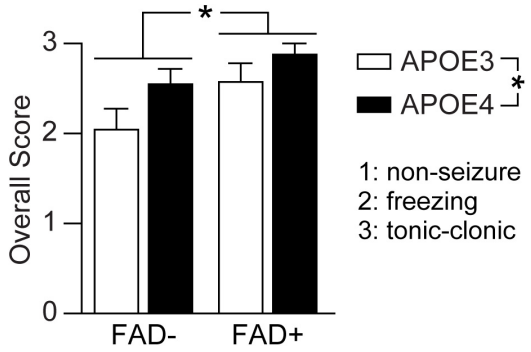

FIGURE 3 | *APOE* genotype and FAD mutations independently modulate pentylenetetrazole-induced seizures. Seizure score was evaluated in EFAD mice after pentylenetetrazole (PTZ, 60 mg/kg) treatment using a scale of 3 (tonic-clonic), 2 (freezing) and 1 (non-seizure). When assessed by two-way ANOVA, *APOE* and FAD modulated seizure score, however there was no interaction. Data is expressed as mean ± S.E.M. Data evaluated by two-Way ANOVA. $F(1, 65)$ = *APOE* genotype: 5.59, $^*p = 0.021$; FAD: 6.32 $^*p = 0.014$; *APOE* × FAD interaction: 0.331, $p = 0.5670$. $n = 6$ male E3FAD–, 11 female E3FAD–, 8 male E4FAD–, 8 female E4FAD–, 9 male E3FAD+, 8 female E3FAD+, 9 male E4FAD+, 11 female E4FAD.

mimic the number of seizures we observed with cage change day in EFAD mice.

Higher Seizures With *APOE4* for Pentylenetetrazole (PTZ)-Induced Seizures

As neither E3FAD− or E3FAD+ had seizures during cage change day, our next goal was to characterize the effect of *APOE* genotype on seizure susceptibility after chemical induction using PTZ (60 mg/kg). For these experiments we used the same cohort of mice subjected to auditory stimulus testing.

We paralleled our analysis of cage change day seizures, through first evaluating the role of *APOE* genotype in modulating seizure frequency after PTZ injection (i.e., E4FAD− and E4FAD+ vs. E3FAD− and E3FAD+). As found in other studies (Brault et al., 2011; Garcia-Cabrero et al., 2013; Bezzina et al., 2015; Van Erum et al., 2020) PTZ administration resulted in a range of seizure-like behaviors from freezing and myoclonic twitches to tonic-clonic seizures. Overall, the incidence of the combined seizure behaviors was higher with *APOE4* compared to *APOE3* (97.2%, 35/36 E4FAD− and E4FAD+ mice vs. 73.5%, 25/34 E3FAD− and E3FAD+ mice, **Figure 2A**). In addition to exhibiting a higher frequency of tonic-clonic seizures (80.5%, 29/36 *APOE4* vs. 58.8%, 20/34 *APOE3*, $p < 0.05$, Chi-square test), only 1 out of the remaining 7 *APOE4* mice showed no seizures compared to 9 out of the 14 *APOE3* mice.

We next evaluated if sex or FAD genotype within the *APOE4* group (E4FAD− and E4FAD+) modulated PTZ-induced seizures. There was similar frequency of total seizure occurrence in both male (94.1%, 16/17) and female (100%, 19/19) *APOE4* mice ($p = 0.47$, male E4FAD− and E4FAD+ vs. female E4FAD− and E4FAD+, Fisher's exact test). In addition, sex did not alter the distribution of seizures (tonic-clonic seizures compared to total seizures, 12/16 male, 17/19 female, **Figure 2B**). E4FAD− (93.8%, 15/16) and E4FAD+ (100%, 20/20) mice also exhibited comparable levels of total seizure occurrences ($p = 0.44$, E4FAD− vs. E4FAD+ mice, Fisher's exact test). However, when evaluated as a distribution of total seizures, a higher proportion of E4FAD+ mice (95%, 19/20) developed tonic-clonic seizures when compared to the E4FAD− group (66.6%, 10/15) (**Figure 2C**).

As with *APOE4*, a similar frequency of total seizures was observed between male (86.7%, 13/15 male) and female *APOE3* mice (63.2%, 12/19 female) ($p = 0.12$, male E3FAD- and E3FAD+ vs. female E3FAD− and E3FAD+, Chi-square test) with similar seizure distribution (9/13 male, 11/12 female, **Figure 2D**). E3FAD− (64.7%, 11/17) and E3FAD+ (82.3% 14/17) mice also exhibited comparable levels of total seizures ($p = 0.24$, E3FAD− vs. E3FAD+ mice, Chi-square test). Although there was a higher frequency of tonic-clonic seizures in E3FAD + mice (92.8%, 13/14) compared to the E3FAD− group (63.6%, 7/11), this was not statistically significant ($p = 0.07$, **Figure 2E**). Our study may have lacked power to detect differences in PTZ-induced tonic-clonic seizures between E3FAD- and E3FAD + mice. Therefore, we performed additional analysis by assigning a score (3 = tonic-clonic, 2 = freezing and 1 = no seizure) to each mouse after PTZ injection (**Figure 3**). When assessed by two-way ANOVA, a significant main effect of *APOE* and FAD were detected, indicating that both variables modulated the behavioral effects of PTZ. However, there was no *APOE* x FAD interaction, supporting our initial analysis that after PTZ treatment, the incidence of seizures is higher with *APOE4* and with FAD mutations.

Collectively, these results demonstrate that compared to *APOE3*, *APOE4* is associated with higher incidence of PTZ-induced seizures. Further, in contrast to cage change day, our data indicate that FAD mutations/Aβ levels are associated with a higher distribution of tonic-clonic seizures in *APOE4*, and potentially *APOE3* mice.

DISCUSSION

Compared to *APOE3*, *APOE4* is associated with tonic-clonic seizures when assessed during cage change day and greater seizure incidence after PTZ injection. Although sex did not modulate seizure incidence in EFAD mice, there was an important impact of FAD genotype. For tonic-clonic seizures in *APOE4* mice, FAD mutations were associated with lower incidence when measured during cage change day, but a higher distribution after PTZ injections. Collectively, our data support that *APOE4*-associated seizures are an important component of the behavioral phenotype in aging-and AD-relevant mice. Thus, research focused on evaluating the cellular basis of these seizures could provide mechanistic insight onto how *APOE* and FAD mutations modulate neural circuit function and connectivity.

Higher Seizure Incidence With *APOE4*: AD Relevance

Seizures have emerged as an important component of the AD phenotype, with multiple groups reporting higher seizure

incidence in patients with dementia and AD (reviewed in Palop and Mucke, 2009; Friedman et al., 2012; Pandis and Scarmeas, 2012; Lam and Noebels, 2020). Rather than a cause of AD, seizures represent a manifestation of altered neuronal function, which may exacerbate brain dysfunction and disease progression caused by the complex repertoire of AD pathologies. Although tonic-clonic seizures could be managed with anti-epileptic medications, sub-clinical seizures may be overlooked in AD patients and the underlying causes of the neuronal hyperexcitability will remain. Thus, understanding the extent AD risk factors modulate seizures could enable mechanistic research on their underlying causes that in turn can be translated to effective biomarker and therapeutic applications for AD patients in the clinic. In a disease as complex as AD, the threshold for neuronal dysfunction to produce seizures may be modified by genetic and lifestyle risk factors. In this study, we report that seizures are higher with one of the greatest genetic risk factors for AD, *APOE4*, in both the absence and presence of FAD mutations (see section "Seizure Incidence Is Modified by FAD Mutations but Not Sex" for discussion on sex and FAD). These data are consistent with reports suggesting that epilepsy risk is higher with *APOE4* (Liang et al., 2019), including after trauma (Diaz-Arrastia et al., 2003; Harden, 2004). Further, *APOE4* is associated with an earlier age of onset for intractable seizures (Briellmann et al., 2000; Gambardella et al., 2005; Kauffman et al., 2010), greater memory dysfunction with chronic temporal lobe epilepsy (Gouras et al., 1997; Gambardella et al., 2005; Busch et al., 2007) and higher epileptiform activity after hyperventilation (Ponomareva et al., 2008). Accordingly, targeted replacement mice with *APOE4*, which are similar to the E4FAD− mice, also showed higher seizures and a faster progression through PTZ-induced seizures (Hunter et al., 2012). It is therefore conceivable that compared to *APOE3*, *APOE4* would increase the incidence of seizures and the onset of epileptiform activity in AD patients, however, there are currently no reports of such an association. Thus, further pre-clinical and clinical studies are warranted to clarify whether the neuronal changes that increase seizure phenotypes with *APOE4* is independent of AD or interact with AD pathology to manifest as a different behavior in patients.

Seizure Incidence Is Modified by FAD Mutations but Not Sex

Previous studies in *APOE* knock-in mice have demonstrated that the effects of *APOE4* on a number of functions are particularly prominent in female mice. For example, detrimental effects of *APOE4* on learning and memory behaviors, Aβ levels, cerebrovascular function and neuroinflammation are typically higher in female mice (Balu et al., 2019). Interestingly, we did not observe an effect of sex for tonic-clonic seizures. Therefore, the changes in neuronal circuits that result in the manifestation of tonic-clonic seizures may be more proximal to the biological effects of apoE4, rather than an interaction with sex hormones.

FAD mice are known to exhibit epileptiform activity and lower threshold to seizure induction with auditory or PTZ stimuli; however, reports of tonic-clonic seizures are lacking (Palop and Mucke, 2009). 5xFAD mice were used to generate EFAD mice and are therefore similar to EFAD + mice but express mouse *APOE* and have an earlier onset of Aβ deposition. As for other FAD mice, to our knowledge there are no reports of tonic-clonic seizures in 5xFAD mice. However, when defined by electroencephalography recordings there are seizures in 5xFAD mice, possibly as early as 4 months (Abe et al., 2020), but that become prevalent at older ages (>10 months) (Paesler et al., 2015; Abe et al., 2020; Angel et al., 2020). In addition, one proposal is that the abnormal epileptiform activity predisposes 5xFAD mice to convulsive seizures with further stress, as has been demonstrated with genetic approaches (Paesler et al., 2015; Angel et al., 2020). Our data that 0% of E3FAD+ mice experience seizures are consistent with these findings, however, E4FAD+ mice did undergo a tonic-clonic seizures. These results indicate that in the presence of FAD mutations, compared to *APOE3 APOE4* may have lowered the threshold for the onset of tonic-clonic seizures, as proposed for additional stressors in 5xFAD mice.

Although our data support that compared to *APOE3*, *APOE4* is associated with higher tonic-clonic seizures, the precise interaction between *APOE4* and FAD mutations remained poorly understood. During cage change day, there were higher tonic-clonic seizures in E4FAD− mice compared to E4FAD+ mice. This result is somewhat surprising, since typically the assumption is that the combination of *APOE4* and FAD mutations would result in higher dysfunction than *APOE4* alone. One potential explanation for lower seizures in E4FAD+ mice compared to E4FAD− mice is that the FAD mutations have changed the types of seizures that are occurring with *APOE4* (e.g., to higher epileptiform activity and partial seizures as in FAD mice). On the other hand, it is possible that we have missed the detection of seizures in FAD + mice during, before or after cage changes. Alternatively, disruption of neuronal circuits involved in tonic-clonic seizures (e.g., brain stem, amygdala) with *FAD* and *APOE4* could also blunt or alter the response to stimuli produced during routine cage change. Indeed, our data obtained following PTZ injection suggest that there is higher neuronal dysfunction with FAD mutations as revealed by E4FAD+ mice exhibiting higher distribution of tonic-clonic seizures than E4FAD− mice, an effect that was also trending in *APOE3* mice.

It is also conceivable that distinct neural circuits are recruited between seizures induced during cage changes and elicited by PTZ. Stimuli produced during cage changes (sounds, new environment, handling) results in stress and anxiety in rodents, as evident from changes in behavior, hormone levels and heart rates (Duke et al., 2001; Meller et al., 2011; Rasmussen et al., 2011). In fact, placing a mouse in a new environment in ways that are similar, if not identical to the cage change procedure in our study is used as an assay of tonic-clonic seizures susceptibility (Todorova et al., 1999; Leussis and Heinrichs, 2006; Hunter et al., 2012; Qi et al., 2018). In this regard, tonic-clonic seizures during cage changes are the result of stress/anxiety signals inducing neuronal hyperexcitability. On the other hand, PTZ induces seizures by directly impacting neuronal activity, and although the precise mechanism of action is unknown (Hansen et al., 2004), it is thought to involve antagonism

of GABA-A receptors. Therefore, PTZ-induced tonic-clonic seizures are a more direct maker of alterations in neuronal functional connectivity (i.e., balance of GABAergic and glutamatergic inputs). Due to the different ways that they induce seizures, there are several potential explanations for the seemingly opposite effect of FAD mutations on *APOE4* associated cage change and PTZ-induced tonic-clonic seizures. For example, the combined effects of *APOE4* and FAD may have disrupted neuronal circuits to an extent that stress cannot induce seizures, yet the remaining neurons are more sensitive to chemical-induced seizures. Alternatively, there are greater memory impairments in E4FAD+ mice than E4FAD− mice, and so EFAD− mice may anticipate what the stimuli of cage change represents. Finally, E4FAD− and E4FAD+ mice may exhibit different levels of susceptibility to stress-induced effects through aging as a result of distinct mechanisms of adaptation occurring at the neural circuit level.

Potential Mechanisms Underlying *APOE4*-Associated Seizures

Our data also point to potential cellular mechanisms underlying the impact of *APOE4* on tonic-clonic seizure incidence *in vivo*. There are general and specific considerations for discussing this concept, all of which continue to be the focus of several research groups (reviewed in Mahley et al., 2007; Liu et al., 2013; Flowers and Rebeck, 2020). The question of how a single amino acid difference between apolipoprotein E3 (apoE3, cysteine at 112) and apolipoprotein E4 (apoE4, arginine at 112) results in modulation of such a wide range of functions in the brain is proving extremely complicated to answer and is likely context dependent. Apolipoprotein E is produced by cells in the periphery and in the brain. Within the central nervous system apoE is produced primarily by glia (astrocytes and microglia), but also by pericytes and neurons and all apoE is found on lipoprotein particles in the interstitial fluid. Therefore, one initial question surrounds the levels and lipidation state of apoE-containing lipoproteins. One suggestion is that apoE4-containing lipoproteins are lower in levels, less lipidated and/or smaller than apoE3-containing lipoproteins, which could have a profound impact on neural circuit connectivity and function. For example, changes in apoE levels and lipidation could disrupt lipoprotein functions in the interstitial fluid such as homeostasis of cholesterol and lipids, binding to debris and other substrates, and as an adaptor molecule. In addition, the structural properties of apoE4 are thought to result in altered activation and recycling of the apoE receptors in all cell types, and/or the generation of intracellular toxic apoE4 fragments in neurons. Thus, through these fundamental processes, apoE can alter neuronal network excitability directly, or indirectly through effects on inflammation, cerebrovascular function, and general homeostatic functions. Intertwined are an equally complex set of research questions that include but not limited to whether *APOE4* is a toxic gain or loss of function and whether *APOE4* imparts advantages on brain function during specific developmental windows that are detrimental in the context of aging and in response to stressors (Mahley et al., 2007; Liu et al., 2013; Flowers and Rebeck, 2020). That apoE impacts such a myriad of cell types and functions in normal and stress conditions is at the heart of why dissecting role of apoE in brain function is extremely complex. Specifically, in the context of seizures, all these changes during aging between *APOE3* and *APOE4* will likely converge to cause hyperexcitable neuronal networks in different brain regions that are important for tonic-clonic seizure manifestation.

APOE4 has been linked to changes in neuron structure and activity in multiple brain regions including the amydagala, cortex, and the hippocampus. Of particular interest is the emerging concept that apoE4 disrupts inhibitory network function (reviewed in Najm et al., 2019). For example, in *APOE*-targeted replacement mice, compared to *APOE3*, with *APOE4* there are lower levels of GABAergic somatostatin-positive interneurons in the hippocampus, an effect that appears driven by apoE production by neurons (Najm et al., 2019). Thus, the loss of GABAergic interneurons could contribute to network hyperexcitability and higher pyramidal cell firing (Nuriel et al., 2017). In humans, there is reduced deactivation of the default mode network with *APOE4* in task-based assays (Pihlajamaki and Sperling, 2009) in association with higher hippocampal (Dickerson et al., 2005) and entorhinal activation (Bondi et al., 2005). Collectively, these observations suggest that lower GABAergic activity with *APOE4* could reduce the threshold for seizures even though *APOE4* is known to disrupt excitatory neuronal activity as well (Mahley et al., 2007; Liu et al., 2013; Flowers and Rebeck, 2020). Overall, future studies are needed to establish the extent by which *APOE4* associated seizures are directly or indirectly dependent on neuronal excitability in different cortical and subcortical brain regions.

Limitations and Future Directions

Although we provided data on seizure incidence, an important question that remains unclear is the underlying neuronal mechanisms that are disrupted with *APOE4* to contribute to the development of seizures. Addressing this question is ultimately critical from mechanistic standpoint as well for testing in human patients. Future detailed experiments could focus on tracking epileptiform activity and seizure incidence in EFAD mice across the lifespan. We focused on older mice as we had not observed seizures during cage change day in younger mice, however there are some complications with utilizing older FAD mice (discussed in Tai et al., 2021), particularly for E4FAD+ mice. In E4FAD+ mice, Aβ pathology (soluble and extracellular) initiates around 4 months and so there is advanced pathology (e.g., Aβ, neuroinflammation, blood-brain barrier deficits) by 8 months of age. The high pathology may have altered brain function in ways that are too advanced to detect changes that are more relevant for early stages of Aβ deposition and evaluate the impact of *APOE4* and FAD on PTZ-induced seizure induction, which require younger mice. Ultimately, however, to provide more detailed mechanistic insight, pharmacological or genetic manipulations targeting either apoE, functions modulated by apoE or defined

cell types and circuits could be conducted to reveal a connection with seizure activity. A discussion on the limitations of mouse models is beyond the scope of this manuscript (reviewed in Tai et al., 2021), however, it will be important to evaluate whether a seizure phenotype also manifests in APP-knock in *APOE4* mice, to validate that findings are not due to the FAD mutations.

CONCLUSION

Our data demonstrate that unlike *APOE3*, *APOE4* is associated with tonic seizures, when evaluated during cage changes. There is also a higher incidence of tonic-clonic seizures with *APOE4* compared to *APOE3* following PTZ injection. However, in contrast to cage changes, the distribution of seizures to the tonic-clonic phenotype is higher with FAD mutations. These data support that *APOE4* is associated with higher tonic-clonic seizures, and that FAD mutations impact tonic-clonic seizures in a paradigm dependent manner.

DATA AVAILABILITY STATEMENT

All data supporting the conclusions of this manuscript will be made available to any qualified researcher without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago.

AUTHOR CONTRIBUTIONS

LL, FM, KT, and LT conceived the study, performed the experiments, and wrote the manuscript. All authors approved the submitted version.

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REVIEW
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Post-stroke Neurogenesis: Friend or Foe?

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The substantial clinical burden and disability after stroke injury urges the need to explore therapeutic solutions. Recent compelling evidence supports that neurogenesis persists in the adult mammalian brain and is amenable to regulation in both physiological and pathological situations. Its ability to generate new neurons implies a potential to contribute to recovery after brain injury. However, post-stroke neurogenic response may have different functional consequences. On the one hand, the capacity of newborn neurons to replenish the damaged tissue may be limited. In addition, aberrant forms of neurogenesis have been identified in several insult settings. All these data suggest that adult neurogenesis is at a crossroads between the physiological and the pathological regulation of the neurological function in the injured central nervous system (CNS). Given the complexity of the CNS together with its interaction with the periphery, we ultimately lack in-depth understanding of the key cell types, cell–cell interactions, and molecular pathways involved in the neurogenic response after brain damage and their positive or otherwise deleterious impact. Here we will review the evidence on the stroke-induced neurogenic response and on its potential repercussions on functional outcome. First, we will briefly describe subventricular zone (SVZ) neurogenesis after stroke beside the main evidence supporting its positive role on functional restoration after stroke. Then, we will focus on hippocampal subgranular zone (SGZ) neurogenesis due to the relevance of hippocampus in cognitive functions; we will outline compelling evidence that supports that, after stroke, SGZ neurogenesis may adopt a maladaptive plasticity response further contributing to the development of post-stroke cognitive impairment and dementia. Finally, we will discuss the therapeutic potential of specific steps in the neurogenic cascade that might ameliorate brain malfunctioning and the development of post-stroke cognitive impairment in the chronic phase.

Keywords: stroke, adult neurogenesis, hippocampus, SVZ, SGZ, aberrant, cognitive impairment

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INTRODUCTION

Stroke is a major cause of death and disability worldwide (Feigin et al., 2020). Clinical interventions to restore blood flow, mechanical or pharmacological clot removal, are the only two therapies currently approved for patient use. Both treatments are limited to the acute phase of the disease: rtPA can be given only within 4.5 h of stroke onset (Gilligan et al., 2005; Krause et al., 2019), while

the window for thrombectomy has recently been extended (Campbell et al., 2019) but is still narrow. However, advances in prevention and healthcare have progressively reduced stroke mortality (Benjamin et al., 2017) and, as a consequence, stroke can now be considered a chronically disabling disease, with many stroke survivors displaying a variety of motor, cognitive, and psychiatric deficits long-term after stroke onset. These clinical manifestations depend on several factors which include extension and region of the injured brain, timing and also possible therapeutic interventions (Iadecola, 2013).

The brain has an intrinsic capability to self-repair after stroke (Zhao and Willing, 2018) and, in fact, some of these deficits, largely motor ones, show a spontaneous recovery along the chronic phase (Nakayama et al., 1994; Jørgensen et al., 1995; Kwakkel et al., 2003; Kwakkel and Kollen, 2013; Cassidy and Cramer, 2017). Indeed, in response to an acute injury, the brain displays a high degree of plasticity to reorganize its function and structure at different levels, from molecular, cellular and behavioral mechanisms, to changes in anatomy, neurochemistry and, importantly, in the generation of new neurons by neurogenesis. In this context, stroke has been reported to drive a neurogenic burst with affects the two adult neurogenic niches, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Arvidsson et al., 2002; Parent et al., 2002; Parent, 2003; Thored et al., 2006; Kernie and Parent, 2010). These exciting findings suggested that the neurogenic burst is an adaptive response for promoting brain recovery and replacing lost neurons. However, it may not be always so. Since the neurogenic cascade is a highly regulated process, exposure to the ischemic environment may result in multiple dysfunctional outcomes. In fact, it has been proposed that, maladaptive neurogenesis could in some instances contribute as a secondary process to brain malfunctioning.

In contrast with the frequent spontaneous motor recovery during the chronic phase, cognitive function tends to worsen long-term after stroke (Levine et al., 2015; Mijajlović et al., 2017). As such, one of the most troubling late complications in stroke patients is the prevalence of the so-called post-stroke cognitive impairment, which involves a series of syndromes that range from mild cognitive impairment to the most severe form, post-stroke dementia, characterized by alterations in different cognitive domains including attention, executive function, language, orientation, and memory. In fact, more than one-third of patients may develop cognitive impairment or even dementia after stroke (Tatemichi et al., 1994; Pendlebury and Rothwell, 2009; Brainin et al., 2015). However, mechanisms underlying post-stroke cognitive impairment and dementia remain quite unknown (Brainin et al., 2015; Mijajlović et al., 2017). Although cognitive deficits after stroke are considered size and location-dependent (Al-Qazzaz et al., 2014; Zhao et al., 2018), studies in both humans and animals provide evidence supporting that brain areas far from the ischemic insult and primarily undamaged may be involved; for instance, stroke may impair the hippocampal function and promote a memory decline even when the primary brain injury does not affect the temporal lobe (Prins et al., 2005; Blum et al., 2012). Still, little is known about the pathophysiological mechanisms through which ischemic infarcts may cause a secondary alteration of the function of distal areas. Several mechanisms have been proposed to account for the variety of secondary changes later after stroke, in susceptible regions such as the hippocampus, that may participate in the development of post-stroke cognitive dementia, such as a low grade chronic hypoxia, an increase in oxidative stress (Li et al., 2013; Ma et al., 2013), chronic activation of the inflammatory response, a secondary dysfunction of the vasculature altering the blood-brain barrier (BBB) or a damage into the white matter (demyelination, axonal loss and degeneration of oligodendrocytes). An interesting hypothesis is that maladaptive hippocampal SGZ neurogenesis is also a major contributor to the development of dementia after stroke (Wang et al., 2010; Candelario-Jalil et al., 2011).

SVZ NEUROGENESIS IN STROKE: A POTENTIAL MECHANISM FOR SELF-REPAIR?

In physiological situations, newborn neurons generated in the SVZ migrate through the rostral migration stream (RMS) toward the olfactory bulb (OB) where they finally differentiate into interneurons to contribute to olfactory functions (Alvarez-Buylla and Garcia-Verdugo, 2002; Ming and Song, 2011). An amount of experimental evidence supports that, in pathological situations such as an ischemic stroke, the brain activates a process of endogenous self-repair and repopulation of the damaged area by stimulating SVZ neurogenesis (Lindvall and Kokaia, 2015).

The enhancement of SVZ neurogenesis after global ischemia was first described in 1998 (Liu et al., 1998). After this initial work, many studies confirmed this finding in both rodents and humans (Eriksson et al., 1998; Jin et al., 2001; Zhang et al., 2001). Post-stroke SVZ neurogenesis has been widely studied in experimental models of cerebral ischemia with striatal affectation, such as the intraluminal middle cerebral artery occlusion (MCAO) in rodents, which shows a clear temporal profile of the different neurogenic steps (Parent et al., 2002; Parent, 2003; Kernie and Parent, 2010): in this model, the literature describes an increase in the proliferation of SVZ precursors that migrate to the lesion site and differentiate to functional neurons around the infarct (Jin et al., 2001; Zhang et al., 2001, 2004a,b; Arvidsson et al., 2002; Parent et al., 2002; Parent, 2003; Thored et al., 2006; Kernie and Parent, 2010; Lindvall and Kokaia, 2015). This increase, produced by a shortening of the cell cycle, is transient, starts 2 days after stroke onset, reaches a maximum in 1–2 weeks after the beginning of the damage (Jin et al., 2001; Zhang et al., 2001, 2004a,b; Arvidsson et al., 2002), recovering its basal levels over six weeks after the damage (Thored et al., 2006). In addition to changes in cell cycle, it has been reported that stroke transiently changes the division of neural stem cells (NSCs) from asymmetric to symmetric, thus increasing their stock in the SVZ (Zhang et al., 2004a,b).

In a longitudinal analysis performed on a mouse model of cortical ischemia by permanent MCAO induced by ligature, we found that cortical stroke has a triphasic effect on the

total number of cells in proliferation at the SVZ, first with an early acute reduction of proliferation on post-stroke day 1, a second slow increase with a maximum on post-stroke day 14 and, finally, a reduction of proliferating cells at 28 days after ischemia onset. Our results also showed that this process was bilateral in all the conditions and at all the time points studied, supporting an important effect of ventricular cerebrospinal fluid (CSF) composition at this level (Palma-Tortosa et al., 2017). This bilaterality in the SVZ had also been described after cerebral ischemia by intraluminal MCAO (Jin et al., 2001). Related to this, our group has recently demonstrated that TLR4 activation with ligands such as high mobility group box 1 (HMGB1), which is released into the CSF after stroke, drives increased proliferation of neural progenitor cells (NPCs) in this setting, while also promoting differentiation of type-C progenitor cells into migrating neuroblasts (Palma-Tortosa et al., 2019).

SVZ has been identified as the main source of neuroblasts generated after stroke. Neuroblasts migrate ectopically from the pre-defined RMS to the injured area (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002), a process which is described in several animal models (Christie and Turnley, 2012; Moraga et al., 2015; Bravo-Ferrer et al., 2017) and also in human studies (Minger et al., 2007; Martí-Fàbregas et al., 2010). Depending on where the damaged area is located, striatum or cortex, the ectopic migration routes differ in the path followed: whereas neuroblasts migrating into the striatum do so directly from the SVZ, adjacent to the striatum, neuroblasts traveling into the ischemic cortex do so through the corpus callosum (Ohab and Carmichael, 2008). This ectopic migration begins 3-4 days after stroke onset, is maintained until approximately 4 months of the damage (Thored et al., 2006), and is observed, similarly to the migration through RMS, as chains of neuroblasts with elongated cell bodies associated with astrocytes and blood vessels (Yamashita et al., 2006). The damaged brain tissue appears to be the main responsible in the redirection of neuroblasts. In this context, SVZ neural stem cells may receive damage-induced stimuli by two ways, either through changes in CSF composition, and/or through the diffusion of signals from the injured area to the SVZ by the damaged parenchyma or blood vessels. In this context, several molecular signals and cellular interactions that re-direct neuroblasts toward the ischemic region have been described (Young et al., 2011). For instance, inflammatory cells located in the infarct may secrete several types of chemoattractants such as stromal-derived factor 1α (SDF-1α) and monocyte chemoattractant factor 1 (MCP-1), prompting neuroblasts to migrate up chemotactic gradients along blood vessels and astrocytic processes toward the injury. Intrinsic changes of the neuroblasts in combination with an active process of tissue remodeling including the generation of new blood vessels are necessary to provide a scaffold that allows neuroblasts to reach the infarct.

Interestingly, in our longitudinal study, we also analyzed the physiological, eutopic RMS route (from the SVZ to the OB) (Palma-Tortosa et al., 2017). Our results revealed an important increase in this migration but only 24 h after the insult, with a recovery to basal levels from 2 to 28 days after stroke. This enhanced eutopic migration might explain the early reduction in the number of proliferating cells at the SVZ during the first hours after the insult. On its turn, the ectopic, damage-induced migration showed an increase from day 14 after the insult (Palma-Tortosa et al., 2017). In contrast with the effects of stroke on the proliferation in the SVZ, migration is unilateral, possibly due to cues originating from the infarcted and/or peri-infarct area, as already demonstrated (Lee et al., 2006; Bagley and Belluscio, 2010).

A great deal of evidence supports that post-stroke neurogenesis in the SVZ could be involved in the functional improvement that stroke patients undergo during the first months after the establishment of the lesion (Lindvall and Kokaia, 2015). SVZ newborn neurons after stroke could contribute to this improvement in different ways. For instance, newly generated neuroblasts may migrate toward the ischemic boundary region, where they can differentiate into fully mature neurons to replace lost neurons. However, despite the clear increase in neuroblast proliferation and migration after stroke, only a very small percentage of immature neurons reaches the damaged area and only around a 0.2% of these immature neurons fully maturate and integrate into the infarcted region (Arvidsson et al., 2002). This may be due to different factors, such as a hostile inflammatory environment due to ischemia, a deficit of functional connections and/or necessary trophic support (Ming and Song, 2011). Interestingly, an alternative/additional beneficial effect may be derived from the fact that neuroblasts have also been described to form new astrocytes that contribute to the formation of the glial scar, to protect neurons from glutamate-induced excitotoxicity or even to release neurotrophic factors that contribute to tissue repair (Arvidsson et al., 2002; Zhang et al., 2004a,b; Thored et al., 2006; Jin et al., 2010; Butti et al., 2012; Wang et al., 2012; Faiz et al., 2015). Consistent with this positive role, post-stroke SVZ neurogenesis inhibition after stroke impedes recovery after ischemia and exacerbates neurological deficits (Jin et al., 2010), whereas transplantation of neural precursor cells causes a neurological improvement indicating that this process participates in post-stroke recovery (Bacigaluppi et al., 2009). All this evidence therefore supports that post-stroke SVZ neurogenesis could be an adaptive brain plasticity process implicated in restoring brain functionality. However, for an efficient repair, the neurorestorative potential of the SVZ neurogenesis after stroke should probably be combined with the promotion of other regenerative processes that, together, will enhance the survival and integration of newborn neurons into the damaged neuronal networks.

THE HIPPOCAMPAL NEUROGENIC BURST AFTER STROKE: A MALADAPTIVE RESPONSE PROMOTING HIPPOCAMPAL MALFUNCTIONING?

According to the literature, the SGZ in the hippocampus is the other neurogenic region of the adult brain where neurons are

continuously generated throughout life. New granule cells are born in the SGZ of the DG, from where they migrate into the granule cell layer (GCL) and become functionally integrated into neuronal networks. The generation and integration of these newborn neurons seems to be strictly regulated, which is of paramount importance considering that hippocampal neurogenesis is a fundamental process for the formation and retrieval of spatial memories and, specifically, for hippocampal pattern separation (Zhao et al., 2008; Sahay et al., 2011; Akers et al., 2014; Rangel et al., 2014; Kempermann et al., 2015; Gonçalves et al., 2016).

After brain ischemia, the proliferation and differentiation of neuronal progenitors in the DG is also strongly stimulated, leading to a significant increase in neurogenesis (Liu et al., 1998; Arvidsson et al., 2001a,b; Kernie and Parent, 2010). In experimental models of global cerebral ischemia, an approximately 10-fold increase in the SGZ proliferation has been observed (Takagi et al., 1999; Kee et al., 2001; Yagita et al., 2001). This increased proliferation has also been detected after focal ischemia using the MCAO models by either permanent or transient occlusion of the middle cerebral artery (Jin et al., 2001; Türeyen et al., 2004). Therefore, even remote cortical infarcts not affecting the hippocampal formation are able to promote a significant augmentation in hippocampal neurogenesis (Keiner et al., 2010; Walter et al., 2010; Cuartero et al., 2019). Importantly, the increased neurogenic response after ischemia is observed, more or less at the same extent, in both ipsilateral and contralateral sides (Jin et al., 2001; Takasawa et al., 2002; Cuartero et al., 2019), as previously commented for the SVZ. In general, this increase in DG proliferation starts approximately one week after stroke, peaks at days 10 to 14 depending of the MCAO model, and returns to basal levels within several weeks (4-5 weeks) after onset. Although many of the new cells die, the majority of surviving cells are believed to adopt a neuronal fate in a rate similar to that observed in physiological conditions (Kempermann et al., 2015): at later times after stroke onset, they differentiate into granular cells, moving from the SGZ to the GCL and shifting their expression from immature markers like doublecortin (DCX) to mature neuronal markers, like calbindin and NeuN (Kee et al., 2001; Sharp et al., 2002; Tanaka et al., 2004), initially suggesting that hippocampal newborn neurons in the ischemic brain follow a time course of neuronal maturation similar to that described in physiological conditions.

Therefore, stroke increases the production of new neurons in the SGZ, but how the hippocampal neurogenic response influences disease outcome, positively or negatively, is still controversial. In global cerebral ischemia models, in which a massive death is observed in the hippocampus, new hippocampal CA1 neurons are detected after the injury (Nakatomi et al., 2002; Daval et al., 2004; Schmidt-Hieber et al., 2004; Bendel et al., 2005; Oya et al., 2009; Wojcik et al., 2009). Although the origin of these new CA1 neurons is not very clear, some studies have suggested that neuroblasts migrating from both the SGZ and the posterior periventricular region could be the source of these new CA1 pyramidal neurons (Nakatomi et al., 2002; Oya et al., 2009; Khodanovich et al., 2018). But the fact is that, after focal ischemia, newborn neurons in the DG do not have the capacity to migrate toward the ischemic boundary region, as observed for SVZ-derived neuroblasts. Thus, SGZ neuroblasts generated after stroke cannot replace lost neurons or promote regeneration by locally secreting trophic factors in the injured area. Instead, these newborn granule cells integrate into the granular cell layer where they will contribute to the hippocampal function. The question is whether this higher number of new granule neurons positively correlate with a better post-stroke outcome. As we will discuss in the next sections, recent evidence indicates that this may not be the case. Although the hippocampal neurogenic burst could be in principle a compensatory response, a significant proportion of newborn neurons display abnormal properties and become aberrantly integrated into the pre-existing hippocampal circuits. Therefore, adding more neurons with altered aberrant features in the DG might compromise the normal functioning of the global hippocampal network.

POST-STROKE NEUROGENESIS REGULATION: INTERNAL CHECKPOINTS AND EXTRINSIC FACTORS

Neurogenesis can be controlled at different steps including NSC maintenance, proliferation, fate specification and differentiation, migration, fully maturation and, finally, newborn neuron integration into the local circuitry (Gage, 2000; Llorens-Bobadilla et al., 2015). All these stages are strictly regulated by intrinsic and extrinsic mechanisms which together determine the outcome of the neurogenic process (Faigle and Song, 2013; Bjornsson et al., 2015; Shin et al., 2015). Therefore, after a pathological insult like a cerebral ischemia, alterations in both intrinsic and extrinsic programs might contribute to the different steps of the neurogenic response in the DG.

Intrinsic Modulators: The Heterogeneous Nature of NSCs

For hippocampal neurogenesis, the number and the type of NSCs/NPCs are key regulators in steady-state conditions. NSCs are located in the SGZ of the DG, at the interface of the hilus and the granular cell layer. Through different intermediate progenitors, they finally generate immature neuroblasts that eventually convert into young neurons (Kempermann et al., 2004, 2015; Ming and Song, 2011). Prototypical NSCs (also called type-1 or radial-glia-like progenitors) are mostly quiescent with only a few of them being mitotic. Upon activation, type-1 mainly divides asymmetrically to give rise to another NSC and a transiently amplifying population of intermediate neuronal precursors (type-2 cells). Type-2 progenitors represent an important stage of clonal expansion and linage choice: they prevalently use a symmetric division mode (Encinas et al., 2011; Pilz et al., 2018) and comprise cell states that mark the transition from a glial/stem-like phenotype (type-2a) to a neuronal phenotype (type-2b) (Steiner et al., 2006). Type-2b

progenitors then generate proliferating neuroblasts (type-3 cells) that, after migrating a short distance into the granule cell layer, stop being proliferative to become immature neurons (Ming and Song, 2011).

In the context of cerebral ischemia, several studies have characterized the contribution of the different types of NSCs/NPCs to the increased post-stroke proliferation (Keiner et al., 2010; Walter et al., 2010). Six hours after the induction of cortical infarcts, an increase in the proliferation of type-1 and type-2a cells has been detected. In addition, type-2b and type-3 cells also showed increased proliferation 24–72 h after MCAO (Keiner et al., 2010; Walter et al., 2010). Following MCAO, the maximal number of proliferating cells was found after seven to fourteen days and then, proliferative activity decreased 2–5 weeks after the lesion. Therefore, it would seem that this expansion might be due, in part, to increased type-1 NSCs recruitment out of quiescence after stroke. However, this picture may not be so simple since recent studies suggest that NSCs are functionally heterogeneous and plastic in nature depending on their microenvironment. In physiological situations or even in normal aging, different kinds of hippocampal type-1 or radial-glia-like progenitors, called type α-, β-, and Ω-cells, can be distinguished by unique morphological features, specific expression markers and by their proliferative capacity and quiescence (Gebara et al., 2016; Martín-Suárez et al., 2019). Further evidence for this heterogeneity comes from recent single-cell RNA sequencing studies which also shed light on the identity of NSCs residing in the SVZ (Llorens-Bobadilla et al., 2015; Luo et al., 2015; Dulken et al., 2017; Zywitza et al., 2018; Mizrak et al., 2020) or the SGZ (Shin et al., 2015; Artegiani et al., 2017; Berg et al., 2019; Bottes et al., 2020). These studies have provided important clues which reflect the heterogeneous nature of NSCs and NPCs in both niches but also suggest distinctive features of NSCs in the two neurogenic regions. In the SVZ, several NSC and progenitor subpopulations can be defined by the expression of specific markers. However, in the SGZ niche, NSCs seem to be so heterogeneous that discrete subpopulations cannot be identified; they rather represent a heterogeneous cellular continuum that progressively downregulates genes shared with astrocytes involved in quiescence while they upregulate activation genes.

An important point is whether this NSC heterogeneity also exists after stroke, and even more, if all NSCs are equally activated or, if on the contrary, just a subpopulation of them is sensitive to the ischemic stimulus. Although this has not been explored yet in the post-stroke hippocampus, some recent data may suggest that NSC heterogeneity exists in the hippocampus after some pathological situations like epileptic disorders, or in the SVZ after ischemic injury. In the first scenario, by performing intrahippocampal injections of low and high doses of kainic acid (KA) to model epileptiform activity (EA) or mesial temporal lobe epilepsy (MTLE), Sierra et al. (2015) demonstrated that the levels of neuronal hyperexcitation provoke a differential dramatic shift in the function and morphology of hippocampal NSCs. Although these models promoted both an increase in NSC activation and, interestingly, a long-term NSC depletion, they elicited different self-renewal modes. Thus, in the MTLE model, upon hyperactivation, NSCs develop a hypertrophic and multibranched reactive phenotype, which makes them enter into the cell cycle in larger numbers, but also produces a change in the way that they divide. Indeed, they display a symmetric division and, instead of maintaining the neural progenitor pool, they contribute to increase astrogliosis by generating reactive astrocytes as main daughter cells. On the contrary, neuronal hyperactivity in the EA model induces augmented NSCs activation without developing a reactive phenotype or changing to a symmetric division and, then, the generation of newborn neurons is enhanced. In the context of cerebral ischemia, a process similar to the one described for the MTLE model has been observed for SVZ NSCs. NSCs can abandon neurogenesis almost completely and transform into reactive NSCs that migrate toward the damaged cortex and mostly generate reactive astrocytes (Faiz et al., 2015). Along this line, by using single cell RNA-seq, Llorens-Bobadilla et al. (2015) demonstrated that, after stroke, NSCs in the SVZ display different states with unique molecular signatures and that, importantly, exhibited differential responses to the ischemic insult. They found that dormant and quiescent NSCs are more responsive to an injured environment. Interestingly, they also found that, for instance, only a subset of NSCs responds to interferon-γ (IFNγ). This could imply that multiple molecularly distinct groups of NSCs coexist in the SVZ neurogenic niche, with the ability to respond differently to pathological stimuli. In addition, this heterogeneous response might also be influenced by temporal dynamics or even by regional differences within the niche (Mizrak et al., 2019). The question arising is: *are similar mechanisms guiding a heterogeneous SGZ NSCs activation upon ischemic injury?* Further studies using single cell transcriptomics are required to deepen our knowledge on the heterogeneous nature of SVZ and SGZ neural precursor population after stroke. These studies will provide insights on the intrinsic regulatory mechanisms controlling NSCs activation upon stroke in both niches and about the type of stimuli that mediates NSCs priming and activation.

Extrinsic Modulators

Apart from the features of neurogenesis which are intrinsic to the cells that populate the niche, the microenvironment plays a fundamental role in guiding the neurogenic process. Quiescent NSCs in both SGZ and SVZ display an enriched expression of genes related to cell-cell adhesion and cell-microenvironment interaction, suggesting that intrinsic and extrinsic signals are actively involved in maintaining stem cell quiescence (Shin et al., 2015; Morizur et al., 2018). Supporting this, when transplanted into a non-neurogenic area, hippocampal neural stem cells lose their ability to differentiate into neurons (Shihabuddin et al., 2000), indicating that the intrinsic neurogenic potential of NSCs is controlled by the neurogenic niche. Therefore, local signals from the niche as well as remote signals coming, for instance, from CSF or the blood might contribute to the regulation of post-stroke neurogenesis. Subsequently, several mechanisms could account for post-stroke aberrant neurogenesis:

(1) *Mediators of post ischemic excitotoxicity or cortical spreading depression*, such as K^+ and glutamate elevations, could be involved, as both have been implicated in an enhanced proliferation of immature cells while at the same time directing toxicity in mature cell types (Shi et al., 2007). Granule cell development is regulated by activity-dependent mechanisms, especially NMDA receptor-mediated input. Therefore, an excessive NMDA receptor activation might contribute to the increased excitatory postsynaptic currents (EPSCs) observed in newborn neurons after stroke due to postsynaptic receptor insertion (Ceanga et al., 2019).

(2) *Injury-induced circulating factors* might directly affect neural progenitors that are in physical contact with the vasculature (Mignone et al., 2004; Tavazoie et al., 2008). For instance, stroke upregulates the levels of circulating vascular endothelial growth factor (VEGF) that easily permeates the characteristic BBB of neurogenic niches. This induces local endothelial cells to increase Notch signaling that, in turn, stimulates neurogenesis (Lin et al., 2019). Another factors identified after stroke and which might directly affect NSC proliferation are, among others, fibroblast growth factor-2 (FGF-2) (Yoshimura et al., 2001, 2003), insulin-like growth factor-1 (IGF-1) (Yan et al., 2006) and brain-derived neurotrophic factor (BDNF) (Chen et al., 2005). Furthermore, several mediators like SDF-1α, MCP-1, and matrix metalloproteinases (MMP) have been implicated in influencing neuroblast positioning and extracellular matrix remodeling (Rahman et al., 2020).

(3) Another influence to be considered is the *inflammatory response* elicited by the injured brain, whereby astrocytes and microglia activation could lead to the secretion of a variety of growth factors and immune modulators able to affect progenitor proliferation and survival. In addition, several studies associate pro-inflammatory cytokines released after stroke with proliferation, migration, differentiation, and survival of neural precursors (Tobin et al., 2014). In this line, Meng et al. demonstrated that IL-6 promotes the proliferation and differentiation of NPCs in the adult SVZ, inducing functional improvement after stroke. Furthermore, the blockade of IL-1 receptor after experimental ischemic stroke resulted in functional improvement of the mice as well as in increased NSCs proliferation of NSCs. In turn, it enhanced neuroblast migration and increased the number of new neurons formed in the peri-infarct cortex (Pradillo et al., 2017).

Despite the extensive characterization of post-stroke neurogenesis in both SVZ and SGZ and of the factors involved, the exact upstream and downstream signaling pathways driving this transient increase in the neurogenic response is still unknown. In this sense, further studies are necessary to dissect each step of the neurogenic cascade after stroke, trying to answer remaining questions like: *do the same mediators act in both adult niches after stroke?; do all hippocampal NSCs equally respond to the injured brain?; does a differential microenvironment account for the morphological differences in ipsi- and contralesional newborn neurons?*

THE ABERRANT PHENOTYPE OF POST-STROKE HIPPOCAMPAL NEWBORN NEURONS

Morphological Alterations of Newborn Neurons After Stroke

In the hippocampus, the maturation stage of newborn neurons can be identified based on the expression of specific molecular markers but also by the progressive development of unique morphological features (van Praag et al., 2002; Zhao et al., 2006). Mature granule neurons generally have only one primary apical dendrite emerging from the soma which is vertically oriented toward the molecular layer (ML). Although basal dendrites can be observed in humans and non-human primates (Llorens-Martín et al., 2015), rodent mature granule cells lack basal dendrites under physiological conditions (Shapiro et al., 2005, 2007). The apical dendrite remains poorly bifurcated until it reaches the ML, where it branches extensively in order to receive inputs from the entorhinal cortex (EC) through "the perforant pathway" and establishes excitatory synapses (van Praag et al., 2002; Zhao et al., 2006; Kelsch et al., 2008). Therefore, the growth of the apical dendrite seems to be a critical factor for the correct integration of newborn neurons (Shapiro et al., 2007; Llorens-Martín et al., 2015). During maturation, newborn neurons also progressively lengthen their axons and send them toward CA3 through the mossy fiber terminals and also to the CA2 area (Zhao et al., 2006; Kohara et al., 2014). This characteristic morphology of granule neurons has been so-called Y-shape to distinguish from other morphologies found after pathological situations (Llorens-Martín et al., 2015). Indeed, the elongation, orientation, location and branching of newborn neurons in the DG are sculpted by many factors and are very vulnerable to injury. For example, in rodents, environmental enrichment and wheel-running modify the morphology and connectivity of newborn neurons (Kempermann et al., 1997; Gonçalves et al., 2016).

An "aberrant morphology" of newborn granule neurons has been described after different pathological situations (Llorens-Martín et al., 2015; Bielefeld et al., 2019). Since information coming from the EC reaches the hippocampus via the DG, abnormalities in the granule cells might have important consequences for the global hippocampal network function. The concept of aberrant neurogenesis was first described in the context of seizure-associated plasticity that leads to long-lasting structural changes in hippocampal morphology. In the setting of cerebral ischemia, we and others have characterized morphological features of newborn hippocampal neurons after different times after stroke onset. By using retroviral labeling to visualize new granule cells, it has been found that different stroke models lead to alterations of newborn neuron morphology (Niv et al., 2012; Woitke et al., 2017; Cuartero et al., 2019; Sheu et al., 2019), suggesting that integration of these abnormal neurons

could promote aberrant hippocampal circuitry rearrangements and, therefore, might contribute to hippocampal cognitive deficits observed after cortical ischemia. From all newborn neurons generated after stroke, a subset displayed morphological alterations. In the studies by Niv et al. (2012) and Woitke et al. (2017), wherein retroviral infection was carried out 4 days after ischemia and the morphological evaluation approximately 6 weeks post-infection, the aberrant features of newborn neurons (5–10%) included the presence of ectopic newborn neurons, abnormal basal dendrites directed toward the hilus and an increase in dendritic complexity with a subsequent increase in the total dendritic length. Importantly, all these alterations seemed to be dependent on the initial lesion size, because the percentages of aberrant neurons in the filament MCAO model was higher than in the cortical photothrombotic model (Niv et al., 2012). We have also characterized the presence of aberrant newborn neurons after stroke by using the ligature permanent MCAO model and a different timing for retroviral infection and morphological evaluation (Cuartero et al., 2019). Thus, after GFP retrovirus infusion into the ipsilesional hippocampus 14 days after surgery and morphology evaluation 35 days post-infection, approximately 28% of the newborn neurons displayed shortening of the apical dendrite, decrease in the dendritic length, and a differential pattern of arborization. Furthermore, aberrant neurons displayed an increased degree of branching in the proximal domain of the dendritic tree and a retraction of the distal domain in the ML, which can also be observed in immature DCX^+ neurons. Similar results, with reduction in the total dendritic length and total dendritic branches, were observed in the study of Sheu and collaborators (Sheu et al., 2019). In this work, retroviral infection was performed 7 days after the filament MCAO model, and mice were euthanized at different time points after retroviral labeling to assess the maturation process of the new generated neurons.

All these studies corroborate the presence of an altered morphology in part of the new granule neurons after stroke and, importantly, based on the normal presence of mushroom spines, all these studies suggest that these neurons might be stably integrated into the hippocampal network. However, the aberrant phenotype observed seems to clearly differ between different studies (**Figure 1**). A plausible explanation for these different phenotypes is that the generation of new aberrant neurons after stroke is a dynamic process so that, depending on the timing of infection and/or visualization, newborn neurons might display specific aberrant features. Indeed, we also tested this possibility by delivering the GFP-expressing retrovirus 35 days after stroke, when post-stroke neurogenesis had almost returned to physiological levels (Cuartero et al., 2019). In this setting, one month after infection, part of the aberrant features previously detected at earlier times points was no longer observed (such as the pattern of arborization or the reduction in mean apical dendrite length). Nevertheless, a population of newborn neurons still displayed apical dendrite growth alterations. This might suggest that aberrant neurogenesis after stroke persists at a lower intensity at later times points and, also, that the factors that induce morphological remodeling, albeit likely decreasing over time, are still present at later time points.

As commented above, the neurogenic burst after stroke affects both the ipsi- and contralesional hemispheres equally, producing an increased proliferation and generation of newborn neurons with similar temporal dynamics, suggesting that the same mediators act in both hemispheres for increasing hippocampal neurogenesis in this scenario. Therefore, if in terms of proliferation, both ipsilateral and contralateral hemispheres behave similarly, *do newborn neurons display an aberrant phenotype in the contralesional side?* We assessed this question in our study by a comparison of both ipsi- and contralesional hippocampi after stroke. Importantly, we found that a different remodeling process seems to occur simultaneously at each side of the hippocampus after ischemic stroke. Indeed, by analyzing immature and mature newborn neurons, we found a different phenotype of newborn neurons at the ipsilesional when compared with the contralesional hippocampus that seems to affect both synaptic inputs and outputs. First, contralesional neurons display an elongation of apical dendrite and, consequently, an increase in the distal dendrite branch that could have important consequences for incoming information from the EC. Second, our data suggest that stroke also promotes a differential bilateral remodeling in the connectivity between DG and CA3. Each new cell in the DG projects a mossy fiber that reaches the CA3 region within approximately 2 weeks, contacting with 11–15 pyramidal cells (Toni et al., 2008). Interestingly, in CA3, we observed a hyper- or a hypo-integration pattern of immature newborn neurons, at the contra- and ipsilesional hemispheres, respectively. These data could imply an increased synaptic rearrangement in the contralesional CA3, which could coexist or replace the previous DG-CA3 synapses. Although we did not confirm this result in mature neurons, newly generated neurons transiently display enhanced synaptic plasticity (Schmidt-Hieber et al., 2004; Ge et al., 2007b; Dieni et al., 2016), suggesting that immature new neurons may also have the ability to deprive pre-existing synapses, which may have therefore important consequences on the hippocampal function.

Altered Electrophysiological Properties of New Granule Cells After Stroke

Although the total number of newborn neurons in the adult hippocampus is very small compared to that of the granule cells being born during development, new immature granular cells display specific cellular properties that differentiate them from the mature ones (Schmidt-Hieber et al., 2004; Ge et al., 2007b; Ming and Song, 2011; Dieni et al., 2016; Gonçalves et al., 2016). For example, immature newborn neurons exhibit hyperexcitability in the first month after birth (Mongiat et al., 2009; Dieni et al., 2013, 2016). As a consequence, they are very efficient in generating action potentials (Marín-Burgin et al., 2012). Furthermore, immature granular neurons also display enhanced synaptic plasticity with a lower threshold for the induction of long-term potentiation (LTP) upon perforant pathway stimulation (Schmidt-Hieber et al., 2004; Ge et al., 2007b) and enhanced LTP at mossy fiber connections into CA3 pyramidal neurons (Gu et al., 2012). This enhanced synaptic

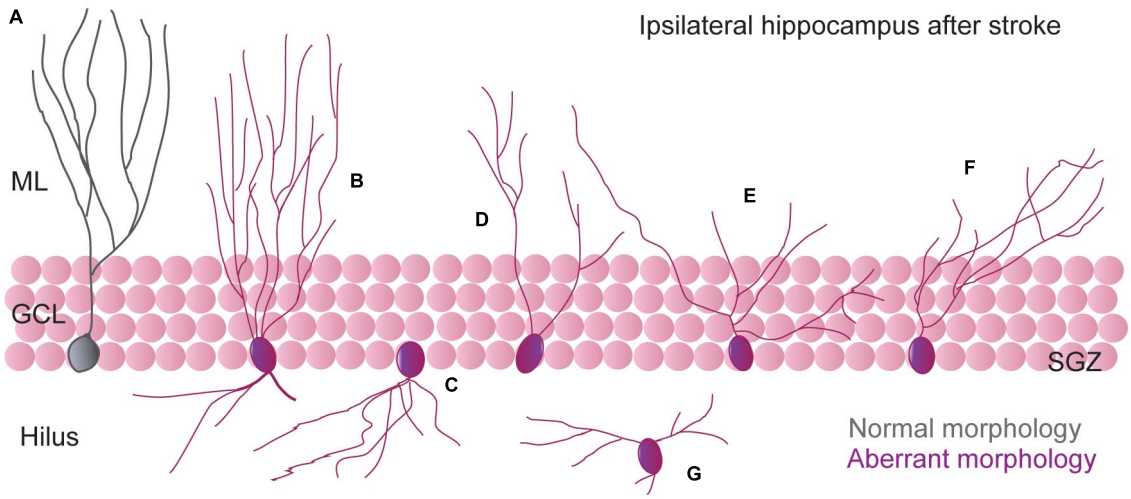

FIGURE 1 | Schematic illustration of most representative aberrant dendritic morphology of newborn neurons generated in the DG after stroke. **(A)** Morphology of regular ("non-aberrant") neurons. **(B)** Neurons with additional basal dendrites toward the hilus (bipolar cells) and an increase in dendritic complexity (Niv et al., 2012; Woitke et al., 2017). **(C)** Neurons with abnormal basal dendrites directed toward the hilus (Niv et al., 2012; Woitke et al., 2017). **(D–F)** Neurons with a dramatic reduction in apical dendrite length and an increased proximal dendritic branch density (Cuartero et al., 2019; Sheu et al., 2019). **(G)** Ectopic neurons (Niv et al., 2012; Woitke et al., 2017).

plasticity is partially due to a reduction in the GABAergic inhibition in immature neurons (Ge et al., 2007a).

Therefore, just by increasing the number of newborn neurons, as occurs after ischemia, important consequences for the hippocampal network might be expected. However, the presence of newborn neurons with an altered morphology might be an indication that stroke not only promotes an augmentation in the newborn neurons but also alters some of their main unique features. Then, *does stroke also alter intrinsic electrophysiological properties of hippocampal newborn neurons?* Recently, Ceanga et al. (2019) aimed to answer this question by evaluating how stroke influences the electrophysiological development of newborn hippocampal neurons. Using doublecortin-dsRed mice to label immature neurons, they found that stroke altered the intrinsic electrophysiological properties of a subset of newborn neurons, with an increase in the number of hyperexcitable immature neurons as earlier as 2 weeks after stroke onset. Of note, these young neurons displayed features that resembled those observed in the mature ones, therefore suggesting that stroke causes an accelerated maturation of new granule cells to become incorporated into the hippocampal network. Under physiological conditions, intrinsic maturation and synaptic excitability of newborn neurons are tightly coordinated processes. In fact, immature granule neurons are hyperexcitable in part to compensate for the low excitatory innervation (Ming and Song, 2011; Toni and Schinder, 2015). However, after stroke, immature neurons displayed an increase in the amplitude of spontaneous and miniature EPSCs indicating that immature newborn neurons also receive increased excitatory inputs. Therefore, stroke seems to result in an uncoupling of synaptic hyperexcitability which further potentiates the intrinsic hyperexcitability of immature newborn neurons. Of note, a fundamental function of immature newborn neurons is the inhibition of mature granule cells by regulating GABAergic interneurons that modulate mature granule cells activity (Drew et al., 2016). Consequently, increasing hippocampal newborn neurons after cerebral ischemia could be a mechanism for reducing hyperactivity by driving a local inhibition of mature granule cells. However, newborn granule cells are still hyperexcitable despite receiving large excitatory inputs (Ming and Song, 2011). Hence, stroke results in an accelerated development of intrinsically hyperexcitable new granule cells that receive unusually large excitatory inputs, therefore promoting higher hyperactivation of the hippocampal circuits. Interestingly, the morphological features of the immature neurons which displayed altered intrinsic patters of maturation after stroke were completely normal. This fact indicates that, after cerebral ischemia, normal neuronal morphology does not preclude disturbances of intrinsic excitability. Therefore, both normal and aberrant morphological newborn neurons could promote hippocampal malfunctioning after stroke.

MORE NEW GRANULE CELLS AFTER STROKE: A MALADAPTIVE RESPONSE?

The hippocampus is one of the main adult brain regions implicated in cognitive functions. Through adult neurogenesis, newborn neurons are continually added to the hippocampal circuits, contributing to the encoding of new hippocampus-dependent memories (Jørgensen et al., 1995). However, neuronal integration also produces remodeling of pre-existing hippocampal network and, therefore, increasing neurogenesis may also promote the destabilization and even the clearance of previous stored memories (Nakayama et al., 1994). Numerous examples have been provided for the role of physiological DG

neurogenesis in hippocampus-dependent learning and memory processes (Kempermann et al., 2015; Gonçalves et al., 2016). Under physiological conditions, higher rates of neurogenesis in the dentate gyrus may improve cognition (Sahay et al., 2011) and may also mediate forgetting of previously acquired memories (Akers et al., 2014), but the same may not necessarily be true under a pathological situation.

Although aberrant neurogenesis has been proposed as contributing to cognitive impairment in different pathological situations, such as epilepsy, schizophrenia, and neurodegenerative diseases (Zhao et al., 2008; Ming and Song, 2011; Aimone et al., 2014; Schreglmann et al., 2015), in the context of cerebral ischemia, the functional consequences of hippocampal neurogenesis and its relation with post-stroke cognitive impairment is still controversial. Part of this controversy is probably due to differences in the cerebral ischemia model used, in the temporal experimental design, as well as in the behavioral paradigm chosen for the evaluation of cognitive function. The positive role of hippocampal neurogenesis in stroke came mostly from studies in which the ischemic injury, caused by both global (Nakatomi et al., 2002; Bendel et al., 2005) or focal ischemia models (Luo et al., 2007; Li et al., 2009; Kernie and Parent, 2010), directly affects the hippocampus, although some positive effects have also been described in MCAO models without hippocampal alteration. In these settings, maneuvers that increase hippocampal neurogenesis such as different types of rehabilitative training (for instance, running), have also shown to correlate with better functional outcomes (Wurm et al., 2007; van Praag, 2008; Zhao et al., 2008). On the contrary, as commented above, we and others have demonstrated that some newborn neurons after stroke display aberrant functional and morphological features (Niv et al., 2012; Woitke et al., 2017; Ceanga et al., 2019; Cuartero et al., 2019; Sheu et al., 2019), and also that the occurrence of aberrant neurogenesis correlates with hippocampus-dependent deficits after focal cortical stroke. Supporting this, interventions directed to increase neurogenesis after stroke, like free running, exacerbate cognitive impairments while increasing hippocampal neurogenesis. Although it is true that hippocampal aberrant neurogenesis seems to be transient and decreases over time (Cuartero et al., 2019), the important fraction of aberrant neurons generated during the stroke-induced neurogenic burst will likely remain abnormally integrated into the hippocampal circuits. Therefore, aberrant neurogenesis would have long lasting effects after stroke.

In order to clarify the role of hippocampal neurogenesis after stroke, different strategies have been used to suppress post-stroke neurogenesis. Several studies have suggested that a decrease in neurogenesis has a negative impact on cognitive function after cerebral ischemia, either using brain ionized radiation (Raber et al., 2004; Zhu et al., 2009; Shiromoto et al., 2017) or anti-mitotic agents like cytosine-β-D-arabinofuranoside to eliminate NPCs (Arvidsson et al., 2002; Zhang et al., 2004a). However, both depletion strategies might promote a non-specific elimination of all dividing cells, affecting different cells types like microglia, astrocytes or endothelial cells in addition to neural progenitor cells. In order to target NSCs/NPCs more specifically, different genetic models have been used in the context of ischemia. In the studies by Li and collaborators (Jin et al., 2010; Wang et al., 2012), authors generated a transgenic mouse expressing herpes simplex virus-1 thymidine kinase (HSV-TK) under the control of the DCX promoter, in which ganciclovir treatment produces the depletion of DCX-expressing cells in the SVZ and the SGZ. Supporting a neuroprotective role of neurogenesis after stroke, they found that depletion of DCX cells increases infarct volume, promotes an exacerbation of short-term sensory motor deficits and impairs long-term recovery after stroke, although they did not evaluate post-stroke cognitive function. The causal relationship between neurogenesis and the post-stroke cognitive function was evaluated by Sun and cols. (Sun et al., 2013) by using a transgenic mouse in which HSV-TK was under the control of the nestin promoter. In this case, neurogenesis inhibition impaired spatial learning and memory in the Barnes maze after stroke. Although all together these studies suggest that post-stroke neurogenesis is necessary for recovery of cognitive function after stroke, several considerations must be taken in to account. First, the genetic models used in these studies targeted both post-stroke SVZ and

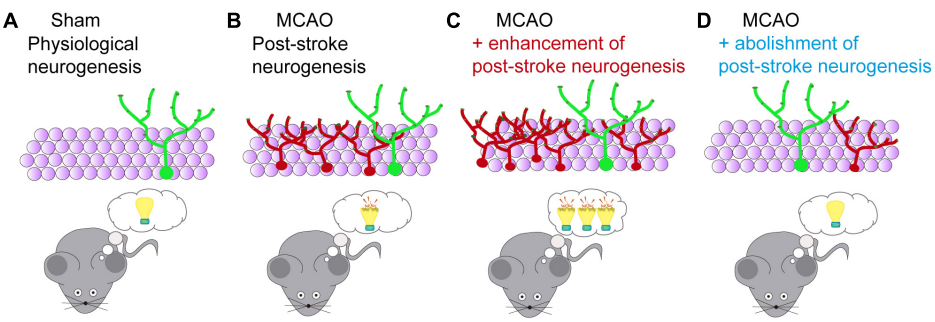

FIGURE 2 | Functional consequences of modulation of post-stroke neurogenesis after stroke. **(A)** The hippocampus is one of the main adult brain regions implicated in cognitive functions. Through adult neurogenesis, newborn neurons are continually added to the hippocampal circuits, contributing to the encoding of new hippocampus-dependent memories. **(B)** Stroke-induced neurogenesis in the SGZ positively correlates with memory impairment after cerebral ischemia. **(C)** Enhancement of post-stroke neurogenesis, for instance, by running, exacerbates hippocampal cognitive deficits after ischemia. **(D)** Post-stroke memory impairment is reduced by abolishment of ischemia-induced aberrant neurogenesis (Cuartero et al., 2019).

SGZ neurogenesis; therefore, confounding results might come from a differential contribution of SVZ and SGZ neurogenesis in recovery after stroke, for instance, by affecting the infarct volume size, since progenitors from the SVZ are known to be recruited to the ischemic lesion as commented above. Most importantly, in both studies, neurogenesis suppression was performed prior to the MCAO procedure, which might promote basal improper cognitive function by reducing basal neurogenesis levels when the brain is still healthy instead of blocking just post-stroke neurogenesis.

In an attempt to solve some of these limitations, we designed a specific strategy for abolishing neurogenesis after MCAO (Cuartero et al., 2019) without altering basal neurogenesis. For such a purpose, we used nestin-Cre^{ERT2}/NSE-DTA mice. In these mice, tamoxifen-inducible Cre is expressed by NSCs under the nestin promoter (Line 4, with display a higher recombination in the SGZ) and the loxP-STOP-loxP-IRES-DTA gene cassette is knocked into the NSE (Eno2; enolase 2) gene (Imayoshi et al., 2006, 2008). After tamoxifen treatment, Cre recombinase deletes the STOP sequence in the NSC pool. Throughout maturation, the NSE promoter becomes active, inducing the expression of the diphtheria toxin A (DTA), resulting in cellular programmed death. Thus, the generation of fully mature newborn granule neurons is dramatically decreased in these mice. Using tamoxifen administration after stroke (starting at day 7), we were able to reduce post-stroke hippocampal neurogenesis, and both proliferating cells and immature newborn neurons returned to similar levels that in sham mice. By abolishing neurogenesis, we clearly detected that MCAO mice displayed an improvement in contextual and spatial memory recall performance when tested long-term after stroke (**Figure 2**), supporting that the inhibition of aberrant hippocampal post-stroke neurogenesis avoids the onset of remote memory deficits after stroke (Cuartero et al., 2019), and strongly supporting the involvement of aberrant hippocampal neurogenesis in post-stroke cognitive impairment and dementia.

FUTURE CHALLENGES: TARGETING POST-STROKE NEUROGENESIS

The post-stroke neurogenic burst in both SVZ and SGZ niches may have different functional consequences in stroke outcome. While increasing SVZ neurogenesis could promote functional recovery, augmentation of hippocampal neurogenesis could further exacerbate the development of post-stroke cognitive impairment. Therefore, in order to consider neurogenesis as a potential therapeutic post-stroke target, it would be necessary to design specific strategies in a *niche-dependent fashion*. Thus, instead of a complete modulation of the post-stroke neurogenic response, strategies should be directed to enhance or inhibit specific steps of the post-stroke neurogenesis.

One potential specific maneuver after stroke might be the enhancement of the survival and integration of SVZ-derived neuroblasts in the injured brain. In this context, given the very low degree of survival of neuroblasts that migrate to the damaged tissue and their low differentiation into mature neurons, the major emphasis from a therapeutic standpoint should probably be put on approaches that improve these processes. Thus, different strategies have focused on trying to regenerate damaged tissue by either increasing endogenous neurogenesis (Wu et al., 2017) or by stem cell therapy or transplantation, resorting to a variety of stem cell sources (Bang et al., 2016; Gervois et al., 2016; Hess et al., 2017).

On its hand, at the level of the SGZ, strategies should focus toward the prevention of the cases of maladaptive neurogenesis leading to aberrant morphologies of newborn granule neurons and their detrimental consequences.

Although we are still a long way from having a whole picture of the post-stroke neurogenesis process, current investigations will likely allow us to dissect the multi-step neurogenic response in both niches, gaining insight on how all these processes are initiated and maintained, and paving the way to develop new therapeutic avenues for stroke patients.

AUTHOR CONTRIBUTIONS

MC, AG-C, and MM conceptualized the study. IL and MM were responsible for the funding acquisition. MC, AG-C, and MM contributed to the writing (original draft). MC, AG-C, CT-L, VM, EF, SV-R, JG-S, IL, and MM contributed to the writing (review and editing). All authors read and approved the final manuscript.

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Therapeutic Potential of GABAergic Signaling in Myelin Plasticity and Repair

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Oligodendrocytes (OLs) produce myelin to insulate axons. This accelerates action potential propagation, allowing nerve impulse information to synchronize within complex neuronal ensembles and promoting brain connectivity. Brain plasticity includes myelination, a process that starts early after birth and continues throughout life. Myelin repair, followed by injury or disease, requires new OLs differentiated from a population derived from oligodendrocyte precursor cells (OPCs) that continue to proliferate, migrate and differentiate to preserve and remodel myelin in the adult central nervous system. OPCs represent the largest proliferative neural cell population outside the adult neurogenic niches in the brain. OPCs receive synaptic inputs from glutamatergic and GABAergic neurons throughout neurodevelopment, a unique feature among glial cells. Neuron-glia communication through GABA signaling in OPCs has been shown to play a role in myelin plasticity and repair. In this review we will focus on the molecular and functional properties of $GABA_A$ receptors ($GABA_A$Rs) expressed by OPCs and their potential role in remyelination.

Keywords: $GABA_A$ receptors, oligodendrocyte precursor cells, NG2 glia, myelination, remyelination, β-carbolines, neurosteroids

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INTRODUCTION

The oligodendrocyte precursor cells (OPCs) are a dynamic glial population widely distributed in the central nervous system which differentiate into new oligodendrocytes (OLs) participating in myelin remodeling (Serwanski et al., 2018; Bonetto et al., 2020). OPCs express the NG2 antigen and the α receptor for platelet-derived growth factor (PDGFRα) and arise sequentially in three waves during early neurodevelopment (Nishiyama et al., 1999, 2009, 2016; Guo et al., 2021). The first wave derives from $Nkx2.1^+$ progenitors (E12.5) from the ganglionic eminence and anterior entopeduncular area in the ventral brain. The second wave (E16.5) arises from $Gsh2^+$ progenitors from the lateral and caudal ganglionic eminences in the ventral brain. Finally, a third wave of OPCs is generated postnatally from the dorsal $Emx1^+$ progenitor and contributes to ∼80% of the OLs in the dorsal brain (Kessaris et al., 2006; Tripathi et al., 2011; Guo et al., 2021). Thus, OPCs quickly generate mature myelinating OLs within the early postnatal weeks and throughout life, but the differentiation rate declines with age. New OLs in the adult brain actively participate in myelin remodeling, and remyelination through OPC differentiation is of interest to treat demyelinating neuropathologies (Watanabe et al., 2002; Hill et al., 2013; Serwanski et al., 2018; Bonetto et al., 2020; **Figure 1A**). OPCs express voltage-gated ion channels and membrane receptors that give them a

complex electrophysiological profile and, in contrast to other glial cells, they receive unidirectional synaptic input from neurons (Berger et al., 1991; Bergles et al., 2000; Jabs et al., 2005; Müller et al., 2009; De Biase et al., 2010; Reyes-Haro et al., 2010; Matyash and Kettenmann, 2010; Reyes-Haro et al., 2013; Arellano et al., 2016; Bedner et al., 2020; Labrada-Moncada et al., 2020). Glutamatergic inputs into OPCs processes usually derive from long-range axons, while GABAergic inputs derive from local interneurons with specifically distributed synaptic contacts. Those from fast spiking interneurons are mainly located at OPCs somata and proximal parts of the processes, and those from non-fast spiking interneurons are mainly located at the distal parts of OPCs processes (Lin and Bergles, 2004; Mangin et al., 2008; Müller et al., 2009; Mangin et al., 2012; Balia et al., 2015; Orduz et al., 2015).

$GABA_A$Rs ARE EXPRESSED IN OPCs

The γ-aminobutyric acid (GABA) is considered the main inhibitory neurotransmitter that hyperpolarizes neurons in the brain. However, GABA produces depolarization in OPCs and promotes an increase in intracellular Ca^{2+} through activation of ionotropic $GABA_A$ receptors ($GABA_A$Rs) (Kirchhoff and Kettenmann, 1992; Arellano et al., 2016). Indeed, *in vitro* studies reported functional expression of $GABA_A$Rs, in both OPCs and mature OLs (Gilbert et al., 1984; Hoppe and Kettenmann, 1989; Von Blankenfeld et al., 1991), and showed that their expression in the OL membrane is controlled by their interaction with neurons (Arellano et al., 2016). The main receptor expressed *in vitro* has functional and pharmacological characteristics that distinguish it from those expressed in most neurons (Arellano et al., 2016; Ordaz et al., 2021). Investigating the molecular identity and structure-function of the $GABA_A$Rs is important to develop pharmacological tools to act specifically on OL receptors.

$GABA_A$R is a pentameric protein that consists of a combination of subunits coupled to a Cl^- channel and modulated by clinical compounds, such as barbiturates and benzodiazepines (Olsen and Sieghart, 2008). The $GABA_A$R family includes 19 identified genes that code for the same number of subunits (α1–α6, β1–β3, γ1–γ3, δ, e, ϕ, π, and ρ1–ρ3) widely distributed in the CNS. The most common pentameric array of subunits is 2α, 2β, and 1γ, with α1β2γ2 as the main combination in neuronal synapsis (Olsen and Sieghart, 2008). $GABA_A$Rs containing the γ2 subunits in neurons usually correspond to synaptic receptors while extrasynaptic transmission is commonly mediated through receptors containing the δ subunit (Olsen and Sieghart, 2008). The main $GABA_A$Rs studied in the oligodendroglial lineage include combinations that contain γ subunits. However, not all of them contain the γ2 subunit, instead they contain the γ1 subunit (Ordaz et al., 2021). For example, single-cell RT-PCR studies reported that α2, α3, β3, γ1, and γ2 subunits were expressed in OPCs from the hippocampus of young mice (P17) (Passlick et al., 2013). Initially, the expression of γ subunits in OPCs was controversial because the contradictory action of benzodiazepines on the GABA response (Von Blankenfeld et al., 1991; Bronstein et al., 1998; Williamson et al., 1998). The reason for this ambiguity appears to be that the OL-$GABA_A$R responds to benzodiazepines with classical potentiation in a neurotransmitter concentration-dependent manner (Arellano et al., 2016). Indeed, GABA concentration is critical to occlude (EC_{50} ∼ 100 μM) or promote potentiation ($\leq EC_{30}$) by benzodiazepines such as diazepam or flunitrazepam. Potentiation by benzodiazepines supports the involvement of a γ subunit in the conformation of the OL-$GABA_A$R, at least at the neonatal stage, given that these studies were performed in primary cultures of cells isolated from the neonate forebrain (P0–P2) or OLs from the optic nerve at P10–P12 (Arellano et al., 2016). However, the OL response to GABA displayed blockage by Zn^{2+}, within the μM range, and lack of modulation by indiplon (a positive modulator that acts on receptors containing the γ2 subunit), clearly indicating that the γ2 subunit was not involved in the conformation of the OL-$GABA_A$R. The potentiation of $GABA_A$Rs with loreclezole (an antiepileptic compound that acts as a positive allosteric modulator) suggest the involvement of β2 or β3 subunits (Wafford et al., 1994), while low sensitivity to GABA (EC_{50} ∼ 100 μM) suggests the expression of α3 subunit (Karim et al., 2013). This supports the original idea that the OL-$GABA_A$R contains α3/β2 or β3/γ1 or γ3 subunits. This proposal was further reinforced by single cell RNAseq transcriptome studies in OPCs ($NG2^+$ cells) from P17 mice (Larson et al., 2016), and by the transcriptomic analysis derived from datasets available in public domain resources, for $GABA_A$R subunit gene expression (assessed by RNAseq) in PDGFRα$^+$ cells isolated by fluorescence-activated cell sorting from mice whole brains, as well as from cortex and corpus callosum (CC) cells of adult mice (Ordaz et al., 2021). Thus, all available transcriptomic analyses support the idea that OPCs express the coding sequences for various subunits, where α2 and α3, together with β2, β3, and γ1 subunits, were well represented and highlighted the low or even null expression of γ2, γ3, β1, or δ subunits. Nevertheless, the molecular composition of $GABA_A$R in OLs seems to change with age and probably also depends on their localization in the brain. Although there is scant information, another possible source of diversity depends on the species. In this context, a transcriptomic analysis in human OPCs (PDGFRα$^+$ cells) resulted in a high expression of ε subunits (Serrano-Regal et al., 2020). The expression of γ2-containing receptors has been documented in $NG2^+$ cells of the mouse barrel cortex during the first postnatal month (Vélez-Fort et al., 2010). Nonetheless, their expression is downregulated in older animals, a time-course that correlates well with a parallel decrease of neuron-OPC synaptic contact and a switch from synaptic to extrasynaptic GABAergic signaling transmission. However, studies in OPCs isolated at early stages (P0-P12) of myelination, from the forebrain and the optic nerve, indicated that a receptor devoid of the γ2 subunit is responsible for their GABA sensitivity. To explore the possible configuration of this receptor, based on its pharmacology and the available transcriptomic analyses, a heterologous expression study was carried out combining in different arrangements the subunits that have been proposed in their configuration. The study showed that the combination α3β2γ1 mimicked the characteristics of the endogenous receptor when expressed in *Xenopus laevis* oocytes. Moreover, OPC α3 subunit silencing by siRNA transfection

shifted the EC_{50} for GABA (from 76 to 46 μM), while γ1 subunit silencing reduced the current amplitude by 55%, indicating their involvement in the endogenous receptor conformation (**Figure 1B**; Ordaz et al., 2021). A question of obvious interest that remains unresolved is whether the configuration containing the γ1 subunit corresponds to receptors located in the neuron-OPC synapse during the neonatal stage. The involvement of γ2 subunit in the conformation of synaptic $GABA_A$Rs in neurons is a well-known fact; however, the substitution of this subunit by γ1 has been reported in some cases, and its sub-localization in the OL membrane has not been systematically explored. On the other hand, information about the pharmacological characteristics of the OL-$GABA_A$R could provide tools that would allow a specific modulation. Thus, β-carbolines, described originally as inverse agonists acting on the benzodiazepine site, differentially and potently enhance the response in OLs when compared to those expressed by neurons (Cisneros-Mejorado et al., 2020; Ordaz et al., 2021). The potentiating effect of β-carbolines has also been demonstrated previously in different neuronal $GABA_A$Rs (Sieghart, 2015). This effect is observed when the classic benzodiazepine site is blocked or eliminated. However, the OL-$GABA_A$R responds directly to diverse β-carbolines applications with an enhancement of the response to GABA. Thus, the identification of β-carbolines as selective positive modulators of OL-$GABA_A$Rs, as well as the molecular identity of the binding site, may help to study the role of GABAergic signaling during myelination (**Figure 1B**; Ordaz et al., 2021).

$GABA_A$Rs-MEDIATED SIGNALING PROVIDES A REGULATORY PATHWAY FOR OPCS DEVELOPMENT

GABA plays an important signaling role in neurodevelopment and synaptogenesis, thus, GABAergic synaptic input to neuronal precursor cells is known to promote the survival and maturation of neuronal progenitors (Tozuka et al., 2005; Song et al., 2013), while a non-canonical function of GABA has been highlighted as a synaptogenic element shaping the early establishment of neuronal circuitry in mouse cortex (Oh et al., 2016). For example, the subventricular zone is a neurogenic niche where $GFAP^+$ /$nestin^+$ cells generate neuroblasts (Doetsch et al., 1999; Garcia et al., 2004). GABA is spontaneously released from neuroblasts and diffuses to activate $GABA_A$Rs functionally expressed by $GFAP^+$ /$nestin^+$ cells. This signaling limits their proliferation, maintaining a balance between neuroblast production and migration in the subventricular zone (Liu et al., 2005). It has been proposed that interactions between axons and the exploratory processes of OPCs could lead to myelination in a similar way to those between dendrites and axons that eventually lead to synapse formation (Almeida and Lyons, 2014). In fact, OPCs express $GABA_A$Rs and receive synaptic input from interneurons early in neurodevelopment (Lin and Bergles, 2004; Zonouzi et al., 2015; Labrada-Moncada et al., 2020). The activation of $GABA_A$Rs induces membrane depolarization and $[Ca^{2+}]_i$ elevation in OPCs and pre-myelinating OLs (Tong et al., 2009; Arellano et al., 2016; Labrada-Moncada et al., 2020), similar to what has been observed in immature neurons (Ben-Ari et al., 2007). However, the mechanism induced by activation of $GABA_A$Rs in OPCs involves Na^+ influx through non-inactivating Na^+ channels, which in turn triggers Ca^{2+} influx via Na^+/Ca^{2+} exchangers (NCXs). This unique Ca^{2+} signaling pathway is further shown to be involved in the migration of OPCs (Tong et al., 2009). The $[Ca^{2+}]_i$ increase promotes differentiation and survival of OPCs through voltage-gated calcium channel CaV1.2 activation (Pitman et al., 2020), and controls their migration through influx via NCXs (Tong et al., 2009). Thus, OPC depolarization by GABA has multifactorial consequences. For example, incubation of the $GABA_A$R agonist muscimol (100 μM) in primary cultures of OPCs decreased the number of $BrdU^+$/OPCs, suggesting that GABA signaling can directly influence their proliferation (Zonouzi et al., 2015). Moreover, loss of $GABA_A$R-mediated synaptic input to OPCs by hypoxia seems to promote the proliferation of these cells and a delay in OL maturation resulting in cerebellar white matter (WM) demyelination during the early postnatal stage (Zonouzi et al., 2015). Recently, we explored whether GABAergic signaling included other glial cells within the cerebellar WM (Labrada-Moncada et al., 2020). The cellular composition of WM is dominated by glial cells and axons, and neuronal somata represents less than 1% of the cells (Sturrock, 1976; Reyes-Haro et al., 2013). First, using calcium imaging analysis we tested the effect of the $GABA_A$R agonist muscimol (50 μM) on cerebellar WM cells and found that 39% of them responded with an intracellular Ca^{2+} increase. No response to baclofen was observed, suggesting that GABA-mediated Ca^{2+} signaling occurs through $GABA_A$Rs at early postnatal development (P7–P9). Then, astrocytes were labeled with sulforhodamine B (SRB) and we observed that muscimol responding cells did not incorporated SRB. To further explore the identity of these cells, electrophysiological analysis was made. In agreement with Ca^{2+} imaging studies, muscimol did not generate any current response in the recorded astrocytes indicating lack of the functional expression of $GABA_A$Rs. In contrast, muscimol-mediated currents were elicited in $NG2^+$ cells, indicating that OPCs were the main cell type in cerebellar WM electrically responsive to GABA through the activation of $GABA_A$Rs (Labrada-Moncada et al., 2020).

Altogether, these observations strongly suggest that $GABA_A$R-mediated signaling represents a specific regulatory pathway to control migration, proliferation and maturation of OPCs during early postnatal development of the cerebellar WM.

THERAPEUTIC POTENTIAL OF $GABA_A$Rs IN REMYELINATION

Premature infants (23–32 weeks gestation) are at high risk of developing diffuse white matter injury (DWMI), a leading cause of neurodevelopmental disabilities often linked to chronic hypoxia (Back, 2006; Anjari et al., 2009). DWMI is also known as bilateral periventricular leukomalacia and is associated with subcortical WM damage characterized by a marked loss of OPCs (Back et al., 2001, 2002) resulting in important behavioral,

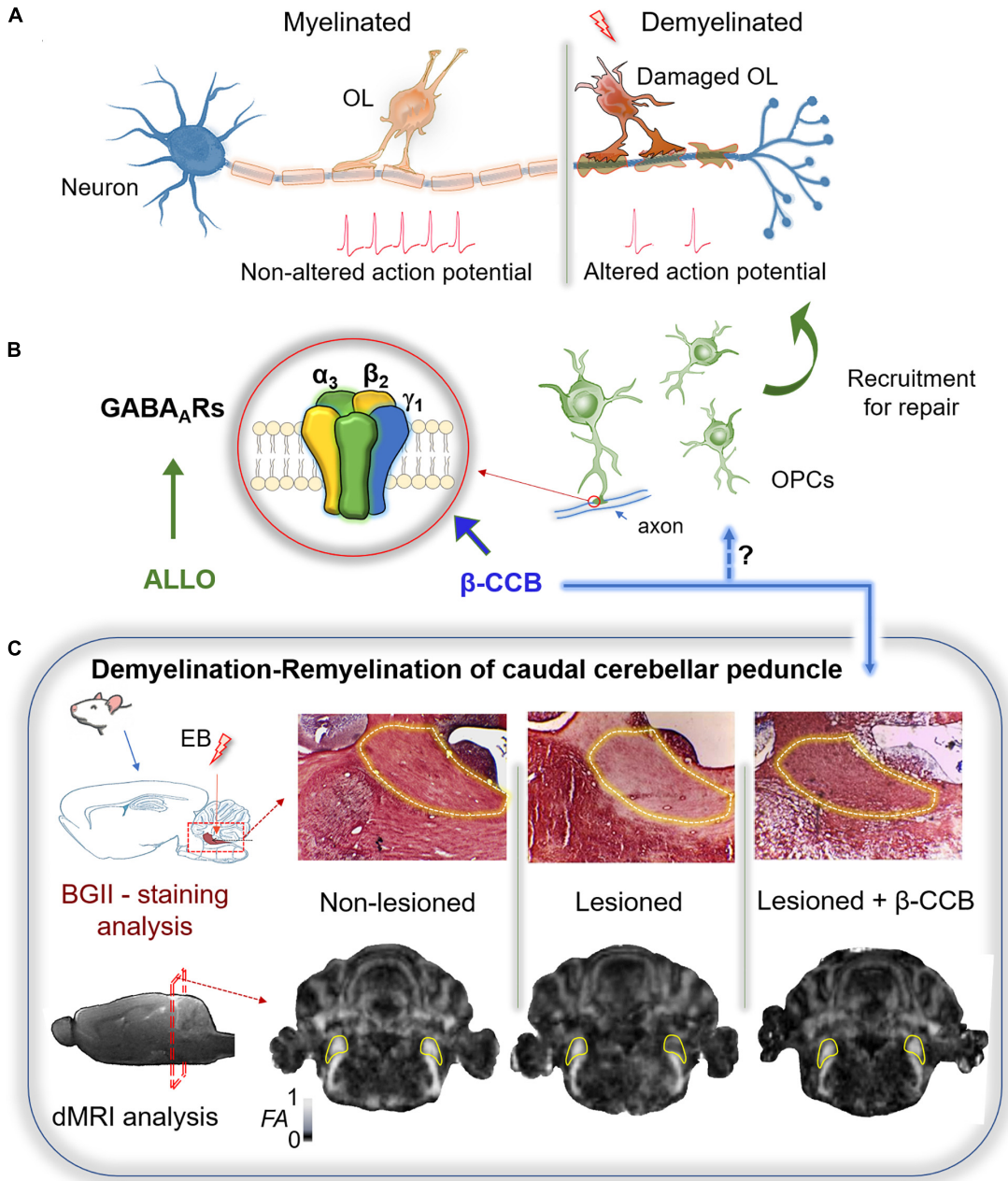

FIGURE 1 | Therapeutic potential of GABAergic signaling on remyelination. **(A)** Myelin sheath accelerates the action potential propagation. The loss of oligodendrocytes (OLs) after white matter injury results in demyelination and altered conduction of the nerve impulse. **(B)** Remyelination requires of OPCs recruitment. These cells express mainly a GABA$_A$R with a defined subunit arrangement (α3, β2, γ1) that is potentiated by some β-carbolines (particularly β-CCB). Allopregnanolone (ALLO) also modulates positively several GABA$_A$Rs and has been shown to promote myelination in different models of demyelination. **(C)** Demyelination of the rat caudal cerebellar peduncle (c.c.p.; yellow line area in both tissue and MRI sections) was used as an experimental model to test the effect of β-carbolines on remyelination. Stereological injection of ethidium bromide (EB) in the c.c.p. induced its demyelination. Both black gold II (BGII) staining, and diffusion-weighted MRI (dMRI) analysis, were performed in three groups of animals: non-lesioned (vehicle-injected), lesioned (injected unilaterally in the right c.c.p.), and lesioned that were systemically treated with β-CCB. In the lesioned group both BGII-staining and fractional anisotropy (FA) decayed in the ipsilateral side compared against the control contralateral side. The β-carboline administration promoted remyelination as confirmed by both experimental analyses, suggesting that OPC-GABA$_A$R positive modulation by β-CCB favors the repairing process (modified from Cisneros-Mejorado et al., 2020).

cognitive and motor deficits (Allin et al., 2008; Larroque et al., 2008). GABAergic signaling markers are reduced in the cortex and WM of preterm infants diagnosed with DWMI (Robinson et al., 2006), and studies have also reported a reduction of cortical GABA concentration in a pre-clinical mouse model of DWMI (Komitova et al., 2013). A therapeutical strategy to overcome

demyelination is to enhance OPC proliferation and maturation to improve functional outcomes (Scafidi et al., 2014).

In this context, GABAergic signaling was tested on the myelination rate in a DWMI murine model induced by neonatal hypoxia treatment. First, cerebellar WM hypomyelination was revealed by electron microscopy and immunolabeling with myelin basic protein (MBP) and neurofilament (NF200) antibodies. This was accompanied by an increase in OPC proliferation ($Ki67^+$ /$Olig2^+$ cells) and a decrease in mature OLs ($CC1^+$ cells). OPCs also showed a reduced GABAergic synaptic input from interneurons. Second, $GABA_A$R-mediated signaling was tested with a pharmacological approach *in vivo* where the administration of bicuculline, a selective $GABA_A$R antagonist, increased the number of OPCs by threefold but decreased the amount of mature OLs. In contrast, tiagabine and vigabatrin, inhibitors of the GABA transporter and the GABA transaminase, respectively, decreased OPCs proliferation and increased the number of mature OLs. Thus, enhancing GABA availability by administration of tiagabine and vigabatrin ameliorated the effects of hypoxia and resulted in oligodendrogenesis enhancement and progression of OPCs to myelinating OLs (Zonouzi et al., 2015).

NEUROSTEROIDS AND $GABA_A$Rs IN REMYELINATION

The peripheral benzodiazepine receptor (PBR) is expressed in peripheral organs and regulates the transport of cholesterol to mitochondria for the synthesis of pregnenolone, a progesterone-derived neurosteroid that is metabolized to allopregnanolone (ALLO) (reviewed by Gavish et al., 1999), a positive allosteric modulator of $GABA_A$Rs that is produced *de novo* by both neurons and glial cells, and acts with 20-fold higher potency than benzodiazepines and barbiturates (Majewska et al., 1986; Purdy et al., 1991; Belelli and Lambert, 2005; Sripad et al., 2013). During late pregnancy, high levels of ALLO maintain the increased GABAergic tone required to attenuate the hypothalamic pituitary-adrenal axis activation and suppress the stress response (Neumann et al., 1998; Brunton et al., 2014). A recent study, performed in pregnant and postpartum rats, used lysolecithin-induced demyelination in the CC to explore a correlation between GABAergic signaling and remyelination (Kalakh and Mouihate, 2019). The results showed augmented proliferation of OPCs and myelination index in the demyelinated CC of pregnant rats when compared to virgin and postpartum rats. Furthermore, Western blot studies showed higher expression of myelin oligodendrocyte glycoprotein (MOG) and 2′,3′-Cyclic nucleotide 3′-Phosphodiesterase (CNPase) in focally demyelinated CC from pregnant rats. Thus, it seems that pregnancy generates a pro-myelinating environment in response to a focal demyelination injury (Kalakh and Mouihate, 2019). To test if the increased GABAergic tone associated with pregnancy was involved, bicuculline was injected together with lysolecithin. Bicuculline administration worsened the demyelination lesion and reduced OPC density in pregnant rats, suggesting that $GABA_A$R-mediated signaling promotes remyelination. Since increased GABAergic tone is modulated by ALLO during pregnancy, the contribution of endogenous ALLO on de/re-myelination was tested by inhibiting its synthesis with finasteride, an inhibitor of 5α-reductase. The administration of finasteride also resulted in a large demyelination lesion and the reduction of OPC population compared to vehicle-treated pregnant rats, in a similar manner to the results obtained with $GABA_A$R antagonism (Kalakh and Mouihate, 2019). Immunofluorescence analysis showed that the expression of $GABA_A$R-γ2 subunit was absent in the saline-injected CC of pregnant, virgin or postpartum rats. However, a subset of OPCs ($NG2^+$ cells) in the vicinity of the demyelination lesion were immunoreactive to this subunit, and Western blot studies showed an increased expression in the CC of pregnant rats when compared to virgin or postpartum animals (Kalakh and Mouihate, 2019). Overall, these results suggest that $GABA_A$Rs containing the γ2 subunit are upregulated in OPCs during remyelination in adult animals. Altogether, these results suggest that OPC proliferation may be promoted through ALLO-modulated $GABA_A$Rs in the demyelinated CC of pregnant rats.

In another study testing the promyelinating action of ganaxolone, a synthetic analog of ALLO with increased bioavailability, was used in an experimental model of preterm birth (Shaw et al., 2019). Brain WM volume is reduced in preterm children, which correlates with an increased risk of developing attention deficient hyperactivity disorder (AHDH) and anxiety. Fetal neurodevelopment requires exposure to neurosteroids provided by the placenta during pregnancy, and preterm birth is accompanied by a drastic drop of ALLO. This was experimentally reproduced in guinea pigs after *in utero* administration of finasteride. The administration of ganaxolone to preterm guinea pigs improved myelination of the CA1 region of the hippocampus and subcortical WM, suggesting that $GABA_A$R modulation by neurosteroids may be a potential therapeutical tool to overcome myelination deficits in early neurodevelopment (Shaw et al., 2019).

β-CARBOLINES AND $GABA_A$Rs IN REMYELINATION

The β-carbolines are part of a heterogeneous family of compounds found in several fruits, tobacco, alcohol and coffee, among others. They are also present in the mammalian cerebrospinal fluid and brain (for review see Polanski et al., 2011). β-carbolines have been assessed in behavioral tests due to their potential modulatory effect on $GABA_A$R and success in a variety of illnesses (Cowen et al., 1981; Novas et al., 1988; Medina et al., 1989; Rowlett et al., 2001; Venault and Chapouthier, 2007). In addition to the inverse agonist action with a negative effect, it has been reported that β-carbolines act on a second binding site with a positive modulatory effect (Novas et al., 1988; Sieghart, 2015).

In some $GABA_A$Rs this effect seems to involve a low-affinity binding site described for diazepam (Walters et al., 2000; Sieghart, 2015). Indeed, N-butyl-β-carboline-3-carboxylate (β-CCB) acts on OL-$GABA_A$Rs with a strong enhancement on the GABA response (Arellano et al., 2016), which is not observed in neuronal

cells isolated from brain cortex (Cisneros-Mejorado et al., 2020). Thus, β-CCB action as a positive modulator of GABAergic neuron-OL signaling was tested on remyelination using a murine model of demyelination/remyelination (Woodruff and Franklin, 1999; Cisneros-Mejorado et al., 2020). Ethidium bromide was stereotaxically injected into the caudal cerebellar peduncle (c.c.p.) of rats to induce demyelination, and the resulting lesion was histologically characterized with black-gold II staining (BGII) and longitudinally characterized by magnetic resonance imaging (MRI) to detect microstructural changes (Cisneros-Mejorado et al., 2020). As expected, decreased fractional anisotropy (FA) and increased radial diffusivity were evident following c.c.p. lesioning. The MRI analysis correlated well with a decrease in myelin content as revealed by BGII staining. However, when systemic β-CCB was administered daily for 2 weeks in lesioned animals, an increase in the FA was observed in parallel with a radial diffusivity decrease. These changes also correlated with recovery of myelin staining with BGII (**Figure 1C**). Animal behavior was unaffected by β-CCB as revealed by open field exploration, freezing, signs of pain, anxiety or apparent aggression. These observations strongly suggest remyelination enhancement by β-CCB treatment.

DISCUSSION

$GABA_A$R-mediated signaling plays a key role during embryonic and early postnatal neurodevelopment of OPCs. Indeed, $GABA_A$R activation is involved in the regulation of proliferation, differentiation, axon-glia recognition and myelination onset (Zonouzi et al., 2015; Arellano et al., 2016; Hamilton et al., 2017). A subset of OPCs prevail in the adult brain; these cells are known as NG2 glia and functional expression of $GABA_A$R-mediated signaling has also been reported (Vélez-Fort et al., 2010). Experimental evidence from different studies suggests that $GABA_A$R-mediated signaling to OPCs is important to improve myelination or remyelination in demyelinating diseases (Zonouzi et al., 2015; Kalakh and Mouihate, 2019; Shaw et al., 2019; Cisneros-Mejorado et al., 2020). Specific control and/or targeting of $GABA_A$Rs expressed in the OPCs will help to understand their role in its physiology, and particularly to comprehend the role of GABAergic signaling in the myelination/demyelination/remyelination process of the brain. In this direction, β-carbolines and neurosteroids, particularly β-CCB and ALLO, are promising therapeutical candidates to selectively target OL $GABA_A$Rs and promote remyelination.

AUTHOR CONTRIBUTIONS

DR-H, AC-M, and RA contributed to the manuscript, approved the submitted version, and designed the content of the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Cell and Developmental Biology

REVIEW
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Neurorepair and Regeneration of the Brain: A Decade of Bioscaffolds and Engineered Microtissue

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Repairing the human brain remains a challenge, despite the advances in the knowledge of inflammatory response to injuries and the discovery of adult neurogenesis. After brain injury, the hostile microenvironment and the lack of structural support for neural cell repopulation, anchoring, and synapse formation reduce successful repair chances. In the past decade, we witnessed the rise of studies regarding bioscaffolds' use as support for neuro repair. A variety of natural and synthetic materials is available and have been used to replace damaged tissue. Bioscaffolds can assume different shapes and may or may not carry a diversity of content, such as stem cells, growth factors, exosomes, and si/miRNA that promote specific therapeutic effects and stimulate brain repair. The use of these external bioscaffolds and the creation of cell platforms provide the basis for tissue engineering. More recently, researchers were able to engineer brain organoids, neural networks, and even 3D printed neural tissue. The challenge in neural tissue engineering remains in the fabrication of scaffolds with precisely controlled topography and biochemical cues capable of directing and controlling neuronal cell fate. The purpose of this review is to highlight the existing research in the growing field of bioscaffolds' development and neural tissue engineering. Moreover, this review also draws attention to emerging possibilities and prospects in this field.

Keywords: bioscaffolds, biomaterials, brain repair, tissue engineering, stem cells

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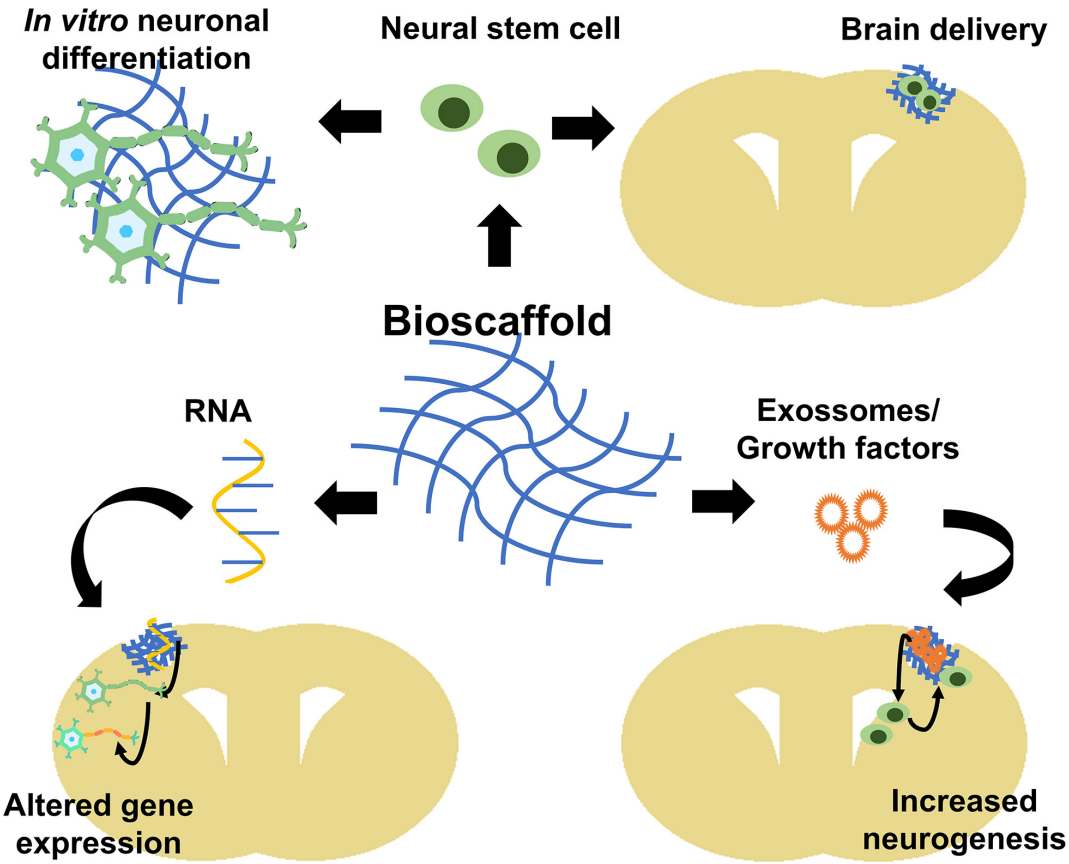
BRAIN INJURIES

Brain injuries are a significant cause of mortality and morbidity across the world. Injuries are divided into two types: (i) traumatic brain injury (TBI), caused by an external force to the head, such as a bump, blow, or penetrating object, and (ii) injury associated with a neurologic illness or condition, such as stroke, brain cancer, and other neurogenerative diseases (Stephenson et al., 2018; Cabrera, 2021). In 2016, neurological disorders were the world's leading cause of disability-adjusted life-years, defined as the sum of years of life lost and years lived with disability, afflicting 276 million people and the second leading death cause, killing 90 million people (GBD 2016 Neurology Collaborators, 2019). The outcome of brain injuries is cell death, with high chances of functional and cognitive limitations, such as movement deficits, mood disorders, headaches, disturbances of memory, emotion, and behavior, and increased risk of development of neurodegenerative diseases (Riggio, 2011; Sulhan et al., 2020). Brain injuries reduce the quality of life of the injured person and their families, besides its high cost to healthcare systems (Humphreys et al., 2013).



GRAPHICAL ABSTRACT | Bioscaffolds potential applications in tissue engineering. Bioscaffolds can be used to grow stem cells and target their differentiation *in vitro* **(upper, left)** or be used as stem cell delivery route in a brain injury **(upper, right)**. Bioscaffolds can also contain si/miRNAs that will modify locally neural cells gene expression **(lower, left)** or contain exosomes/growth factors for paracrine signaling such as stimulating neurogenesis and increase neural stem migration to injury area **(lower, right)**. This cover has been designed using resources created by Vitaly Gorbachev from Flaticon.com.

CELLULAR AND MOLECULAR RESPONSES TO BRAIN INJURIES

Inflammatory Response

Inflammation is a complex biological process in the body in response to cell and tissue damage (Chen et al., 2017). The definition of neuroinflammation is an inflammatory process within the brain or spinal cord (DiSabato et al., 2016; Wang Y. et al., 2020). Neuroinflammation is a common feature in many neurological diseases such as brain trauma, stroke, multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD) (Stephenson et al., 2018). Neuroinflammation will vary in type and range depending on the context, duration, and course of the primary insult. Inflammation can be transient and self-limited, facilitating tissue repair or persistent and dysregulated, leading to a chronic inflammatory state, resulting in tissue degeneration (Tansey et al., 2007).

There are several possible mechanisms of inflaming. Here, we provide a general overview of the process. The inflammatory processes may be initiated by the endogenous host-derived cell debris [damage-associated molecular patterns (DAMPs)] originated from acute cell death or that accumulate with age due to increased production or impaired elimination (Sochocka et al., 2017). DAMPs bind on pattern recognition receptors (PRRs), leading to cellular activation, that triggers inflammatory response. The PRRs comprise a family of membrane-bound toll-like receptors (TLRs), C-type lectin receptors (CLRs), cytoplasmic receptors, RIG-like receptors (RLRs), and NOD-like receptors expressed mainly on resident microglia (Dokalis and Prinz, 2019). Resident microglia are central players in this process because of their active role in immune surveillance. Microglia remove cell debris and become activated, releasing inflammatory proteins, like Interleukin 1 beta (IL1β), Interleukin 6 (IL 6), tumor necrosis factor alpha (TNF α), chemokines (such as C-C motif ligand 2 or CCL2 and C-X-C motif ligand 1 or CXCL1), reactive oxygen species (ROS) proteases and prostaglandins (Petrovic-Djergovic et al., 2016; Szalay et al., 2016; Mundim et al., 2019). Astrocytes become reactive, a process characterized by changes like hypertrophy and increased glial acid fibrillary protein (GFAP) expression (Wang et al., 2018). Reactive astrocytes proliferate and migrate through the injury site. Astrocytes secrete matrix metalloproteinases (MMPs) that degrade extracellular matrix (ECM) and facilitate their

migration, but also degrade the basal lamina and promote blood–brain barrier (BBB) breakdown (Abdul-Muneer et al., 2016). With the disruption of the BBB, circulating neutrophils, monocytes, T cells, and dendritic cells invade the brain parenchyma and potentiates inflammation, creating a positive loop (Takeshita and Ransohoff, 2012).

The inflammatory response stop mechanism is called "inflammation resolution." Inflammation resolution naturally occurs after acute or chronic inflammation and relies on the synthesis of specialized pro-resolving lipid mediators (SPM) by endothelial cells, macrophages, and neutrophils (Shang et al., 2019). SPM are a class of cell signaling molecules (Carracedo et al., 2019) that includes resolvins, protectins, maresins, and lipoxins (Qu et al., 2015). They result from the metabolism of polyunsaturated fatty acids released from omega-3-rich membranes by lipoxygenase, cyclooxygenase, or cytochrome P450 monooxygenase enzyme. During neuroinflammation resolution, anti-inflammatory cytokines as Interleukin 10 (IL 10) and trophic factors are released, promoting tissue regeneration (**Figure 1**; Dokalis and Prinz, 2019). However, if the inflammatory process remains unresolved, it can lead to chronic central nervous system (CNS) inflammation and neurodegeneration.

Extracellular Matrix (ECM) Remodeling and Glial Scar

After an injury, the presence of ROS, free radicals, and pro-inflammatory cytokines makes the perilesional area a hostile environment for cell survival (Sulhan et al., 2020). Reactive astrocytes migrate to the injury site, where they secrete inflammatory factors and MMPs that remodel the ECM, creating a barrier between the injured and the healthy tissue (Jang et al., 2020). The glial scar consists predominately of reactive astrocytes, microglia/macrophages, and ECM molecules, mainly chondroitin sulfate proteoglycans (CSPG) (Rolls et al., 2009). The glial scar contains the spread of neurotoxic molecules and prevents the expansion of neuronal damage and degeneration. Thus, the glial scar is essential for preventing extra cell degeneration in injury's acute phase (Rolls et al., 2009).

Astrocytes are well known for providing neuron trophic support. In the injury site, astrocytes maintain that function, producing and secreting several metabolites, including glucose, nutrients, and growth factors such as insulin-like growth factors (IGFs), nerve growth factors (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (Rolls et al., 2009). Thus, astrocyte migration to the injury site is crucial for perilesional neurons to survive (Liu and Chopp, 2016). The glial scar fills the ECM gaps in the lesion area, providing an environment where the vascularization network can regrow. Astrocytes and matrix components stimulate the local angiogenesis by recruiting endothelial cells and fibroblasts into the lesioned area (Rolls et al., 2009). Reactive astrocyte conditional ablation in transgenic mice leads to increased local tissue disruption, severe demyelination, and neuron and oligodendrocyte death (Bush et al., 1999; Faulkner et al., 2004; Vercelli and Boido, 2015), indicating that the glial scar might have an essential role in the injury acute phase. However, once a certain homeostasis level is reached, the glial scar impedes axon growth, necessary for repair. In that manner, the glial scar possesses a dual role, and its manipulation has to be well planned since its beneficial or detrimental role appears to be a matter of timing (Rolls et al., 2009).

The Glial Scar and Brain Repair: Effects on Plasticity and Neurogenesis

Brain plasticity refers to any process that leads to the recreation of functional neuronal circuits and function regain. Plasticity involves short-distance axon sprouting, leading to new connections and alteration in the strength of existing connections (Sharma et al., 2013). These changes can allow signals to bypass areas of damage through newly created circuits and reassign areas of the CNS to new functions. After brain injuries, such as a stroke, neurons in the perilesional area upregulate signaling pathways that promote axonal growth and synapse formation (Dancause and Nudo, 2011). There is an enhancement of dendritic spine turnover, providing a substrate for new connections. Neurons in perilesional tissue can project new axonal by several millimeters into nearby cortical areas where new functional synaptic connections are formed (Nagappan et al., 2020).

The glial scar tissue is well known for its inhibitory effect on axonal growth. One of the main studied glial scar growth-inhibitory components is CSPG. CSPG has been shown to induce neurite retraction and growth cone collapse *in vitro* (McKeon et al., 1991). Also, *in vitro* studies comparing astrocytic cell lines revealed decreased axonal growth when astrocytes produced more CSPG (Fidler et al., 1999). Degradation of CSPG by chondroitinase AC allowed for axon growth at the lesion site, although there was increased local astrocyte activation (Coulson-Thomas et al., 2008).

Other brain ECM components, many of them present at the glial scar, can inhibit neuroregeneration, mainly by inhibiting axonal growth, remyelination, and plasticity, summarized in **Table 1**.

The adult mammalian brain has two main areas known to produce new neurons: the subgranular zone (SGZ) of the hippocampus dentate gyrus, in which newborn neurons migrate laterally and integrate the hippocampus's granular zone, and the subventricular (SVZ) located in both lateral ventricles. Neural stem cells (NSC) located in the SVZ are pluripotent stem cells (iPSC) that can differentiate into astrocytes, oligodendrocytes, and neurons. Newborn neurons (neuroblasts) from the SVZ migrate a long distance through the rostral migratory stream (RMS) to the olfactory bulbs, where they differentiate into mature interneurons (Alvarez-Buylla and Garcia-Verdugo, 2002). The discovery of adult neurogenesis in mammalian brains shed light on new possibilities for brain repair. In rodent models of brain trauma and stroke, there is increased cell proliferation in the SVZ and recruitment of neuroblasts that migrate along blood vessels toward the injury (Saha et al., 2013; Liang et al., 2019). Reactive astrocytes are essential players in this process. Astrocytes are critical regulators of adult neurogenesis. Astrocytes are one of the primary sources of molecules such as bone morphogenetic protein (BMP) and WNT, which regulate NSC proliferation

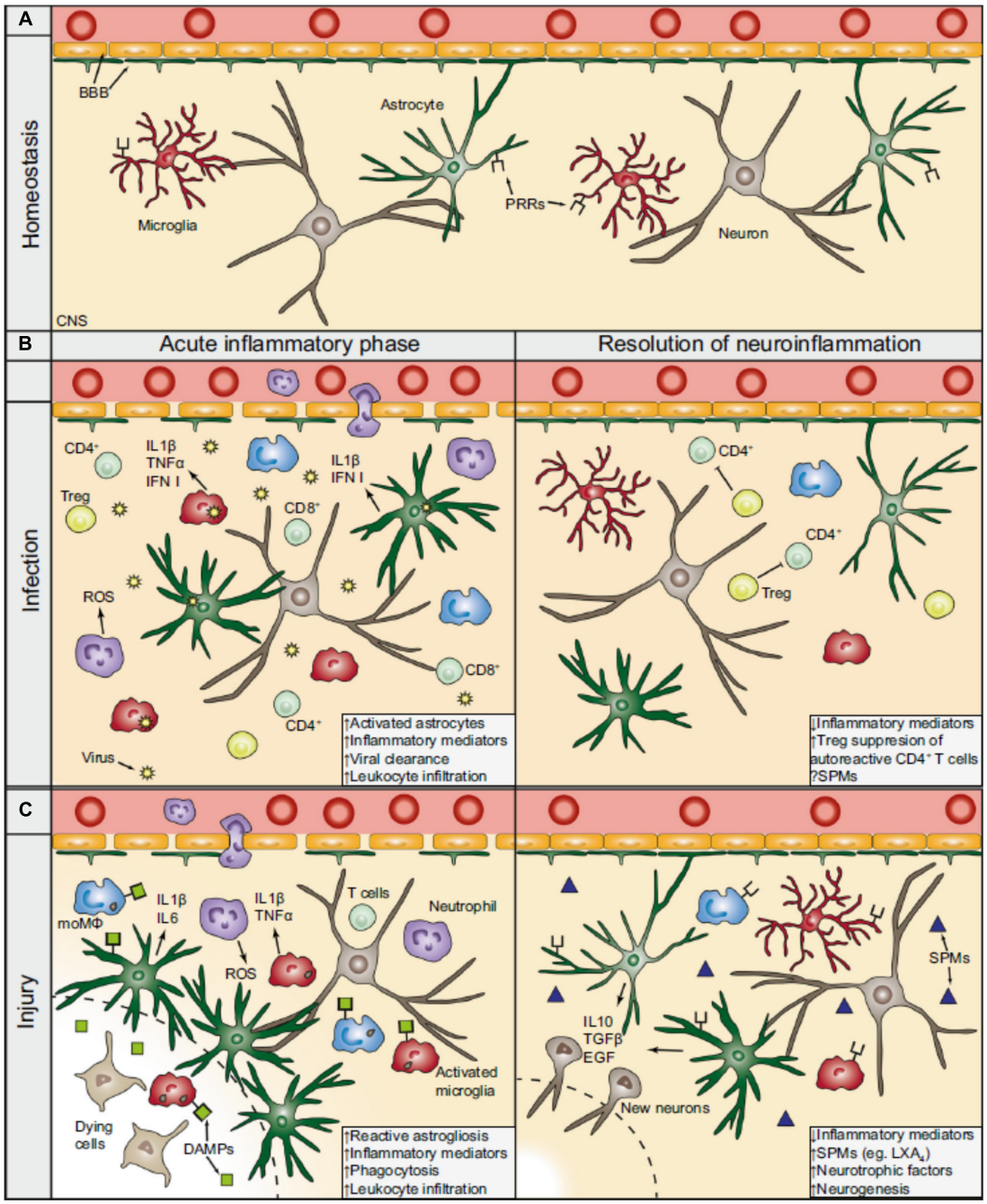


FIGURE 1 | The inflammatory sequel during CNS viral infection and injury. **(A)** During homeostasis, the BBB separates the periphery from the CNS by tightly regulating the entrance of circulating molecules and nutrients. Astrocytes are part of the BBB and functionally support neurons, while microglia constantly survey the CNS parenchyma for potential factors that could compromise its integrity. **(B)** During viral infection, intracellular PRRs (not shown) recognize virus-derived material and activate resident CNS cells to produce cytokines (e.g., interferons), which recruit peripheral immune cells. CD4+ and CD8+ T cells (lime) play a prominent role for the effective clearance of the virus. During the resolution phase and after the clearance of the virus, Tregs (yellow) action is important in silencing autoreactive CD4+ T cells and promotes resolution. The contribution of SPMs following viral CNS infections remains largely unexplored. **(C)** In a generalized model of CNS injury, DAMPs are released from dying and stressed cells which bind to PRRs thereby activating the resident CNS cells. The BBB is consequently compromised, hypertrophic astrocytes surround the lesion core (dashed line), and microglia become activated. In turn, glial cells can release pro-inflammatory mediators (e.g., IL 1β), which attract peripheral leukocytes and augment the inflammatory response. During the resolution of the neuroinflammation following CNS injury, anti-inflammatory cytokines (e.g., IL 10) and trophic factors (e.g., EGF) are released, promoting neuronal tissue regeneration. SPMs, such as LXA4, that are produced at the injured parenchyma facilitate, as well, in the resolution phase. BBB, blood-brain barrier; CNS, central nervous system: PRRs, pattern recognition receptors; moMΦ, monocyte-derived macrophages (blue); ROS, reactive oxygen species; Treg, regulatory T cells; IFN, interferon; SPMs, specialized pro-resolving mediators; DAMPs, damage-associated molecular patterns; IL, interleukin; EGF, endothelial growth factors; LXA4, lipoxin A4. Extracted from Dokalis and Prinz (2019). Republished with Springer Nature permission.

TABLE 1 | Proteins in the CNS extracellular matrix that contribute to the inhibition of neuroregeneration after injury.

Inhibitory protein	Function	Complementary receptors
Nogo-A	Remyelination inhibitor via the RhoA pathway	Nogo-66 terminus: NgR1, p75, TROY, and LINGO1 Amino-Nogo terminus: unknown
MAG	Remyelination inhibitor via the RhoA pathway	NgR2, GT1b, NgR1, p75, TROY, and LINGO1
OMgp	Remyelination inhibitor via the RhoA pathway	NgR1
Versican (CSPG2)	Important during inflammation as it interacts with inflammatory leukocytes and inflammatory cells recruiting chemokines. It also stabilizes perineuronal nets to stabilize synaptic connections.	N-terminus: hyaluronan in the extracellular matrix (ECM) C-terminus: Ligands in ECM, especially tenascin
NI-35	Non-permissive growth factor in myelin	Unknown
Ephrin B3	Inhibits remyelination	EphA4
Semaphorin 4D (Sema 4D)	Inhibits remyelination	PlexinB1
Semaphorin 3A (Sema 3A)	In scars in both PNS and CNS injuries	Nrp1, Nrp2, L1cam, Nrcam

Nogo: Neurite outgrowth inhibitor; NgR1: Neuronal Nogo-66 receptor 1; LINGO1: Leucine rich repeat and Immunoglobulin-like domain-containing protein 1; p75: neurotrophin receptor; TROY: Tumor necrosis factor receptor superfamily member 19; RhoA: Ras homolog family member A; MAG: Myelin-associated glycoprotein; GT1b: Trisialoganglioside protein; OMgp: Oligodendrocyte-myelin glycoprotein; CSPG2: chondroitin sulfate proteoglycan core protein 2 or versican; ECM: extracellular matrix; NI-35 A: CNS myelin-associated neurite growth inhibitor; EphA4: Ephrin type-A receptor 4; Nrp1: Neuropilin 1; Nrp2: Neuropilin 2; L1cam: L1 cell adhesion molecule; Nrcam: Neuronal cell adhesion molecule. Extracted from Nagappan et al. (2020).

and differentiation. NSC are attracted to the injury site by chemoattractive agents like CCL2 (C-C motif ligand 2), CCL11 (C-C motif ligand 11), CXCL12 (C-X-C motif ligand 12), and Prokineticin 2 (PROK2), mostly produced by astrocytes (Yan et al., 2007; Filippo et al., 2013; Moon et al., 2013; Mao et al., 2016; Wang et al., 2017; Zamproni et al., 2017; Mundim et al., 2019).

The glial scar matrix components also influence NSC cells under both physiological and pathological conditions. The developing CNS is enriched in proteoglycans which control developmental processes: neuronal migration and homing. In the adult brain, CSPG contributes to the maintenance of the neurogenic niches. Sulfated proteoglycan structures and, especially CSPG, were reported to affect NSC fate, survival, and maturation (Rolls et al., 2009). In a mouse model of brain trauma, SVZ-derived neuroblasts migrate toward the injured cortex but do not enter the area corresponding to the glial scar. Galindo et al. (2018) showed that, *in vivo*, neuroblasts migrated around the glial scar and attributed the inhibition of penetration into the scar to the presence of CSPG. *In vitro*, CSPG impaired neuroblast migration by altering cell protrusion and adhesion dynamics through Rho GTPase inhibition.

Although mobilization of NSC toward the injury occurs, many cells die or stray from the migratory path. Many NSC that reach the injury area fail to integrate into new neuronal circuits and die. For this reason, adult endogenous neurogenesis is insufficient for complete brain repair (Lu et al., 2017).

WHY USE BIOSCAFFOLDS TO REPAIR THE BRAIN?

To answer this question, we must first address the concept of repair. The term "repair," when used to describe damaged tissue healing, means to restore tissue architecture and function and comprises two processes: regeneration and replacement. Regeneration occurs when the damaged tissue grows into new tissue and is restored to its normal state. Replacement occurs when a different tissue, usually connective tissue, is deposited over the damaged tissue, producing a scar (Krafts, 2010). The CNS anatomy, physiology, and pathobiology complexity make repair exceptionally challenging. Rebuilding the brain means rebuilding the complex brain tissue architecture and its intricate and extensive vascular networks, not only morphologically but also functionally (Xu et al., 2011). The disability provoked by cerebral lesions justifies the need to explore new therapeutic solutions (Nih, 2020).

As with most tissues in the body, the brain has mechanisms to regenerate itself, such as, previously mentioned, endogenous neurogenesis and neuroplasticity (Sharma et al., 2013). However, these processes are limited after injury (Modo, 2019). One of the main reasons explaining the limitation is the hostile microenvironment formed in brain injuries or diseases. The lack of a healthy ECM and the presence of the glial scar impairs neuronal survival, axonal sprouting, and synaptogenesis (Erning and Segura, 2020).

Tissue engineering is a newly emerging field that combines biomaterials, stem cells, and chemical and physical cues to produce engineered tissue-like structures with the ultimate goal of replacing *in vivo* tissues and organs (Chandra et al., 2020). Biomaterials refer to a class of materials that have been engineered to integrate with a biological system and provide beneficial effects by directing or controlling cell interaction (Detsch et al., 2018). In brain injuries, biomaterials are mainly used for two purposes: as bioscaffolds, to provide mechanical support to the injured brain while providing cues for new neural

circuits formation, or as carriers, to deliver content such as stem cells, growth factors, exosomes, and gene vectors to the site of injury (Tuladhar et al., 2018). By replacing the virtual cavity formed after a brain injury, bioscaffolds can provide a tissue−appropriate physical and trophic environment for new neural cells and circuitry to survive and integrate into the host tissue (Tuladhar et al., 2018).

BRAIN ECM COMPOSITION VERSUS BIOMATERIAL CHARACTERISTICS

The ECM is fundamental for regulating several neural processes, including neurite outgrowth, synaptogenesis, synaptic stabilization, and injury-related plasticity, both in development and adulthood (Lam et al., 2019). Brain ECM is synthesized by both neurons and glia, comprising 20% of the adult brain's total volume (Lam et al., 2019). The main components include glycosaminoglycans (chondroitin sulfate, heparan sulfate, and hyaluronic acid), proteoglycans (neurocan, brevican, versican, and aggrecan), glycoproteins (tenascin-R), and low levels of fibrous proteins (collagen, fibronectin, and vitronectin) (Lam et al., 2019). Also, brain ECM has a different composition in different compartments, such as the vascular basement membrane composed of collagen, laminin, fibronectin, and proteoglycans; the perineuronal matrix, made primarily of CSPG, and the interstitial matrix containing mainly proteoglycans, hyaluronic acid, and small amounts of collagen, elastin, laminin, and fibronectin (Lau et al., 2013). Due to the lack of fibrous proteins like collagen, the brain scar composition is softer than the healthy tissue (Moeendarbary et al., 2017).

The brain EMC structure imposes some characteristics for the biomaterial to be used in the brain. The material must be biocompatible and possess mechanical properties close to the brain tissue (stiffer materials lead to increased gliosis, softer materials lead to poor material stability at the implant site). The material should induce no or minimal inflammatory response. In this way, once long-term implants can cause a chronic inflammatory reaction, degradability is also desirable, and degradation products should be non-cytotoxic as well. Once the brain is confined to the skull, the biomaterial must present minimal swelling to avoid a rise in intracranial pressure (Tuladhar et al., 2018; Mitragotri and Lahann, 2009).

The presence of a rigid skull also influences the biomaterial delivery route, making injectable and shape-adaptable materials like hydrogels preferred over solid scaffolds that require invasive surgical procedures for the implant (Tuladhar et al., 2018).

BIOMATERIALS ARE USED AS BIOSCAFFOLDS IN THE BRAIN

Biomaterial scaffolds can be derived from both natural and synthetic materials (Chen et al., 2010). Natural materials include ECM proteins (collagen, fibrin, laminin), polysaccharides (alginate, chitosan), and decellularized tissue ECM. Synthetic materials include metals, ceramics, and inorganic polymers. Natural polymers are composed of naturally occurring biological substances and have properties closely resembling the native brain ECM. Natural materials possess bioactive molecules that can induce bioscaffold remodeling by the host, supporting *de novo* tissue formation and less prone to generating an immune response. However, its physicochemical properties are difficult to control (Modo, 2019; Tuladhar et al., 2018).

On the other hand, synthetic polymers are more tunable and can be more easily functionalized to achieve desirable characteristics (Tuladhar et al., 2018). Physicochemical properties and geometric conformation are precise, and they can be produced on an industrial scale. The absence of biological material reduces contamination risk but limits its ability to induce a regenerative response (Modo, 2019).

Biomaterials can assume different forms as particles, fibers and hydrogels (Tuladhar et al., 2018). Hydrogels are formed by physical or chemical cross-linking of hydrophilic polymers or by self-assembly systems. Their mechanical properties are usually similar to brain tissue. As previously mentioned, for brain repair, hydrogels are easier to deliver than solid scaffolds. They can be injected in liquid form, fill the irregular injury cavity and then polymerize, forming a gel (Lacalle-Aurioles et al., 2020).

BIOSCAFFOLDS FOR BRAIN REPAIR

As we previously mentioned, bioscaffolds' primary role is to provide a substrate where cells can anchor. The ideal bioscaffold should match the brain biochemical environment (water content and pH), the brain biophysical environment (viscoelastic properties and porosity), the ECM three-dimensional (3D) architecture on a biologically relevant length scale, and stimulate cell infiltration into it (Maclean et al., 2018).

Bioscaffolds' mechanical forces can regulate the cell biological environment and control how cells interact with each other and with the ECM (Yuan et al., 2020).

Mechanical forces can influence cell functions such as migration, proliferation, differentiation, and apoptosis (Oksdath et al., 2018). For the CNS, bioscaffold electroconductive properties are usually desirable. It is well established that an electroconductive surface can increase neuronal differentiation, stimulate axon growing and facilitate synapsis formation (Herland et al., 2011; Pires et al., 2015). Fibrous scaffolds, particularly those with oriented fibers, can regulate and guide axon sprouting and synapse formation (**Figure 2**; Schaub et al., 2016; Zamproni et al., 2019). Since there is a tremendous variety in biomaterials and scaffold types being studied for their interaction with neural cells, **Table 2** provides an overview of the most recent research on this field. Most studies involving biomaterials and neural cells are *in vitro*. There is a lack of *in vivo* data, which explains the absence of commercially available human therapy platforms so far. One of the concerns regarding implanting biomaterials in the brain is the foreign body response (Lotti et al., 2017; Mariani et al., 2019). Although some materials have been shown to modulate inflammation, for example, high molecular weight hyaluronic acid decreases microglia and glial scarring at the injury site (Austin et al., 2012), there is a concern of adverse immune reactions resulting in exacerbate inflammation, healing impairment, fibrotic encapsulation, and

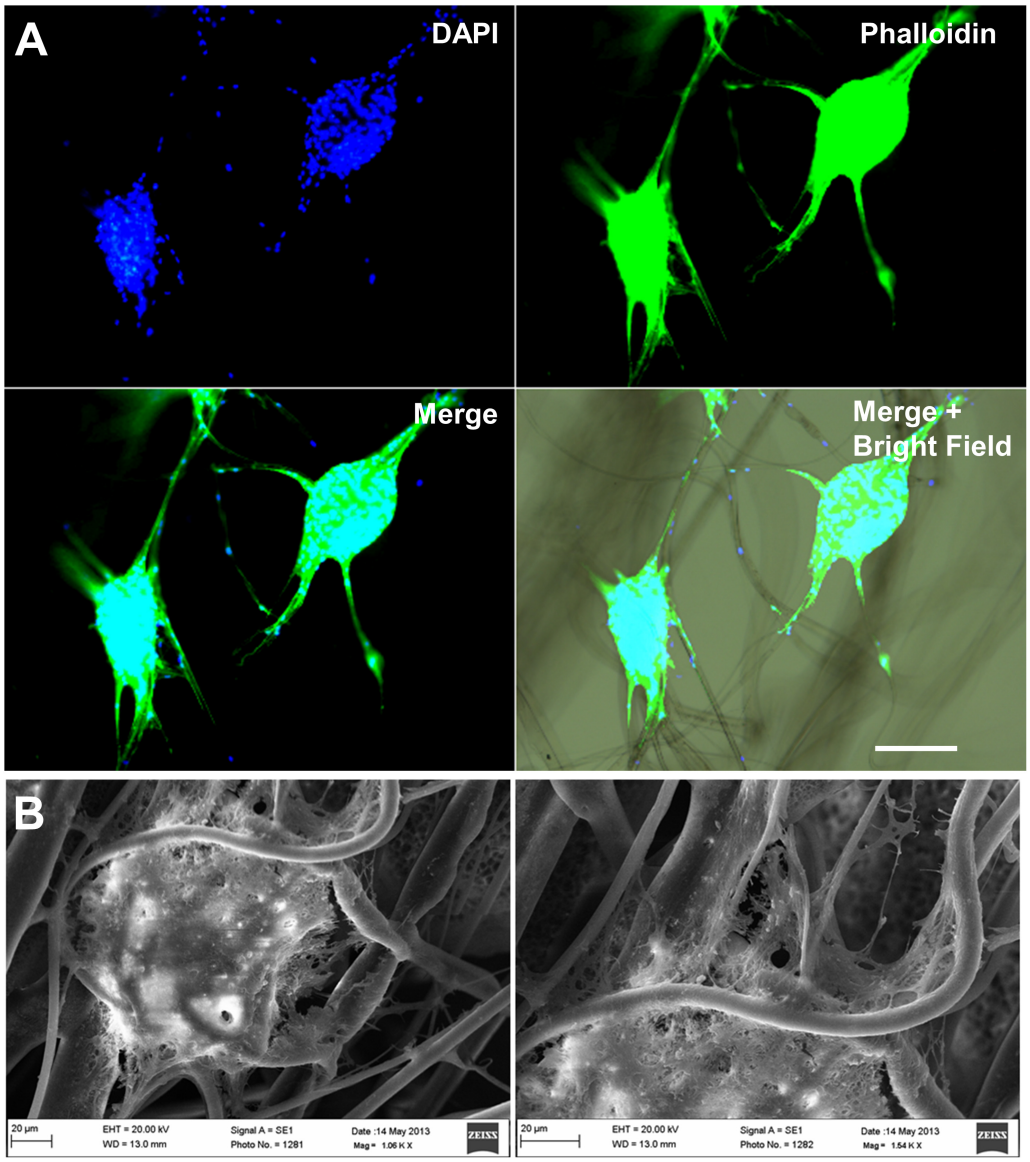


FIGURE 2 | Fibrous bioscaffolds can direct neural stem cell migration. Mouse neural stem cells neutrospheres cultured over polylactic acid fibers migrate following the direction of the scaffold's fibers. **(A)** DAPI plus phalloidin staining, Scale bar = 100 μm. **(B)** Scanning electron microscopy, Scale bar = 20 μm. Unpublished data from Zamproni et al. (2019).

isolation and rejection of medical devices (Mariani et al., 2019). One of the strategies to overcome this issue is incorporating of bioactive molecules (cytokines or growth factors) that can modulate the inflammatory response. To date, bioscaffolds are being investigated together with stem cells, growth factors, and exosomes to increase therapeutic possibilities (Qu et al., 2020).

BIOSCAFFOLDS AND STEM CELLS

Stem cells are cells with self-renewal capacity and the potential to differentiate into different cell types (Zakrzewski et al., 2019). Stem cell-based therapies are hugely explored for the CNS. Stem cell sources include embryonic stem cells (ESC), mesenchymal stem cells (MSC), induced iPSC and NSC (Zakrzewski et al., 2019).

Stem cells can exert therapeutic properties by differentiating into appropriate cell types at the injury site or, more often, by secreting neurotrophic factors that can promote neuroprotection, angiogenesis, and neurogenesis (Zakrzewski et al., 2019).

As previously mentioned, the injured brain disrupted ECM does not offer a proper microenvironment for cell anchoring, proliferating, and differentiating (Li et al., 2016; Wan et al., 2015), and this poses a challenge for conventional stem cell delivery. MSC intravenous or intracardiac administration after a TBI in rats showed that <0.0005% of the cells injected were

TABLE 2 | Selected PubMed indexed papers published in 2020, focusing on bioscaffolds and neural cell interactions.

Cell	Scaffold	Outcome	References
Embryonic mouse cortical neural cells	Silk fibers	-Increased neurite extension -Guided axonal elongation -Guided cell migration from cellular spheroids along the fibers	Mercado et al., 2020
Rat hippocampal neurons	Graphene	-Induced neuronal networks formation -Increased GABAergic activity	Rauti et al., 2020
Mouse NG108-15 cells	Graphene oxide/silk fibers	-Increased cell proliferation -Increased neurite extension	Magaz et al., 2021
Neuroglioma cells	Poly (3,4-ethylenedioxythiophene)/chitosan fibers	-Increased cell proliferation -Increased axon density	Du et al., 2020
Mouse NG108-15 cells	Poly (3,4-ethylenedioxythiophene)-polystyrene sulfonate (PEDOT: PSS)/silk fibers	-Increased cell proliferation -Increased neurite extension	Magaz et al., 2020
Human neuroblastoma cells	Poly-(-caprolactone (PCL) nanofibers	-Increased neurite extension	Elnaggar et al., 2021
Human neuroblastoma cells	Poly(3,4-ethylenedioxythiophene) (PEDOT)/Carbon nanotubes	-Increased neuronal markers	Dominguez-Alfaro et al., 2020
Rat hippocampal cells	Aragonite skeleton of the coral *Trachyphyllia geoffroyi*	-Promoted elongation of astrocytic processes -Increased GFAP expression in astrocytes	Morad et al., 2020
Rat adipose tissue-derived neuron-like cells	Corning(§PuraMatrix(^{TM} hydrogel	-Increased cell proliferation -Increased neuronal markers expression	Darvishi et al., 2020
Rat pheochromocytoma cells	Porcine brain decellularized ECM	-Increased neuronal differentiation	Reginensi et al., 2020
Human Glioblastoma cells (U-87MG)	Carbon nanotubes	-Reduced cell growth	Parikh et al., 2020

found at the injury site after 3 days (Turtzo et al., 2015). Delivering stem cells in bioscaffolds can surpass this issue once bioscaffolds provide the biomechanical support for cells until they can produce an ECM, increasing cell survival (Li et al., 2016). Scaffolds seeded with MSC improved cell retention compared to MSC alone in a mouse stroke model (Zamproni et al., 2019). Stem cells in biological scaffolds can be implanted into damaged sites to secrete neurotrophic factors, improve axon regeneration, promote myelinization, and reduce scar formation (Cooke et al., 2010). **Table 3** provides a list of the most recent research focusing on stem cell delivery through bioscaffolds.

Moreover, bioscaffolds can direct stem cell fate by providing physical-chemical cues to enhance stem cell differentiation in one specific cellular type. Scaffolds' physical cues include mechanical properties, pore sizes, porosity, surface stiffness, 3D structures, and mechanical and electrical stimulation. Scaffolds chemical cues include cell-adhesive ligands and exogenous growth factors (Xing et al., 2019). **Table 4** summarizes the most recent research on bioscaffolds directing stem cell fate into neuronal types.

BIOSCAFFOLDS FOR GROWTH FACTOR DELIVERY

Biomaterials are promising drug delivery vehicles for their ability to provide local, time-controlled release, which is particularly important in the brain since the BBB imposes intravenous drug delivery restrictions. Also, a biomaterial platform provides sustained drug release in a single application (Ziemba and Gilbert, 2017). Bioscaffolds can be used to deliver growth factors and help to create a pro-regenerative environment. Some of the factors include erythropoietin (EPO), BDNF, fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) (Chan et al., 2017). Liu et al. (2019) combined a collagen/chitosan scaffold with FGF to promote recovery in a spinal cord injury model in rats. The authors found significant improvements in locomotor function and electrophysiological examinations 8 weeks after scaffold implantation. Rats receiving collagen/chitosan scaffold/FGF group presented improved nerve fibers tract regeneration in magnetic resonance imaging. Sandoval-Castellanos et al. (2020) developed a platform to increase neurite extension using heparin binding-functional amine groups. NGF and BDNF were bound to heparin by electrostatic interaction. Both NGF and BDNF, alone or combined, supported neurite growth. Maximum dorsal root ganglion neurite growth *in vitro* was found at 1 ng/mL NGF alone, without a BDNF addictive effect. Leipzig et al. (2010) compared interferon-gamma with BDNF and EPO surface-immobilized to a methacrylamide chitosan scaffold to promote rat NSC differentiation. Interferon-gamma was shown to be the best single growth factor for the induction of neuronal differentiation. Also, NSC exposed to interferon-gamma/chitosan scaffold resulted in more neurons than soluble interferon-gamma. Skop et al. (2016) engineered chitosan-based scaffolds by covalently linking heparin using genipin, which then served as a linker to immobilize FGF. Fetal rat NSC cultured over the

TABLE 3 | Selected PubMed indexed papers published in 2020, focusing on bioscaffolds-stem cell delivery for CNS therapy.

Stem cell source	Scaffold	Disease Model	Outcome	References
Embryonic rat neural stem cells	Collagen/heparan sulfate porous scaffolds	Rat TBI model	-Improved regeneration of neurons, nerve fibers, synapses, and myelin sheaths -Reduced brain edema and cell apoptosis -Recovered rat motor and cognitive functions	Zhang et al., 2021
Human fetal brain- and spinal cord-derived neural stem cells	Aligned collagen sponge scaffolds	Rat complete spinal cord section	-Stem cell long-term cell survival -Stem cell neuronal differentiation -Reduced inflammation -Reduced glial scar formation -Recovered rat locomotor functions	Zou et al., 2020
Rat adipose tissue mesenchymal stem cells	RADA4GGSIKVAV (R-GSIK), a self-assembling nano peptide scaffold	Rat TBI model	-Reduced reactive astrocytes -Reduced microglial cells -Reduced TLR4, TNF, and IL6	Sahab Negah et al., 2020
Human umbilical cord mesenchymal stem cells	Collagen hydrogels	Rat Parkinson disease model	-No differences in proteomics between treated and control group	Santaella et al., 2020
Human umbilical cord mesenchymal stem cells	Collagen scaffolds	(1) Rat and dog complete spinal cord section (2) Patients with a medullar lesion	-Increased motor scores -Enhanced amplitude, and shortened latency of the motor evoked potential -Reduced injury area in magnetic resonance imaging	Deng et al., 2016
Adipose-derived mesenchymal stem cells overexpressing brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3)	Silk fibroin/chitosan scaffold	Rat complete spinal cord section	-Reduced scar tissue -Decreased inflammation -Increased nerve fiber formation	Ji et al., 2020
Rat neural stem cells	Matrigel	Rat complete spinal cord section	-Decreased reactive astrogliosis -Improved functional recovery	Wang J. et al., 2020
Human embryonic stem cell derived-neural stem cells	Hyaluronic acid hydrogel	Rat complete spinal cord section	-Increased oligodendrocyte differentiation -Improved locomotor function	Zarei-Kheirabadi et al., 2020
Mouse-induced pluripotent stem cell-derived neural stem cells	Fibroblast growth factor and chondroitin sulfate hydrogel	Mice stroke model	-Improved vascular remodeling -Improved cortical blood flow -Improved sensorimotor function	McCrary et al., 2020
Embryonic rat neural stem cells	Collagen/silk fibroin scaffold 3D bioprinted	Rat complete spinal cord section	-Reduced glial scar -Increased regenerative axons -Improved functional recovery -Improved electrophysiologic tests	Jiang et al., 2020

FGF/chitosan scaffold proliferated and remained multipotent for at least 3 days without FGF addition to the medium. NSC seeded on this scaffold showed high expression of stem cell markers (BLBP and SOX2) and presented decrease GFAP astrocytic marker expression compared to cells maintained on fibronectin-coated plates with FGF supplemented media. These data suggest that FGF/chitosan scaffolds are efficient in maintaining NSC stemness.

BIOSCAFFOLDS COMBINED WITH EXOSOMES

Exosomes are small membrane vesicles secreted by eukaryotic cells for intercellular communication and signaling (Ludwig and Giebel, 2012). Exosomes cargo includes cytokines and growth factors, signaling lipids, mRNAs, and microRNA that can influence cell response to injury, infection, and disease (Phinney and Pittenger, 2017). Exosomes have been studied for brain repair and now are being combined with bioscaffolds for therapy. This association rationale is that the scaffold may prolong exosome retention and sustain exosome delivery at the injury site (Tsintou et al., 2021).

Zhang et al. (2017) investigated if exosomes from MSC cultured in 3D collagen scaffold were superior for brain trauma recovery than exosomes from MSC cultured on conventional conditions. They delivered exosomes intravenously in a mice model of brain trauma. Both exosome types promoted endogenous angiogenesis and neurogenesis, reduced neuroinflammation, and significantly improved rat functional recovery. However, 3D cultured MSC-exosomes provided a better outcome in spatial learning than conventional MSC-exosomes.

Hsu et al. (2020) developed an alginate scaffold with human umbilical cord MSC exosomes to treat nerve injury-induced pain. The neuroprotective and neurotrophic effects of the exosomes were evaluated *in vitro*. The exosomes induced PC12 (pheochromocytoma) cells neurite outgrowth and protected PC12 and HEK293 (human embryonic kidney) cells against

TABLE 4 | Selected PubMed indexed papers focusing on bioscaffolds directing stem cell fate.

Stem cell source	Scaffold	Outcome	References
Mouse CGR8 embryonic stem cells	Poly ε-caprolactone (PCL)/gelatin scaffolds	-Promoted neural differentiation -Promoted efficient secretion of dopamine	Kheradmand et al., 2020
Human-induced pluripotent stem cell- and embryonic stem cell-derived neural stem cells	Poly(ethylene glycol) diacrylate-crosslinked porous scaffolds	-Increased neural cells functional maturity	Murphy et al., 2020
Human-induced pluripotent stem cells	Fibrin hydrogel	-Increased Olig2, MBP, Sox10, and PDGFRα expression -Increased oligodendrocyte differentiation	Nazari et al., 2020
Human olfactory ecto-mesenchymal stem cells	Chitosan-aniline pentamer/gelatin/agarose scaffolds	-Promoted differentiation into motor neuron-like cells	Bagher et al., 2019
Rat hippocampal neural stem cells	Poly-ε-caprolactone (PCL) fibers	-Increased cell proliferation -Increased astrocyte and oligodendrocyte differentiation	Patel et al., 2019
Neural stem cells	Poly (L-lysine) modified silk fibroin film	-Increased cell proliferation -Decreased apoptosis -Increased neuronal differentiation	Zhao et al., 2018
Mouse mesenchymal stem cells	Graphene foam	-Promoted dopaminergic neuronal differentiation	Tasnim et al., 2018

formaldehyde acid treatment. Right L5/6 spinal nerve ligation was performed in Sprague-Dawley rats to induce mechanical allodynia and thermal hyperalgesia. Exosomes in scaffolds were wrapped around ligated L5/6 spinal nerves for treatment. Treated rats performed better in functional scores and presented signs of enhanced myelinization of injured axons. Treatment also attenuated upregulation of c-Fos, GFAP, Iba1 (ionized calcium-binding adapter molecule 1), TNFα, and IL-1β, while enhancing IL-10 and GDNF (glial cell line-derived neurotrophic factor) in the ipsilateral dorsal root ganglion.

BIOSCAFFOLDS AND GENE THERAPY

Gene therapy is fast-growing, and many CNS disorders are potential candidates for treatment approaches that involve the correction of genetic abnormalities (Choong et al., 2016). However, the use of viral vectors in gene therapy still poses some concern, and the development of new, highly efficient, low cytotoxic gene therapy strategies are required (Costard et al., 2020). Biomaterials and bioscaffolds may offer a safer alternative in delivering genetic material to cells and can be, in the future, the key for genic therapy in humans (Gower and Shea, 2013). Costard et al. (2020) used MgAl-NO_3 layered double hydroxide as a non-viral vector to deliver nucleic acids (pDNA, miRNA, and siRNA) to MSC using a 3D scaffold approach. Nucleic acids were complexed with MgAl-NO_3 layered double hydroxide and incorporated in collagen-nanohydroxyapatite scaffolds. The fabricated platform allowed successful MSC transfection.

The bioscaffold-gene therapy combination strategy has been investigated for the development of angiogenic platforms. Angiogenesis is a critical process required in the regeneration of many tissue and systems, including CNS regeneration (Lee et al., 2020). Laiva et al. (2018) developed a system by combining nanoparticles carrying a gene encoding for stromal-derived factor-1 alpha (SDF-1α) with a collagen-CSPG scaffold to enhance the MSC angiogenic response. They found that MSC on the scaffold exhibited early over-expression of SDF-1α mRNA combined with the activation of the angiogenic markers VEGF and CXCR4 (C-X-C chemokine receptor type 4). The conditioned media from these cells promoted a 20% increase in endothelial cell viability, a 33% increase in endothelial cell tubule formation, and a 50% increase in endothelial cell migration in a wound-healing model. Pro-angiogenic genes were also upregulated in endothelial cells exposed to conditioned media of MSC in scaffold.

Huntington's Disease (HD) is an inherited autosomal-dominant neurodegenerative disease. Although genetic mutation responsible for HD is well know, there is still no treatment to stop or slow disease progression. Sava et al. (2020) developed chitosan nanoparticles loaded with anti-huntingtin siRNA to treat an HD mouse model using the intranasal route. The authors developed four formulations of nanocarriers able to lower huntingtin mRNA expression by at least 50%.

ENGINEERED MICROTISSUE

Microtissue Engineered Neural Networks

Microtissue engineered neural networks (micro-TENNs) were developed at the University of Pennsylvania for supporting neuronal survival and neurite extension (Struzyna et al., 2015). Micro-TENNs consist of neuronal populations with long axonal tracts entrapped into tubular hydrogels of 180–500 μm diameter and up to 2.0 cm length. Micro-TENNs are fabricated by filling a cylindrical mold with a longitudinally centered needle with liquid hydrogel. Once the gelification occurs, the needle is removed, creating a hollow micro-column that will be filled with an

ECM solution. The ECM solution is responsible for providing an environment suitable for neuronal adhesion and axonal outgrowth (Struzyna et al., 2017). Micro-TENNs reconstitute the architecture of long-distance axonal tracts. They may serve as an effective substrate for re-establishing long-distance axonal connections and reconstruction of damaged brain pathways. Micro-TENNS have very small diameter being easily delivered into the brain with minimally invasive procedures.

Micro-TENNs were injected into rats' brains using a stereotaxic device to connect deep thalamic structures with the cerebral cortex. The authors found that micro-TENN neurons survived at least 1 month and maintained their extended axonal architecture along the cortical-thalamic axis. They also found micro-TENN neurons extend neurites into the host cortex, with successful synapse formation (Struzyna et al., 2015). In another approach, Micro-TENNs were used to align an astrocytic network with mimicking the glial tube existent along the RMS. Those astrocytic networks successfully improved NSC migration and directly directed the cells from the neurogenic niche until the injured area (Winter et al., 2016).

Biomaterial Based Cerebral Organoids

A cerebral organoid is an *in vitro* miniature organ resembling the brain. Organoids production relies on self-organizing cell properties, recapitulating early developmental events (Di Lullo and Kriegstein, 2017; Hoang and Ma, 2021). They are usually derived from iPSC and cultured for months with a set of growth and trophic factors that emulate organogenesis. The organoid organization is ideal for understanding cell interactions in a complex environment and offers great potential in disease modeling and regenerative medicine (Di Lullo and Kriegstein, 2017).

Bioscaffolds' major role in tissue engineering is to control the biochemical and physical microenvironment of the cells. In organoids, cellular self-assembly leads to the secretion of ECM components and trophic factors by the cells themselves. However, it is desirable to control the initial conditions in organoid formation (Wan, 2016). In cerebral organoids, the most commonly used bioscaffold is Matrigel®. The generation of brain organoids based on Matrigel® systems allowed to generate more sophisticated models that can capture region-specific features of the human brain, like cortical plate formation (Lancaster et al., 2017), forebrain (Kadoshima et al., 2013; Lancaster et al., 2017), midbrain and hypothalamic development (Jo et al., 2016; Qian et al., 2016). Matrigel® droplets have been standardized for numerous brain organoid disease models such as microcephaly, AD, and PD (Hoang and Ma, 2021).

Lancaster et al. (2017) combined organoids with poly(lactide-co-glycolide) copolymer (PLGA) fiber microfilaments as a scaffold to elongate the embryoid bodies and found that the organoids engineered with microfilaments presented several advantages over the traditional organoid formation. The presence of PLGA microfilaments elongated and enhanced neuroectoderm formation and improved cortical development with microfilament-engineered cerebral organoids (enCORs) presenting large lobes of brain tissue. EnCORs showed a very reproducible neuronal induction and presented an almost complete lack of non-neural tissue with decreased amounts of endoderm and mesoderm layers. By reconstituting the basement membrane with Matrigel®, the authors could polarize the cortical plate and recapitulate an architecture similar to radial units, a characteristic not previously recapitulated *in vitro*.

Three-Dimensional Bioprinting

As previously stated, the human brain is the most complex structure in the human body. In this context, 3D bioprinting offers a solution for designing specific individualized constructs while controlling tissue architecture. 3D bioprinting combines one or more cell types with a supportive bioscaffold, named bioink, to fabricate structures that resemble the native tissue topographically (Thomas and Willerth, 2017).

Despite the massive advance in the field in the last decade, it is still impossible to print whole tissues or organs that can be implanted. Some promising results have been shown for the spinal cord. Joung et al. (2018) printed a mixture of iPSC-derived spinal NSC and oligodendrocyte progenitor cells in a gelatin and fibrin bioink to fabricate a spinal cord. Bioprinted NSC was able to differentiate and extend axons throughout the scaffold. These neuronal networks' activity was confirmed by physiological spontaneous calcium flux studies. Jiang et al. (2020) designed a 3D silk fibroin scaffold with cavities that simulate the normal spinal cord anatomy. They transplanted the scaffold combined with NSC in Sprague-Dawley rats submitted laminectomy. Rats receiving the combination of scaffold plus cells presented functional neurological scores significantly higher. They also performed better in electrophysiological studies, and magnetic resonance imaging revealed spinal cord continuity and injury cavity filling. The bioprinted spinal cords also decrease the glial scar while increasing regenerative axons.

Although implantable brain tissue is not yet available, it is feasible to produce smaller and less complex brain structures to study physiological cell-to-cell or cell-to-material interactions. 3D bioprinting can improve *in vitro* platforms for modeling neurological diseases, neural regeneration, and drug development. Li et al. (2020) developed a 3D brain-like co-culture construct where neurospheroid 3D structures were fabricated in an astrocyte-laden resembling a NSC niche environment. Then, the authors used a photo-cross-linkable bioink to bioprint neurospheroid layers. Neurospheroids into the 3D net were able to differentiate into neuronal cells. Sharma et al. (2020) used a fibrin-based bioink formulation combined with drug-releasing microspheres and human iPSC-derived NSC to print neural tissues. Microspheres were loaded with guggulsterone, a molecule capable of promoting NSC differentiation into dopaminergic neurons. Combining these three elements, they achieved a high viable tissue (95% viable cells 7 days post-printing) that expressed neural markers TuJ1 (class III beta-tubulin), Forkhead Box A2 (FOXA2), tyrosine hydroxylase (TH), GFAP, and the oligodendrocyte progenitor marker (O4). Quantitative polymerase chain reaction (qPCR) analysis also demonstrates the presence of *NURR1* (nuclear receptor related 1, gene expressed in midbrain dopaminergic neurons), *LMX1B* (LIM homeobox transcription factor 1-beta), *TH*, and *PAX6* (Paired box protein 6) after 30 days.

CONCLUSION AND FUTURE DIRECTIONS

Although spontaneous tissue regeneration is limited in the CNS, tissue engineering strategies to overcome the biological and physical challenges imposed by brain injury are gradually being developed.

Bioengineering already offers a series of commercially available products for tissues like skin, bone, and cartilage, but this is not the case for the CNS, and up to now, there are no suitable bioengineered therapeutic solutions to amend injuries to the CNS.

This review highlighted the CNS therapeutic approaches involving bioscaffolds. Since several pathologies can affect the CNS, it is rational to believe that these approaches will be complementary rather than competing, and the constructs should match patient needs. In PD, for example, a platform that stimulates dopaminergic neuron differentiation is required, whereas, in amyotrophic lateral sclerosis (ALS), the challenge is to replace long neuronal tracts. The advancement of precision medicine and new scaffold fabrication methods such as 3D printing will allow individualized treatment. Bioscaffolds and scaffold-based constructs should evolve in the next years with increasing complexity and functionality, impacting medical research.

However, significant challenges must be addressed. It is still impossible to produce fully vascularized tissue units, which is essential to increase constructs' thickness and complexity while ensuring cell survival. Also, biomaterials' long-term effects in the CNS and their interaction with the immune system must be addressed. Finally, it is essential to understand developmental biology to better design "smart bioscaffolds" capable of stimulating neurogenesis and neural network formation.

AUTHOR CONTRIBUTIONS

LZ and MM wrote the manuscript. MP revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Microglia Function on Precursor Cells in the Adult Hippocampus and Their Responsiveness to Serotonin Signaling

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Microglia are the resident immune cells of the adult brain that become activated in response to pathogen- or damage-associated stimuli. The acute inflammatory response to injury, stress, or infection comprises the release of cytokines and phagocytosis of damaged cells. Accumulating evidence indicates chronic microglia-mediated inflammation in diseases of the central nervous system, most notably neurodegenerative disorders, that is associated with disease progression. To understand microglia function in pathology, knowledge of microglia communication with their surroundings during normal state and the release of neurotrophins and growth factors in order to maintain homeostasis of neural circuits is of importance. Recent evidence shows that microglia interact with serotonin, the neurotransmitter crucially involved in adult neurogenesis, and known for its role in antidepressant action. In this chapter, we illustrate how microglia contribute to neuroplasticity of the hippocampus and interact with local factors, e.g., BDNF, and external stimuli that promote neurogenesis. We summarize the recent findings on the role of various receptors in microglia-mediated neurotransmission and particularly focus on microglia's response to serotonin signaling. We review microglia function in neuroinflammation and neurodegeneration and discuss their novel role in antidepressant mechanisms. This synopsis sheds light on microglia in healthy brain and pathology that involves serotonin and may be a potential therapeutic model by which microglia play a crucial role in the maintenance of mood.

Keywords: microglia, serotonin, hippocampus, BDNF, fluoxetine, neuroinflammation

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INTRODUCTION

In the adult brain, microglia are the resident macrophages and, as such, a unique cell population interacting with neurons, astrocytes, oligodendrocytes, and the various signaling molecules. Characterized by Iba-1 and CD11b immunoreactivity (expressed in resting and activated cells; Franco and Fernández-Suárez, 2015), microglia exhibit a diverse, dynamic morphology that allows a quick response to changes in the environment. Under physiological conditions, highly branched microglial cells constantly sense the environment to maintain homeostasis, modulate synapse maturation and connectivity, and regulate neuronal activity (Kettenmann et al., 2011). In the hippocampus, in particular, microglia display a vigilant phenotype (Grabert et al., 2016);

they take part in learning-dependent synaptic plasticity and neural network excitability, and release of growth factors and neurotrophins, e.g., brain-derived neurotrophic factor (BDNF) (Parkhurst et al., 2013), involved in memory formation. As part of the limbic system, the hippocampus plays a central role in learning, especially in the encoding and retrieval of episodic and spatial memories (Buzsaki and Moser, 2013). Importantly, microglia contribute to the lifelong generation of new neurons in the hippocampus. Set in the dentate gyrus, neural stem cells (NSCs) retain fate plasticity and respond to a variety of local cues and extrinsic stimuli that foster a neuronal fate. Most of the newly generated cells die before maturation into granule neurons (Dayer et al., 2003) as a strategy balancing cell proliferation vs. cell death. In their role in phagocytosis of damaged cells and debris, recent studies attribute non-activated microglia to the control over the neuronal cell pool by removal of apoptotic progenitor cells (Sierra et al., 2010).

A prominent local component of the neurogenic niche is serotonin (5-HT). Modulating both proliferation and survival of newly generated cells, serotonin is a key regulator of adult neurogenesis (Alenina and Klempin, 2015) and, together with BDNF, is involved in antidepressant mechanisms (Mattson, 2008; Molendijk et al., 2011; Kronenberg et al., 2018a). Accumulating evidence from rodent studies and *in vitro* modeling indicates that microglia interact with local hormones and neurotransmitters by the expression of various receptors (Pocock and Kettenmann, 2007). Among them are metabotropic glutamate receptors, the chemokine fractalkine receptor (CX3CR1) (Sellner et al., 2016), and various serotonin receptor subtypes, particularly 5-HT2B (Krabbe et al., 2012). Expressed on microglia subpopulations (Kettenmann et al., 2011), receptors' attraction to neuronal secretion of signaling molecules assists surveillance of the microenvironment (Szepesi et al., 2018).

Upon stimulation, microglia become activated, proliferate, lose their ramified morphology, and display the first innate immune defense (Beynon and Walker, 2012). They rapidly act by secretion of distinctive inflammatory cytokines, e.g., interleukins (ILs), interferons (IFNs), and tumor necrosis factors (TNFs) that in turn modulate the release of neurotransmitters and neurotrophins. Depending on the microenvironment, cytokines function pro- (i.e., IL-1β, IL-6, IL-18, and TNF-α) or anti-inflammatory (i.e., IL-4 and IL-10) (Suzumura, 2013; Franco and Fernández-Suárez, 2015). BDNF exerts primarily anti-inflammatory and neuroprotective effects (Chen et al., 2016). Dysregulation of the immune defense function leads to neuroinflammation and neuronal cell death. Excessive glutamate release is particularly neurotoxic (Lewerenz and Maher, 2015). Microglia-mediated "neuroinflammation" is increasingly recognized to contribute to the development and progression of neurodegenerative diseases and psychiatric disorders. Structural changes in neuroplasticity, altered intrinsic signaling, i.e., of serotonin and BDNF, and impaired neurogenesis are observed in stress-related events, Alzheimer's disease (AD), or major depression. This review will summarize microglia function on precursor cells in the adult hippocampus, their contribution to neuroplasticity, and modulation by physiologic stimuli. We will synopsize how their behavior is altered upon activation leading to neurodegeneration and will discuss microglia response to serotonin signaling and 5-HT receptor function on microglial cells *in vitro* and *in vivo*. We will complete by describing the role of microglia in serotonin-mediated antidepressant action, e.g., in response to the selective serotonin reuptake inhibitor (SSRI) fluoxetine. Key findings are summarized in **Table 1**.

Microglia Function in Neuroplasticity—BDNF Signaling and Physiologic Stimuli

Neuroplasticity in the adult hippocampus enables its structure to adapt to environmental challenges and novel experiences by rewiring upon learning and to respond to trauma or injury. Specifically, the discovery that new neurons are continuously generated has stirred hope for new therapeutic strategies to improve cognitive function and to treat neurodegenerative disorders. Microglia contribute to adult neurogenesis and memory formation (Gemma and Bachstetter, 2013). In close proximity to neurons and dendritic spines, microglia control synapse connectivity *via* secretion of TNF-α and adenosine triphosphate (ATP) that in turn promote astrocyte-mediated neurotransmission (**Figure 1**; Pascual et al., 2012). Activation of the ATP receptor subtype P2X4 drives BDNF release from microglia, which might display a central pathway in microglia–neuron signaling (Trang et al., 2011). BDNF is crucially involved in neuronal maturation and neurotransmission *via* binding to tropomyosin-related kinase receptor B (TrkB) located on neurons (Mattson, 2008). Microglial release of BDNF directly affects nearby synapse connectivity and promotes neuronal TrkB phosphorylation that enhances microglia–neuron interplay in learning (Parkhurst et al., 2013). BDNF released by activated microglia alters neuronal excitability by causing synaptic disinhibition (Ferrini and De Koninck, 2013). In their major role in synaptic pruning, microglia actively engulf and remove dysfunctional synapses from neuronal cell bodies in the uninjured brain (Paolicelli et al., 2011). In vicinity to NSCs in the subgranular zone, they remove apoptotic progenitor cells within the first days of cell birth (Sierra et al., 2010) through the phagocytosis secretome (Diaz-Aparicio et al., 2020), thereby balancing synaptogenesis and cell death.

Novel experiences and external stimuli influence NSC/progenitor behavior, and activity-dependent changes in neuroplasticity occur, including a robust increase in precursor cell proliferation upon running (van Praag et al., 1999; Kronenberg et al., 2003) and cell survival upon exposure to an enriched environment (ENR) (Kempermann and Gage, 1999). The neurogenic regulatory effect of running is mediated through central serotonin (Klempin et al., 2013), with circulatory factors, i.e., the angiotensin-converting enzyme 2 (Klempin et al., 2018) or cathepsin B released by skeletal muscle (Moon et al., 2016) contributing to increased precursor proliferation. Physical exercise increases microgliosis in the dentate gyrus of wild-type mice that is further enhanced in the absence of brain serotonin (Klempin et al., 2013), or in the lack of bradykinin B2 receptor (Wasinski et al., 2018). Physical activity increases the number of newborn microglia in the adult mouse

TABLE 1 | Summary of recent findings on microglia function in neuroplasticity and neuro-inflammation in the hippocampus, with focus on serotonin and antidepressant action.

Microglia in neuroplasticity and neuroinflammation		
Kettenmann et al., 2011; Grabert et al., 2016		Microglia represent a diverse and vigilant phenotype with high numbers in the dentate gyrus
Pocock and Kettenmann, 2007; Szepesi et al., 2018	5-HT, 5-HTR	Microglia interact with local neurotransmitters and hormones
Gemma and Bachstetter, 2013		Microglia contribute to adult neurogenesis
Pascual et al., 2012	TNF-α, ATP, glutamate	Synapse plasticity *via* microglial release of TNF-α and ATP triggering nearby astrocytes to release glutamate
Trang et al., 2011	ATP, BDNF	ATP-P2X4 drives BDNF release from microglia
Parkhurst et al., 2013	BDNF	Microglia-mediated synaptogenesis *via* BDNF
Ferrini and De Koninck, 2013	BDNF	In neuroinflammation, microglia-mediated BDNF signaling causes synaptic disinhibition
Sierra et al., 2010; Diaz-Aparicio et al., 2020		Microglia phagocytosis of apoptotic newborn cells in the dentate gyrus through the phagocytosis secretome
Klempin et al., 2013	Tph2, Iba-1	Running-induced microgliosis in wild-type hippocampus that is further enhanced in mice lacking brain serotonin
Wasinski et al., 2018	B2R, Iba-1	Running-induced microgliosis in hippocampus of bradykinin B2 receptor knockout mice
Ehninger and Kempermann, 2003; Ehninger et al., 2011	Iba-1	Physical exercise increases newborn microglia numbers in cortex, but decreases the amount in adult amygdala
Ali et al., 2019	Iba-1	Long-term ENR enhances microgliosis in adult hippocampus and amygdala, hypertrophied and ramified microglia morphology
de Sousa et al., 2015; de Oliveira et al., 2020	Iba-1	Increased microglia complexity in CA3, reduced diversity in molecular layer in ENR
Johnson et al., 2003; Szuhany et al., 2015	BDNF	Physical exercise strongly induces BDNF release in rodents, and humans
Moon et al., 2016	BDNF	Skeletal muscle releases cathepsin B during running in monkeys that affects BDNF levels in the brain
Tuchina et al., 2018		Interplay of the endocrine, immune and limbic systems during stress
Goronzy and Weyand, 2013		Senescent myeloid cells decrease process motility and chemotaxis
Pickering and O'Connor, 2007	TNF-α, IL-1, IL-18 in AD	Enhanced release of pro-inflammatory cytokines in disease progression
Shen et al., 2018	AD	Dysfunctional microglia in disease progression
Ng et al., 2018	IL-1β, IL-6 in AD, major depression	Enhanced peripheral levels in patients
Burbach et al., 2004	BDNF in AD	In AD inflammation, release of BDNF by microglia in close proximity to plaques
Floden et al., 2005	TNF-α, glutamate in AD	b-amyloid-induced microglia-mediated cell death *via* the release of TNF-α and glutamate
Makar et al., 2009	BDNF, IL-10	BDNF promotes IL-10 release in multiple sclerosis
Borsini et al., 2015	Cytokines	Distinctive cytokines acting on cell proliferation and differentiation *in vitro*
Kelly et al., 2001; Lim et al., 2013	IL-10	Anti-inflammatory; promotes synaptic plasticity and long-term potentiation
Cacci et al., 2008; Willis et al., 2020	IL-10 IL-6	Potent suppression of pro-inflammation and robust support of adult neurogenesis
Paolicelli et al., 2011; Sellner et al., 2016; Bolós et al., 2018	Fractalkine/CX3CR1 *Cx3cr1*	Prominent chemokine regulator of neuron–microglia communication in the postnatal and adult dentate gyrus; important for synaptic pruning
Bachstetter et al., 2015; Milior et al., 2016	Fractalkine/CX3CR1 in AD, chronic stress	Deficiency results in microglia-induced pro-inflammation and impaired neurogenesis
Monje et al., 2003; Bastos et al., 2008; Fujioka and Akema, 2010	LPS, BrdU and neuronal markers	Dose- and time-dependent effects on cell proliferation, survival and neuronal fate in the adult dentate gyrus, *in vivo/in vitro*
Ekdahl et al., 2003	LPS, BrdU	Negative correlation of activated microglia-newborn cells
Mizoguchi et al., 2014 Zhang et al., 2014 Wu et al., 2020	LPS BDNF-TrkB	(LPS-induced) microglia activation, transformation can be reduced by BDNF or TrkB agonist treatment; BDNF sustains $Ca2^+$ elevation
Serotonin–Microglia function		
Stagaard et al., 1987; Vetreno et al., 2017	5-HT, Tph2, VMAT, SERT, Iba-1, CD11b	Serotonin depletion increases microgliosis in dorsal raphe, and subcommissural organ
Krishna et al., 2016	LPS, 5-HT	Transient increased microglia numbers and a depressive-like phenotype upon chronic LPS
Carabelli et al., 2020	LPS, Omega-3, 5-HT	Fish oil reverses depression-like behavior, increases serotonin in the hippocampus
Albertini et al., 2020	5-HT	Microglial processes in close proximity to serotonergic axons in the adult hippocampus
Seifert et al., 2011	5-HT, $Ca2^+$	Transient enhanced $Ca2^+$ signaling in response to serotonin *in vitro*
Glebov et al., 2015	5-HT2A/B, 5-HT4, $Ca2^+$	Serotonin stimulates secretion of exosomes from microglia cells
Krabbe et al., 2012; Etienne et al., 2019	5-HTR, 5-HT2B, LPS, TNF-α, IL-6	Serotonin promotes microglia-induced targeted motility, but attenuates phagocytosis activity

(Continued)

TABLE 1 | Continued

Serotonin–Microglia function		
Kolodziejczak et al., 2015	5-HT2B	Serotonin–microglia neurotransmission in development
Béchade et al., 2021	*5-ht2b*	In the lack of *5-ht2b,* overexpression of cytokines and prolonged neuroinflammation
de las Casas-Engel et al., 2013	5-HT7	Microglia-mediated serotonin neurotransmission to maintaining anti-inflammatory state
Mahé et al., 2005; Wixey et al., 2018	5-HT7	Present on human microglial MC-3 cells
Quintero-Villegas and Valdés-Ferrer, 2019	5-HT7, IL-6, AD	Promotes synaptogenesis and inflammatory priming *via* IL-6
Lim et al., 2009	FLX	Diminished microglia activation in ischemia
Liu et al., 2011	FLX, TNF-α, IL-6	Reduction in TNF-α and IL-6 secretion, *in vitro*
Jin et al., 2009	FLX, TNF-α, IL-1β	Fluoxetine-induced neuroprotection in the dentate gyrus following kainate-mediated neuronal cell death
Dhami et al., 2013	FLX, TNF-α, IL-1β	Reduction in the release of pro-inflammatory cytokines, and glutamate, *in vitro*
Alboni et al., 2016	FLX, TNF-α, IL-1β Iba-1, CD11b	Treatment on microglia activation and cytokine release differs depending on environmental conditions
MacGillivray et al., 2011	FLX, SERT, CD11b	Inhibition of SERT increases CD11b expression accompanied by loss of dopaminergic neurons
Zimniak et al., 2020	FLX	Attenuates symptoms in COVID-19 patients

5-HT, 5-hydroxytryptamine; AD, Alzheimer's disease; ATP, adenosine triphosphate; B2R, bradykinin receptor 2; BDNF, brain-derived neurotrophic factor and its receptor TrkB (tropomyosin-related kinase receptor B); BrdU, bromodeoxyuridine (cell proliferation marker), microglia marker CD11b Integrin αM, and Iba-1 (Ionized calcium binding adaptor molecule 1); ENR, enriched environment; FLX, fluoxetine; Interleukins, IL-1 to IL-18; LPS, lipopolysaccharide; SERT, serotonin transporter; TNF-α, tumor necrosis factor; Tph2, tryptophan hydroxylase 2; VMAT, vesicular monoamine transporter.

cortex (Ehninger and Kempermann, 2003), while a reduction was observed in the adult amygdala upon running and ENR (Ehninger et al., 2011). While Iba-1 expression is reduced up to 2 months in ENR, long-term ENR conditions enhance microgliosis in adult hippocampus and amygdala accompanied by hypertrophied and ramified microglia morphology (Ali et al., 2019). Microglia proliferation and morphological transformation are characteristics of the vigilant phenotype that allows rapid adaptation to a demanding microenvironment. Thereby, cellular physiology including $Ca2^{+}$ signaling and highly branched processes supports the sensor ability, while an amoeboid shape with dynamic extensions facilitates cellular locomotion toward the site of neural damage and factor release (Nayak et al., 2014). As a result of positive stimuli, physical exercise, and ENR, altered microglia phenotypes display neuroprotective functions. In ENR, microglia morphology in adult rodent brain shows increased complexity in CA3 (de Sousa et al., 2015) but decreased diversity in the molecular layer (de Oliveira et al., 2020). In animal models for several diseases, physical activity induces anti-inflammatory effects revealed by decreased microglia activation and Iba-1/CD11b expression, a ramified morphology, or normalization in synaptic density in CA3 (Andoh and Koyama, 2020). Exercise also strongly enhances BDNF signaling in mammals that in turn exerts positive cognitive effects (Johnson et al., 2003; Szuhany et al., 2015; Moon et al., 2016). Together, these studies show that microglia contribute to neuroplasticity and synaptic rewiring in the adult hippocampus and respond to physiologic stimuli that could ameliorate from pathologies.

Microglia Function in Neuroinflammation and Neurodegeneration

Neuroplasticity of the adult brain can also be negatively regulated, inducing structural changes and impaired neurogenesis as has been observed in stress-related events (Tuchina et al., 2018), and is associated with age-related cognitive decline and neurodegenerative and psychiatric disorders in humans. Upon inflammation or following infection, an acute immune response comprises the release of pro-inflammatory cytokines and phagocytosis of damaged cells, mainly neurons (Suzumura, 2013). Notably, activation of endogenous microglial cells goes along with migration of blood-derived cells into the brain, such as in ischemia (Kronenberg et al., 2018b). Chronically activated microglia, increased cell density and hyper-ramified morphology (Dubbelaar et al., 2018), and the enhanced release of pro-inflammatory cytokines, e.g., TNF-α, IL-1β, and IL-18, are observed in response to stress, major depression, or AD, leading to disease progression and brain damage (Pickering and O'Connor, 2007). This "hyperactivation of the immune response" might be due to inefficiency in the phagocytosis phenotype of microglia. Indeed, similar characteristics are observed for aging, senescent myeloid cells, where an impaired immune response results from decreased process motility and chemotaxis (Goronzy and Weyand, 2013). Dysfunctional microglia might be a hallmark of late-stage AD development (Shen et al., 2018). In particular, microglial cells are in close proximity to β-amyloid plaques, one of the characteristics of disease progression; their processes engulf β-amyloid that leads to enhanced pro-inflammatory signaling, e.g., of TNF-α inducing cell death and the release of BDNF (Burbach et al., 2004; Floden et al., 2005). Although BDNF is anti-inflammatory and considered as a therapeutic target, increased BDNF signaling might negatively contribute to the aberrant axonal growth in AD in its role as modulator of neuronal and synapse maturation in healthy conditions. However, in an animal model of multiple sclerosis, BDNF promotes IL-10 that reduces clinical severity (Makar et al., 2009). Notably, increased peripheral levels of IL-1β, and of IL-1β and IL-6, but unchanged TNF-α, were reported in patients with AD or major depression, respectively (Ng et al., 2018).

Microglial release of inflammatory factors in the dentate gyrus differentially affects precursor cell proliferation, survival,

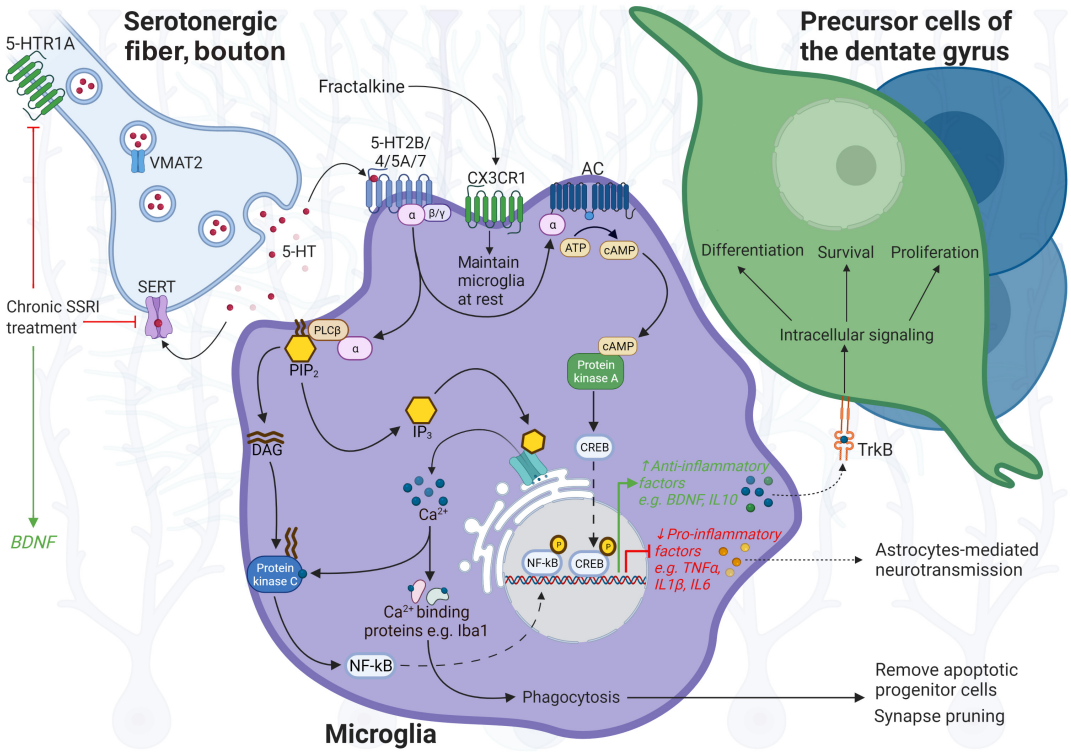

FIGURE 1 | Illustration of microglia function in neuroplasticity of the hippocampus. In close proximity to precursor cells and neurons in the dentate gyrus, resting microglia control the neuronal cell pool by removal of apoptotic progenitor cells and synapse pruning, regulate synaptic plasticity and neural network excitability *via* ATP, and the release of TNF-α and BDNF, and respond to serotonin (5-HT) neurotransmission. Microglia express 5-HT receptors, most prominently 5-HT2B in response to serotonin, and CX3CR1 in response to neuronal fractalkine/CX3CL1 signaling that allows surveillance of the niche, and communication with neurons to maintain homeostasis. In particular, serotonin neurotransmission can direct microglia function toward neuroprotection or permit the response to inflammation. Dense tracts of serotonergic fibers terminate in the hippocampus. Upon receptor binding, 5-HT2B, coupled to Gαq/G11 protein, activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol-4,5-bisphosphonate (PIP2), and mediates cellular effects through increasing levels of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 promotes $Ca2^{+}$ excretion from endoplasmic reticulum, which activates Iba-1 involved in motility and phagocytosis activity of microglia, and is affected by 5-HT2B. IP3-induced $Ca2^{+}$ release can also stimulate phospholipase C (PLC) (likewise *via* DAG), activating nuclear factor kappa beta (NF-kB) and in turn controls the expression of pro-inflammatory genes, e.g., TNF-α, IL-1β, and IL-6. Activated by 5-HT4 and 5-HT7 coupled to Gαs, the enzyme adenylate cyclase (AC) synthesizes the second messenger elevating cyclic AMP (cAMP) from ATP that activates protein kinase A (PKA); cAMP response element-binding protein (CREB) then controls transcription of genes involved in the anti-inflammatory response, BDNF or IL-10, exerting effects through their receptors, TrkB and IL-10R, located on precursor cells (TrkB) and neurons. 5-HT5A interactions with Gαi protein inhibit AC and downstream cascades. Upon harmful stimuli, microglia secrete pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, and actively remove cell debris. In prolonged neuroinflammation, microglia–neuron communication is altered, leading to neurodegeneration and cognitive deficits. In response to SSRIs, targeting SERT and presynaptic 5-HT1A auto-receptors on serotonergic neurons, 5-HT availability is enhanced in the synaptic cleft, which may also modulate BDNF levels. Increased release of pro-inflammatory cytokines may be counter-balanced by increased 5-HT levels upon SERT inhibition through fluoxetine—having anti-inflammatory properties. 5-HT, 5-hydroxytryptamine; BDNF, brain-derived neurotrophic factor and its receptor TrkB (tropomyosin-related kinase receptor B); Iba-1, ionized calcium binding adaptor molecule 1; SERT, serotonin transporter; TNF, tumor necrosis factor; VMAT2, vesicular monoamine transporter 2. *BioRender* was used to build the image.

and differentiation (Borsini et al., 2015). Secretion of anti-inflammatory IL-10 is involved in synaptic plasticity and long-term potentiation (Kelly et al., 2001; Lim et al., 2013) and counteracts the pro-inflammatory phenotype of chronically activated microglia (Cacci et al., 2008). In traumatic brain injury, repopulated microglia can adopt a phenotype that drives repair, specifically by promoting adult neurogenesis *via* soluble IL-6 receptor (Willis et al., 2020). Increased release of pro-inflammatory cytokines is observed upon decreased microglial CX3CR1 expression in response to fractalkine/CX3CL1 signaling deficiency, which results in a dramatic reduction in adult neurogenesis in chronic stress (Milior et al., 2016) and in AD (Bachstetter et al., 2015). Microglia *Cx3cr1* knockout mice display a transient early postnatal increase in synaptogenesis due to deficiency in synaptic pruning in the dentate gyrus (Paolicelli et al., 2011) that is independent of fractalkine signaling (Sellner et al., 2016), accompanied by reduced neuronal maturation of precursor cells and impaired learning and memory in the adult (Bolós et al., 2018). To model systemic inflammation *in vitro/in vivo*, bacterial lipopolysaccharide (LPS) is administered, resulting in increased microglia density and the release of pro-inflammatory factors IL-1β/IL-6 and TNF-α. A dose- and time-dependent decrease in proliferation and survival of precursor cells is observed *in vivo* in adult mouse and rat, respectively (Bastos et al., 2008; Fujioka and Akema, 2010), which is accompanied by a depressive-like state

(Tang et al., 2016). Thereby, a dramatic reduction in newborn neurons is correlated with an increase in activated microglia (Ekdahl et al., 2003). *In vitro* experiments reveal that the number of precursor cells adopting a neuronal fate is significantly reduced when co-cultured with activated microglia expressing IL-6, while LPS added directly to precursor cells has no effect on neurogenesis (Monje et al., 2003). A single dose of LPS also significantly decreases the density of p-TrkB and BDNF protein in dentate gyrus and CA3 of young-adult male mice (Zhang et al., 2014). Likewise, an age-related decline in BDNF-TrkB signaling is accompanied by increased microglia activation (Wu et al., 2020). *Vice versa*, microglia activation, phenotypic transformation, and release of pro-inflammatory cytokines can be reduced by local supplement of BDNF *in vivo/in vitro* (Wu et al.) or TrkB agonist treatment *in vivo* (Zhang et al., 2014). BDNF induces sustained elevation of intracellular $Ca2^{+}$ signaling and inhibits microglial production of nitric oxide (NO) (Mizoguchi et al., 2014).

Serotonin–Microglia Interplay

Serotonin is the most widespread monoamine of the central nervous system, key signaling molecule in neuroplasticity of the hippocampus, and target in antidepressant therapy. Briefly, synthesized in neurons of the brain stem dorsal and median raphe nuclei (DRN, MRN) by the rate-limiting enzyme tryptophan hydroxylase 2, serotonin is packed into synaptic vesicles by the vesicular monoamine transporter (VMAT) 2, and upon release, re-uptake is regulated by the selective serotonin transporter, SERT (Gaspar et al., 2003). Earlier studies on brain serotonin–microglia interaction were done upon stimuli or pharmacological depletion of serotonergic neurons that results in increased microglia density, characterized by Iba-1 and CD11b expression, in DRN (Vetreno et al., 2017), or microgliosis in the subependymal layer of the subcommissural organ in adult rats (Stagaard et al., 1987). Neuroinflammation induced by systemic LPS reduces serotonin levels in the hippocampus that is accompanied by a depressive-like phenotype in rats (Carabelli et al., 2020). Chronic LPS activation only transiently increases microglia numbers and alters striatal and prefrontal serotonin signaling alongside depressive-like behavior (Krishna et al., 2016). Omega-3 administration leads to increased serotonin levels in the hippocampus and reverses the behavioral phenotype (Carabelli et al., 2020). Serotonin fiber pathways project into numerous brain areas and spinal cord. Target areas in the dentate gyrus, precursor cells and neurons, express various 5-HT receptors that control the response from efferent activity at different cell stages within the neuronal lineage (Brezun and Daszuta, 2000; Klempin et al., 2010). Recent studies establish that neurotransmitter receptors are not specific for neurons, but can be found on glial cells, and molecules are detected through diffuse non-synaptic transmission in the extracellular space (Pocock and Kettenmann, 2007). Serotonin, in particular, is released *via* boutons *en passant*, and ultrastructure imaging reveals brain serotonin–microglia interplay in the hippocampus with microglia processes in close proximity to serotonergic axons (Albertini et al., 2020). Seven groups (5-HT1–5-HT7) and their subtypes, with 5-HT3 as an exception, belong to the G-protein-coupled receptor family regulating different signaling pathways; almost all of them are expressed on distinct microglia subpopulations (Krabbe et al., 2012; Glebov et al., 2015).

Accumulating evidence attributes 5-HT2B receptor subtype an important role in microglia–neuron communication in rodent brain development (Kolodziejczak et al., 2015), and in microglia-mediated serotonin transmission. *In vitro* studies reveal enhanced microglia response to injury in acute mouse brain slices (Krabbe et al., 2012), and transiently boosted $Ca2^{+}$ signaling in cultured resting microglia upon serotonin administration (Seifert et al., 2011). Specifically, activation of 5-HT2B leads to enhanced motility and oriented growths of microglial processes that is important in response to injury but decreases the phagocytosis activity (Krabbe et al., 2012; Etienne et al., 2019). In the lifelong absence of microglial *5-ht2b*, peripheral LPS injection causes cytokine overexpression and prolonged neuroinflammation *in vivo* that goes along with increased morphology transformation and hyper-ramification (Béchade et al., 2021). These studies suggest that serotonin is involved in the alterations of microglial phenotype as is known for peripheral macrophages (de las Casas-Engel et al., 2013). Together with 5-HT2, microglial expression of 5-HT4 is involved in the release of exosomes from microglia that is dependent on elevated cytosolic $Ca2^{+}$ signaling (Glebov et al., 2015). Microglial secretion of cytokines modulated by serotonin neurotransmission might lead to maintenance of an anti-inflammatory state (de las Casas-Engel et al., 2013); indeed, secretion of pro-inflammatory factors TNF-α or IL-6 was unchanged during LPS stimulation in the presence of serotonin (Krabbe et al., 2012). Functional 5-HT7 receptors are present on human microglial MC-3 cells (Mahé et al., 2005; Wixey et al., 2018). 5-HT7 expressions on both neurons and microglia promote synaptogenesis and induce inflammatory priming *via* IL-6 production. In an AD animal model, reduced neurotoxicity of β-amyloid was observed in hippocampus upon administration of LP-211, a 5-HT7 agonist (Quintero-Villegas and Valdés-Ferrer, 2019). Collectively, these studies suggest serotonin's role in keeping microglia in a resting, surveillance, and anti-inflammatory state.

Serotonin–Microglia Interplay Upon Fluoxetine

Dysregulation of serotonin signaling is associated with neurogenic decline, age-related memory loss, and psychiatric disorders. SSRIs increase serotonin neurotransmission targeting SERT and specific 5-HT (auto-) receptors (Descarries and Riad, 2012) that leads to clinical improvement and is linked to a delayed increase in adult neurogenesis as shown in rodents (Malberg et al., 2000; Santarelli et al., 2003). BDNF has been implicated in the pro-neurogenic effects; SSRI-induced increases in serum BDNF have been detected in rodents (Nibuya et al., 1996), and similarly in depressed patients (Molendijk et al., 2011); however, BDNF protein in hippocampus of mice is not elevated (Petermann et al., 2020). Increasing evidence indicates that neurodegenerative diseases and psychiatric disorders are characterized by an immune-inflammatory state and that antidepressants not only improve mood but also

possess anti-inflammatory properties. It is suggested that hyperactive microglia and increased pro-inflammatory cytokine levels result in elevated SERT expression as a consequence or interdependency to elevated serotonin levels. SSRIs target SERT function that in addition to inhibiting serotonin re-uptake might activate anti-inflammatory intracellular pathways (Walker, 2013): In LPS-induced primary microglia culture, incubation with serotonin significantly alters TNF-α production (Tynan et al., 2012). Likewise, pre-treatment with five different SSRIs, including fluoxetine, substantially inhibits IL-1β or IL-6 secretion (Liu et al., 2011) and microglial production of TNF-α and NO, with cyclic adenosine monophosphate signaling involved in the regulation of an anti-inflammatory response (Tynan et al., 2012). Co-cultured with cortical neurons, microglial release of the pro-inflammatory factors IL-1β, TNF-α, and glutamate was reduced upon fluoxetine and citalopram (Dhami et al., 2013). *In vivo* pre-treatment with fluoxetine or paroxetine attenuates LPS-induced increases in TNF-α serum levels (Ohgi et al., 2013). In models of neurodegenerative disease, fluoxetine administration reduces microglia activation in ischemia (Lim et al., 2009) and leads to recovery from kainate-induced cell death in the dentate gyrus (Jin et al., 2009). Depending on environmental challenges, cytokine release in hippocampus is differentially affected by fluoxetine resulting in increased pro-inflammatory IL-1β expression in ENR conditions, but decreased TNF-α production upon stress. However, microglia density and Iba-1/CD11b expression in hippocampus remain unchanged (Alboni et al., 2016). In contrast, in substantia nigra, SERT inhibition by fluoxetine increases microglia activation and CD11b immunoreactivity, leading to loss of dopaminergic neurons (MacGillivray et al., 2011). Together, microglia activity and release of cytokines can be modulated by serotonin neurotransmission, e.g., SERT-mediated clearance of released serotonin upon fluoxetine (Robson et al., 2017) and altered intrinsic cellular signaling. However, whether SERT is expressed on microglia lacks evidence.

An overreaction of the immune system, a "cytokine storm" (Ragab et al., 2020) is also associated with the pathophysiology following SARS-CoV-2 infection that might contribute to long-term neurological impairments. Preliminary results reveal that fluoxetine treatment specifically decreases viral protein expression in COVID-19 patients (Zimniak et al., 2020). Thus, SSRI treatment with anti-inflammatory effects given early might prevent both severe progression of the disease and chronic despair.

DISCUSSION

Over the past few years, it has become apparent that endogenous microglia of the adult brain take part in neuroplasticity of the hippocampus by controlling the neuronal cell pool, regulating synaptic plasticity in learning *via* release of TNF-α and BDNF, and responding to physical exercise (**Table 1**). Resting microglia express 5-HT2B and CX3CR1, constantly survey the niche's microenvironment, and communicate with neurons to maintain homeostasis. In particular, serotonin neurotransmission can direct microglia function toward neuroprotection, or permit the response to inflammation (Pocock and Kettenmann, 2007). Upon harmful stimuli, microglia perform an innate immune response; secrete pro-inflammatory cytokines TNF-α, IL-1β, and IL-6; and actively remove cell debris, similar to peripheral macrophages. When toxic molecules are removed from the nervous tissue, microglia become "alternatively activated", change their phenotype to anti-inflammatory (IL-10), and start restoring homeostasis (Lobo-Silva et al., 2016). However, in chronic diseases, neuron–microglia communication is somewhat altered, causing a prolonged inflammatory state, leading to impaired chemotaxis and phagocytosis. Hyperactivation of the immune response also impairs survival and differentiation of progenitor cells, which, together with impaired serotonin and BDNF signaling, are characteristics of major depression. With SSRIs such as fluoxetine targeting both signaling pathways and, in addition, enabling an anti-inflammatory response, microglia might display an add-on therapeutic target to improve psychiatric disorders, cognitive decline, or viral-induced neurological deficits. Nonetheless, considering the various factors involved and the vast heterogeneity of human microglial cells (Böttcher et al., 2019), there is a long road ahead.

AUTHOR CONTRIBUTIONS

AT, OT, and FK have equally contributed to designed and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Cell and Developmental Biology

REVIEW
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The Role of Astrocytes in the Neurorepair Process

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Astrocytes are highly specialized glial cells responsible for trophic and metabolic support of neurons. They are associated to ionic homeostasis, the regulation of cerebral blood flow and metabolism, the modulation of synaptic activity by capturing and recycle of neurotransmitters and maintenance of the blood-brain barrier. During injuries and infections, astrocytes act in cerebral defense through heterogeneous and progressive changes in their gene expression, morphology, proliferative capacity, and function, which is known as reactive astrocytes. Thus, reactive astrocytes release several signaling molecules that modulates and contributes to the defense against injuries and infection in the central nervous system. Therefore, deciphering the complex signaling pathways of reactive astrocytes after brain damage can contribute to the neuroinflammation control and reveal new molecular targets to stimulate neurorepair process. In this review, we present the current knowledge about the role of astrocytes in brain damage and repair, highlighting the cellular and molecular bases involved in synaptogenesis and neurogenesis. In addition, we present new approaches to modulate the astrocytic activity and potentiates the neurorepair process after brain damage.

Keywords: astrocytes, brain damage, neurorepair, synaptogenesis, neurogenesis

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ASTROCYTES: A CELL TYPE WITH MULTIPLE ROLES

Astrocytes are glial cells responsible for homeostasis, nutrition and protection of the central nervous system (CNS) (Nedergaard et al., 2003). They were first described in 1846 by Rudolph Virchow as a population of homogeneous cells that provide support for neuronal functions (Molofsky and Deneen, 2015; Perez-Nievas and Serrano-Pozo, 2018). For more than a century, the role proposed for astrocytes was merely their ability to support CNS cells. Innovative thinking has emerged in the last few decades, when the classic view of astrocytes as passive supporting cells changed to a crucial active element in the CNS (Halassa and Haydon, 2010; Kettenmann et al., 2013; Rusakov, 2015).

Astrocytes exhibit a star-shaped morphology with extensively branched processes terminating in fine structures, called perisynaptic astrocytic processes (PAPs), that structurally and functionally interact with synapses (Araque et al., 1999). In a mouse, a single astrocyte can cover up to 100,000

synapses, whereas in the human brain that number is more than one million (Halassa et al., 2007). This property of astrocytes has been confirmed for different experimental models and different regions of the CNS and peripheral nervous system (PNS) (Wilhelmsson et al., 2006).

Based on their function, distribution and/or morphology, astrocytes can be divided into two subpopulations: fibrous astrocytes and protoplasmic astrocytes (Wang and Bordey, 2008). Fibrous astrocytes are highly prevalent in white matter, have long non-branched processes with endfeet involved in Ranvier's nodes. The protoplasmic astrocytes are mainly found in the gray matter and their morphology displays branched processes that involve synapses, and whose endfeet cover the blood vessels. Astrocytic processes interact with the elaborate network of synaptic terminals, dendrites, and dendritic spines (Wang and Bordey, 2008).

Fibrous and protoplasmic astrocytes show differences patterns of protein expression, however, glial fibrillary acidic protein (GFAP) is the main intermediate filament expressed in both subtypes (Marin-Padilla, 1995; Wilhelmsson et al., 2006; Brozzi et al., 2009; Saur et al., 2014). It is important to note that different isoforms of GFAP can be expressed in astrocytes, and the additional intermediate filament proteins Nestin and Vimentin may also contribute to the astrocytic cytoskeleton (Wang and Bordey, 2008; Kamphuis et al., 2012). In different neuroinflammatory pathologies (e.g., infection, ischemia, neurodegenerative diseases, and brain trauma), an increase in GFAP expression can be observed (Hol and Pekny, 2015; Pekny et al., 2016; Escartin et al., 2019; Smit et al., 2021). GFAP is commonly used as an astrocyte marker, although it is important to note that some other cell types inside (e.g., ependymal cells) and outside (e.g., hepatic stellate cells) the CNS cells can also have GFAP (Liu et al., 2006; Wang and Bordey, 2008), and that some quiescent astrocytes do not have detectable levels of GFAP (Kuegler et al., 2012).

Astrocytes have multiple functions in the CNS and are fundamental to the dynamics of tissue functioning. First, the classic function attributed to astrocytes is the mechanical support of nervous tissue, which is done through their endfeet which forms a network and help to anchor astrocytes to blood vessels, neurons, and other cells. This strategic positioning of astrocytes with blood vessels and synapses, allows these cells to regulate blood flow according to synaptic demand. In addition, the endfeet can also enhance the physical barrier properties of astrocytic processes, limiting the signal and communication between neighboring synapses and, at the same time, favoring the specificity of neurotransmission (Abbott et al., 2010). In addition to functioning as a physical barrier, astrocytes can also influence the permeability of blood vessels by releasing factors, such as cytokines and vasoactive agents, that control the balance of water, ions and other molecules in the CNS (Abbott et al., 2010; Michinaga and Koyama, 2019). In this way, astrocytes are also part of the blood-brain barrier (Beenhakker and Huguenard, 2010).

Another important function of astrocytes is the regulation of brain metabolism, which is carried out through the extensions of protoplasmic astrocytes that surround neurons, maintaining a microenvironment suitable for their metabolic functions (Cornell-Bell et al., 1990). Astrocytes are sensitive to neuronal metabolic demand through neurochemical signaling, which involves the release of glutamate, ATP, nitric oxide (NO), hydrogen peroxide (H_2O_2), potassium through pre- and post-synaptic neuronal terminals (Petzold and Murthy, 2011; Rose et al., 2017). For example, astrocyte metabolism specializes in glutamate uptake and its metabolism to glutamine. Once produced, glutamine is released from astrocytes to neurons, where it can be deaminated into glutamate and used as a transmitter. The energy metabolism of astrocytes is coupled to that of neurons by the lactate shuttle hypothesis. This way, the energy of glucose from the bloodstream is made available to the brain. The glucose uptake depends on the activation of the Na^+/K^+ pump on the surface of the astrocytes. The glycolytically- produced lactate is transferred to neurons, where it provides energy by fueling oxidative phosphorylation (Pellerin, 2008).

One of the most important functions of astrocytes is the neurotransmitter uptake and release. The privileged positioning of astrocytes allows an efficient coverage of the synapses, where these cells clear the excess of transmitters and optimize neurotransmission. For instance, the rapid uptake of gamma-aminobutyric acid (GABA) prevents the inhibitory signal from spreading to other regions (Kinney and Spain, 2002). Likewise, the glutamate transporters excitatory amino acid transporter 1 (EAAT1) and excitatory amino acid transporter 2 (EAAT2), capture the neurotransmitter in glutamatergic synapses. Therefore, the removal of glutamate by astrocytes from the synaptic cleft guarantees the functioning of synaptic transmission, placing astrocytes as protagonists, instead of merely supporters of synapses (Kinney and Spain, 2002). In addition, astrocytes participate in the synaptic modulation by releasing neurotransmitters and modulators, such as glutamate, purines (ATP, adenosine, and guanine), GABA and D-serine (Bezzi and Volterra, 2001).

Several studies by Clarke and Barres (2013) showed that astrocytes have essential functions in the formation of functional synapses during the development of the CNS. The first evidence that astrocytes participated in the formation of synapses comes from research done with mouse retinal ganglion cells (RGCs). These cells, when grown in the absence of glial cells, formed very few synapses but when grown in the presence of astrocytes or in astrocyte-conditioned medium (ACM), they were able to form ten times more excitatory synapses with an increased functionality (Barres et al., 1988; Meyer-Franke et al., 1995). With this evidence it became clear that astrocytes secrete signals that control the development of synapses (Clarke and Barres, 2013).

Studies have shown that during embryonic development, the neurons need to be physically contacted by astrocytes before becoming receptive to synaptogenic signals secreted by this cell type. This suggests that there is a multi-step process that occurs during synaptic development, beginning with astrocyte-neuron contact that modifies the neuron's maturational state making it able to make synapses. There are several contact-mediated signaling transducers described, such as integrin-protein kinase C (Hama et al., 2004),

neurexin (Barker et al., 2008), gamma protocadherins (Garrett and Weiner, 2009), and neuroligins (Stogsdill et al., 2017). However, the main astrocyte-associated synapse-regulating pathways identified involve interaction of astrocyte-secreted signals with neurons. These signals have diverse roles, including the induction of synapse formation, alteration in presynaptic and postsynaptic function, via recruitment of receptors, induction of synapse maturation, and even synapse stabilization linked to memory formation (Huang et al., 2019; Chen et al., 2020).

Astrocytes also play a central role in responding to brain damage. During brain damage, significant biochemical and morphological changes are observed (Wilhelmsson et al., 2006; Zamanian et al., 2012; Pekny and Pekna, 2014). Reactive astrocytes show hypertrophy of their main cellular processes and changes in their protein profile (Wilhelmsson et al., 2006; Bardehle et al., 2013; Choi et al., 2014). Fibrous and protoplasmic astrocytes exhibit structural differences in their processes after mechanical injury: The fibrous astrocytes exhibit condensed retracted processes (Sun et al., 2010); In contrast, protoplasmic astrocytes exhibited an increase in the length and complexity of the branch of their processes after injury (Kajihara et al., 2001; Wilhelmsson et al., 2006). There is evidence demonstrating that astrocytes can activate the maturation and proliferation of adult neural stem cells through growth factor production, which is critical for the tissue regeneration after damage (Cornell-Bell and Finkbeiner, 1991).

The current scientific evidence indicates that astrocytes guide the neurorepair process, neurogenesis and are essential to reestablish local homeostasis after brain damage (Acosta et al., 2017). In this review, we summarize the current knowledge about the role of reactive astrocytes in brain repair by highlighting the molecular and cellular bases involved in neurogenesis and synaptogenesis. In addition, we highlight new approaches that increase the activity of glial astrocytes responsible for the recovery from injuries or diseases in the CNS.

THE ROLE OF ASTROCYTES IN BRAIN DAMAGE AND DISORDERS

During the 1970s, the term "reactive astrocytes" was first forged after the discovery of the intermediate filament protein GFAP (Eng et al., 1971). Based on robust evidence from experimental animals, a definition of reactive astrocytes has been proposed covering four main characteristics: (1) a spectrum of molecular, cellular and functional changes that occur in astrocytes in response to CNS injuries and diseases, (2) they vary with the severity of the lesion, (3) they are regulated by inter and intracellular signaling molecules, (4) and can be beneficial or harmful to neighboring cells (Sofroniew, 2009). Thus, astrocyte reactivity can be classified as mild to moderate, diffuse, or severe (Episcopo et al., 2013).

Reactive astrocytes is a common response to CNS injuries/diseases, encompassing a spectrum of changes ranging from hypertrophy (increased cell size) to cell proliferation (Sofroniew and Vinters, 2010). Indeed, the evaluation of the reactivity of astrocytes in animal models for brain damage and disorders so far has been done through changes in the amounts of GFAP protein and in the level of expression of the GFAP gene (Lewis et al., 1984).

Reactive astrocytes has been evolutionary developed as defensive reaction (Falsig et al., 2008; Schildknecht et al., 2012) and therefore, a variety of inter and intracellular signals can trigger reactive astrocytosis (Eddleston and Mucke, 1993). Cytokines, modulate the function of astrocytes by inhibiting or promoting astrocytosis. The best researched cytokines are interleukin-1beta (IL-1β), gamma interferon (IFNγ) and transforming growth factor beta 1 (TGF-β1) (John et al., 2003). The role of IL-1β in inducing reactive astrocytes has been confirmed by experiments using primary astrocyte cultures, where an increase in IL-1β induced the expression of multiple proinflammatory genes such as the inducible form of nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNFα) (Liu et al., 1998; Falsig et al., 2006).

TNFα is also markedly up-regulated in injured or inflamed brains in animal models and in humans (Selmaj et al., 1991; Taupin et al., 1993). Furthermore, it has also been shown that the positive regulation of TNFα precedes the increase of GFAP in the brain, a result that correlates this cytokine with the astrocytosis process (Rostworowski et al., 1997). In studies with primary astrocyte culture, it has been shown that the administration of IL-1β can induce the production of TNFα, that activates the transcription of the nuclear factor kappa B (NF-κB), responsible for increasing the production of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and chemokines, such as interleukin-8 (IL-8) and IP-10 (Lee and Brosnan, 1997; Henn et al., 2011; Kleiderman et al., 2016b; Efremova et al., 2017). Interestingly, TNFα along with IL-1α, IFNγ and C1q are sufficient to induce a markedly pro-inflammatory astrocytic profile, thus confirming the crucial role of these cytokines for promoting astrocytosis (Falsig et al., 2006; Christiansen et al., 2011; Liddelow et al., 2017; **Figure 1**).

Moreover, although IL-1β, IFN-γ, and TNFα can change the neural microenvironment toward a pro-inflammatory onset (Falsig et al., 2006; Christiansen et al., 2011; Henn et al., 2011; Kuegler et al., 2012; Efremova et al., 2017), transforming growth factor beta 1 (TGFβ1) can play another role in astrocytes. In CNS injury models, inhibition of TGFβ1 activity does not significantly alter GFAP expression but prevents reactive astrocytes from producing glial scarring. This process is responsible for generating a barrier that separates the injured area from healthy nervous tissue (Logan et al., 1994). In addition, the administration of TGFβ1 in primary astrocyte cultures downregulates the expression of several pro-inflammatory genes and induces the expression of multiple extracellular matrices and cytoskeletal molecules. For example, TGFβ1 inhibits the astrocytic expression of major histocompatibility complex class II (MHC class II), ICAM-1 and VCAM-1 molecules, as well as the astrocytic production of TNFα, thus modulating a different pathway for the pro-inflammatory role (Dong and Benveniste, 2001; **Figure 1**).

Interestingly, the sustained IL-1β signaling also can induce the expression of nerve growth factor (NGF) by astrocytes

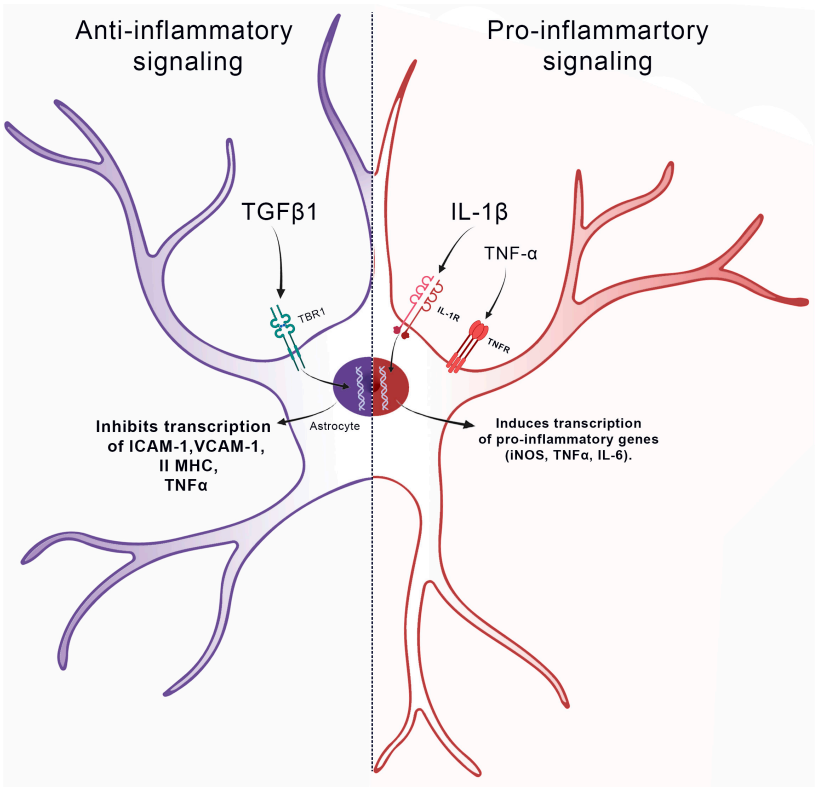

FIGURE 1 | Role of cytokines in promoting astrocytosis. IL1β, IL-6, TNF, and IFNγ induce a pro-inflammatory signaling pathway in the astrocyte, stimulating the gene transcription of iNOS, IL-6, and TNF-α. On other hand, TGF-β1 induces an anti-inflammatory signaling pathway in the astrocyte, inhibiting the gene transcription of ICAM-1, Vcam-1, II MHC, and TNF-α.

in vivo, which can contribute positively to the maintenance and proliferation of damaged neurons (Sun et al., 2010). In fact, in astrocytic cell culture, IL-1β also induces the expression of a subset of genes including interleukin 6 (IL-6), ciliary neurotrophic factor (CNTF) and NGF, which are primarily associated with neuronal and glial growth and survival. Moreover, activated astrocytes also release other neuroprotective factors like glutathione and cysteine (Gutbier et al., 2018). These data indicate a differential and possibly counterbalance effects of IL-1β signaling in astrocytosis, given that it can promote early inflammatory responses and can trigger regenerative cellular signaling such as the expression of CNTF and NGF. This data indicates that astrocytes, in certain circumstances, become highly cell-protective and beneficial with anti-inflammatory, neuroprotective functions, thus facilitating neuronal recovery and repair (Cornell-Bell and Finkbeiner, 1991; Kajihara et al., 2001; Marchetti and Abbracchio, 2005).

THE ROLE OF ASTROCYTES IN NEUROGENESIS DURING BRAIN DAMAGE

Neurogenesis comprises the set of processes necessary to generate new cells and differentiate them into neurons, which in adult brain, normally is observed in the dentate gyrus (subgranular zone) and in the lateral ventricle (subventricular zone) (Altman and Das, 1965; Eriksson et al., 1998; Alvarez-Buylla and Lim, 2004; Lie et al., 2005; Ming and Song, 2005). Adult neurogenesis is a dynamic process influenced by changes in the microenvironment, such as neurotransmitter release or neurotrophins, but also occurs in response to pathological stimuli (Emsley et al., 2005; Ming and Song, 2005). Neural stem/progenitor cell (NSPC) behavior is affected by seizures and in ischemia and depression; and cells also respond to environmental changes and physical exercises (Kempermann et al., 1997; Kokaia and Lindvall, 2003; Lledo et al., 2006; Parent et al., 2006; Warner-Schmidt and Duman, 2006). Thereby, the specific anatomical and cellular characteristics of the neurogenic niche seems to play an essential role for NSPCs, since they are near to capillary endothelial cells, astrocytes, and ependymal cells (Lim et al., 2000; Palmer et al., 2000; Shen et al., 2004; Ma et al., 2005).

Stem cells of the adult dentate gyrus exhibit radial glia-like properties, express GFAP, and represent the quiescent stem cell population (Alvarez-Buylla et al., 2001). Upon a stimulus, radial glia-like stem cells undergo asymmetric division, whereby they are able to self-renew and generate a progenitor cell to become a neuron or astrocyte (Bonaguidi et al., 2011). The progenitor cells are highly proliferative and amplified and give rise to new

neurons (Suh et al., 2007; Goncalves et al., 2016). Surrounding mature astrocytes also help the differentiation and integration of neurons in the hippocampal circuit. Blocking vesicular release of astrocytes has been shown to impair the survival of new neurons and dendritic maturation, reducing branching and the number of dendritic spines in new neurons-that is important for *N*-methyl-D-aspartate (NMDA) receptor functioning-suggesting that astrocytes are important regulators of adult neurogenesis in many stages of the process (Sultan et al., 2015). In addition, β-arrestin-1 (b-arr1) secreted by astrocytes in the dentate gyrus, participates in adult neurogenesis by regulating the production of excretory factors derived from the astrocyte niche and the expansion of precursor cell numbers, thus maintaining homeostasis in the hippocampal niche (Tao et al., 2015).

In the adult brain, astrocytes are not neurogenic under regular conditions, nevertheless, some astrocytes maintain neurogenic potential. The investigation of neurogenic and non-neurogenic astrocytes reveals that distinct subtypes of astrocytes populate different brain areas and present distinct morphological and biochemical features (Bachoo et al., 2004). The majority of astrocytes *in vivo* apparently lose the ability to generate new neurons (Mori et al., 2005). Neural/glial antigen 2 (NG2) positive cells are the major stem/progenitor cell population outside neurogenic regions, that can become neurons *in vitro* (Belachew et al., 2003). In the hippocampus, the brain-derived neurotrophic factor (BDNF) is the main factor involved in the maturation of neurons and in the phenomenon of synaptic plasticity, and thus released by both microglia and astrocytes (Chmielnicki et al., 2004; Ferres-Coy et al., 2013). Nowadays, it is clear that astrocytes can be differentiated in neurons or can release factors that will act in neurogenic niches to stimulate the proliferation and differentiation of NPSCs. Due to that, understanding the difference of glia populations and the factor produced and released during neurorepair may unlock new perspective for adult neurogenesis after brain damage.

The Wnt pathway plays an important role during the development of the CNS and the maintenance of the structure of synapses in addition to the functions of neurons in the mature brain (Clevers et al., 2014; Kumawat and Gosens, 2016; Oliva et al., 2018). Wnt signaling supports the maintenance of cellular homeostasis in the heart and blood vessels, in addition to being the target in some pathologies such as AD, cancer, schizophrenia and multiple sclerosis (van Amerongen and Nusse, 2009; Cerpa et al., 2010; Inestrosa and Arenas, 2010; Gay and Towler, 2017). Currently, there are at least three Wnt signaling pathways: the canonical Wnt/β-catenin pathway, the Wnt/polarity pathway (or planar cell polarity pathway Wnt/PCP pathway) and Wnt/Ca^{2+} pathways (Niehrs, 2012; Humphries and Mlodzik, 2018).

Wnt proteins are generally classified into canonical ligands of the Wnt pathway and non-canonical ligands (Libro et al., 2016). Studies mainly focus on the canonical Wnt/β-catenin signaling pathway in which Wnt signaling depends on the cytoplasmic level of free β-catenin and binds to the transmembrane receptor Frizzled protein (Fz), and to the low-density protein co-receptor related to the lipoprotein receptor (LRP5/6).

Astrocytes in the adult hippocampus express Wnt-3. *In vitro* studies have shown they stimulate Wnt/β-catenin signaling in isolated adult hippocampal progenitor cells (AHPs) and induce the differentiation into neurons. Differentiation induced by a co-culture with astrocytes was reduced in the presence of the soluble Wnt inhibitor of proteins 2 and 3 related to Frizzled- (sFRP2/3) (Lie et al., 2005). In addition to Wnt signaling derived from astrocytes, there is an autocrine Wnt signaling activity in AHPs (Wexler et al., 2009).

Wnt/β-catenin signaling enhanced neurogenesis by regulating pro-neuronal genes Nurr-1, Pitx-3, Ngn-2, and NeuroD1 (Singh et al., 2018; **Figure 2**). Specifically, Wnt-3 and Wnt-3a protein in astrocytes of rats decreased progressively in the dentate gyrus between 2 and 22 months of age, which is accompanied by a decrease in NeuroD1 (Okamoto et al., 2011). NeuroD1, therefore, constitutes a basic helix-loop-helix transcription factor of paramount importance in the generation of granular cells in the embryonic brain (in development) and the mature brain (Gao et al., 2009). We suggest that a decline of Wnt-3/3a in astrocytes may cause a decrease on the expression of pro-neuronal genes and, therefore, a decrease in adult neurogenesis in aging animals (**Figure 2**).

The Wnt signaling pathway is also involved in Parkinson's disease (PD). Current evidence points to glial reactivity but whether the activation of glial cells can protect or exacerbate the loss of dopaminergic neurons is currently the subject of debate (Episcopo et al., 2013). The Wnt/β-catenin signaling pathway appears to play a central role in development of dopaminergic neurons in the ventral middle brain (VM) (Gordon and Nusse, 2006; L'Episcopo et al., 2014). The periventricular region of the adult midbrain aqueduct (Aq-PVR) has been shown to harbor neural stem/progenitor cells with dopaminergic potential *in vitro*, but it is believed that restrictive mechanisms *in vivo* limit their regenerative capacity. Using *in vitro* mNPC culture systems, L'Episcopo et al. (2014) demonstrated that aging is a critical factor that restricts neurogenic potential by deregulating Wnt/β-catenin signaling. Co-culture assessments of young-adult progenitor cells and young-adult astrocytes identified a decline in glial-derived factors, including Wnts, while Wnt activation strategies effectively reversed the deficit and enhanced dopaminergic differentiation (L'Episcopo et al., 2014). Astrocytes also participate in the endogenous modulation of regenerative processes through the release of Wnt, which may represent an alternative treatment for CNS injuries, such as ischemia. NSPCs that reside in regions such as the subventricular zone (SVZ) in the adult brain can proliferate and differentiate into other cell types and compensate for the damage caused by ischemia. During development, these cells are largely influenced by the Wnt pathway, according to a previous work by Kriska et al. (2016), where the Wnt pathway/β-catenin is a factor that promotes neurogenesis under the expansion of gliogenesis in neonate mice (Kriska et al., 2016).

In the current studies of this same group, the impact of the Wnt pathway on NSPC modulation was evaluated in transgenic animals and middle cerebral artery occlusion (MCAO) ischemia model animals. In this work, the effects of the Wnt pathway on the modulation of NSPCs were not identified *in vitro* experiments derived from a healthy brain. However, when culturing cells derived from ischemic brains, inhibition of the Wnt pathway

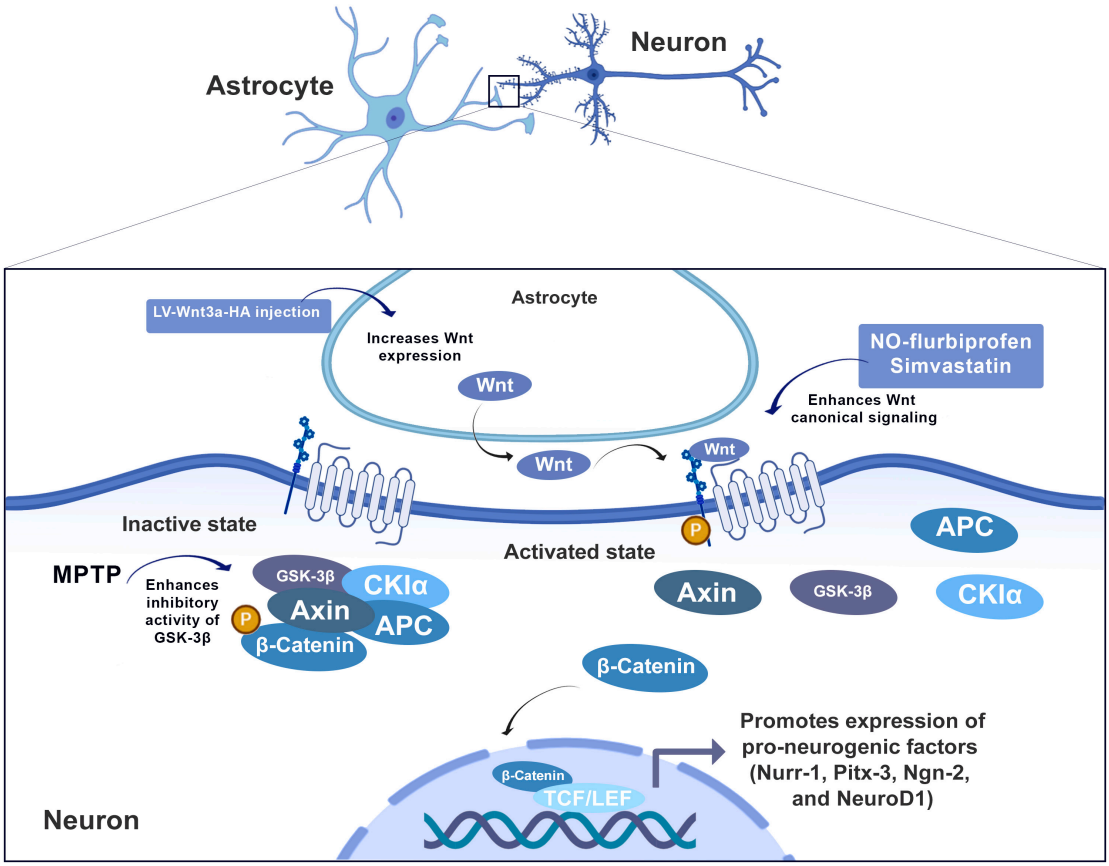

FIGURE 2 | The canonical pathway: Wnt binds to the transmembrane receptor Frizzled protein (Fz) and to the low-density protein co-receptor related to the lipoprotein receptor (LRP5/6) to activate the intracellular Dishevelled protein (Dvl), which therefore inhibits the activity of the complex composed of cytoplasmic glycogen synthase kinase 3β (GSK-3β), adenomatous polyposis coli (APC), and Axin, leading to dephosphorylation, aggregation and nuclear translocation of β-catenin. Within the nucleus, β-catenin combines with T cell transcription factor (TCF)/lymphoid reinforcement factor (LEF) to form a complex, leading to transcriptional activation and expression of specific genes. Wnt/β-catenin signaling enhanced neurogenesis by regulating proneural genes (Nurr-1, Pitx-3, Ngn-2, and NeuroD1).

resulted in fewer neuron-type cells. In addition, electrophysiology analyzes indicated that blocking this pathway affects the distribution of K^+ and Na^+ channels (Kriska et al., 2021). Interestingly, immunohistochemistry analyzes demonstrated an increase in the count of positive cells for labeling Doublecortin (DCX), GFAP and proliferating cell nuclear antigen (PCNA) (Kriska et al., 2021).

Other studies also seek to understand the role of the Wnt pathway in NSPCs after ischemic damage. Wang et al. (2017) demonstrated that some MicroRNAs can play a crucial role in neurological repair, such as miRNA-148b which is overexpressed in the SVZ of ischemic mice. By inhibiting or mimicking miRNA-148b in culture, the effect is to suppress or increase the Wnt/β-catenin pathway. The inhibitor of miRNA-148b promoted the proliferation of NSPCs in neurons and young astrocytes and this action can be silenced with the knockdown of Wnt-1 (Wang et al., 2017). In MCAO models in rats, the injection of this miRNA was able to reduce ischemic damage and improve neurological function (Wang et al., 2017). Another example, Wnt1 levels increased significantly over a period of 1–6 h in the penumbra region in ischemic rats in an MCAO model in the studies by Chong et al. (2010). This fact was also recently described by Jean LeBlanc et al. (2019) demonstrating that β-catenin levels increase in endothelial cells already within 3 h after MCAO model.

Similarly, Wnt3 derived from astrocytes has been related to the differentiation of NSPCs by increasing the expression of synapsin-I and tubulin-III in a paracrine manner. Overexpression of Wnt3 in elderly primary astrocytes using lentivirus expression significantly improved neurogenesis (Okamoto et al., 2011). Interestingly in the SVZ region also the expression of β-catenin and Wnt3a reduced in the subacute phase after ischemia (Wei et al., 2018). Therefore, these studies identify Wnt as a signaling pathway through which astrocytes from specific regions of the CNS regulate adult neurogenesis. In addition, these findings show that Wnts are key regulators of adult neurogenesis *in vivo*, suggesting that they are a central pathway for determining the fate of NSPCs.

The Notch signaling is a common evolutionarily conserved pathway that plays major roles in the regulation of proliferation and differentiation of CNS cells (Lai, 2004;

Siebel and Lendahl, 2017). Notch signaling are guaranteed by four different Notch receptors (Notch1-4), which is characterized as a single-pass transmembrane receptor composed by a large extracellular portion and a small intracellular region (Brou et al., 2000). Canonical Notch pathway is mediated by endogenous ligands such as Jagged1-2 and Delta1-4, cell surface proteins that activate the Notch receptor and promotes the proteolytic cleavage -realized by γ-secretase- of the notch intracellular domain (NICD) (Oswald et al., 2001). NICD translocate from the cytoplasm to cell nucleus, where it regulates the expression of several different target genes, mostly Hairy enhancer of split isoforms 1 and 5 (Hes1 and Hes5), through interaction with the RBP-J transcription factor (Jarriault et al., 1995).

Interestingly, there is evidence showing an important role for Notch signaling pathway in promoting reactive astrocyte formation during brain injury. In fact, Shimada et al. (2011) have demonstrated that reactive astrocytes in the peri-infarct area expresses NICD 1, and inhibition of Notch signaling by administration of a γ-secretase inhibitor markedly decrease the number of proliferative reactive astrocytes in this area. Moreover, many different studies have demonstrated an association between Stat3, an important transcription factor that is activated in reactive astrocytes, and Notch signaling. Indeed, it appears that changes in Notch expression can alter Stat3 activity, thus pointing to a modulatory effect of Notch signaling in the promotion of reactive astrocytes (LeComte et al., 2015; Yang et al., 2019).

Another factor secreted by astrocytes during the neurorepair process is D-serine. This amino acid is an important co-agonist of the NMDA receptor. Calcium-dependent release of D-serine by astrocytes participates in long-term potentiation (LTP) of the hippocampus (Henneberger et al., 2010; Sultan et al., 2013; **Figure 3**). D-serine administration *in vitro* increases the proliferation of stem/progenitor cells of the SVZ and stimulates the survival of new neurons suggesting autocrine regulation (Huang et al., 2012). However, it is still not entirely clear whether the release of D-serine plays a role in regulating adult neurogenesis *in vivo*.

Radzishevsky et al. (2013) report that D-serine is secreted by both astrocytes and neurons, whereby the release of D-serine from astrocytes is triggered by activation of the glutamate receptor agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Schell et al., 1995). In the hippocampus, the astrocytic niche spans from the hilus over the subgranular zone to the molecular layer, and is therefore ideally located to relay signaling between synaptic activity and the neurogenic niche (Wang et al., 2018).

We have shown above that D-serine, plays an important role in cell proliferation and stimulation of the survival of new neurons. Therefore, it is also important to understand the role of D-serine in a pathological context and how its function implies on neurogenesis and astrocyte function. Psychiatric disorders are characterized by having a polygenic character, that is, they are influenced by distinct genetic variants (Sullivan and Geschwind, 2019). Disrupted-in-Schizophrenia-1 (DISC1) gene is known as risk factor for mental illnesses and present in many psychiatric disorders (Ma et al., 2013). More precisely, DISC1 is considered an important risk factor on neurodevelopment and the use of its mutant form (C-terminus-truncated form) has been used as a molecular tool for the study of the DISC1 pathway in astrocytes (Niwa et al., 2016). Some studies such as Terrillion et al. (2017) reported that an important pathway of DISC1 astrocytes has an important role in stabilizing and binding to the serine racemase (SR) enzyme, which is responsible for the conversion of L-serine to D-serine in astrocytes. The mutant expression of human DISC1 in astrocytes has been shown to reduce endogenous levels of DISC1 in rodents in a negative dominant manner, which results in impaired binding between DISC1 and the serine racemase resulting in increased ubiquitination of the enzyme and reduced production of D-serine by astrocytes. Biochemical changes like these are related several times to an increased response to NMDAR's non-competitive antagonism by MK-801 which is reversed when in the presence of exogenous D-serine, thus it is suggested that D-serine has a relationship functional with the expression of the DISC1 mutant in astrocytes where the reduction of D-serine production causes changes in behavior (Terrillion et al., 2017).

Other studies have linked DISC1 to maintaining an adequate dendritic morphogenesis of newly formed neurons during adult hippocampal neurogenesis and in the differentiation of newly generated granular cells (Enomoto et al., 2009). In addition, it is shown that astrocytes play a critical role in regulating neurogenesis through D-serine secretion (Sultan et al., 2015). Thus, the hypothesis that the expression of mutant DISC1 in astrocytes would decrease the production of D-serine in the hippocampus is reinforced, leading to a hippocampal neurogenic deficit and consequently behavioral impairments. Specifically, Terrillion et al. (2017) reveal that the expression of mutant DISC1 in astrocytes increased the animals' anxious behavior, attenuated the social interaction and the preference for social novelty and caused cognitive deficits in rodents. Behavioral changes associated with reduced proliferation of neural progenitor cells and reduced dendritic afforestation of newly formed neurons in the region of the dentate gyrus are observed, which reiterates the data observed when there is a reduction in D-serine in rats with mutation to DISC1. In addition to these observations, treatment with D-serine alleviated the damage in behavior by restoring the typical development of newly formed neurons. Findings like these demonstrate at first glance that the expression of DISC1 in mature astrocytes may be involved in the regulation of neurogenesis and in behavioral parameters that are dependent on the hippocampus activity (Terrillion et al., 2017).

THE ROLE OF ASTROCYTES IN SYNAPTOGENESIS DURING BRAIN DAMAGE

Synaptogenesis is a process responsible for the formation of synaptic contacts and helps to maintain and eliminate synapses over time. This is a succession of structural events that occurs in neurons that depends on the differentiation of synaptic terminals in specialized membranes that have specific functions. The synapse represents an important functional unit of brain circuits

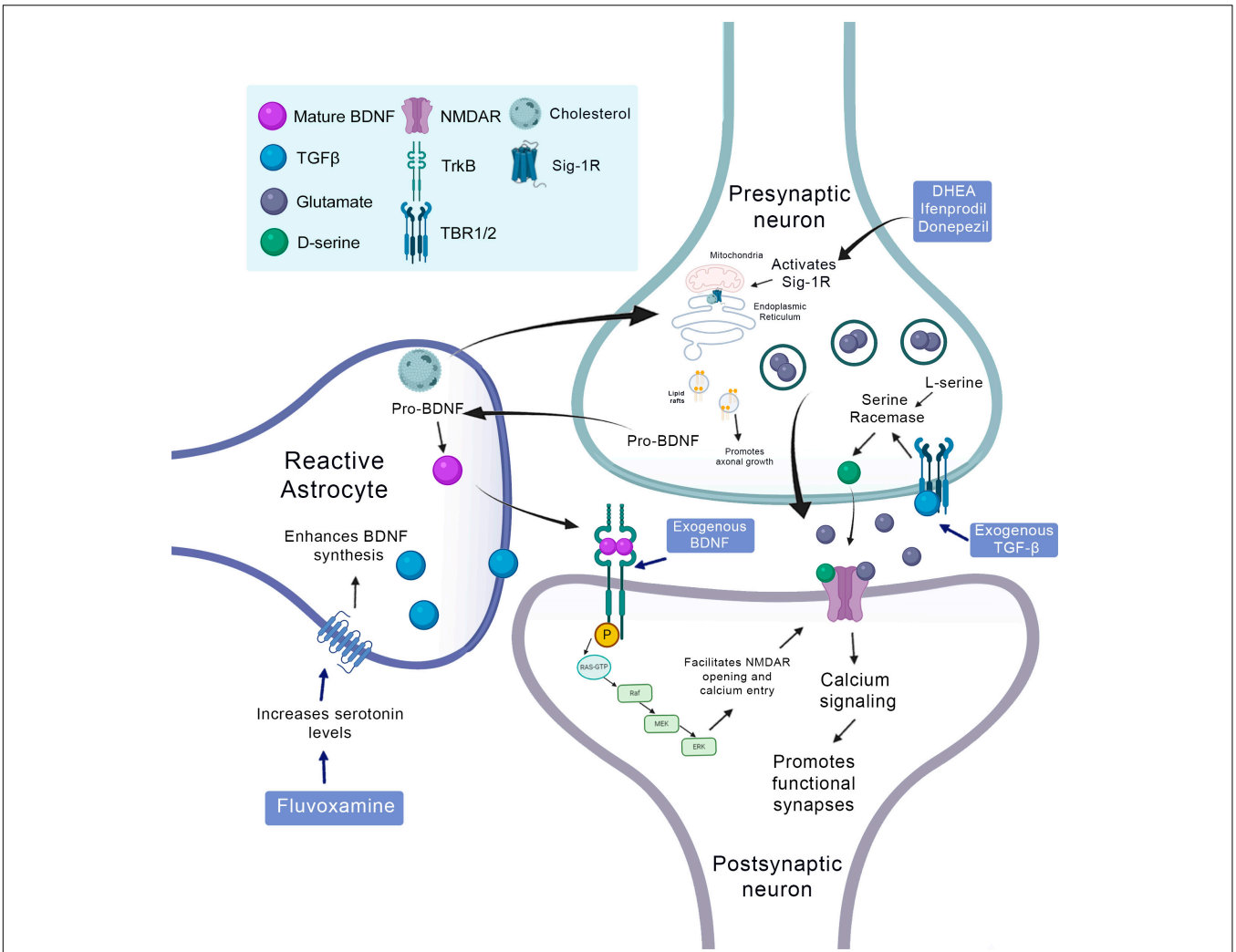

FIGURE 3 | Synaptogenic factors involved in neurorepair in the context of the CNS injury and therapeutic strategies to promote it. Cholesterol released by the astrocyte binds to the sigma-1 receptor and promotes axonal growth. BDNF is produced in the presynaptic neuron and is transformed into mature BDNF in the astrocyte, binds to the TRKB receptor in the postsynaptic neuron and induces its phosphorylation and facilitates the opening of NMDA receptors. The TGF released by astrocytes binds to its TBR1/2 receptor at the presynaptic terminal and activates the serine racemase that converts L-serine to D-serine, which is released in the synaptic cleft and acts on NMDA receptors promoting the formation of functional synapses. The use of fluvoxamine increases the levels of serotonin that will stimulate synthesis and BDNF by the astrocyte. DHEA, ifenprodil and donepezil, are agonists of the sigma-1 receptor. The administration of exogenous BDNF can stimulate phosphorylation of the TRKB receptor and its consequent activity, just as the exogenous administration of TGFβ can stimulate the TBR1/2 receptor.

that forms the basis of neural networks. The differentiation of synapses is continually undergoing dynamic changes in response to the needs of the circuits, a process called synaptic plasticity. The effective transfer of information between neurons not only contributes to the formation of functional synapses but can also eliminate those that are unproductive or uncompetitive (Walsh and Lichtman, 2003).

The impairment of neural network function after brain damage is the main cause of disability after brain damage (Desgeorges et al., 2015). Thus, the neurorepair process must be efficient to build a new neural network and reestablish the brain function (Huang et al., 2012). In this context, astrocytes have a critical role in the construction of new synapses and in their maintenance. Astrocytes regulate synapses by direct contact with neurons or secreting soluble factors that act in pre and postsynaptic sites, therefore modulating the structure and function of inhibitory and excitatory synapses (Ullian et al., 2001; Christopherson et al., 2005; Nishida and Okabe, 2007; Stogsdill et al., 2017). Astrocyte structures that make connections with several synapses, are known as PAP. A combination of a PAP with the pre and postsynaptic compartments is known as the tripartite synapse (Araque et al., 1999). Through their fine processes, astrocytes can sense and adhere to synapses and coordinate with the neighboring astrocytes to tile and completely cover the neuropil. Astrocytes do not cover all synapses; however, synapse association of astrocytes is a dynamic process that can be altered by neuronal activity (Genoud et al., 2006; Bernardinelli et al., 2014).

In a tripartite synapse, neurotransmitters released from neurons also bind to receptors on the adjacent PAP, activating signaling pathways in astrocytes that modulate synaptic behavior (Papouin et al., 1715; Araque et al., 1999). In addition to contacting neurons, astrocytes are interconnected by communicating junctions, specialized channels that allow nutrients and ions to diffuse between astrocyte networks, further expanding the range and magnitude of synaptic regulation of neurons by these cells (Pannasch and Rouach, 2013). Recently, the notion of a "tetrapartite synapse," that includes the extracellular matrix as an important component is emerging (Dityatev and Rusakov, 2011; Smith et al., 2015; Cope and Gould, 2019). Thus, reactive astrocytes can also secrete signals that will assist in the restoration of synapses after CNS injury and can provide a target to promote plasticity and neurorepair (Emirandetti et al., 2006; Tyzack et al., 2014).

Cholesterol-bound apolipoprotein E (APOE) was the first synaptogenic molecule identified by astrocytes. In the CNS, cholesterol is largely produced by astrocytes. It increases presynaptic differentiation in RGC cultures by promoting the synthesis and maturation of synaptic vesicles (Mauch et al., 2001; Goritz et al., 2005). The presynaptic terminals need an adequate number of proteins and lipids to carry out their activities, the binding of cholesterol and synaptophysin, for example, is necessary for the biogenesis of synaptic microvesicles. Cholesterol depletion inhibits this biogenesis probably because it interferes with the formation of the synaptic vesicle curvature (Thiele et al., 2000).

Cholesterol-dependent lipid raft mobilization can help neurorepair through axonal growth after stroke. Stroke-induced increased the cholesterol-binding sigma-1 receptor in astrocytes is beneficial; treatment with a sigma-1 receptor agonist 2 days after MCAO enhances behavioral recovery and neurite outgrowth without decreasing infarct size, suggesting a neural repair, rather than neuroprotection, mechanism (Ruscher et al., 2011). This may be due to increased export of cholesterol to neurons via the sigma-1 receptor. Similarly, increasing high-density lipoprotein cholesterol (HDL-C) by administration of the liver X receptor agonist GW3965 24 h after tMCAO in mice improves recovery, causing synaptogenesis and angiogenesis (Cui et al., 2013). This may partially be explained by the importance of cholesterol in synaptogenesis and neurorepair after injury in the CNS (Gleichman and Carmichael, 2014; **Figure 3**).

Another molecule secreted by reactive astrocytes during neurorepair processes is thrombospondin (TSPs), which is the main synaptogenic factor secreted by astrocytes (Christopherson et al., 2005). They are large proteins located in the extracellular matrix (Adams and Lawler, 2011). Gray-matter protoplasmic astrocytes express TSP1 and 2, while astrocytes originating in the SVZ and fibrous astrocytes express TSP4 (Eroglu et al., 2009; Benner et al., 2013). The addition of purified TSP in neuron culture increased the number of synapses compared to the number of those with ACM, whereas removal of ACM eliminated most of the synaptogenic activity of ACM. According to these *in vitro* findings, studies using TSP1/2 double knockout (KO) mice showed that these animals exhibited less excitatory cortical synapses, also indicating that TSPs are important for the development of synapses *in vivo*. Interestingly, in the rodent cortex, TSPs are expressed by immature astrocytes only during the first week of postnatal development, a period that corresponds to the beginning of the formation of excitatory synapses on this region (Christopherson et al., 2005).

The receptor to which TSPs bind to induce synaptogenesis is a type 1 transmembrane protein located in neurons, which is a subunit of the calcium channel α2δ-1. This protein has a special domain known as Von Willebrand Factor A (VWF-A) domain, which is the exact place of interaction with TSPs (Eroglu et al., 2009). Therefore, when TSPs bind to this domain, it is believed that this interaction leads to a change in the original conformation of the α2δ-1 receptor, which triggers important processes for the occurrence of synaptogenesis (Risher and Eroglu, 2012). Recently, Risher and colleagues demonstrated in mice the mechanism of action of binding TSPs to the α2δ-1 receptor. It was observed that in the cerebral cortex of these animals this interaction controls synaptogenesis via activation of Rac1 in the post-synaptic neuron, which in turn promotes the remodeling of the actin cytoskeleton in nascent synaptic contact (Risher et al., 2018).

In addition, it has been shown that astrocytes release together with TSPs, another molecule, the innate immune molecule pentraxin 3 (PTX3) (Falsig et al., 2004). This molecule increases the quantity and synaptic grouping of AMPA receptors, therefore playing a key role in promoting functionally active CNS synapses (Fossati et al., 2019). TSPs are the most well-known matrix proteins that are associated with brain tissue repair and synaptogenesis after brain injury. In stroke, TSP-1 is upregulated in the peri-infarct zone within 3 days, while an increase in TSP-2 is observed 1 week later. The relevance of TSP on this process was confirmed by the findings of Liauw et al., 2008 who showed that TSP1 and 2 are necessary for synaptic plasticity and functional recovery of animals after stroke. They induced focal cerebral ischemia in mice and observed that TSP levels increased after brain injury. KO mice for TSP-1 and TSP-2 exhibited deficits on synaptic density and axonal germination when compared to wild animals. They demonstrated that deficits in TSP-1/2 lead to difficulty in recovery after cerebral ischemia mainly due to the role of these proteins on the formation of synapses and in axonal appearance. In addition, they also confirm that TSPs are partially secreted by astrocytes after the stroke, which was evidenced by the co-localization of TSP-1 and TSP-2 with S100 and GFAP, respectively (Liauw et al., 2008; Zhang et al., 2020).

Another article published by Tyzack et al. (2014) further emphasized the importance of TSPs in neurorepair, highlighting a new mechanism for their release. They showed direct evidence that reactive perineuronal astrocytes have great relevance for maintaining the neuronal circuit after distant axotomy. They also revealed a new function as an astrocytic signal transducer and transcription-3 activator (STAT3). STAT3 regulates the formation of the perineuronal astrocytic process and the re-expression of a synaptogenic molecule, TSP-1, in addition to supporting neuronal integrity. It became clear that, through this new route, TSP-1 is responsible for astrocyte-mediated remote recovery from excitatory synapses in axotomized motor neurons in adult mice (Tyzack et al., 2014).

Astrocytes also secrete other proteins important for the formation of new excitatory synapses, such as cysteine-rich acid secreted protein (SPARC) and Hevin, which is also known as SPARC 1-like protein (SPARCL1). These proteins are expressed by the astrocytes of the superior colliculus. Hevin induces the formation of synapses between the RGCs of cultured rats. It is important to note that SPARC antagonizes Hevin's synaptogenic function (**Figure 3**; Kucukdereli et al., 2011).

Kucukdereli et al. (2011) have demonstrated that Hevin null mice have fewer excitatory synapses whereas SPARC null mice showed an increase in synaptic connections in the superior colliculus. They concluded that Hevin is a positive regulator and SPARC is a negative regulator of synapse formation and that through the regulation of relative levels of Hevin and SPARC astrocytes can control the formation, maturation, and plasticity of synapses *in vivo* (Kucukdereli et al., 2011). Hevin makes the connection between two neuronal receptors, neurexin at the presynaptic site and neuroligin at the postsynaptic. Neurexin and neuroligin are well known neuronal synaptogenic molecules that can interact with each other. Thus, the presence of Hevin between these two proteins increases the potential for synaptogenesis. In addition, mice without Hevin are unable to remodel synapses in the visual cortex in response to visual deprivation, confirming Hevin's importance for this type of plasticity during the critical period (Singh et al., 2016). However, a recent study, using rigorous genetic manipulations, showed that Hevin does not require neurexins and neuroligins for his activity, confronting the results mentioned above. Thus, Hevin selectively increases excitatory synaptogenesis and synaptic transmission by a new mechanism that can be independent of neurexins and neuroligins (Gan and Sudhof, 2020; **Figure 4**).

It is known that after the end of the development of the CNS, there is a reduction in the levels of both Hevin and SPARC. On the other hand, when we have an injury or development of some disease in the CNS, both in reactive astrocytes and in microglia, the amount of these proteins is significantly increased. This was demonstrated in a study developed by Liu et al. (2005), where changes on the production of mRNA and SPARC protein in the hippocampus of adult mice were evaluated after transections of the entorhinal afferents to assess whether SPARC was involved in regulating changes on plasticity induced by adult brain injury. Therefore, they concluded that SPARC was actually involved in denervation-induced neuronal plasticity (Liu et al., 2005). In other studies, a significant increase in Hevin (Lively and Brown, 2007) and SPARC was also observed in reactive astrocytes after injury and stroke (Liu et al., 2005; Lloyd-Burton et al., 2013; Jones et al., 2018).

Neurons in the hippocampus grew more synapses when co-cultured with astrocytes, which is possibly mediated by Agrin – a well-known astrocyte-derived synaptogenesis promoter. It was first described at the neuromuscular junction, as an extracellular matrix protein (Smith and Hilgenberg, 2002; Tournell et al., 2006). Hilgenberg et al. (2006) identified proteins that served as ligands for Agrin, such as membrane tyrosine kinases and the Na/K ATPase.

Some articles discuss the role of protein in synaptogenesis after brain injury, such as a study by Falo et al. (2008), which examined both time and profiles of the Agrin in the deferred hippocampus during reactive traumatic brain injury (TBI)-induced synaptogenesis. They concluded that Agrin increased in lesion-targeted sub-regions, a response associated with synaptic terminals and in sites with reactive astrocytes and that Agrin mRNA transcription was increased in TBI models during the period of rapid synapse formation. In general, these observations identify distinct spatial and temporal differences in Agrin that are associated with the effectiveness of synaptic plasticity. The current results suggest that Agrin plays an important role in the successful synaptic reorganization and neurorepair after TBI (Falo et al., 2008). Recently, the effect of Agrin on neurorepair and synaptogenesis after stroke was investigated. The effect of exercise on this process was also studied. The poststroke exercise improved the recovery of behavioral function and agrin played a critical role in the synaptogenesis process. Due to that, Agrin can be considered a potential therapeutic target for the treatment of stroke and other diseases of the nervous system (Zhang et al., 2020).

Another indication for the synaptogenesis process modulated by astrocytes is the Specificity protein 1 (Sp1). This protein that binds to DNA, can activate or inhibit the transcription of genes in mammalian cells. It belongs to the Krüppel-like protein/factor transcription family (SP/KLF) (Li and Davie, 2010). Several studies show the beneficial role of this protein in relation to diseases or injuries in the CNS, such as in Parkinson's disease, AD, spinal cord injury and brain trauma. It was also demonstrated that Sp1 has its levels regulated positively in stroke with consequent neuronal protection (Simard et al., 2006; Citron et al., 2015; Miras-Portugal et al., 2016; Wang and Song, 2016; Chuang et al., 2017).

Despite all these studies, the functional role of Sp1 in astrocytes has remained unclear. Recently, Hung and collaborators used $Sp1^{-/-}$ mice to study the function of this protein in astrocytes. They showed that Sp1 can be of great importance for astrocyte function. They found that Sp1 in astrocytes regulates the expression of several genes that are involved in the neuronal development process such as neurite outgrowth and synaptogenesis (Augusto-Oliveira et al., 2020; Hung et al., 2020).

Finally, neurotrophins are molecules of great importance for the development of synapses in both the central and PNSs (Reichardt, 2006; Cohen and Greenberg, 2008). It has been shown in more recent studies that neurons are not the only source of BDNF in the CNS, this neurotrophin can also be found in oligodendrocytes and astrocytes (Dai et al., 2003; Jean et al., 2008). Thus, astrocytes are key elements in BDNF signaling in the brain: pro-BDNF is produced and released by neurons, accumulates in astrocytes, is converted to a mature form of BDNF and is secreted by protein-mediated exocytosis of the membrane associated with the vesicle (VAMP2) (Bergami et al., 2008). BDNF secreted by astrocytes induces phosphorylation of neuronal TrkB, which is essential for the maintenance of LTP and memory retention (Carroll et al., 1993; Emsley and Hagg, 2003). BDNF also regulates the probability of opening the channels at NMDA and GABA receptors, by phosphorylation. NMDA

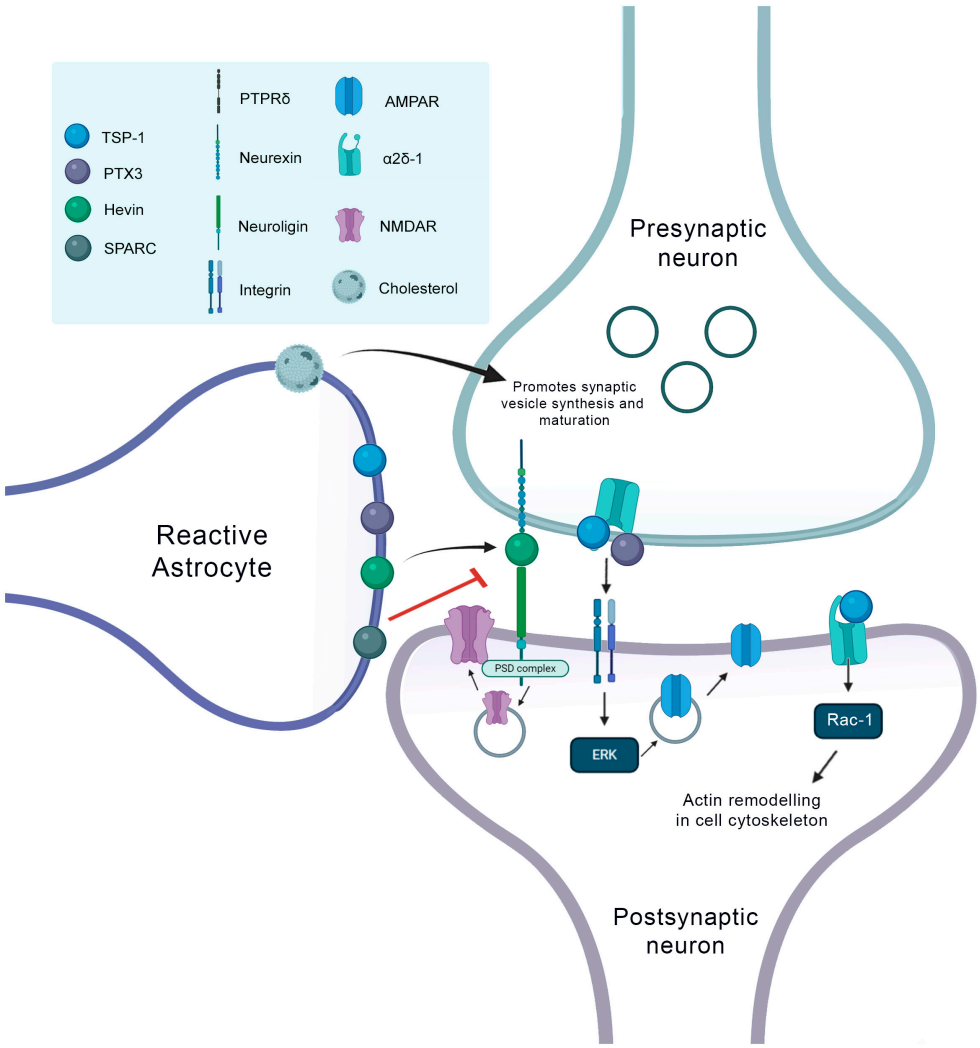

FIGURE 4 | Synaptogenic factors involved in neurorepair in the context of the CNS injury. At the presynaptic terminal, TSP1 and PTX3 bind to the α2δ1 receptor. This binding of TSP at the presynaptic site increases the number of synapses, but they are silent, so the PTX3 molecule released together with TSP, promotes the functional maturation of these synapses by recruiting AMPA receptors. The binding of TSP to postsynaptic receptors, activates the Rac1 protein and stimulates actin remodeling to promote synaptogenesis. Hevin makes the connection between neurexin and neuroligin and thus increases the recruitment of the PSD95 and NMDAR subunits. Hevin's effect is antagonized by SPARC. Cholesterol increases presynaptic differentiation promoting the synthesis and maturation of synaptic vesicles.

receptors via ErK1/2 and GABA receptors via protein kinase C (PKC) (Jovanovic et al., 2004).

Gomez-Casati et al. (2010) also identified the synaptogenic potential of BDNF produced by astrocytes. Genetically modified mice, in which the erbB signaling was eliminated in the support cells, failed to form synapses between the hair cells and the sensory neurons of the vestibular organ, it was also shown that this support showed reduction of BDNF and when these cells re-expressed BDNF synapses were recovered. These results show *in vivo* the relevance of erbB and BDNF receptors for vestibular synaptogenesis (Gomez-Casati et al., 2010). In addition, BDNF causes a rapid increase in intracellular calcium through the Trk-PI3K (phosphoinositide 3 kinase) pathway that leads to the formation of a dendritic column (Amaral and Pozzo-Miller, 2007; **Figure 5**).

The importance of this neurotrophin in neurorepair was highlighted by Béjot et al. (2011). They demonstrated that ischemic stroke models induce an increase in BDNF protein levels 8 days after infarction (Béjot et al., 2011). In accordance, BDNF was also increased in reactive astrocytes 1 day after middle cerebral artery occlusion transitory (tMCAO) in a different report (Zamanian et al., 2012).

Besides, de Pins et al. (2019) have shown that BDNF released by astrocyte increases spine density and dendritic growth in transgenic mice model of Alzheimer's disease (AD), which is accompanied by cognitive improvements on these animals. In another study by Vignoli et al. (2016), the importance of this neurotrophin was also highlighted. They showed that the interruption of astroglial processing and BDNF secretion leads to deficits and the interruption of memory processes,

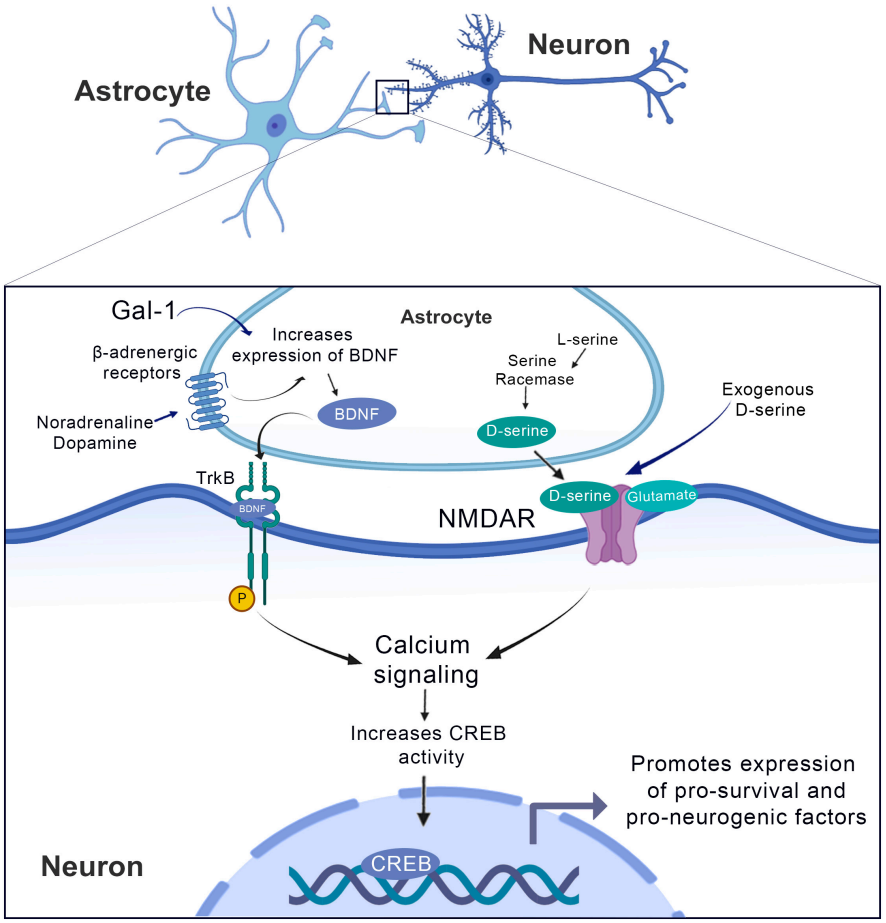

FIGURE 5 | The brain-derived neurotrophic factor (BDNF) supports neurogenesis in normally non-neurogenic brain regions. Gal-1 treatment induces the production of BDNF from cortical astrocytes from this increase in BDNF, an activation of the TrkB receptor occurs, which in turn increases calcium signaling and CREB activity, promoting the expression of pro-survival and pro-neurogenesis factors. Similarly, we observed the effect of noradrenaline and dopamine by increasing the BDNF and following the cascade downstream. In turn, the effect of D-serine is on NDMA receptors, increasing the intracellular calcium signaling favoring the increase of CREB activity.

demonstrated by conducting object recognition behavior tests (Vignoli et al., 2016).

ASTROCYTE-BASED STRATEGIES TO PROMOTE NEUROREPAIR

Astrocyte-Driven Neurogenesis

As previously discussed, one of the most important signaling pathways correlated with adult neurogenesis is the Wnt/β-Catenin pathway. Hence, one therapeutic approach to enhance adult neurogenesis correlated with astrocytes function is the use of drugs that have Wnt/β-Catenin pathway as a target. For instance, Shruster et al. (2012) have demonstrated a positive effect of Wnt/β-Catenin-related astrocytic-driven neurogenesis using gene therapy. Using injections of lentivirus expressing Wnt3a-HA (LV-Wnt3a-HA), the authors have demonstrated that Wnt/β-Catenin activation can promote enhanced functional recovery after ischemic injury and increase the number of immature neurons in the SVZ and striatum. Moreover, the authors have shown that the neurogenesis enhancement was accompanied by reduced propagation of the neuronal injury.

Furthermore, another treatment that has demonstrated a positive effect on Wnt/β-Catenin-related astrocytic-driven neurogenesis is simvastatin administration. Robin et al. (2014) have demonstrated that oral simvastatin treatment enhances Wnt signaling in the adult hippocampus, increasing the number of newborn neurons in the dentate gyrus through enhancement of intermediate precursor cells (IPCs) in the subgranular zone. These results are in accordance with previous evidence that the statins pharmacological class can increase neurogenesis in the dentate gyrus and reduce delayed neuronal death in CA3 hippocampal region (Lu et al., 2007). Moreover, whereas Wu and collaborators shows that simvastatin-related neurogenesis is mediated by the upregulation of pro-survival environmental changes created with the upregulation of trophic factors such as BDNF and vascular endothelial growth factor

(VEGF) (Wu et al., 2008). Robin et al. (2014) demonstrate that simvastatin actions are correlated with Wnt signaling enhancement by depleting isoprenoids, rather than through a cholesterol-dependent mechanism (**Figure 5**).

There is also evidence on the role of this pathway from a nigrostriatal neurodegeneration model, used to mimic the pathology of PD. In this model, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is administered to mice. It is converted by astrocytes to the final toxic agent, MPP+ (1-methyl-4-phynylpyridinium), an inhibitor of the mitochondrial respiratory chain (Efremova et al., 2015; Schildknecht et al., 2015). The oral administration of the NO-donating non-steroidal anti-inflammatory drug flurbiprofen (NO-flurbiprofen) was shown to be neuroprotective in this model. L'Episcopo showed that the damage involves attenuated Wnt signaling via upregulation of GSK-β and β-catenin degradation. Under these conditions, the neurogenic effect of Wnt signaling was reduced. Inhibition/silencing of GSK-3β, Wnt1 exposure, or astrocyte coculture restored neurogenesis in MPTP-treated mice (**Figure 4**). In fact, treatment with NO-flurbiprofen can activate Wnt/β-Catenin signaling, resulting in GSK-3β downregulation and consequently reduction of the inflammatory SVZ microenvironment in PD. Thus, this promotes neuroprotective effects on neural progenitor cells against mitochondrial dysfunction and cell death, enhancing proliferation and neurogenesis in the SVZ.

As discussed previously, astrocytes manage the microenvironment of synapses by secreting several growth factors such as BDNF, Fibroblast growth factor-2 (FGF-2) and VEGF (Wang et al., 2011; Egervari et al., 2016). Many studies have developed strategies to promote the upregulation of BDNF synthesis and secretion to promote synaptic plasticity and neurogenesis. In fact, Benraiss et al. (2001) showed that adenoviral injection with BDNF constructs in the SVZ is capable of increasing the BDNF in several brain areas outside the hippocampus, such as the olfactory bulb and the striatum. Moreover, intracerebroventricular injection of BDNF leads to an increase in newborn neurons in several areas adjacent to the ventricles, such as striatum, septum and thalamus (Pencea et al., 2001).

Besides the injection of exogenous BDNF, there are relevant data that Galectin-1 (Gal-1), a soluble carbohydrate-binding protein that is widely expressed in both neurons and glia, can enhance astrocytic BDNF production and improve functional outcomes in rats following brain ischemia. In fact, Qu et al. (2010) have demonstrated that exogenous Gal-1 treatment induces the production of BDNF from cortical astrocytes *in vitro* and *in vivo*, which may contribute to brain ischemia through a neurogenic process (**Figure 5**). Using a rat model of focal ischemia induced by photochemical injury, the authors have shown that 7-days continuous infusion of Gal-1 into subarachnoid space promoted long-term improvement in neurological outcomes along with cell death reduction (Qu et al., 2010). This protective role of Gal-1 was also proved important in another rodent model of focal ischemia, where treatment with this compound was responsible to promote functional recovery and facilitation of neurogenesis (Ishibashi et al., 2007).

Moreover, another treatment that increases astrocytic BDNF synthesis is stimulation of adrenergic receptors. These play several important roles on astrocytes (Christiansen et al., 2011) and treatment of rodent astrocyte cultures with β-adrenergic agonists markedly increases BDNF expression (Zafra et al., 1992; Schwartz and Nishiyama, 1994; **Figure 4**). Furthermore, Jurič et al. (2006) have demonstrated that noradrenaline and 5-hydroxytryptamine (5-HT) are able to induce an increase in astrocytic BDNF levels in cortical astrocytes (Jurič et al., 2006). Regarding the mechanisms underlying the increase in BDNF levels after noradrenergic stimulation, the authors have demonstrated that the stimulatory effect was abolished by either β1 or β2 selective antagonists, thus confirming the important activity of these receptors on this effect (Jurič et al., 2008). The authors speculate that the stimulation of β-adrenergic receptors leads to the activation of cAMP /PKA cascades, thus leading to an increased phosphorylation of cAMP response element binding protein (CREB), a transcription factor involved in the production of BDNF. Additionally, α-adrenergic receptors stimulation can also promote an increase in astrocytic BDNF levels, presumably by the activation of the PLC-mediated pathway with further activation of calcium-dependent kinases such as Ca^{2+}/calmodulin-dependent protein kinase (CaMK) that targets CREB to phosphorylation (**Figure 5**).

Another important growth factor for promoting astrocyte-driven neurogenesis is the CNTF. CTNF is produced and secreted by astrocytes in neurogenic niches and can promote adult neurogenesis, increasing proliferation and maintenance of newborn neurons (Carroll et al., 1993; Emsley and Hagg, 2003). In fact, there is evidence that exogenous application of CNTF in mice can promote proliferation of neural precursor cells in both neurogenic areas (SVZ) and dentate gyrus (Emsley and Hagg, 2003). The authors hypothesize that this observed effect is correlated with the activation of CNTF receptor, widely distributed on these neurogenic niches. Interestingly, recent studies have shown novel approaches to increase CNTF levels by pharmacological inhibition of focal adhesion kinase (FAK) and Janus kinase (JAK) downstream signaling cascade (Jia et al., 2018). Indeed, the authors have demonstrated that systemic FAK and intrastriatal JNK inhibition enhances SVZ neurogenesis entirely through CNTF, whereas astrocyte-specific deletion of FAK can lead to an increase in CNTF and SVZ neurogenesis as well. Thus, this FAK-JAK-CNTF pathway seems to be a useful therapeutic target to promote astrocytic-driven neurogenesis.

Besides the secretion of growth factors that are crucial to the management of the synaptic microenvironment, another class of molecules secreted by the astrocytes that are also important to this control is the gliotransmitters. As described earlier, an important gliotransmitter secreted by astrocytes surrounding excitatory synapses is D-serine, a neural modulator that acts as an co-agonist of the NMDA receptor in glutamatergic synapses (Schell et al., 1995). Interestingly, since the modulation of glutamatergic synapses can lead to an increase in neuronal activity in neurogenic niches such as the dentate gyrus, D-serine can also exhibit this ability to promote adult hippocampal neurogenesis. In fact, as demonstrated by Sultan et al. (2013), 8 days intraperitoneal administration of D-serine increased cell

proliferation and adult hippocampal neurogenesis. Moreover, the authors also have demonstrated that D-serine *in vitro* treatment increased cell number and survival in adult hippocampal neural progenitors suggesting a direct effect of D-serine on adult neural stem cells. The authors hypothesize that this effect in newborn neurons is correlated with an increase in NMDA receptors activity, given that much evidence points to glutamate synaptic activity as responsible for this effect (Platel et al., 2010; Kelsch et al., 2012). Furthermore, besides its effects on neurogenesis, exogenous D-serine has important effects on dendritic spine maturation being relevant for the functional integration of newborn neurons. Sultan et al. (2015), using two distinct conditional transgenic mice to manipulate exocytosis from astrocytes during the maturation stage of new neurons, found that this blockage leads to a markedly impairment on dendritic maturation of newborn neurons, which was partially alleviated by the administration of exogenous D-serine. Thus, modulation of D-serine levels on astrocytes can be a useful target to promote neurogenesis.

Astrocyte-Driven Synaptogenesis

Astrocytes are key components of the tripartite synapse, being crucial to the energetic balance as well as to synaptic formation and maturation. Astrocytes secrete several factors that contribute to the synaptic plasticity and synaptogenesis, and these can be used in therapeutic approaches to enhance astrocyte-driven synaptogenesis in the brain (Dityatev and Rusakov, 2011). As discussed above, one important factor that is released from astrocytes and can enhance presynaptic formation and transmitter release, contributing to dendrite development is the cholesterol-bind Apolipoprotein (Mauch et al., 2001; Goritz et al., 2005). In fact, Goritz et al. (2005) have demonstrated that astrocyte-secreted cholesterol is crucial for dendritic differentiation, continuous synaptogenesis and functional stability of evoked transmitter release.

Moreover, one important target for astrocyte-secreted cholesterol is the Sigma-1 receptor, which is bound to lipid rafts in complex with glucose-related protein 78/binding immunoglobulin protein (GRP78/BiP), an endoplasmic reticulum (ER) chaperone of the mitochondria-associated ER membrane (MAM) domain (Hayashi and Su, 2007). Several Sigma-1R agonists have demonstrated neuroprotective and synaptogenic effects. For instance, stimulating Sigma-1Rs in PC12 cells with ifenprodil or donepezil enhanced neurite outgrowth, whereas the inhibition or knockdown of Sig-1R abolished this synaptogenic effect (Ishima et al., 2008; Ishima and Hashimoto, 2012). Furthermore, another important class of Sig-1R agonists are neurosteroids, such as dehydroepiandrosterone (DHEA), the most abundant endogenous neurosteroid in the CNS (Su et al., 1988). In fact, Hajszan et al. (2004) have demonstrated that subcutaneous injections of DHEA (1 mg/d for 2 days) increased CA1 spine synapse density in ovariectomized rats, which can partially be attributed to the agonistic effect of this compound in Sig-1 receptors, although other unknown mechanisms have not yet been elucidated (**Figure 6**).

Moreover, there is evidence suggesting that indirect enhancement of cholesterol trafficking to the brain can also promote further upregulation of Sigma1-R activation leading to a marked increase in synaptogenesis and dendritic spine outgrowth. In fact, Cui and colleges have demonstrated that treatment with GW3965, a synthetic liver X receptor agonist, a member of the nuclear receptor family of transcription factors, elevates high-density lipoprotein cholesterol (HDL-c) and promotes increased synaptic protein and axonal density. This effect is partially explained by Sig-1R agonism of cholesterol, although another mechanism could be related with the activation of liver X receptor (LXR), given that LXR activation increase cerebral blood flow and influx of energy substrates into the brain and increase the clearance of toxic products, maintaining a pro-synaptogenic microenvironment (Sandoval-Hernández et al., 2016).

Another important astrocyte-secreted molecule that modulates synaptogenesis is the TGF-β. As discussed above, TGF-β signaling cascade can regulate the formation of excitatory and inhibitory synapses in the brain. Notably, Diniz et al. (2012) have demonstrated that TGF-β signaling pathway is involved in astrocytes synaptogenic modulation through a D-serine dependent cascade. Using treatment of cortical neurons and astrocytes with exogenous TGF-β, the authors have demonstrated an increase on extracellular levels of D-serine, which can modulate NMDA receptors activity and further promote markedly modulation of synaptogenesis and synaptic plasticity. Interestingly, the authors have demonstrated that pharmacological inhibition assays for serine racemase and D-serine abolishes this TGF-β/D-serine synaptogenic effect. Thus, TGF-β signaling pathway can be a useful therapeutic approach to promote synaptogenesis (**Figure 6**).

Moreover, besides the secretion of TGF-β, another important astrocyte-secreted molecule that is crucial to pro-synaptogenic effect is BDNF. BDNF plays a major role in several cellular processes such as growth, maturation and maintenance of neuronal cells in the brain (Miranda et al., 2019). Furthermore, BDNF can be secreted by astrocytes in an activity-dependent manner, promoting pro-synaptogenic changes in the synaptic cleft microenvironment, mostly from excitatory synapses such as the glutamatergic ones (Gomez-Casati et al., 2010). Hence, therapeutic strategies focused on increasing BDNF levels in the brain can be useful in promoting synaptogenesis. Indeed, several studies have demonstrated a positive effect of BDNF direct injection, gene transduction or delivery via non-viral carriers in different animal models of PD. The treatment could promote not just pro-synaptogenic signaling but also neuroprotective signals that prevented the loss of dopaminergic neurons in the substantia nigra, a crucial part to the development of PD pathogenesis (Tsukahara et al., 1995; Hung and Lee, 1996; Klein et al., 1999; Sun et al., 2005; Hernandez-Chan et al., 2015).

Besides that, there is evidence that suggests a marked increase in BDNF levels in the brain after treatment with antidepressants that selectively inhibits the reuptake of serotonin (called "SSRI"). In fact, Einoch et al. (2017) have demonstrated that treatment with the SSRI fluvoxamine increased the activity of the BDNF-CREB pathway in rat prefrontal cortex, hippocampus and *in vitro* cortical neuronal cultures, whereas the inhibition of PI3K abolished this positive

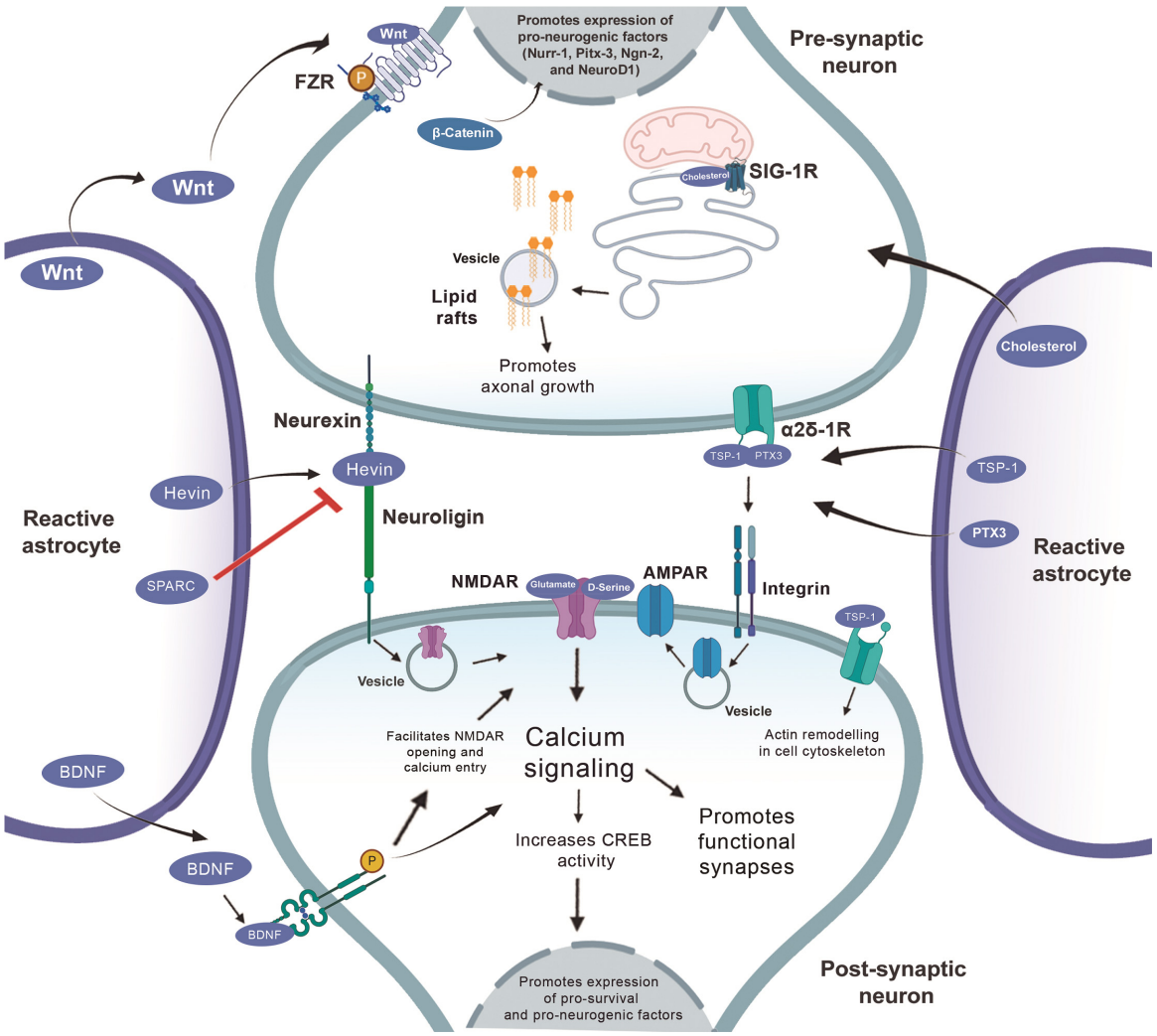

FIGURE 6 | Role of reactive astrocytes in neurorepair in the context of injury and the neurogenic and synaptogenic factors involved in this process. Wnt secreted by the reactive astrocyte interacts with the Frizzled receptors (FZR) and activates the *b*-catenin that will act inside the nucleus promoting the expression of pro-neurogenic genes (Nurr-1, Pitx-3, Ngn-2, and NeuroD1). BDNF activity at TRkB receptors increases calcium signaling, both by increasing CREB activity which, by acting on the nucleus, also promotes the expression of pro-survival and pro-neurogenesis factors, in addition to promoting the formation of functional synapses. D-serine acts similarly via NMDA receptor and CREB activation to promote such factors. TSP1 and PTX3 bind to the α2δ1 receptor, this binding at the presynaptic site increases the number of synapses, but they are silent, so the PTX3 molecule released together with TSP promotes the functional maturation of these synapses through the recruitment of AMPA receptors. The binding of TSP to postsynaptic receptors, activates the Rac1 protein and stimulates the remodeling of actin to promote synaptogenesis. Hevin makes the connection between neurexin and neuroligin and, thus, increases the recruitment of the PSD95 and NMDAR subunits. Hevin's effect is antagonized by SPARC. The cholesterol released by the astrocyte binds to the sigma-1 receptor and promotes axonal growth.

effect. Hence, novel strategies focusing on increasing BDNF levels in the brain may be an interesting approach to promote synaptogenesis (**Figure 6**).

Astrocyte-Related Therapeutic Strategies to Induce Neurorepair

In the last 10 years, cell lineage reprogramming has emerged as a novel opportunity to drive cellular regeneration. Initial studies made use of pluripotent stem cells, that were then differentiated to neurons or astrocytes (Takahashi and Yamanaka, 2006; Kim et al., 2011; Han et al., 2012; Ring et al., 2012; Chandrasekaran et al., 2016; Marx et al., 2020). Then, it was found that cell types can be directly transdifferentiated to the neural lineage and that astrocytes in brain may convert into neurons (Vierbuchen et al., 2010; Robel et al., 2011; Ladewig et al., 2013; Kim et al., 2014; Flitsch and Brüstle, 2019; Gatto et al., 2021). Mechanisms found in cell culture were also transferred to transgenic mouse models: direct expression of reprogramming genes in striatal astrocytes converted them into fully functional neurons (Torper et al., 2013). This creates an interesting approach to promote astrocyte-related neurorepair, given the fact that in the injury context, inducing astrocyte-to-neuron trans-differentiation can lead to neural tissue repair.

Furthermore, several molecules have been used to promote astrocyte-to-neuron conversion. For instance, Heinrich and collaborators showed that increasing of Neurog2 and Dlx2 in cortical astrocytes, both transcription factors that are related to neuronal cell-fate of neural progenitors, can produce glutamatergic and GABAergic neurons (Heinrich et al., 2010). Additionally, Guo et al. (2014) have demonstrated that reactive astrocytes of an AD mouse model can be directly reprogrammed to functional neurons *in vivo* using retroviral NeuroD1, another transcription factor related to neuronal lineage. Interestingly, the authors showed that reactive astrocytes were mainly reprogrammed into glutamatergic neurons after NeuroD1 translation.

Rivetti di Val Cervo et al. (2017) have demonstrated the induction of functional dopamine neurons from astrocytes. Using three transcription factors, NEUROD1, ASCL1 and LMX1A, and the microRNA miR218, the authors have demonstrated direct reprogramming of both human astrocytes *in vitro* and mouse astrocytes *in vivo* into dopaminergic neurons. Interestingly, the reprogramming efficiency *in vitro* was improved by administration of small molecules that activate TGF-β and Wnt signaling pathways. Hence, this indicates that modulating both astrocyte-to-neuron conversion and activation of pro-neurogenic pathways is an interesting approach to induce neurorepair.

Another interesting strategy to promote neurorepair that is also related to cell reprogramming is the conversion of astrocytes into neuroblasts to further induce new neurons formation, a process that is called "dedifferentiation" (Kleiderman et al., 2016a). Several *in vitro* studies have demonstrated that many astrocytes in response to injury can re-enter in the cell cycle and differentiate itself in neuroblast, which then is destined to form new neurons (Buffo et al., 2008; Yang et al., 2009; Robel et al., 2011). Promoting adult astrocytes dedifferentiation involves several different pathways, epigenetic silencing of mature astrocyte genes and removement of silencing marks from progenitor genes to further promote a pro-neuroblast cell environment (Robel et al., 2011). For example, increased acetylation of H3K9K14 near NeuroG1 and NeuroG2 genes is required during astrocyte dedifferentiation in mouse primary neuronal cultures (Hirabayashi et al., 2009).

Interestingly, several pieces of evidence have shown that altering the production of many transcription factors and downstream targets can change astrocyte fate and induce dedifferentiation. For instance, Nanog Homeobox, POU Class 5 Homeobox 1 (OCT4), Forkhead Box G1 (FOXG1), SRY-Box 2 (SOX2), and Cell Cycle Exit and Neuronal Differentiation 1 (CEND1) are transcription factors that during situations with increased activity can promote dedifferentiation of adult astrocytes into NSC (Corti et al., 2012; Niu et al., 2013; Aravantinou-Fatorou et al., 2015). Hence, cell reprogramming either by direct astrocyte-to-neuron conversion or astrocyte dedifferentiation into adult neural stem cells is a promising therapeutic approach to promote neurorepair in the context of injury, being crucial on the development of new research to further investigate and elucidated the mechanisms by which these therapies works and its usability in regeneration medicine.

CONCLUSION

Astrocytes play a central role in the neurorepair process. They are not only responsible for cleaning the system after injuries and controlling the inflammatory process, but they are also responsible for orchestrating the process of neurogenesis and synaptogenesis. Astrocytes are widely spread throughout the cerebral tissue and can be regenerated after brain damage. Their privileged location in the brain puts them in contact with neurons and endothelial cells, which allows them to control the neuronal differentiation, the synaptic sprouting, and its integration in the networks. Advances in understanding the mechanisms associated with astrocyte activation and its impact on the neurorepair process are fundamental for the development of new therapeutic approaches for astrocyte-induced neurogenesis.

AUTHOR CONTRIBUTIONS

RC, GC, BM, LM, OO-L, RMG, FK, FS, AB, and MP wrote the manuscript. RG, ML, and MP revised and approved the manuscript. All the authors contributed to the article and approved the submitted version.

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frontiers in Cell and Developmental Biology

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Autocrine Effects of Brain Endothelial Cell-Produced Human Apolipoprotein E on Metabolism and Inflammation *in vitro*

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Reports of *APOE4*-associated neurovascular dysfunction during aging and in neurodegenerative disorders has led to ongoing research to identify underlying mechanisms. In this study, we focused on whether the *APOE* genotype of brain endothelial cells modulates their own phenotype. We utilized a modified primary mouse brain endothelial cell isolation protocol that enabled us to perform experiments without subculture. Through initial characterization we found, that compared to *APOE3*, *APOE4* brain endothelial cells produce less apolipoprotein E (apoE) and have altered metabolic and inflammatory gene expression profiles. Further analysis revealed *APOE4* brain endothelial cultures have higher preference for oxidative phosphorylation over glycolysis and, accordingly, higher markers of mitochondrial activity. Mitochondrial activity generates reactive oxygen species, and, with *APOE4*, there were higher mitochondrial superoxide levels, lower levels of antioxidants related to heme and glutathione and higher markers/outcomes of oxidative damage to proteins and lipids. In parallel, or resulting from reactive oxygen species, there was greater inflammation in *APOE4* brain endothelial cells including higher chemokine levels and immune cell adhesion under basal conditions and after low-dose lipopolysaccharide (LPS) treatment. In addition, paracellular permeability was higher in *APOE4* brain endothelial cells in basal conditions and after high-dose LPS treatment. Finally, we found that a nuclear receptor Rev-Erb agonist, SR9009, improved functional metabolic markers, lowered inflammation and modulated paracellular permeability at baseline and following LPS treatment in *APOE4* brain endothelial cells. Together, our data suggest that autocrine signaling of apoE in brain endothelial cells represents a novel cellular mechanism for how *APOE* regulates neurovascular function.

Keywords: apolipoprotein E, brain endothelial cells, metabolism, inflammation, SR9009

INTRODUCTION

Human *APOE* genotype plays an important role in the homeostasis of the central nervous system and has long been linked to neurodegenerative disorders (Huang, 2006; Mahley et al., 2007; Yamazaki et al., 2019; Chen et al., 2020; Lanfranco et al., 2020; Lewandowski et al., 2020; Li et al., 2020). For example, *APOE4* is associated with greater cognitive decline in aging, poorer outcomes following stroke and traumatic brain injury, and is a major genetic risk factor for Alzheimer's disease compared to *APOE3* (reviewed in Mahley et al., 2007; Liu et al., 2013; Flowers and Rebeck, 2020). One way *APOE* genotype could impact neuronal function is through neurovascular disruption, which is found with *APOE4* during aging, in Alzheimer's disease and in respective mouse models (Salloway et al., 2002; Zipser et al., 2007; Poels et al., 2010; Halliday et al., 2013; Zlokovic, 2013; Halliday et al., 2015; Tai et al., 2016; Thomas et al., 2016; Marottoli et al., 2017; Tai et al., 2017; Thomas et al., 2017). Since specialized brain endothelial cells are central for the unique properties of the neurovasculature, it is important to identify mechanisms through which *APOE* alters brain endothelial cell function.

Current research has focused on how the *APOE* genotype of astrocytes and pericytes (Bell et al., 2012; Yamazaki et al., 2020b) impact brain endothelial cells; less explored and more controversial, is whether the *APOE* genotype of brain endothelial cells is important. Indeed, limited *in vitro* data conflict on whether endothelial cells produce apolipoprotein E (apoE) to any significant level and if there are any *APOE* genotype-specific functional differences (Rieker et al., 2019; Yamazaki et al., 2020b). In our opinion, a case can be made for the concept that brain endothelial cell-produced apoE contributes to regulation of neurovascular function. In general, evidence that cells beyond astrocytes and hepatocytes produce apoE to impact cellular function is expanding (Basu et al., 1982; Werb et al., 1986; Mahley et al., 2007; Fernandez et al., 2019; Najm et al., 2019; Yamazaki et al., 2020a,b). In these other cell types, apoE modulates basal and reparative processes, particularly metabolism and inflammation, which may be important for brain endothelial cells given their specialized function and location. As a simplification, brain endothelial cells control bi-directional movement of essential and unwanted molecules to and from the brain, are functionally linked to neuronal activity, integrate signals with multiple cell types and regulate inflammation. Brain endothelial cells are also continually exposed to acute and chronic fluctuations in circulating molecules from the interstitial fluid and plasma in physiological and pathological states. Brain endothelial cells may utilize fundamental metabolic and inflammatory functions of apoE for local homeostasis. Thus, for such an important protein as apoE, and in a cell as specialized as brain endothelial cells, there is a distinct advantage of autocrine signaling. Resolving the question of whether apoE functions in an autocrine manner in brain endothelial cells could provide a novel cellular mechanism for how *APOE* regulates neurovascular function both in physiological and pathological states.

The objective of the present study was to determine the role of human *APOE* genotype in modulating the phenotype of brain endothelial cells *in vitro*. To this end, we isolated primary mouse brain endothelial cells from *APOE3*- and *APOE4*-targeted replacement mice and assessed genotype-specific differences in apoE levels, transcriptomic profiles and cellular functions, including metabolism and inflammation, using biochemical and immunocytochemical assays.

MATERIALS AND METHODS

Supplementary File 1 is a comprehensive "Materials and Methods" section containing protocols for brain endothelial cell isolation, *in situ* hybridization, immunocytochemistry, western blot analysis, RNA sequencing, metabolomics, atomic force microscopy and leukocyte adhesion assays. In addition, in **Supplementary Table 1** we detail specific growth surfaces, sample preparation, modifications to commercially available assay kit protocols and quantification methods for each figure panel. **Supplementary File 1** also contains a results table and five figures (one of which is full western blot images). **Supplementary File 2** is an excel file containing all raw data and statistical analysis tables. Thus, here we provide a brief description of the brain endothelial isolation protocol, and a summary of the assays.

Primary Brain Endothelial Cell Cultures

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Primary cortical mouse brain endothelial cells were isolated from male and female human *APOE3*- and *APOE4*-targeted replacement mice (Taconic, 1548 and 1549, respectively). Due to our focus on *APOE* genotype we isolated cells from juvenile mice to limit the influence of sex hormones. Briefly, cerebral cortices dissected from 28-day-old mice were diced, centrifuged (1,000 × *g*, 5 min, 4°C), resuspended in papain (20 U/ml)/DNase (2,000 U/ml) and lightly triturated through a 19G needle. Brain homogenates were then incubated for exactly 15 min at 37°C, triturated with a 21G needle, mixed thoroughly with 2 ml of 25% BSA per cortex, vortexed and centrifuged (4,000 × *g*, 5 min, 4°C) to separate out the myelin. The supernatant was collected, vortexed, and centrifuged a second time (4,000 × *g*, 5 min, 4°C). Pellets from both centrifugations were combined in complete growth media (EBM-2 containing EGM™-2 MV Microvascular Endothelial SingleQuots™ and 5.5 U/ml heparin), passed through a 100 μm cell strainer and pelleted (1,000 × *g*, 5 min, 4°C). Resuspended cells were plated at approximately 1 cortex (i.e., 2 hemispheres) to 3.466 cm^2 on a growth matrix of fibronectin, collagen and laminin, and placed at 37°C with 5% CO_2. 4 to 6 h after plating, red blood cells and debris were very gently washed away and attached cells incubated overnight at 37°C in heparin-supplemented complete growth media. The following morning, cells were washed once more and then incubated in 8 μg/ml puromycin for 48 h to negatively select for brain endothelial cells. After puromycin was removed, brain endothelial cells were washed and grown to confluence in complete growth media.

Experiments followed the general timeline of isolation on day 0, puromycin added on day 1, puromycin removed on day 3, media changed on day 5 (±5 μM SR9009), lipopolysaccharide

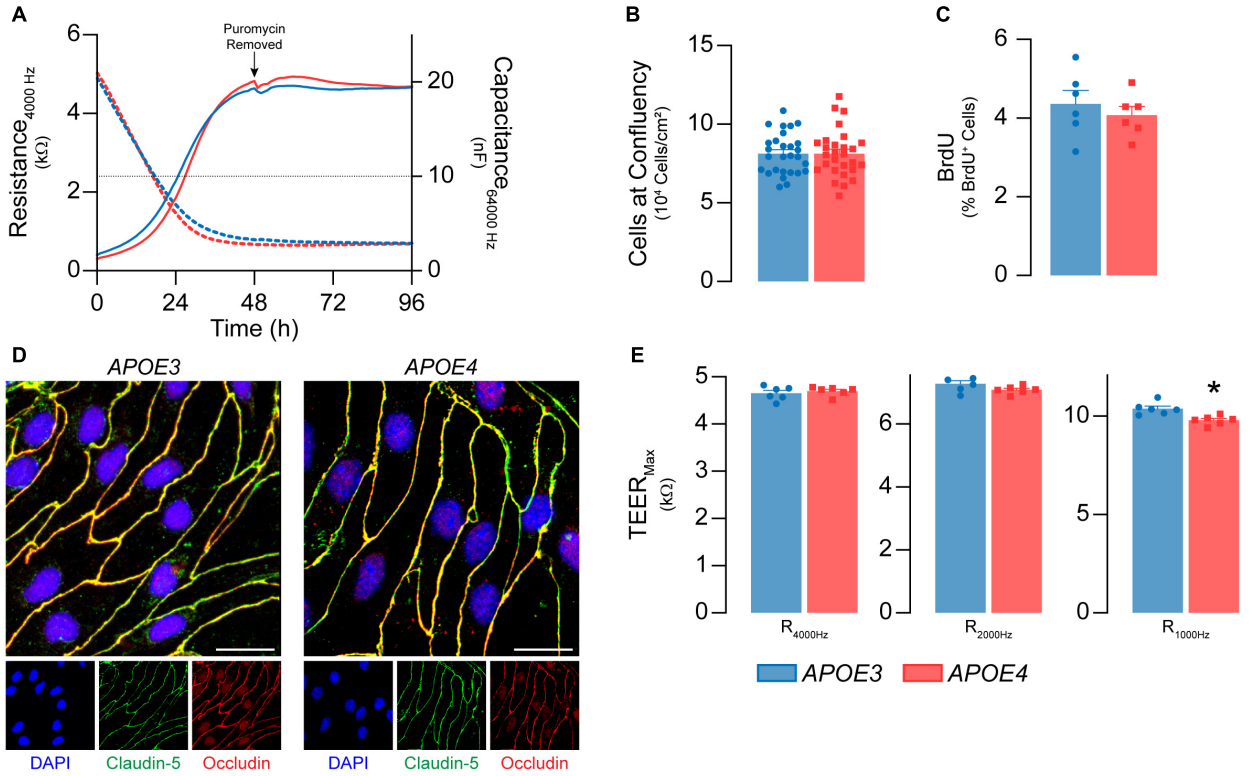

FIGURE 1 | Primary mouse brain endothelial cell characterization at passage 0. **(A)** Following isolation, brain endothelial cells establish a confluent monolayer. Capacitance (dashed lines) measured at 64 kHz reflects cell attachment and spreading and decreases as cell coverage increases; capacitance plateaus below 10 nF (black dotted line) once a monolayer is established (ECIS® ZΘApplied Biosystems). *APOE3* and *APOE4* brain endothelial cell cultures establish a monolayer by 48 h, with no genotype differences. At confluence, there are no differences in **(B)** cell density (n = 28) or **(C)** BrdU$^+$ nuclei (<5%) between *APOE3* and *APOE4* brain endothelial cells. **(D)** *APOE3* and *APOE4* brain endothelial cells express the tight junction protein markers claudin-5 (green) and occludin (red), and exhibit classical endothelial cell morphology (narrow, elongated, tightly packed cells) when assessed by immunocytochemistry, scale bar = 20 μm. **(E)** When impedance is measured at lower frequencies (i.e., 4000, 2000, and 1000 Hz), more current passes between the cells and is therefore a measure of paracellular permeability referred to as transendothelial electrical resistance (TEER). At all frequencies, TEER (solid lines in **A**) progressively increases over the course of 48 h and is then maintained for at least 4 days for both *APOE* genotypes. The frequency that represents paracellular permeability to the greatest extent is cell type-dependent and can be estimated empirically (Stolwijk et al., 2015). 1000 Hz represents the optimal frequency, as the ratio of cell-covered electrode:cell-free electrode is higher (~22) compared to 2000 Hz (~20) and 4000 Hz (14) for both *APOE3* and *APOE4* brain endothelial cells. At 1000 Hz TEER values are lower in *APOE4* brain endothelial cells compared to *APOE3*. Data is expressed as mean ± S.E.M. *$p < 0.05$ by Student's t-test with n = 6 (unless otherwise specified above).

(LPS from *E. coli* O8:K27 (S-form)) spiked into the media on day 6 as required, and experiments conducted on day 7.

Summary of Assays Used for Evaluation of Brain Endothelial Cell Phenotypes

In **Figure 1**, cell confluence was measured via capacitance and paracellular permeability by transendothelial electrical resistance (TEER). In addition, total cell counts (DAPI), proliferation (bromodeoxyuridine), and tight junction proteins were assessed by immunocytochemical analysis. ApoE levels were measured using *in situ* hybridization, immunocytochemistry, western blot, ELISA and native gel analysis (**Figure 2** and **Supplementary Figure 1**). RNA-sequencing analysis was performed to evaluate transcriptomic profiles (**Figure 3**). In **Figure 4**, ATP levels, ATP production rates, percentage of ATP production due to glycolysis or oxidative phosphorylation (Seahorse ATP Rate Assay), glucose uptake rate and lactate production were measured. Mitochondrial activity was also evaluated using assays for membrane potential (tetramethylrhodamine ethyl ester, JC-1), citrate synthase activity, electron transport chain complex levels (western blot) and the ratio of NAD$^+$: NADH. **Figure 5** and **Supplementary Figure 2** contain readouts for cellular reactive oxygen species (2′,7′–dichlorofluorescein diacetate, hydrogen peroxide, peroxynitrite, cellular superoxide anions), mitochondrial reactive oxygen species and calcium levels (MitoSOX, hydroxyl radical levels, Rhod-2), and mitophagy. Levels of antioxidants (heme and bilirubin levels, reduced:oxidized glutathione ratio) were also measured. Markers and outcomes of oxidative stress to DNA (8-oxo-dG and γH2A.X immunocytochemistry), proteins (glutathionylation, carbonylation measured by DNPH binding, autophagy via BacMan 2.0 RFP-GFP-LC3B transfection, chymotrypsin-, trypsin- and caspase-like proteasome activities), and lipids (peroxidation detected by BODIPY® 581/591 C11, TBARS and 4-hydroxynonenal ELISA) were measured in **Figure 6**. Lipid

homeostasis was determined using lipidomics, and by measuring phosphatidylcholine (ELISA), cholesterol and triglycerides levels, cell stiffness (atomic force microscopy) and extracellular lactate dehydrogenase levels (**Figure 7** and **Supplementary Figure 3**). Basal inflammation was evaluated through assays for chemokine and cytokine levels in the media (Miliplex assay), selectin-mediated membrane tethering force and adhesion probability (atomic force microscopy with sialyl-Lewis^x-coated cantilevers), and leukocyte adhesion (**Figure 8**). Further, after LPS treatment, the effect of *APOE* on TEER, apoE levels, chemokine and cytokine levels (Miliplex assay) and leukocyte adhesion were evaluated (**Figure 9** and **Supplementary Figure 4**). The impact of the Rev-Erb agonist SR9009 (5 μM) on apoE levels (media, ELISA), mitochondrial superoxide levels (MitoSOX), cell stiffness (atomic force microscopy) and inflammation (chemokine and cytokine levels, selectin-mediated membrane tethering force and adhesion probability, and leukocyte adhesion) was determined (**Figure 10**). In addition, we assessed whether SR9009 modulated apoE levels (media, ELISA), TEER, chemokine and cytokine levels, cell stiffness, selectin-mediated membrane tethering force and adhesion probability, and leukocyte adhesion with LPS treatment (**Figure 11** and **Supplementary Figure 4**).

Data and Statistical Analysis

For all experiments, data were normalized to either cell count or protein concentration. In addition, for assays that required quantification of media samples, total volume of media was measured at the end of the experiment and incorporated into calculations (media volume × [analyte]/cell number). In every figure, n represents either an individual animal or data from separate isolations of grouped animals (as described in **Supplementary Table 1**). All data are presented as mean ± S.E.M and were analyzed using Student's *t*-test or two-way ANOVA followed by the appropriate multiple comparisons test as described in the figure legends (with GraphPad Prism v9). Outliers were excluded by Grubbs' test with $\alpha = 0.05$. **Supplementary File 2** contains all data used to generate the graphs in this manuscript and statistical comparisons tables. RNA-sequencing data is available from Gene Expression Omnibus; GSE160483.

RESULTS

Confluence, Permeability, *ApoE* Production, and Transcriptomic Analysis

Our goal was to evaluate the role of human *APOE* genotype in modulating the phenotype of brain endothelial cells *in vitro*. Brain endothelial cells are highly specialized, and so we designed our study to limit phenotypic changes (e.g., protein expression, functions, loss of specialization) that can occur with cell passage and longer culture times. Therefore, we implemented an experimental protocol that enabled us to complete all experiments with fully confluent primary mouse brain endothelial cells expressing *APOE3* or *APOE4* at passage 0, within 7 days of isolation. Due to the novelty of the topic, our initial set of experiments were designed to evaluate the role of *APOE* in modulating general cellular characteristics, apoE production and the transcriptomic profile of brain endothelial cells.

APOE3 and *APOE4* Brain Endothelial Cells Form Confluent Monolayers of Contact Inhibited Cells

We first determined whether *APOE* genotype modulated the ability of brain endothelial cells to form a confluent monolayer of contact-inhibited cells, as found *in vivo*. *APOE3* and *APOE4* brain endothelial cells both established a monolayer 3 days after isolation (**Figure 1A**). 7 days post-isolation, cultures of both *APOE* genotypes averaged 8×10^4 cells/cm^2 (**Figure 1B**) and displayed minimal bromodeoxyuridine staining (<5%, **Figure 1C**), consistent with contact-inhibited endothelial cells. A second important feature of brain endothelial cells is the development of a paracellular barrier, due to tight junctions (**Figure 1D**), which we assessed using transendothelial electrical resistance (TEER). TEER values were ∼7-10% lower in *APOE4* brain endothelial cells (7 days post-isolation, **Figure 1E**, $p = 0.0045$). These data demonstrate that *APOE* genotype does not modulate proliferation or cell number at confluence, however TEER is slightly lower in *APOE4* brain endothelial cells.

APOE4 Brain Endothelial Cells Produce Less *ApoE*

For the *APOE* genotype of brain endothelial cells to modulate their function, they would have to produce apoE. We found that *APOE3* and *APOE4* brain endothelial cells express *APOE* by *in situ* hybridization (**Figure 2A**) and confirmed the presence of cell-associated apoE at the protein level by immunocytochemistry (**Figure 2B**). We next determined if *APOE* modulated apoE protein levels (**Figures 2C-E**). Cell-associated monomeric apoE was detected as a doublet (34 and 36 kDa) and was lower in *APOE4* brain endothelial cell lysates when measured by western blot analysis (34 kDa, $p = 0.036$; 36 kDa, $p = 0.0064$, **Figure 2C**). Consistent with cell-associated apoE data, secreted/extracellular monomeric apoE levels (in media) were lower with *APOE4* (34 kDa, $p = 0.0008$; 36 kDa, $p = 0.0014$), as was a multimeric apoE band (∼90-100 kDa, $p = 0.014$) when assessed by western blot analysis (**Figure 2D**) and extracellular apoE levels were approximately 15% lower with *APOE4* when quantified by ELISA (**Figure 2E**, $p < 0.0001$). Evidence suggests that cell-derived apoE4 is less lipidated than apoE3 when produced by other cell types, but we found no qualitative differences in the migration of apoE produced by *APOE3* and *APOE4* brain endothelial cells in native gel analysis (**Supplementary Figure 1**). However, apoE levels were lower in the media from *APOE4* brain endothelial cells when analyzed under native conditions. Collectively, our data demonstrate that the *APOE* genotype modulates the production of apoE by brain endothelial cells (i.e., *APOE3* > *APOE4*).

APOE Genotype Modulates the Transcriptomic Profile of Brain Endothelial Cells

To identify the potential impact of *APOE* genotype on brain endothelial cell biology, we conducted an unbiased RNA sequencing approach (**Figure 3A**). There were 1304 differentially

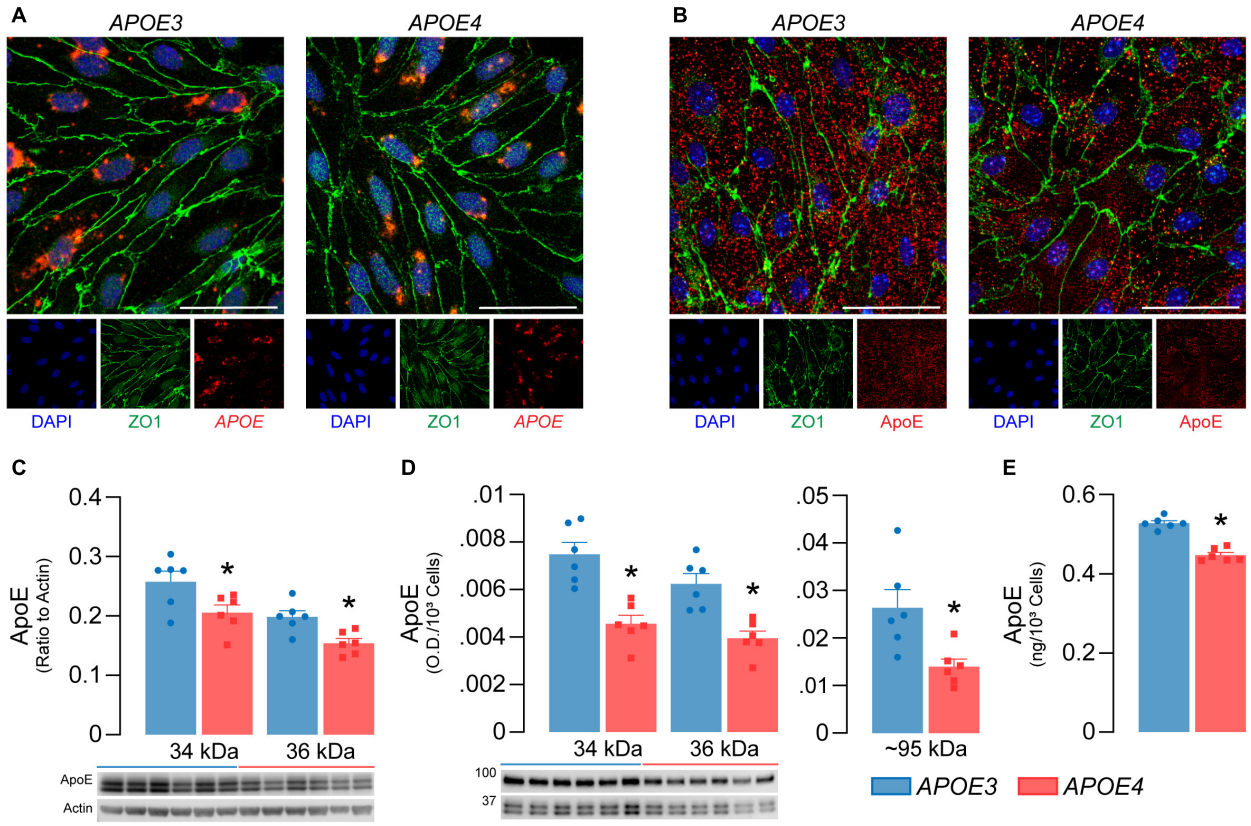

FIGURE 2 | *APOE4* brain endothelial cells produce less apoE compared to *APOE3*. *APOE3* and *APOE4* brain endothelial cells express **(A)** the *APOE* transcript (red) and **(B)** produce apoE protein (red) when assessed by *in situ* hybridization and immunocytochemistry, respectively. Cells were counterstained for the brain endothelial cell marker ZO1 (green) and DAPI (blue); representative confocal Z-stack images were captured at 52X (scale bar = 20 μm). Both **(C)** cell-associated (lysate) and **(D)** secreted (conditioned media) apoE levels are lower with *APOE4* when assessed by western blot analysis. Cell-associated apoE was normalized to actin as a loading control and secreted apoE was loaded as equal volumes and normalized to cell count. **(E)** ApoE levels in the media are ∼15% lower with *APOE4* when quantified by ELISA and normalized to cell count. Data is expressed as mean ± S.E.M. $^*p < 0.05$ by Student's *t*-test, $n = 6$.

expressed genes in *APOE4* brain endothelial cells that fell into both broad (e.g., molecular mechanisms of cancer) and highly specific (e.g., sperm motility) categories using canonical pathway analysis (**Supplementary File 2**). Our manual comparative analysis using several resources (UniProtKB, GeneCards, Pubmed) suggested that *APOE* modulates metabolism, inflammation, signaling, cell cycle/differentiation, ion homeostasis, cytoskeleton, cell adhesion, as well as other general categories including transcription/translation (**Figure 3B**, **Supplementary Table 2**, and **Supplementary File 2**). We focused our subsequent research on metabolism and inflammation as there were many transcripts in these categories, they are essential cellular processes, are often interconnected, and are found disrupted in diseases with vascular dysfunction.

Metabolism

APOE modulated gene expression profiles for different aspects of metabolism related to energy production, mitochondrial function, reactive oxygen species levels and oxidative stress (**Supplementary Table 2**); findings that served as a segue to our functional assays.

APOE4 Brain Endothelial Cells Have Lower Glycolysis and Higher Oxidative Phosphorylation and Mitochondrial Activity Compared to *APOE3*

Endothelial cell metabolism is important in homeostatic conditions and vascular disorders (Dranka et al., 2010; Tang et al., 2014; Bierhansl et al., 2017; Pi et al., 2018). Our transcriptomics data (**Supplementary Table 2**) implied that *APOE* genotype altered the amount of glycolysis compared to oxidative phosphorylation. Therefore, we evaluated the role of *APOE* in modulating ATP levels, glycolysis and oxidative phosphorylation in brain endothelial cells.

We found no differences in total ATP levels between *APOE* genotypes (**Figure 4A**) or rates of ATP production (**Figure 4B**). However, the proportion of ATP produced by glycolysis was lower in *APOE4* brain endothelial cells compared to *APOE3* (∼15%, $p = 0.038$, **Figure 4C**), as were both the rate of glucose uptake (∼25%, $p = 0.001$, **Figure 4D**) and lactate levels in the media (∼12%, $p = 0.046$, **Figure 4E**). These data support that, compared to *APOE3*, *APOE4* brain endothelial cells have higher rates of mitochondrial oxidative phosphorylation compared to glycolysis to produce ATP

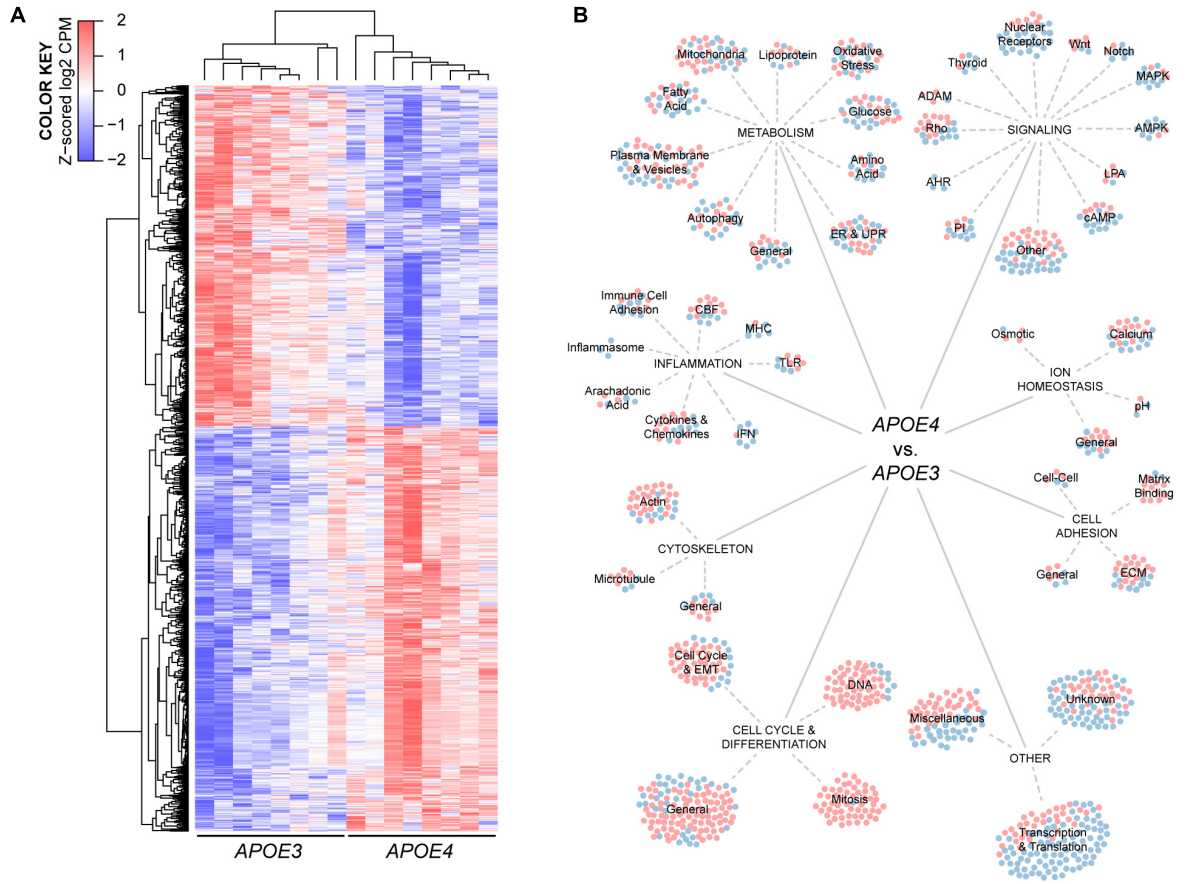

FIGURE 3 | Human *APOE* genotype modulates the transcriptional phenotype of brain endothelial cells. **(A)** Hierarchical cluster analysis of 1304 differentially expressed genes in *APOE4* brain endothelial cells determined by RNA-sequencing analysis, $n = 8$. **(B)** Manual categorization of the differentially expressed genes reveals changes related to metabolism, inflammation, signaling, ion homeostasis, cell adhesion, cytoskeleton, cell cycle as well as other more general functions. Each circle represents a differentially expressed gene, where red indicates higher expression and blue indicates lower expression with *APOE4* compared to *APOE3*.

(**Figure 4C**). Consistent with this idea, markers of mitochondrial activity were higher in *APOE4* brain endothelial cells including lower membrane potential (∼50%, TMRE, $p = 0.028$; JC-1, $p = 0.013$, **Figures 4F,G**), higher citrate synthase activity (∼20%, $p = 0.0045$, **Figure 4H**), greater levels of electron transport chain components (transcripts and proteins, **Figure 4I** and **Supplementary Table 2**) and a higher NAD^+: NADH ratio (NAD/NADH-Glo assay in which a reductase reduces proluciferin to luciferin in the presence of NADH, $p = 0.016$, **Figure 4J**). Overall, our data support that there is higher oxidative phosphorylation and mitochondrial activity in *APOE4* brain endothelial cells.

Higher Mitochondrial Superoxide Levels and Lower Antioxidant Levels With *APOE4*

Mitochondrial activity produces reactive oxygen species, which play several important physiological roles including metabolic adaptation, signaling and stress/inflammatory responses. As there was higher mitochondrial activity in *APOE4* brain endothelial cells, we evaluated whether there was a corresponding increase in reactive oxygen species levels. Surprisingly, total cellular reactive oxygen species levels were ∼32% lower ($p = 0.0051$, **Figure 5A**) with *APOE4*, which was likely driven by low H_2O_2 levels ($p = 0.012$, **Figure 5B**). Levels of total reactive oxygen species were also lower in *APOE4* brain endothelial cells after the addition of exogenous H_2O_2 ($p = 0.036$, **Figure 5C**). These data suggest upregulation of H_2O_2 degradation pathways with *APOE4*. For example, peroxisome activity may be higher due greater oxidative phosphorylation, and we found transcript levels of two H_2O_2 degradation enzymes (Gpx7, Prdx4) were higher in *APOE4* brain endothelial cells (**Supplementary Table 2**). For other common reactive oxygen species, although there were no changes in peroxynitrite levels (**Figure 5D**), cellular superoxide (O_2^-) levels were ∼31% higher ($p = 0.045$, **Figure 5E**) in *APOE4* brain endothelial cells. O_2^- is more proximally linked to mitochondrial activity than other reactive oxygen species and when evaluated directly in mitochondria, there were ∼29% higher O_2^- ($p = 0.0036$, **Figure 5F**) and ∼7% higher hydroxyl radical ($p = 0.02$, **Figure 5G**) levels with *APOE4*, without changes in mitochondrial calcium level or mitophagy (**Supplementary Figure 2**). Therefore, with *APOE4*,

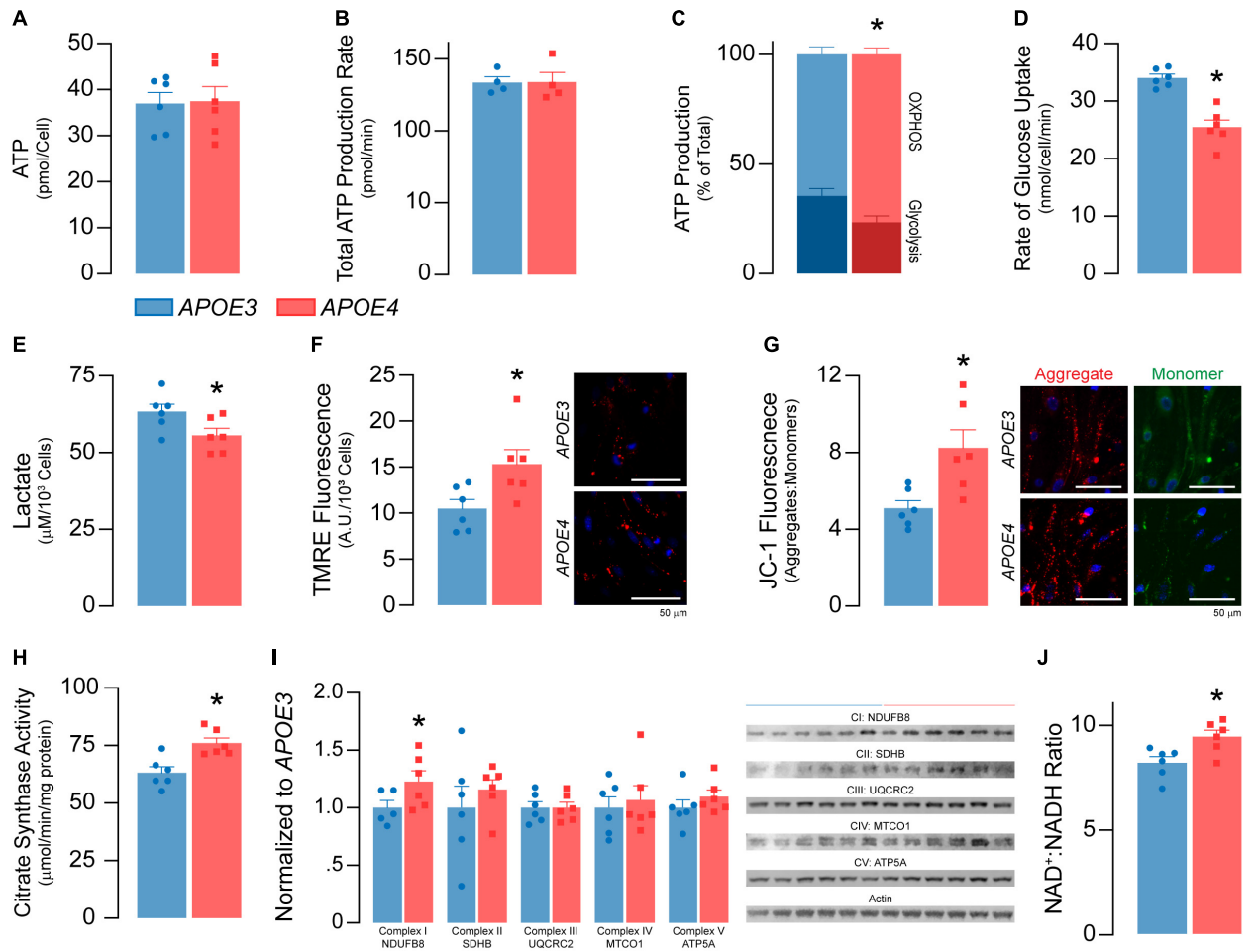

FIGURE 4 | *APOE4* brain endothelial cells have lower glycolysis and higher oxidative phosphorylation and mitochondrial activity compared to *APOE3*. **(A)** ATP levels and **(B)** the rate of total ATP production ($n = 4$) are similar in *APOE3* and *APOE4* brain endothelial cells. Glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) both contribute to ATP production. Comparing the bioenergetic profiles of *APOE3* and *APOE4* brain endothelial cells revealed **(C)** ATP production from OXPHOS is higher and glycolysis is lower with *APOE4* as measured by Seahorse XF Real-Time ATP Rate Assay ($n = 4$). Consistent with lower glycolytic activity, *APOE4* brain endothelial cells also have **(D)** a lower rate of glucose uptake and **(E)** lower levels of lactate in the media compared to *APOE3*. Mitochondrial activity is central to OXPHOS, which may be higher with *APOE4*. We measured mitochondrial activity using complementary approaches. Active mitochondria have a greater net negative charge that can be measured by the accumulation of tetramethylrhodamine ethyl ester (TMRE) and aggregation of JC-1 (monomer, green; aggregate, red) dyes. In *APOE4* brain endothelial cells there is **(F)** higher TMRE staining (∼46%) and **(G)** aggregate:monomer ratio of JC-1 (∼62%). **(H)** Citrate synthase activity, which is important for the first step of the Krebs cycle, is also higher with *APOE4* (∼20%), as are transcripts of electron transport chain components (e.g., mt-Nd1, mt-Nd2, mt-Nd4, mt-Nd5, mt-Cytb, mt-Co1 **Supplementary Table 2**), which **(I)** we validated at the protein level. **(J)** Nicotinaminde adenine dinucleotide (NAD) is a co-factor present in two forms in a cell; NAD^+ (oxidized) and NADH (reduced). NADH is utilized in the electron transport chain to donate electrons for ATP generation and as a co-factor for enzymatic activity. The ratio of NAD^+:NADH is higher with *APOE4*, data that may imply lower NADH levels to supply the cell with energy, or that high levels have been oxidized to sustain higher mitochondrial function. Data is expressed as mean ± S.E.M. $^*p < 0.05$ by Student's *t*-test with $n = 6$ (unless otherwise specified above).

higher mitochondrial activity may have resulted in greater levels of O_2^-.

Levels of reactive oxygen species are intimately linked to antioxidants; transcripts related to two important antioxidant systems, heme and glutathione, were modulated by *APOE* genotype (**Supplementary Table 2**) and we measured markers of both. Heme is produced in a series of reactions involving the mitochondria and can be degraded into the antioxidant and anti-inflammatory molecule bilirubin. In *APOE4* brain endothelial cells, there were lower levels of genes that both produce (Ppox, Alas) and degrade heme to bilirubin (Hmox1, Blvrb) and, importantly, lower levels of intracellular heme ($p = 0.001$, **Figure 5H**) and bilirubin ($p = 0.0038$, **Figure 5I**). Glutathione (GSH) neutralizes reactive oxygen species and is converted to oxidized/disulfide glutathione (GSSG); low GSH:GSSG indicates higher reactive oxygen species levels. In *APOE4* brain endothelial cells the GSH:GSSG ratio was ∼ 32% lower ($p = 0.0002$, **Figure 5J**). Relatedly, we also found lower levels of reduced nicotinamide adenine dinucleotide phosphate with *APOE4*, which can reduce GSSG to form GSH (**Supplementary File 2**).

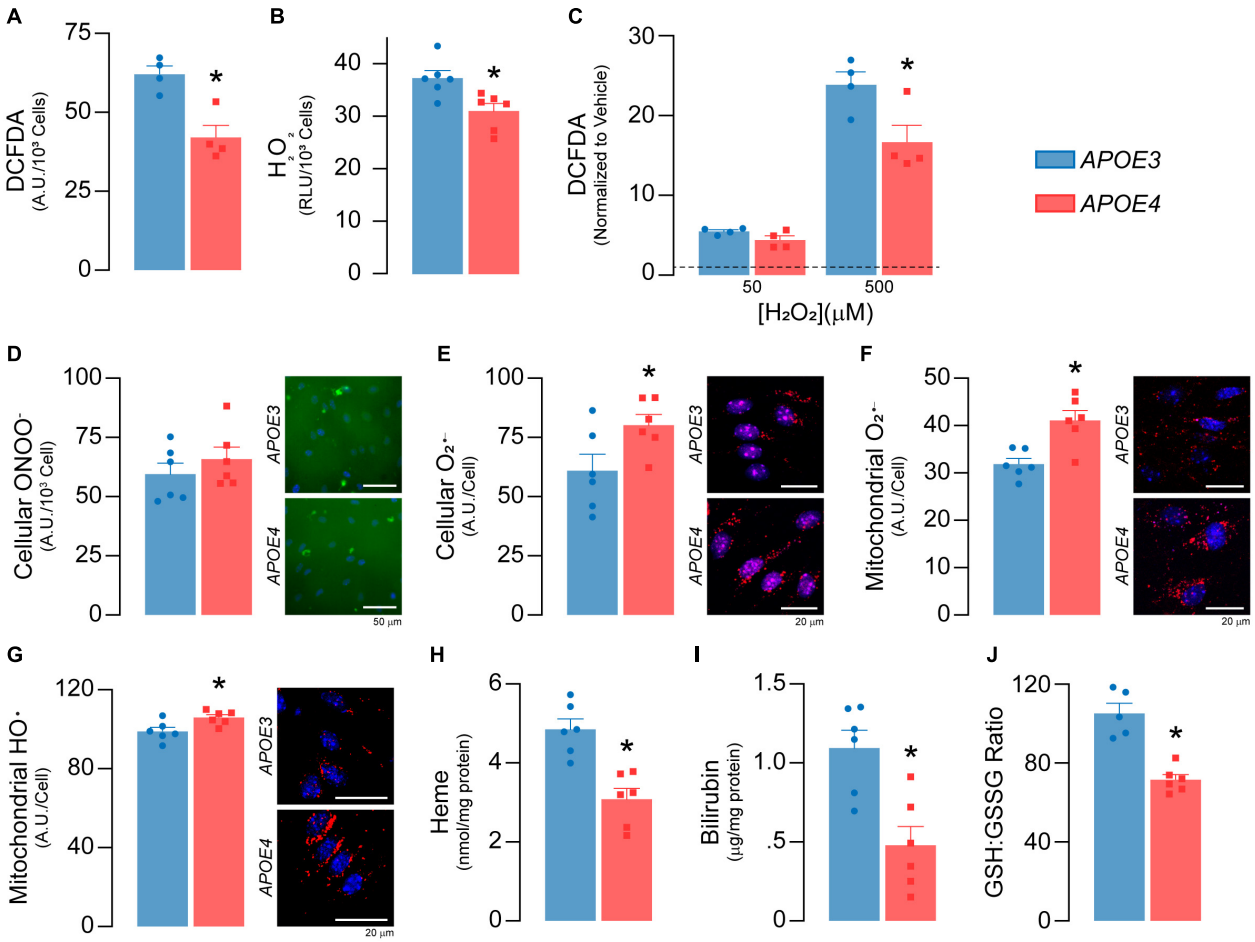

FIGURE 5 | *APOE* genotype modulates reactive oxygen species and antioxidant levels. **(A)** Total reactive oxygen species (ROS) levels were measured with 2′,7′-dichlorofluorescein diacetate (DCFDA; $n = 4$). DCFDA diffuses into the cell where it is deacetylated to a non-fluorescent compound by cellular esterases. Reactive oxygen species then oxidize the deacetylated DCFDA to form 2′, 7′-dichlorofluorescein, which fluoresces green. *APOE4* brain endothelial cells exhibit lower total cellular ROS as evidenced by ∼32% lower DCFDA fluorescence. Of the different types of ROS, H_2O_2 is the most abundant and accounts for the majority of the DCFDA signal and **(B)** specific levels of H_2O_2 are lower in *APOE4* brain endothelial cells. After the addition of exogenous H_2O_2, **(C)** levels of total reactive oxygen species (DCFDA) are still higher with *APOE3* ($n = 4$), suggesting an upregulation of pathways to limit H_2O_2 levels in *APOE4* brain endothelial cells, such as peroxisome or other enzymatic activity. For other ROS, while there are **(D)** no changes in peroxynitrite levels with *APOE4*, **(E)** there are ∼31% higher cellular O_2^- levels. These data are consistent with the idea that higher mitochondrial metabolism can result in the accumulation of O_2^-. Indeed, specifically for mitochondrial ROS, there are **(F)** ∼29% higher O_2^- (MitoSOX Red O_2^- Indicator which accumulates in mitochondria and is oxidized by O_2^-) and **(G)** ∼7% higher hydroxyl radical (OH580 probe which is oxidized by hydroxyl radicals) levels in *APOE4* brain endothelial cells. The effects and levels of reactive oxygen species are in part regulated by antioxidant systems. Heme is an essential iron-containing compound with pleiotropic functions from respiration, oxygen transport and xenobiotic modification to modulation of reactive oxygen species levels. Heme is produced from glycine and succinyl CoA in a series of reactions that starts in the mitochondria, continues in the cytoplasm, and is then completed in the mitochondria. Heme can be degraded into bilirubin, which is an antioxidant and anti-inflammatory molecule. *APOE4* brain endothelial cells have lower levels of **(H)** heme and **(I)** bilirubin, and **(J)** a lower ratio of GSH:GSSG (indicating more oxidative stress; *APOE3* $n = 5$, *APOE4* $n = 6$). Data is expressed as mean ± S.E.M. $^*p < 0.05$ by Student's *t*-test with $n = 6$ (unless otherwise specified above).

Collectively our data demonstrate an altered balance of reactive oxygen species, characterized by higher mitochondrial O_2^- and hydroxyl radicals, lower heme/bilirubin levels, and a lower GSH:GSSG ratio in *APOE4* brain endothelial cells.

Higher Markers of Oxidative Stress in *APOE4* Brain Endothelial Cells

Reactive oxygen species can induce cellular damage to DNA, proteins, and lipids, often termed oxidative stress. As levels of O_2^- were higher in *APOE4* brain endothelial cells, we determined whether markers of oxidative stress were also higher. Although there were no *APOE* genotype effects on markers of DNA damage (**Figures 6A,B**), there were alterations in markers of oxidative stress to proteins. With *APOE4* we found ∼50% lower protein glutathionylation ($p = 0.0007$, **Figure 6C**), which is a protective mechanism to limit oxidative damage to cysteine thiol groups on proteins, and ∼25% higher protein carbonylation, which is an irreversible oxidation of amino acid side chains ($p = 0.045$, **Figure 6D**). Although we did not observe any changes in autophagy (**Figure 6E**), there were higher levels

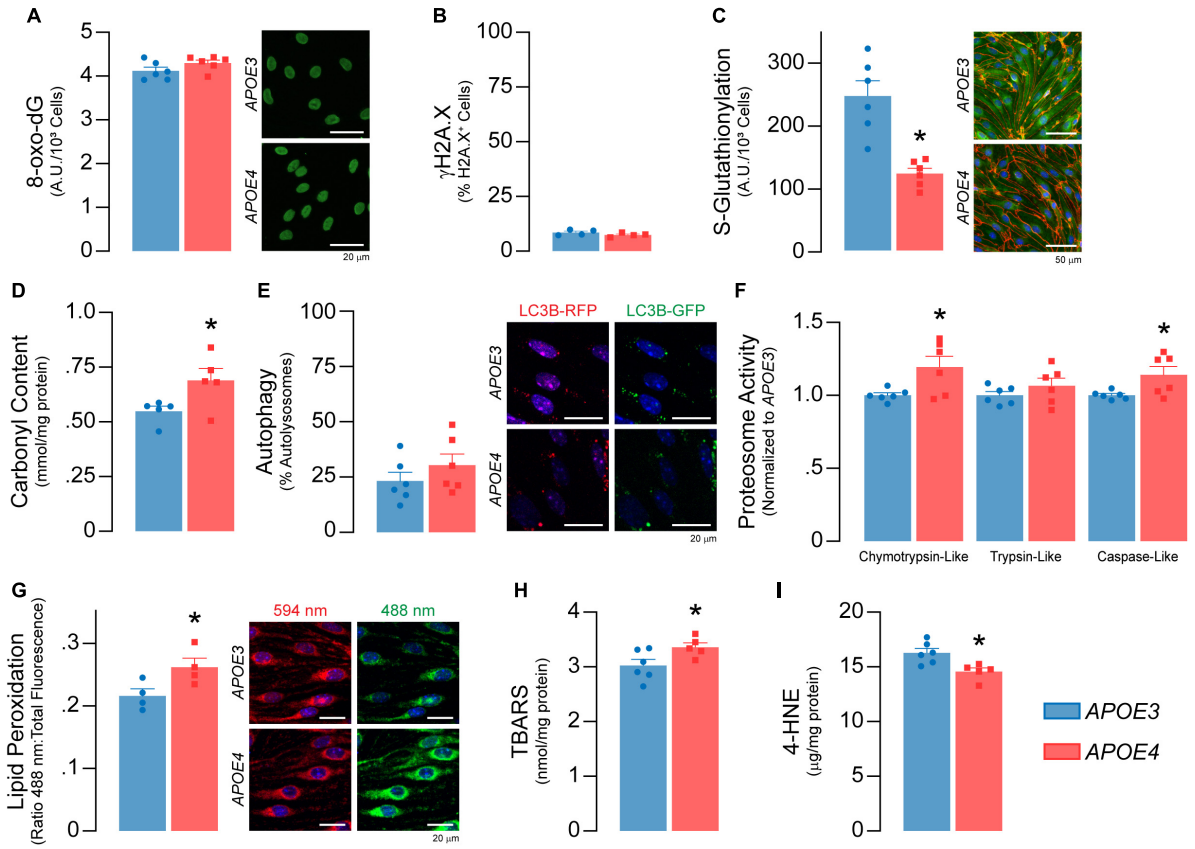

FIGURE 6 | Higher levels of oxidative damage to proteins and lipids in *APOE4* brain endothelial cells. There are no *APOE* genotype effects on markers of DNA damage, including **(A)** levels the oxidized deoxyguanosine derivative 8-oxo-2'-deoxyguanosine and **(B)** the double-stranded DNA breaks marker γH2A.X (n = 4). **(C)** Protein glutathionylation limits irreversible oxidative damage to cysteine thiol groups on proteins. *APOE4* brain endothelial cells have ∼50% lower S-glutathionylated protein levels, that could be due to the lower GSH:GSSG ratio induced by reactive oxygen species (**Figure 5J**) and/or lower levels glutathione transferases (**Supplementary Table 2**; Gstt3, Gstm2, Gstp1, Gstm1, Gstt2). **(D)** The lower protection of proteins with *APOE4* could lead to irreversible oxidation of amino acid side chains referred to as carbonylation. Protein carbonylation is ∼25% higher with *APOE4* as measured by 2,4-dinitrophenylhydrazine (DNPH) binding (n = 5). In addition, while there was no difference in **(E)** autophagy, **(F)** chymotrypsin-like and caspase-like proteosome activity are higher with *APOE4*, potentially to degrade damaged proteins. **(G)** Lipids are sensitive to the effects of reactive oxygen species through peroxidation, which we measured using a ratiometric fluorescent indicator (BODIPY 581/591 C11) that changes from red to green upon lipid peroxidation. In *APOE4* brain endothelial cells, lipid peroxidation is higher, as evidenced by ∼20% higher ratio of green:total fluorescence (n = 4). In addition, **(H)** levels of lipid peroxidation products, called TBA reactive substances, are ∼11% higher (*APOE3* n = 6, *APOE4* n = 5), although **(I)** 4-hydroxynonenal levels are ∼11% lower as assessed by ELISA (*APOE3* n = 6, *APOE4* n = 5). Data is expressed as mean ± S.E.M. *$p < 0.05$ by Student's t-test with n = 6 (unless otherwise specified above).

of a proteasomal 20S subunit (Psmb9, **Supplementary Table 2**) as well as higher chemotrypsin-like (∼19%, p = 0.028) and caspase-like (∼14%, p = 0.034) proteasome activity (**Figure 6F**) with *APOE4*. The higher proteasome activity may reflect higher protein clearance due to oxidative stress and/or a physiological upregulation due to different cellular requirements in *APOE4* brain endothelial cells.

Lipids are sensitive to the effects of reactive oxygen species through peroxidation. We found that lipid peroxidation of an exogenously added sensor (BODIPY® 581/591 C11) was higher with *APOE4* (∼ 20%, p = 0.046, **Figure 6G**), as were levels of TBA reactive substances (∼11% higher, p = 0.045, **Figure 6H**), although 4-hydroxynonenal levels were ∼11% lower (p = 0.011, **Figure 6I**). Often, changes in cellular lipid biology are reflected in membrane structures (e.g., plasma membrane), and in *APOE4* brain endothelial cells there were changes in transcripts related to membrane dynamics/composition (**Supplementary Table 2**). In fact, *APOE* genotype modulated metabolites related to membrane phospholipids when evaluated by lipidomic analysis, including lower levels of those related to phosphatidylcholine, phosphatidylethanolamine, and plasmalogens, whereas some ceramides were higher (**Figure 7A** and **Supplementary File 2**). Consistent with metabolomic data, phosphatidylcholine levels were ∼37% lower in *APOE4* brain endothelial cells (p = 0.0062, **Figure 7B**) and, while there were no changes in cholesterol levels (**Figure 7C**), total (∼45%, p = 0.0007) and free (∼26%, p = 0.0004) triglyceride levels were lower with *APOE4* (**Figure 7D**, also found in the media; **Supplementary Figure 3**). Changes in plasma membrane composition can alter mechanical properties of a cell including overall cell stiffness and integrity. We found that

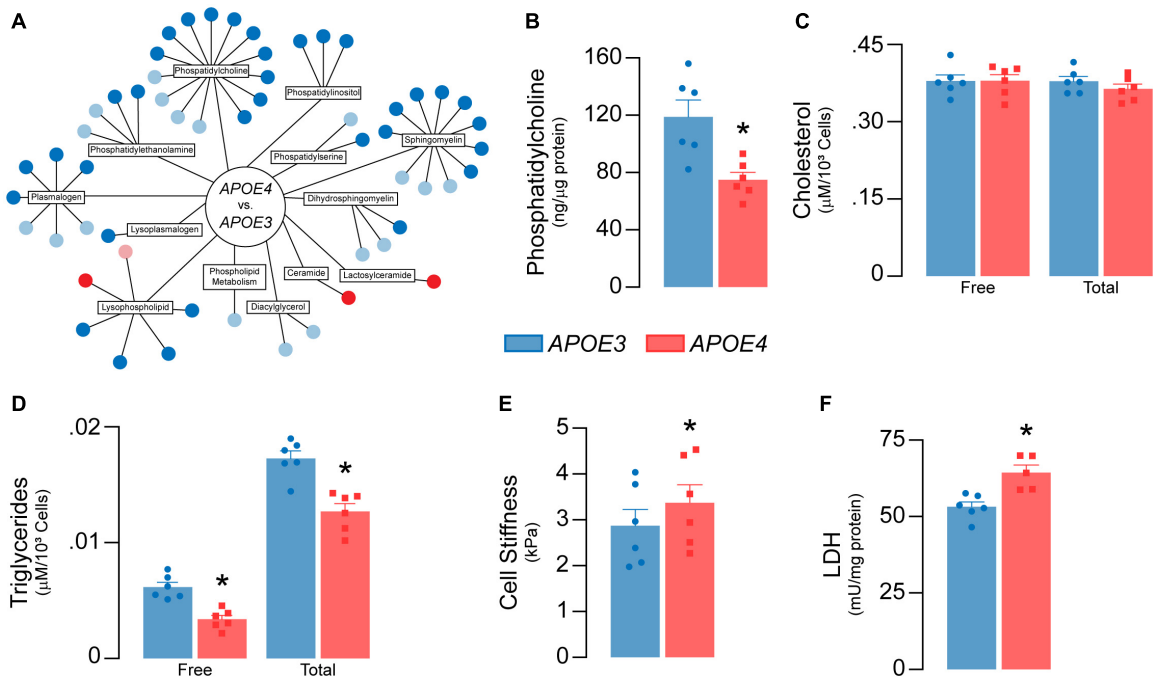

FIGURE 7 | *APOE* genotype modulates lipid metabolism in brain endothelial cells. **(A)** Lipidomic analysis reveals 43 significant differences (dark blue = lower; dark red = higher) and 21 trending differences (light blue = lower; pink = higher) in *APOE4* brain endothelial cells. Each circle represents a unique metabolite. Most of the metabolites are lower with *APOE4* and include phosphatidylcholines (1-myristoyl-2-arachidonyl-GPC (14:0/20:4), phosphatidylethanolamines (1-palmitoyl-2-arachidonyl-GPE (16:0/20:4))), phosphatidylinositols, lysophospholipids (1-oleoyl-GPC (18:1)), and plasmalogens (1-(1-enyl-palmitoyl)-2-arachidonyl-GPC (P16:0/20:4)), with the exception of two ceramides (N-behenoyl-sphingadienine (d18:2/22:0) & actosyl-N-nervonoyl-sphingosine (d18:1/24:1)) that are higher. **(B)** Consistent with lipidomic analysis, phosphatidylcholine levels are lower with *APOE4* when assessed by ELISA. **(C)** Although there are no differences in cellular cholesterol levels, **(D)** cellular triglyceride levels are lower in *APOE4* brain endothelial cells. **(E)** Changes in membrane composition can alter mechanical properties of a cell. In the plasma membrane, this can manifest as changes in stiffness, which can contribute to cell stiffness when measured by atomic force microscopy (Askarova et al., 2013). In this technique, a cantilever tip approaches the plasma membrane, makes contact, and then indents the cell surface; the force required to make the indentation corresponds to cell stiffness. We found that cells stiffness is higher in *APOE4* brain endothelial cells when measured by atomic force microscopy (analyzed by paired *t*-test). **(F)** Lactate dehydrogenase (LDH) is a cytosolic enzyme found in cell culture media when the plasma membrane is damaged, as found with cytotoxicity. Despite the lack of toxicity, levels of lactate dehydrogenase in the media are higher with *APOE4*. Data is expressed as mean ± S.E.M. *$p < 0.05$ by Student's *t*-test with $n = 6$ (unless otherwise specified above).

APOE4 brain endothelial cells were ∼17% stiffer when measured by atomic force microscopy ($p = 0.0002$, **Figure 7E**). In addition, media levels of lactate dehydrogenase (LDH), a cytosolic enzyme found extracellularly when the plasma membrane is damaged, were ∼21% higher with *APOE4* ($p = 0.0036$, **Figure 7F**). Overall, our data support that there is oxidative damage to proteins (lower glutathionylation and higher carbonylation) and lipids (altered membrane composition, higher cell stiffness and permeability of the plasma membrane to LDH) in *APOE4* brain endothelial cells.

Inflammation

There is a tight connection between metabolism and inflammation, and brain endothelial cells are the interface between plasma and brain inflammatory signaling. *APOE4* is associated with a different inflammatory response compared to *APOE3* in non-brain endothelial cells such as astrocytes and microglia (reviewed in Tai et al., 2015), and in endothelial-like cells differentiated from IPSCs (Rieker et al., 2019). In *APOE4* brain endothelial cells, there were higher levels of transcripts related to chemokines and pro-inflammatory cytokines, toll-like receptor signaling, immune cell recruitment/activation and MHC molecules, inflammasome signaling, and antiviral responses (leukocyte adhesion/immune cell activation and blood coagulation/clotting; **Supplementary Table 2**). Based on these transcriptomics data, we conducted further functional assays to determine the extent *APOE* modulates the inflammatory phenotype of brain endothelial cells.

APOE Modulates Basal Inflammation in Brain Endothelial Cells Characterized by Higher Chemokine Levels and Immune Cell Adhesion With *APOE4*

Soluble chemokines and cytokines are major effector molecules of the inflammatory response and can be produced by brain endothelial cells. Nonetheless, our finding that *APOE* genotype modulates chemokine/cytokine transcripts in brain endothelial cells was still surprising. Thus, we evaluated whether levels of 31 common chemokines and cytokines in the media were different between *APOE3* and *APOE4* brain endothelial cells by multiplex ELISA. Seven chemokines and cytokines were

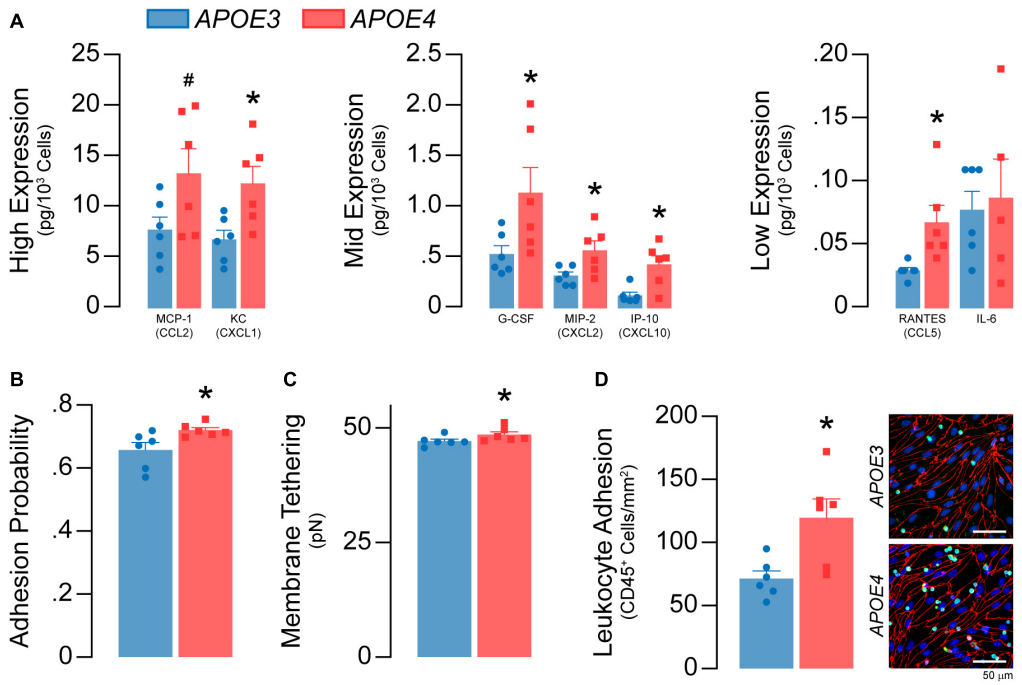

FIGURE 8 | *APOE4* brain endothelial cells have a greater inflammatory phenotype under basal conditions. **(A)** The media of *APOE4* brain endothelial cells has higher levels of MCP-1/CCL2, KC/CXCL1, G-CSF, MIP-2/CXCL2, IP-10/CXCL10, RANTES/CCL5 and IL-6 when assessed by multiplex ELISA. **(B)** Higher inflammation can lead to greater immune cell adhesion that involves selectin-mediated capture. Selectin-mediated binding can be evaluated via atomic force microscopy, using cantilevers coated/biofunctionalized with the selectin ligand sialylic-Lewis^x (sLe^x). In this assay, the biofunctionalized cantilever tip touches and indents the cell, then retracts; binding of sLe^x to selectins on the plasma membrane results in a rupture event when the cantilever is retracted (Askarova et al., 2013). Thus, two parameters are obtained; adhesion probability (i.e., whether a rupture occurs and, therefore, selectin binding) and selectin-mediated membrane tethering force (i.e., the force needed to rupture the tether). In *APOE4* brain endothelial cells there is higher sLe^x-mediated adhesion probability and **(C)** membrane tethering force (analyzed by paired *t*-test). Immune cell adhesion can be directly assessed using a leukocyte adhesion assay. In this assay, unstimulated leukocytes (white blood cells) isolated from the spleens of *APOE3*-targeted replacement mice are spiked into the media of brain endothelial cell cultures. After 60 minutes, loosely adherent and unbound cells are removed and the remaining, firmly adherent cells are quantified by immunocytochemistry (CD45) (Lutz et al., 2017). **(D)** Consistent with AFM data, there are more CD45$^+$ leukocytes adhered to *APOE4* brain endothelial cells. Data is expressed as mean ± S.E.M. *$p < 0.05$ by Student's *t*-test with $n = 6$.

above the limit of detection, 6 of which were higher with *APOE4*: MCP-1/CCL2 (∼71%), KC/CXCL1 (∼81%), G-CSF (∼112%), MIP-2/CXCL2 (∼77%), IP-10/CXCL10 (∼236%), and RANTES/CCL5 (∼135%) (**Figure 8A**). One consequence of higher inflammation is the attachment of immune cells to brain endothelial cells, which is initiated by selectin-mediated capture. Selectin-mediated binding was higher with *APOE4* when assessed by atomic force microscopy using cantilevers coated/biofunctionalized with the selectin ligand sialylic-Lewis^x (sLe^x) (Askarova et al., 2013) ($p = 0.02$, **Figures 8B,C**). Further, ∼68% more exogenously added CD45$^+$ leukocytes adhered to *APOE4* brain endothelial cells ($p = 0.013$, **Figure 8D**) (Lutz et al., 2017). Our data demonstrate that *APOE4* brain endothelial cells have a higher basal inflammatory state characterized by higher chemokine/cytokine levels and immune cell adhesion.

APOE Modulates Inflammation After LPS Treatment, Characterized by Lower TEER, Higher Chemokine Levels and Immune Cell Adhesion With *APOE4*

APOE genotype-specific differences in inflammatory markers are particularly prominent after stimulation with an inflammatory agent (Tai et al., 2015). LPS, a bacterial endotoxin and toll-like receptor 4 agonist, is often utilized to induce inflammation *in vitro*, *in vivo* and even in human studies focused on *APOE* genotype. Therefore, we evaluated whether *APOE* modulated LPS-induced inflammation.

LPS has been reported to induce barrier disruption to brain endothelial cells, which may be modulated by *APOE* genotype. Therefore, we spiked LPS into the media at concentrations previously reported to modulate brain endothelial cell function, 100 ng/ml – 100 µg/ml (Nagyoszi et al., 2010; Kacimi et al., 2011; Li et al., 2012; Zhao et al., 2014; Qin et al., 2015; Serizawa et al., 2015), and measured TEER over the course of 24 h at 1000 Hz (**Figures 9A,B**). For both *APOE* genotypes LPS lowered TEER in an initial phase (0-6 h) followed by a plateau or a recovery (6-12 h) and, finally, a second phase of TEER decline (12-24 h). LPS induced dose-dependent effects on TEER at 12–24 h. For example, TEER values were ∼10, ∼20, and ∼30% lower compared to vehicle with 1, 10 and 1000 µg/ml LPS at 24 h for *APOE4* brain endothelial cells. Importantly, the higher TEER values we found with *APOE3* compared to *APOE4* at baseline (**Figure 1E**) were sustained with LPS treatment (**Figure 9B**). Indeed, when plotted as the log of the LPS dose response curves at 24 h, there is an apparent leftward curve shift with *APOE4*

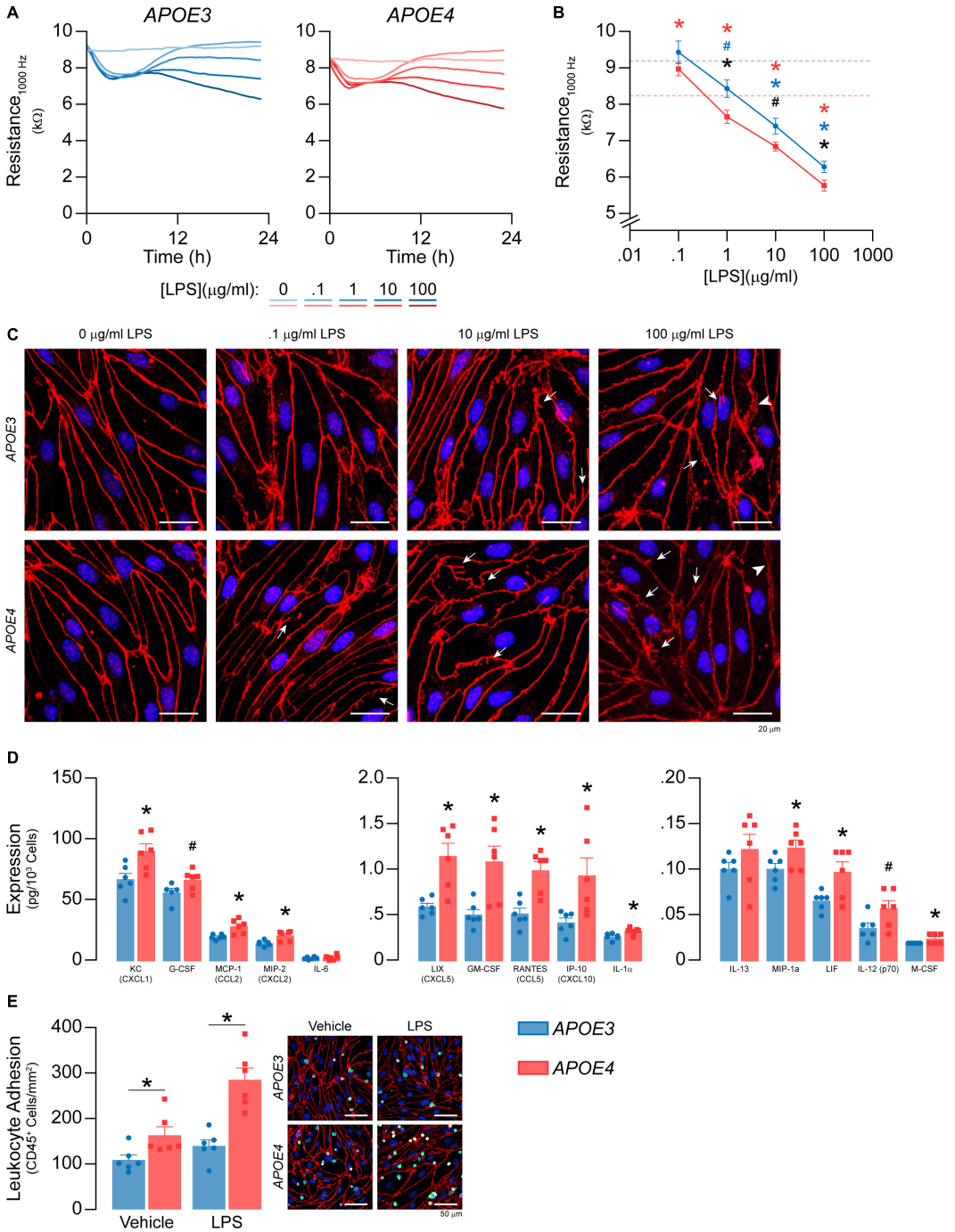

FIGURE 9 | *APOE* modulates inflammation after LPS treatment. **(A,B)** LPS was spiked into the media to a final concentration of 100 ng/ml – 100 μg/ml and TEER was measured over the course of 24 h at 1000Hz. LPS lowers TEER at 1, 10, and 1000 μg/ml in both *APOE3* and *APOE4* brain endothelial cells and at these doses, TEER values are higher with *APOE3* compared to *APOE4* (2-way ANOVA followed by Student's *t*-test; *APOE3* $n = 5$, *APOE4*, $n = 6$). Dashed line represents vehicle control for each genotype (i.e., blue is *APOE3* and red is *APOE4*). **(C)** Qualitatively, high doses of LPS also modulate the brain endothelial cell shape, with morphological changes that include abnormal tight junction protein protrusions (white arrows) and separation of tight junctions from neighboring cells (arrow heads). **(D)** After 24 h of treatment with 100 ng/ml LPS, there are higher levels of KC/CXCL1, G-CSF, MCP-1/CCL2, MIP-2/CXCL2, LIX/CXCL5, GM-CSF, RANTES/CCL5, IP-10/CXCL10, IL-1a, MIP-1a, LIF, IL-12 (p70), and M-CSF. **(E)** To determine whether *APOE* modulated immune cell adhesion after LPS treatment (100 ng/ml, 24 h), we modified the leukocyte adhesion assay. Leukocytes were added with a full media change, rather than spiking, to avoid activation by LPS. Therefore, any *APOE* genotype differences in chemokine and cytokine levels that typically influence immune cell adhesion would be negated during the assay. Nonetheless, there are higher numbers of $CD45^+$ leukocytes adhered to *APOE4* brain endothelial cells both with (~49%) and without (~104%) LPS treatment. Data is expressed as mean ± S.E.M. $^*p < 0.05$ by two-tailed Student's *t*-test and $^\#p < 0.05$ by one-tailed Student's *t*-test; black is *APOE4* vs. *APOE3*, blue is *APOE3* + LPS vs. *APOE3* Vehicle, red is *APOE4* + LPS vs. *APOE4* Vehicle. $n = 6$ (unless otherwise specified above).

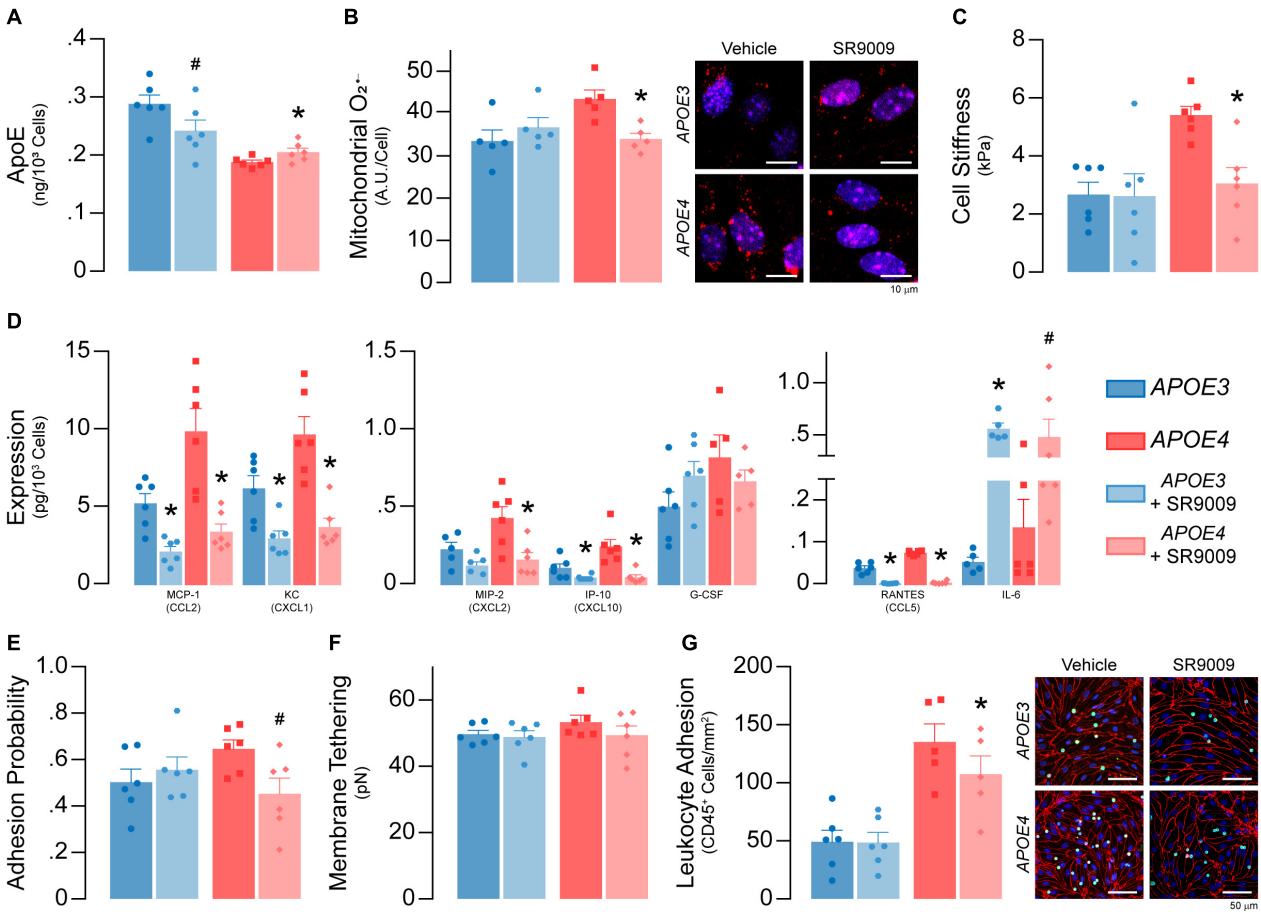

FIGURE 10 | SR9009 treatment impacts metabolism and inflammation in *APOE4* brain endothelial cells. At confluence, brain endothelial cells were treated with 5 µM SR9009 for the 48 h leading up to each assay. **(A)** SR9009 treatment results in lower apoE levels (~16%) with *APOE3* and higher levels (~9%) with *APOE4* when assessed by ELISA. **(B)** SR9009-treated *APOE4*, but not *APOE3*, brain endothelial cells have lower mitochondrial superoxide levels compared to the vehicle ($n = 5$). **(C)** Cell stiffness is lower in SR9009-treated *APOE4*, but not in *APOE3*, brain endothelial cells (analyzed by paired *t*-test). **(D)** MCP-1/CCL2, KC/CXCL1, IP-10/CXCL10 and RANTES/CCL5 levels are lower with SR9009 in both *APOE3* and *APOE4* brain endothelial cells when assessed by multiplex ELISA. SR9009 lowers MIP-2 levels in *APOE4* brain endothelial cells and increases IL-6 levels for both *APOE3* and *APOE4* endothelial cells. **(E)** The adhesion probability is lower in *APOE4* brain endothelial cells with SR9009 treatment but **(F)** membrane tethering force is unaffected (analyzed by paired *t*-test). **(G)** Leukocyte adhesion is lower with *APOE4* following treatment with SR9009 (*APOE3* $n = 6$, *APOE4* $n = 5$). Data is expressed as mean ± S.E.M. $^{*}p < 0.05$ by two-tailed Student's *t*-test and $^{\#}p < 0.05$ by one-tailed Student's *t*-test compared to vehicle control with $n = 6$ (unless otherwise specified above).

compared to *APOE3*. High doses of LPS also modulated brain endothelial cell shape, with morphological changes that included abnormal tight junction protein protrusions and separation of tight junctions from neighboring brain endothelial cells (**Figure 9C**). Interestingly, apoE levels were lower in brain endothelial cell cultures treated with 10 and 100 µg/ml LPS for both genotypes (**Supplementary Figure 4A**).

We next evaluated whether *APOE* modulated chemokine and cytokine levels after treatment with 100 ng/ml LPS (24 h). This dose and time-point were selected to avoid the *in vitro* equivalent of pathological changes in paracellular permeability and so that we could also evaluate immune cell adhesion. After LPS treatment, fifteen chemokines and cytokines were above the limit of detection (**Figure 9D**), 13 of which were higher with *APOE4* by ~ 20-30% (G-CSF, IL-1α, MIP-1α/CCL3, M-CSF/CSF1, KC/CXCL1), 40-50% (MCP1/CCL2, MIP-2/CXCL2, IL-12p70, LIF), and 100% (LIX/CXCL5, GM-CSF, RANTES/CCL5, IP-10/CXCL10). There were also more adhered $CD45^{+}$ leukocytes in *APOE4* brain endothelial cells after LPS treatment ($p = 0.0278$, **Figure 9E**). These data demonstrate that, as for basal inflammation, after LPS treatment (100 ng/ml) there are higher chemokine/cytokine levels and immune cell adhesion with *APOE4*.

Rev-Erb Is a Potential Pathway Modulated by *APOE* Genotype

Our final goal was to use pharmacological probes to identify pathways that could contribute to the *APOE4* brain endothelial phenotype. Through the evaluation of our RNA-sequencing data, we identified several signaling-related transcriptomic profiles as candidates, of which the nuclear receptor family

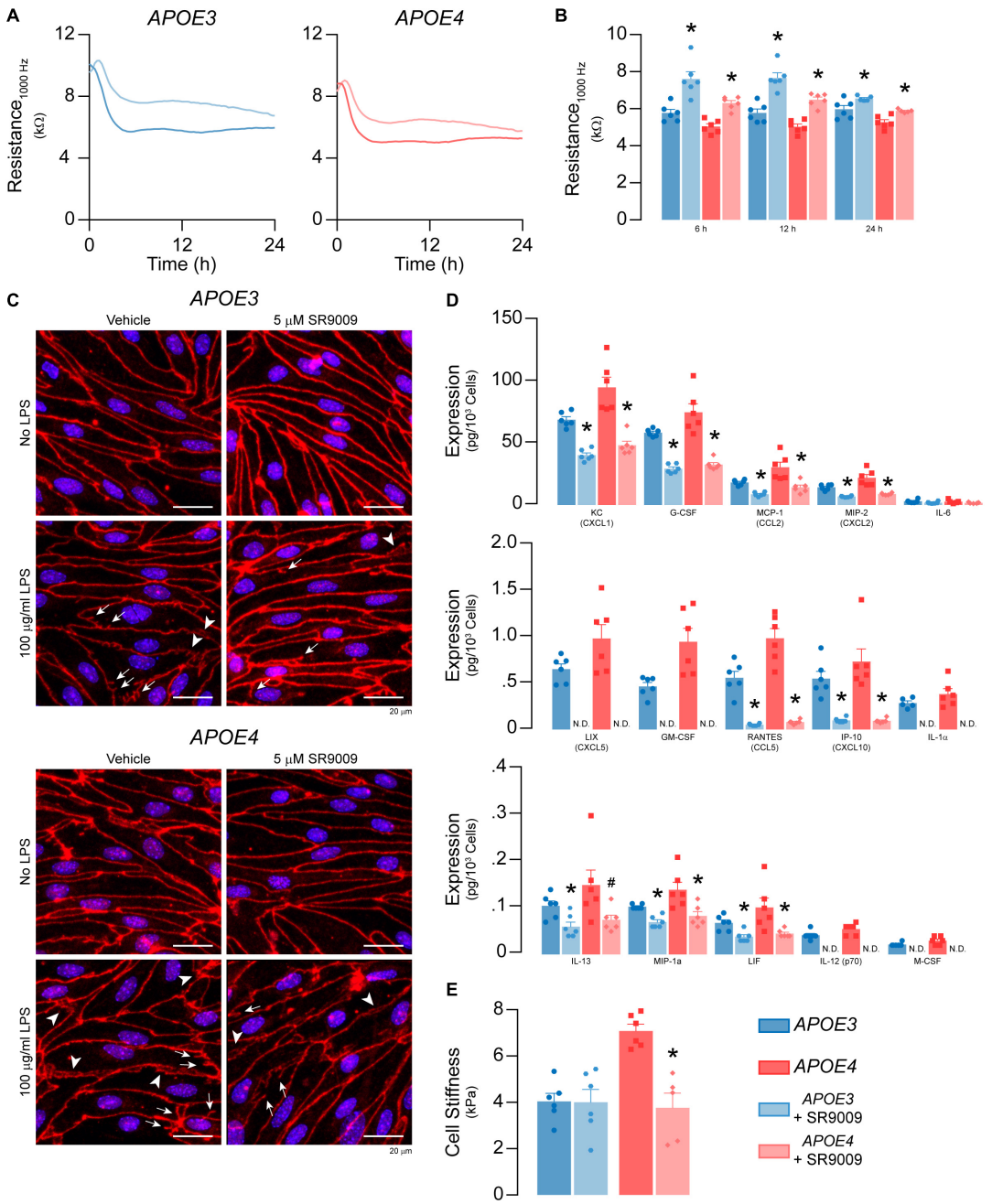

FIGURE 11 | SR9009 mitigates the effects of LPS on metabolism and inflammation in *APOE4* brain endothelial cells. Confluent cells were treated with 5 μM SR9009 for 48 h and LPS was spiked into the media 24 h prior to assays. SR9009 treatment mitigated 100 μg/ml LPS-induced disruption of **(A,B)** paracellular permeability (*APOE3* vehicle $n = 5$, *APOE4* SR9009 $n = 5$, all other groups $n = 6$) and **(C)** changes in tight junction morphology. In cells treated with 100 ng/ml LPS, SR9009 treatment results in **(D)** lower levels of chemokines and cytokines for both *APOE3* and *APOE4* (multiplex ELISA) and **(E)** lower cell stiffness (analyzed by paired *t*-test, $n = 5$ for *APOE4* SR9009, all others $n = 6$), Data is expressed as mean ± S.E.M. $^{*}p < 0.05$ by two-tailed Student's *t*-test and $^{\#}p < 0.05$ by one-tailed Student's *t*-test compared to vehicle control with $n = 6$ (unless otherwise specified above).

was particularly prominent (**Supplementary Table 2**). There are different subclasses of nuclear receptors that hetero- and homodimerize, and agonists of RXR, LXR and PPAR nuclear receptor families can mitigate some dysfunctional changes found with *APOE4* (Koster et al., 2017; Moutinho et al., 2019). In *APOE4* brain endothelial cells there were lower levels of genes for RAR (e.g., Rarg, Rara) and LXR (Nr1h2) nuclear receptors, as well as nuclear receptor coactivators (e.g., Ncoa2, Ncoa7) and transcripts associated with general nuclear receptor activation (e.g., Klf15 Klf2, Klf4). Although these and other signal molecules

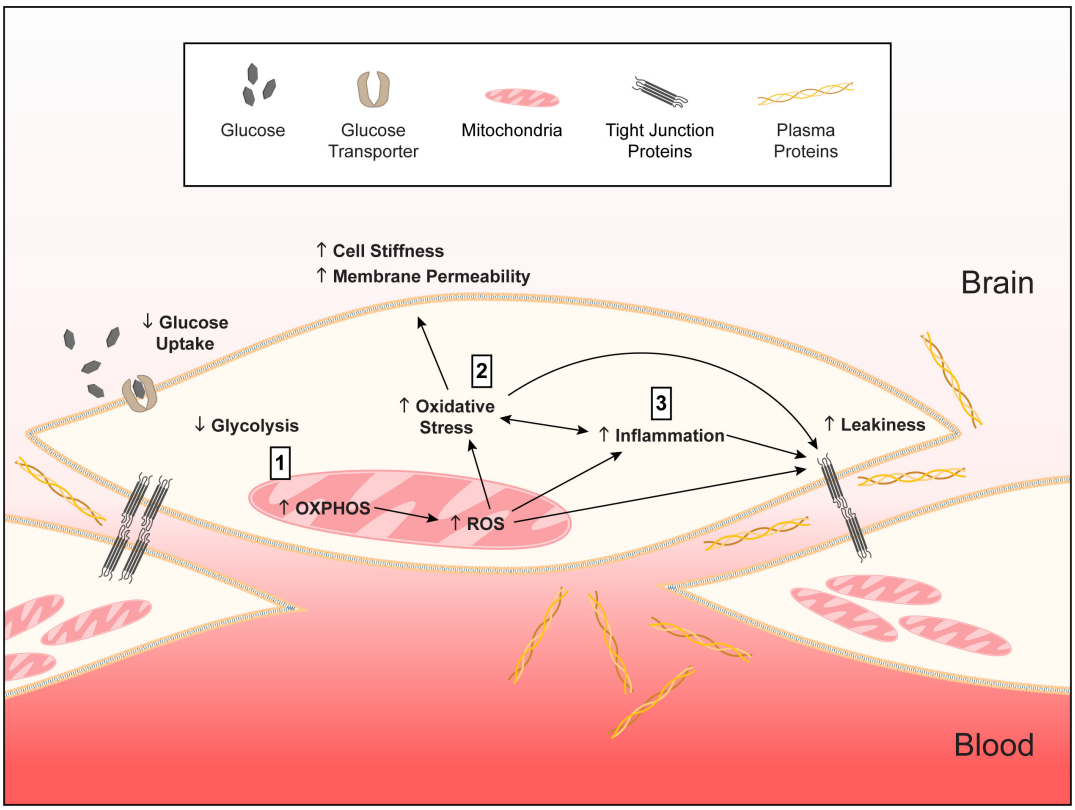

FIGURE 12 | Working model of *APOE*-modulated brain endothelial cell function. ↓ = lower and ↑ = higher for *APOE4* compared to *APOE3*. We propose that **(1)** *APOE4* brain endothelial cells have higher preference for oxidative phosphorylation (OXPHOS) compared to glycolysis. Lower glycolysis is evident in ATP rate assays, lower rate of glucose uptake and higher lactate production. Due to OXPHOS there is higher peroxisome and mitochondrial activity indicated by higher hydrogen peroxide degradation, mitochondrial membrane potential, citrate synthase activity and levels of electron transport chain complexes. Higher mitochondrial activity leads to the generation of reactive oxygen species, particularly superoxide, and lower levels of antioxidant systems, including heme and glutathione. **(2)** Higher reactive oxygen species lead to more oxidative stress to proteins (e.g., carbonylation) that must be cleared by proteasome activity. Oxidative stress to lipids (peroxidation, production of TBARS) alters membrane structures (lower phospholipids, higher ceramides) that manifests as higher cell stiffness and plasma membrane permeability. **(3)** Either in parallel or due to higher mitochondrial activity, inflammation pathways are activated with *APOE4*. *APOE4*-assocaited inflammation is characterized by chemokine production, immune cell adhesion and higher sensitivity of innate receptors to activation (e.g., TLR4). The combination of all these changes leads to higher basal transcellular permeability with *APOE4*. Therefore, autocrine signaling of apoE in brain endothelial cells represents a novel cellular mechanism for how *APOE* regulates neurovascular function. We further propose that this pathway is not necessarily detrimental in basal conditions for *APOE4* and may even be beneficial for responding to infections and other stressors earlier in life. However, chronic changes in metabolic, mitochondrial, or inflammatory pathways in neurodegenerative conditions could lead to brain endothelial cell dysfunction.

warrant follow-up in future studies, we focused on the nuclear receptor Rev-Erb due to several considerations. The first was that transcript levels of both forms of the receptor (Rev-Erbα and β) were lower in *APOE4* brain endothelial cells and to greater extent than other nuclear receptors (**Supplementary Table 2**). Second is that molecules and transcripts related to Rev-Erb were also lower in *APOE4* brain endothelial cells. Although considered an orphan receptor, heme is an agonist of Rev-Erb which was lower in *APOE4* brain endothelial cells (**Figure 5H**) and Rev-Erb also regulates circadian genes, some of which were also lower (Per1, Per2, Per3, **Supplementary Table 2**) with *APOE4*. In addition, Rev-Erb is functionally linked to metabolism and inflammation. We, therefore, determined the effect of SR9009 (Stenabolic), a widely used Rev-Erb agonist (Solt et al., 2012), on select assays that were affected by *APOE4* both under basal conditions (**Figure 10**) and following stimulation with LPS (**Figure 11**).

SR9009 Treatment Impacts Metabolism and Inflammation Under Basal Conditions in *APOE4* Brain Endothelial Cells

We determined whether SR9009 influenced apoE levels, metabolism (mitochondrial O_2^-, cell stiffness) and inflammation (chemokine levels and leukocyte adhesion) under basal conditions. SR9009 treatment (5 μM, 48 h) resulted in lower apoE levels (∼16%) with *APOE3* and higher levels (∼9%) with *APOE4* when assessed by ELISA (**Figure 10A**). For metabolism, compared to vehicle, SR9009 treatment resulted in ∼18% lower mitochondrial O_2^- levels (p = 0.0056, **Figure 10B**) and ∼44% lower cell stiffness compared to vehicle in *APOE4* brain endothelial cells (p = 0.01, **Figure 10C**). For inflammation, in both *APOE* genotypes SR9009 reduced chemokine levels by ∼50-80% (MCP-1/CCL2, KC/CXCL1, MIP-2/CXCL2, IP-10/CXCL10, RANTES/CCL5) compared to vehicle controls (**Figure 10D**).

By contrast IL-6 levels were markedly increased by SR9009 (∼910% higher in *APOE3*, ∼259% higher in *APOE4*). SR9009 also lowered selectin-mediated binding (∼18%; **Figure 10E**) and leukocyte adhesion (21%, $p = 0.0002$, **Figure 10G**) in *APOE4* brain endothelial cells. Thus, SR9009 treatment lowers mitochondrial O_2^-, cell stiffness and inflammation in *APOE4* brain endothelial cells in basal conditions.

SR9009 Modulates LPS-Induced Effects on TEER and Inflammation in *APOE4* Brain Endothelial Cells

We evaluated whether SR9009 mitigated LPS-induced effects on TEER, cytokine/chemokine levels, cell stiffness, selectin binding and leukocyte adhesion. The extent of LPS-induced TEER disruption was lower with SR9009 treatment (100 μg/ml LPS, 24 h, **Figures 11A,B**). For both *APOE3* and *APOE4* brain endothelial cells, TEER was higher with SR9009 treatment at 6 h (∼32% higher *APOE3,* $p = 0.0016$, and ∼25% *APOE4,* $p < 0.0001$), 12 h (∼33% higher *APOE3* $p = 0.0003$ and ∼29% *APOE4,* $p < 0.0001$), and 24 h (∼10% higher *APOE3,* $p < 0.033$ and ∼12% *APOE4,* $p = 0.0037$) compared to vehicle. Consistent with these findings, there were, qualitatively, fewer tight junction protrusions and breaks with SR9009 treatment (**Figure 11C**). The beneficial effects of SR9009 on TEER occurred without preventing the LPS-induced (100 μg/ml) lowering of apoE levels in brain endothelial cells. In fact, SR9009 resulted in ∼15% lower apoE levels compared to vehicle after LPS treatment (100 μg/ml) for both *APOE3* and *APOE4* (**Supplementary Figure 4B**). SR9009 treatment also resulted in lower chemokine/cytokine levels after 24 h incubation with 100 ng/ml LPS for both *APOE3* and *APOE4* brain endothelial cells ranging from ∼40-50% (KC/CXCL1, G-CSF, MCP-1/CCL2, MIP-2/CXCL2, IL-13, LIF) to 80-100% (LIX/CXCL5, RANTES/CCL5, IP-10/CXCL10, IL-1α, IL-12p70, M-CSF/CSF1) (**Figure 11D**). However, in contrast to baseline conditions, SR9009 had no effect on selectin-mediated adhesion, and increased leukocyte adhesion in *APOE4* brain endothelial cells (∼38%, **Supplementary Figures 4C-E**) after LPS treatment (100 ng/ml). SR9009 treatment also resulted in lower cell stiffness in *APOE4* brain endothelial cells (∼47%, 24 h after 100 ng/ml LPS, $p < 0.018$ **Figure 11E**). Thus, SR9009 partially mitigates high-dose LPS-induced disruption of TEER and lowers chemokine levels and cell stiffness with a low-dose of LPS in *APOE4* brain endothelial cells.

DISCUSSION

In this manuscript, we identified that *APOE4* is associated with an altered brain endothelial cell phenotype characterized by differences in apoE levels, metabolism, and inflammation compared to *APOE3*. Further research could provide insight into the contribution of this phenotype to neurovascular dysfunction in aging and neurodegenerative disorders.

Brain Endothelial Cell *APOE* Genotype and Neurovascular Function

Reports of *APOE4*-associated neurovascular dysfunction in neurodegenerative disorders (reviewed in Zlokovic, 2013; Tai et al., 2016) has led to ongoing research to identify underlying mechanisms. ApoE has been found to impact brain endothelial cells indirectly and directly. Indirectly, *APOE* modifies peripheral inflammation, neuroinflammation, metabolites, and disease-specific proteins (e.g., Aβ), all of which can affect brain endothelial cell function (Bell et al., 2012; Tai et al., 2016; Yamazaki et al., 2020a). ApoE produced by pericytes (Yamazaki et al., 2020b) and astrocytes (Nishitsuji et al., 2011) can directly signal to brain endothelial cells *in vitro* and our data supports autocrine effects of apoE in brain endothelial cells. The complex regulation of brain endothelial cell function by apoE provides an opportunity for context-dependent integration. For example, signals from different cell types, over a range of distances from the brain and periphery, in response to homeostatic and stress/pathological conditions can all be transmitted to brain endothelial cells for the control of neurovascular function. Within this framework, apoE production by brain endothelial cells may be important for local signaling. Brain endothelial cells receive signaling inputs from both the interstitial fluid and blood, and therefore an added advantage of autocrine apoE production is the ability to self-regulate, rather than rely on apoE produced by other cell types.

Integrated Working Model of the *APOE* Modulated Brain Endothelial Cell Phenotype

Based on our data and findings in other cell types (Mahley et al., 2007; Liu et al., 2013; Dose et al., 2016; Fernandez et al., 2019; Butterfield and Mattson, 2020; Flowers and Rebeck, 2020; Johnson, 2020), we present a working model of how *APOE* genotype could modulate the basal phenotypic state of brain endothelial cells (**Figure 12**). In this model, we propose that, compared to *APOE3*, *APOE4* brain endothelial cells have higher preference for oxidative phosphorylation over glycolysis, which results in higher mitochondrial activity and generation of mitochondrial reactive oxygen species and lower levels of antioxidants (heme/bilirubin and glutathione). Higher levels of reactive oxygen species with *APOE4* produce oxidative stress to proteins that must be cleared by proteasome activity, and lipids, which results in greater cell stiffness and plasma membrane permeability. In tandem, or due to higher mitochondrial activity, there is higher inflammation with *APOE4* characterized by chemokine production, immune cell adhesion and higher sensitivity of innate receptors to activation (e.g., TLR4). The combination of all these changes leads to higher basal transcellular permeability with *APOE4*.

The *APOE4*-associated brain endothelial cell phenotype is not necessarily detrimental in basal conditions. Indeed, *APOE4* carriers do not have developmental neurovascular malformations, or overt vascular dysfunction early in life. One could even argue that compared to *APOE3*, *APOE4* brain endothelial cells would respond to infections and other stressors earlier in life in a more beneficial manner. However, chronic changes in neurodegenerative conditions could lead to brain endothelial cell dysfunction. For example, risk factors for dementia are associated with metabolic changes, oxidative stress,

peripheral inflammation and neuroinflammation, all of which could exacerbate the *APOE4*-associated brain endothelial cell phenotype as found in other endothelial cell contexts (Sena et al., 2018; Urbano et al., 2019).

Signaling Pathways and *APOE*-Modulated Brain Endothelial Cell Function

There are several potential mechanisms of how *APOE* modulates the brain endothelial cell phenotype. Overall, answers to the question of how a single amino acid difference between apoE3 (cysteine at 112) and apoE4 (arginine at 112) results in functional changes are proving complex, at times enigmatic, and continue to be the focus of several research groups (reviewed in Mahley et al., 2007; Liu et al., 2013; Flowers and Rebeck, 2020). One aspect of this question is whether there are differences in structural properties of apoE. ApoE is post-translationally lipidated prior to secretion, and one suggestion is that apoE4-containing lipoproteins are less lipidated than apoE3-containing lipoproteins, resulting in lower stability and levels. In our study, we found that transcript levels of *APOE4* were lower than *APOE3* (-0.56 log-fold change), as were apoE4 levels. Thus, one explanation for these data is that in brain endothelial cells, the lower stability of apoE4 results in lower levels that in turn further results in a cascade that suppresses the transcription of *APOE*. In tandem, there may also be proteasomal degradation of apoE4, further contributing to modulation of apoE levels. The isoform differences in structure and lipidation are thought to influence a range of fundamental processes even if apoE levels were equivalent. There are many proposed consequences of the lower levels and structural differences of apoE4 including lower ability to maintain cholesterol and lipid homeostasis, less binding to oxidative stress-related products and other substrates, disruption in adaptor molecule function, modulation of intracellular metabolism and organelle dynamics, altered activation and recycling of the apoE receptors and a profound influence on intracellular signaling cascades. It remains plausible that any of these are proximally linked to *APOE*-associated brain endothelial cell phenotypic differences.

Intertwined with the structural and functional differences between the apoE isoforms is cellular signaling. As indicated in our transcriptomics data, several signaling cascades were differentially modulated by *APOE* (**Supplementary File 2**), many which are related to metabolism and inflammation, providing a link with our proposed working model (**Figure 12**). Given that multiple aspects of cellular biology were modulated by *APOE4* genotype, there is high likelihood of a complex interaction among the different signaling pathways; it is unlikely that a single signaling pathway is responsible for all the *APOE*-modulated functional differences. However, in general, there is increasing evidence of a connection between *APOE* genotype and nuclear receptors (reviewed in Koster et al., 2017; Moutinho et al., 2019). For example, agonists for PPAR, LXR and RXR have been shown to modulate either levels and lipidation of apoE and/or *APOE*-modulated inflammation, metabolism, neuronal function, and behavior. The precise mechanistic connection between *APOE* and nuclear receptors is unclear but may be related to the *APOE*-associated phenotype of a cell. For example, higher inflammation and fatty acid oxidation with *APOE4* would be associated with a corresponding set of signaling pathways that could include lower activation of nuclear receptors that suppress these functions.

Our data extends the link between *APOE* and nuclear receptors to Rev-Erb since, with *APOE4*, there were lower levels of Rev-Erbα and β transcripts, heme (Rev-Erb agonist) and circadian genes (Per1, Per2, Per3). Furthermore, SR9009 (Rev-Erb agonist) treatment resulted in improved metabolic and inflammatory phenotypes. To date, no direct link between *APOE* genotype and Rev-Erb has been reported, however there is overlap with the reported functions of Rev-Erb and the metabolic and inflammatory aspects of the *APOE4* brain endothelial phenotype (Raspe et al., 2002; Duez and Staels, 2008; Duez et al., 2008; Le Martelot et al., 2009; Yin et al., 2010; Bugge et al., 2012; Cho et al., 2012; Delezie et al., 2012; Gibbs et al., 2012; Woldt et al., 2013; Mayeuf-Louchart et al., 2017; Pariollaud et al., 2018; Wang et al., 2018; Reitz et al., 2019; Cunningham et al., 2020; Wang et al., 2020). In addition beneficial effects have been reported for SR9009 in a variety of *in vivo* models of disease with metabolic (Raspe et al., 2002; Le Martelot et al., 2009; Cho et al., 2012) and inflammatory components (Delezie et al., 2012). However, specifically for dementia, data are conflicted on whether SR9009 is beneficial or detrimental in Alzheimer's disease-relevant models. On the one hand, the loss of Rev-Erb results in a mania-like phenotype and impaired performance in memory tasks (Mayeuf-Louchart et al., 2017) and, in a model of aging, SR9009 treatment improved and reversed behavioral deficits (Woldt et al., 2013). On the other hand, inhibition of Rev-Erb results in higher synaptic markers in an amyloidosis model (Bugge et al., 2012). In addition, SR9009 can act independently of Rev-erb (Duez et al., 2008), but the mechanisms have not yet been identified. Our own data, albeit *in vitro*, would suggest that, in the context of *APOE4*, agonists of Rev-Erb would result in a beneficial phenotype, particularly for the cerebrovasculature, through modulating metabolism and/or inflammation.

Limitations and Future Directions

Our data provide important phenotypic information on the role of *APOE* genotype in brain endothelial cell function, however, identification of the underlying mechanistic pathways is important. In addition, the use of double and triple cultures of brain endothelial cells, astrocytes and pericytes could enable functional comparisons of the different sources of apoE. Although when evaluated by western blot analysis our brain endothelial cell cultures are GFAP- and desmin-negative, we recognize that a limitation of primary cell isolation, regardless of the cell type, is the presence of non-target cells and it is rare that any protocol produces completely pure cultures.

From a broader perspective, identifying the extent that brain endothelial cell *APOE* genotype impacts vascular function *in vivo*, as well as interactions with Rev-Erb signaling is important. We and others have demonstrated that markers of cerebrovascular leakiness are higher with *APOE4 in vivo* (Salloway et al., 2002; Zipser et al., 2007; Poels et al., 2010; Halliday et al., 2013; Zlokovic, 2013; Halliday et al., 2015; Tai et al., 2016; Thomas et al., 2016;

Marottoli et al., 2017; Tai et al., 2017; Thomas et al., 2017), however the extent that metabolism and inflammatory markers are affected is unknown and are the focus of our ongoing studies. In terms of Rev-Erb, it is interesting to note that under basal conditions we found that SR9009 slightly increased apoE4 levels, which could have contributed to the modulation of select metabolic and inflammatory read-outs. Thus, Rev-ErB may alter levels of genes or proteins involved in apoE metabolism, as found for other nuclear receptor agonists. Alternatively, SR9009 altered cellular metabolism, in turn upregulating genes related to apoE metabolism. We focused on *APOE* functional effects and, therefore, conducting a more detailed study with SR9009 in *APOE4* brain endothelial cells *in vitro* could reveal the underlying mechanism(s) of action. Indeed, a limitation of the current study is a lack of full pharmacological characterization of SR9009, including dose-response evaluation and testing activity in Rev-Erb knock out cells. In addition, and the focus of our ongoing studies, evaluation of SR9009 activity *in vivo* would aid in understanding potential clinical relevance of this class of drugs for *APOE4* associated neurodegeneration. Although we propose that *APOE4* brain endothelial cells are more prone to stress-induced degeneration, beyond select read-outs with LPS, we did not fully explore this concept, which is the focus on our ongoing studies. For example, phenotypic changes we found with *APOE4* in cells from younger mice may be more pronounced in cells isolated from older mice.

The *APOE4* associated brain endothelial cell phenotype may contribute to overall cerebrovascular and neuronal dysfunction in neurodegenerative disorders including dementia. However, it is critical to conduct further research to fully explore this concept. One important aspect is identification of how brain endothelial cell dysfunction impacts neuronal function, and there are multiple potential pathways including altered homeostasis of nutrients, neurotransmitters, metabolites and ions in the interstitial fluid, inflammation, influx of plasma proteins and other peripheral molecules into the brain, as well as modulation of disease specific elements such as clearance of amyloid-β. Related, is evaluation of whether some neuronal populations are more sensitive to the effects of cerebrovascular dysfunction, either due to fundamental differences in neuronal biology or greater disruption of neuronal signaling in specific neurodegenerative conditions. In addition, neurodegenerative disorders involve altered function of multiple cell types including peripheral cells, pericytes, vascular smooth muscle cells, glia as well as neurons. Therefore, full evaluation of how our identified endothelial cell-specific phenotype impacts the function of these other cell types is important, as is the incorporation of *APOE2* genotype, which is protective for neurodegeneration. These types of questions are the focus of our future studies and will be enabled by the development of mouse models to conditionally knock-down *APOE2*, *APOE3* or *APOE4* in endothelial cells.

CONCLUSION

The goal of this manuscript was to test the hypothesis that brain endothelial cells produce apoE to modulate their own phenotype. Our *in vitro* data support this hypothesis and therefore autocrine signaling of apoE in brain endothelial cells represents a novel cellular mechanism for how *APOE* regulates neurovascular function. In basal conditions, *APOE4* brain endothelial cells had altered metabolism consistent with greater oxidative phosphorylation and higher inflammation. The basal differences may predispose *APOE4* brain endothelial cells to dysfunction during aging and in different neurodegenerative disorders, especially as *APOE4* is linked to cognitive decline in aging and Alzheimer's disease, and to poorer outcomes after stroke and brain trauma. Therefore, the autocrine effects of apoE4 on metabolism and inflammation in brain endothelial cells could provide the framework for understanding mechanisms of neurovascular dysfunction in neurodegeneration, and open avenues for the development of therapeutics that target brain endothelial cells.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Gene Expression Omnibus (GSE160483).

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LT and FM conceived the study, performed the experiments, and wrote the manuscript with SL and JL. TT and SL conducted the leukocyte adhesion assays. XG and JL conducted the AFM experiments. ZA, PK, and MM-C conducted the transcriptomics analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.668296/full#supplementary-material

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Notch1 and Galectin-3 Modulate Cortical Reactive Astrocyte Response After Brain Injury

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After a brain lesion, highly specialized cortical astrocytes react, supporting the closure or replacement of the damaged tissue, but fail to regulate neural plasticity. Growing evidence indicates that repair response leads astrocytes to reprogram, acquiring a partially restricted regenerative phenotype *in vivo* and neural stem cells (NSC) hallmarks *in vitro*. However, the molecular factors involved in astrocyte reactivity, the reparative response, and their relation to adult neurogenesis are poorly understood and remain an area of intense investigation in regenerative medicine. In this context, we addressed the role of Notch1 signaling and the effect of Galectin-3 (Gal3) as underlying molecular candidates involved in cortical astrocyte response to injury. Notch signaling is part of a specific neurogenic microenvironment that maintains NSC and neural progenitors, and Gal3 has a preferential spatial distribution across the cortex and has a central role in the proliferative capacity of reactive astrocytes. We report that *in vitro* scratch-reactivated cortical astrocytes from C57Bl/6J neonatal mice present nuclear Notch1 intracellular domain (NICD1), indicating Notch1 activation. Colocalization analysis revealed a subpopulation of reactive astrocytes at the lesion border with colocalized NICD1/Jagged1 complexes compared with astrocytes located far from the border. Moreover, we found that Gal3 increased intracellularly, in contrast to its extracellular localization in non-reactive astrocytes, and NICD1/Gal3 pattern distribution shifted from diffuse to vesicular upon astrocyte reactivation. *In vitro,* Gal3$^{-/-}$ reactive astrocytes showed abolished Notch1 signaling at the lesion core. *Notch1* receptor, its ligands (*Jagged1* and *Delta-like1*), and *Hes5* target gene were upregulated in C57Bl/6J reactive astrocytes, but not in Gal3$^{-/-}$ reactive astrocytes. Finally, we report that Gal3$^{-/-}$ mice submitted to a traumatic brain injury model in the somatosensory cortex presented a disrupted response characterized by the reduced number of GFAP reactive astrocytes, with smaller cell body perimeter and decreased NICD1 presence at the lesion core. These results suggest that Gal3 might be essential to the proper activation of Notch signaling, facilitating the cleavage of Notch1 and nuclear translocation of NICD1 into the nucleus of reactive cortical astrocytes. Additionally, we hypothesize that reactive astrocyte response could be dependent on Notch1/Jagged1-Hes5 signaling activation following brain injury.

Keywords: neurogenic program, astrocyte reactivation, traumatic brain injury, dedifferentiation, neurogenic signaling pathway, NICD, Jagged, Hes5

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INTRODUCTION

Cortical astrocytes are highly specialized glial cells that actively participate in brain functions' homeostasis (Verkhratsky et al., 2017). Injuries to the central nervous system (CNS) challenge astrocytes to resume tissue homeostasis by activating specific cell programs, characterizing a reactive cell-state. There are multiple functional reactive astrocyte profiles, varying depending on lesion type and severity (Verkhratsky et al., 2012; Verkhratsky and Parpura, 2016; Escartin et al., 2021). In the context of brain injury, reactive astrocytes perform a protective role. In the acute phase after brain damage, the reparative response is exclusively neuroprotective and might become both positive and negative in a chronic advanced phase. Neuroprotection includes regulating neural plasticity, facilitating the generation of neurons, axonal sprouting, and controlling the number and function of synapses (Pekny et al., 2019). *In vivo* and *in vitro* studies had shown this reparative response. Reactive astrocytes isolated from injured brains gave rise to neurospheres *in vitro* (Buffo et al., 2008; Shimada et al., 2012; Sirko et al., 2015). *In vivo*, cortical astrocytes reproduced a neurogenic response in transgenic mice with Rbpj-κ-depleted astrocytes submitted to traumatic brain injury (Zamboni et al., 2020) and reactive astrocytes activated a neurogenic program after stroke in Notch-depleted striatal astrocytes transgenic (Magnusson et al., 2020; Santopolo et al., 2020).

Thus, these complex molecular and structural changes could be targeted to promote neuroregeneration. The most recent consensus report on reactive astrocytes highlights the importance of clarifying the contribution of astrocyte-associated signaling pathways to the pathogenesis of specific neurological conditions, as well as in the development of astrocyte-guided therapies (Escartin et al., 2021). In this work, we address Notch1 signaling pathway activation and the role of Galectin-3 (Gal3) in reactive astrocytes following a traumatic injury to the brain.

Notch receptor is a transmembrane protein that signals through cell-cell interactions through its ligand Delta-like or Jagged, in mammals, which is also a transmembrane protein on a neighboring cell. Ligand binding promotes two proteolytic cleavage events in the Notch receptor, releasing the Notch intracellular domain (NICD), which then translocates to the nucleus and cooperates with the DNA-binding protein Rbpj and its co-activator Mastermind-like (MAML) to promote target genes transcription (Hes1, Hes5, Hey). The Notch signaling pathway outcome depends on the cellular context and can result in proliferation, differentiation, and apoptosis. Notch is a critical element of cell fate decision during neurodevelopment, specifying radial glial cell identity (Gaiano et al., 2000; Gaiano and Fishell, 2002) and promoting differentiation of astrocytes, in detriment of oligodendrocytes and neurons (Grandbarbe et al., 2003). Notch has been extensively studied in neurogenesis in the adult brain, as it has a pivotal role in maintaining neural stem cell pool in the neurogenic niches (Ables et al., 2010; Engler et al., 2018). Notch is also implicated in neuropathological contexts, including astrocyte proliferative response to brain injury (Shimada et al., 2011; LeComte et al., 2015; Zhong et al., 2018; Santopolo et al., 2020) and regulation of reactive astrocyte morphology and response upon inflammatory stimuli (Acaz-Fonseca et al., 2019).

Gal3 is a lectin that binds to galactose residues in glycoproteins and glycolipids and oligomerizes through its N-terminal domain. Gal3 oligomerization generates a dynamic and complex structure capable of regulating diffusion, compartmentalization, and endocytosis of glycoproteins and membrane glycolipids. Gal3 contributes to cell-cell and cell-matrix interactions when located at the extracellular matrix, but it is also found in the nucleus and cytoplasm. The ubiquitous Gal3 distribution explains its broad influence in cellular functions such as apoptosis, proliferation, migration, angiogenesis, RNA splicing, and surface to nuclear signal transport (Nieminen et al., 2007; Nabi et al., 2015). Gal3 is expressed at different levels throughout the CNS and was shown to have a widespread expression profile in the cerebral cortex (John and Mishra, 2016). Overexpression of Gal3 was correlated with a reactive cell-state and reactive astrocyte ability to re-create neural stem cell properties *in vitro* (Sirko et al., 2015). Furthermore, Gal3 plays a significant role in neuroinflammation (Yip et al., 2017; Srejovic et al., 2020) and cancer stemness maintenance (Newlaczyl and Yu, 2011; Nangia-Makker et al., 2018).

Acknowledging the extraordinary complexity of astrocyte response to traumatic brain injury, we hypothesized that the interaction of Gal3 and Notch1 in reactive astrocytes is critical for the maintenance and proper function of the adult brain. Here we report that Gal3 modulates Notch1 signaling pathway in reactive astrocytes, and we provide new evidence of the participation of Notch1-Hes5 signaling axis activation in reactive astrocytes.

MATERIALS AND METHODS

Animals

The animals were handled following National Institute of Health (NIH) regulations, and all procedures were approved by the Committee on Ethics in the Use of Animals from Universidade Federal de São Paulo (CEUA n. 7740290318; CEUA n. 2451111116). CEDEME/UNIFESP Animal Facility supplied isogenic C57Bl/6J and Gal3 knockout ($Gal3^{-/-}$) mice (**Supplementary Figure 1**) aged 6 and 45 days. Gal3 knockout ($Gal3^{-/-}$) mice were generated by Fu-Tong Liu group (Hsu et al., 2000) and were obtained from Biotério Central, Faculdade de Medicina, USP (Rede PREMIUM[1]). The animals were housed in standard cages, maintained under controlled light-dark cycles (12/12 h; lights on at 7 a.m.) with food and water available *ad libitum*. We made all efforts to minimize suffering and the number of animals used.

Primary Astrocyte Culture and *in vitro* Model of Astrocyte Reactivity

Astrocyte isolation from mice cortices was adapted from Yang et al. (2009). The animals were anesthetized with intraperitoneal injection of 2% xylazine (10 mg/kg, Syntec, Barueri, SP, Brazil) and 10% ketamine hydrochloride (100 mg/kg, Syntec, Barueri,

[1]https://www.premium.fm.usp.br/

SP, Brazil) and euthanized by decapitation. The brain was removed from the skull and the cortices dissected and placed in Hanks/DNAse solution. The tissue was mechanically dissociated and incubated with 0.25% trypsin (Sigma Aldrich Corporation, Saint Louis, EUA) in Versene/DNAse solution for 20 min. Trypsin activity was blocked with fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil), and cells were homogenized and dissociated in Versene/DNAse solution. The cells were suspended in DMEM/F12 medium containing 100 U/mL penicillin/streptomycin (Gibco, Grand Island, EUA), 200 mM L-glutamine (Sigma Aldrich Corporation, Saint Louis, EUA), and 10% FBS, filtered through a 40 μm cell strainer and plated in T25 flasks coated with poly-l-lysine. Half-medium changes were performed every 2 days.

In vitro model of astrocyte reactivity was adapted from Yang et al. (2009). Astrocytes at first passage were seeded in 13 mm coverslips for microscopy analysis and 60 mm dishes for RNA extraction and flow cytometry assay. After reaching 80–90% confluency, the astrocyte monolayer was scratched with a 10 μm pipette tip. The scratch pattern for coverslips was a "cross" composed of one horizontal and one vertical scratch, and for 60 mm dishes, the pattern was a "grid" composed of several scratches 0.5 cm distant from each other. The scratch assay causes cell detachment and loss of cell-cell contact, comparable to the borders of a traumatic brain injury. Three days post lesion (3dpl), cultures were highly enriched with reactive astrocytes and were processed for immunocytochemistry, total RNA extraction for quantitative PCR (qPCR) and flow cytometry.

Traumatic Brain Injury Model

Traumatic brain injury (TBI) model was previously described in Mundim et al. (2019). Briefly, adult 45-days-old C57BL/6J ($n = 3$) and Gal3$^{-/-}$ ($n = 3$) mice were anesthetized with intraperitoneal injection of 0.2% acepromazine (2.5 mg/kg, Vetnil, Louveira, SP, Brazil), 2% xylazine (20 mg/kg), 10% ketamine hydrochloride (80 mg/kg), and 0.05% fentanyl (0.5 mg/kg, Syntec, Brazil) and were placed in a stereotaxic frame. Mice were submitted to a unilateral penetrating lesion performed with a 22-gauge needle (0.7 mm) in the somatosensorial cortex (stereotaxic coordinates from bregma: AP + 0 mm; ML + 1 mm; DV − 0.7 mm) three times, for 2 min. Three days later (3dpl), mice were deeply anesthetized with intraperitoneal injection of 2% xylazine, 10% ketamine hydrochloride, and 0.05% fentanyl and intracardially perfused with 4% paraformaldehyde (PFA). Brains were removed from the skull, postfixed in 4% PFA overnight at 4°C, submersed in 30% sucrose at 4°C, and frozen using dry ice. Cryostat coronal sections (40 μm) were collected and prepared for immunohistochemistry.

Immunocytochemistry and Immunohistochemistry Analysis

For immunocytochemistry assays, cells were previously seeded on coverslips and submitted to a model of *in vitro* astrocyte reactivity; alternatively, cells were maintained under the same medium conditions as control. After 3dpl, both control and reactive astrocytes were fixed in 4% PFA and permeabilized with PBS-0.1% Triton (PBST) for 5 min. After sequential washes with PBS 1×, the cells were incubated at room temperature for 1 h in blocking solution (5% bovine serum albumin in PBST). Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. The cells were washed with PBS 1× and incubated at room temperature for 2 h with the corresponding secondary antibodies and fluorescence nuclear counterstain DAPI. Primary antibodies: chicken anti-Glial fibrillary acidic protein, GFAP (AB5541, 1:1,000, Millipore, Massachusetts, United States); rat anti-Gal3 (sc-23938, 1:250, Santa Cruz, Texas, United States); rabbit anti-Jagged1 (orb10065, 1:500, Biorbyt Ltd., Cambridge, United Kingdom); mouse anti-Notch1 (ab128076, 1:500, Abcam, Cambridge, United States). It is important to note that the anti-Notch1 antibody ab128076 strongly recognizes the activated form of the intracellular domain of Notch1 protein (NICD1). The unprocessed Notch1 protein is recognized with lower affinity. Secondary antibodies: Alexa Fluor 488-conjugated goat anti-chicken IgG; Alexa Fluor 488-conjugated goat anti-rabbit IgG; Alexa Fluor 594-conjugated goat anti-mouse IgG; Alexa Fluor 647- conjugated goat anti-rat IgG; Alexa Fluor 647-conjugated goat anti-chicken IgG (1:500, Invitrogen, Massachusetts, United States). Coverslips were mounted onto slides with Fluoromount G solution (Electron Microscopy Sciences, Hatfield, Pennsylvania, United States).

For immunohistochemistry assays, after several washes in PBST, the sections were pre-incubated for 1 h at room temperature in 5% normal goat serum. Immunohistochemistry was performed by incubating the sections overnight (2–8°C) with selected primary antibodies. Next day, sections were rinsed in 0.1% PBST and incubated for 90–120 min with the corresponding secondary antibody. Finally, the sections were rinsed and mounted onto slides with Fluoromount G. Primary antibodies: chicken anti-GFAP (1:500), mouse anti-Notch1 (1:500). Secondary antibodies: Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 647-conjugated goat anti-chicken IgG (1:500).

The immunofluorescence was analyzed by confocal microscopy using Leica TCS SP8 microscope (Houston, United States) and Zeiss Observer Z.1. (Jena, Germany) and images were processed using ImageJ software (1.49v[2]).

Vesicular Assay and ROI Analyses

GFAP and Nuclear NICD1 Normalized Fluorescence: For GFAP protein expression in selected areas, first we used the "histogram" and "threshold" tools to establish a minimum intensity value and determine regions of interest (ROI). Next, we measured the mean gray value (GV) and integrated density (IntD) and selected additional ROI to measure the background fluorescence (bk). GV was corrected through the subtraction of the mean bk. GV and IntD were used to compare GFAP fluorescence intensity between groups. For nuclear NICD1 analysis, single cell analysis of protein expression was calculated using normalized fluorescence. First, ROI were drawn around cells and were determined: area (A), mean gray value (GV),

[2]http://rsbweb.nih.gov/ij

and the integrated intensity (IntD). Next, background (bk) fluorescence was measured by drawing a ROI in an area around the cells or nucleus of interest and determined a mean gray value of the background fluorescence (GVbk). Normalized fluorescence was calculated as A-(IntD x GVbk) and used to compare NICD1 nuclear fluorescence intensity between groups. NICD1/Jagged1 colocalization: To determine NICD1/Jagged1 colocalization, ROIs were drawn around cells according to GFAP immunostaining and the "colocalization threshold" tool was used. Vesicular assay: The "threshold" and "create selection" tool and "ROI manager, -AND- option" were used to detect and count the number of vesicles in the nucleus and in the intracellular compartment. It was established a minimum intensity value through the "histogram" tool in each channel to configure a "threshold" and a vesicular size of 0.03 μm (± 0.02 SD) to create ROIs. Cell body perimeter (morphological analysis): The "Simple Neurite Tracer (SNT)" plugin and "perimeter" tool of the convex hull area were used for the reconstruction of each cell and the morphological analysis. GFAP/Notch1 distribution: Lesioned areas were localized by GFAP staining and the perimeters of the lesion were defined using the "enlarge" tool, with a distance of 50 μm between each concentric grid.

Quantitative RT-PCR Analysis

We evaluated mRNA expression profile of Notch signaling pathway members (*Notch1*, *Jagged1*, *Delta-like1, Hes1, Hes5, Mash1*) in control and reactive astrocyte cultures of C57BL/6J and $Gal3^{-/-}$ mice. RNA was extracted according to manufacturer's recommendations using PureLink RNA Micro Kit (cat n. 12183-016, Invitrogen, MA, United States). RNA was quantified and quality was assessed using spectrophotometer NanoVue Plus (GE Healthcare, Buckinghamshire, United Kingdom). Total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (cat n. 4368814, Applied Biosystems, MA, United States). Quantitative RT-PCR (qPCR) was performed using Fast SYBR Green Master Mix (cat n. 4385610, Applied Biosystems) in an Applied Biosystems 7500 Real-Time PCR System. Thermal cycling conditions were 95°C for 20 s, 40 × 95°C for 3 s, and 58°C for 30 s. The dissociation curve was performed at 95°C for 1 min, 60°C for 30 s, and 95°C for 30 s. The primers used were *Dll1*–sense 5′-CCCATCCGATTCCCCTTCG-3′ and antisense 5′-GGTTTTCTGTTGCGAGGTCATC-3′; *Gapdh*–sense 5′-AGGTCGGTGTGAACGGATTTG-3′ and antisense 5′-TGTAGACCATGTAGTTGAGGTCA-3′; *Hes1*–sense 5′-CTATCATGGAGAAGAGGCGAAG-3′ and antisense 5′-CCGGGAGCTATCTTTCTTAAGTG-3′; *Hes5*–sense 5′-CCAAGGAGAAAAACCGACTG-3′ and antisense 5′-TCCAGGATGTCGGCCTTCTC-3′; *Jag1*–sense 5′-GATTGCCCACTTCGAGTATCA-3′ and antisense 5′-CGTTCTGGTCACAGGCATAA-3′; *Mash1*–sense 5′-CTTGAACTCTATGGCGGGTT-3′ and antisense 5′-TAAAGTCCAGCAGCTCTTGTT-3′; and *Notch1*–sense 5′-CCCGCTGTGAGATTGATGTTA-3′ and antisense 5′-CACCTTCATAACCTGGCATACA-3′. Three biological replicates for each group and three technical replicates for each gene were analyzed. Gene expression was normalized to *Gapdh* expression and the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used for relative quantification analysis.

Flow Cytometry Analysis

Cells were detached using a 0.25% trypsin and suspended in astrocyte medium. Cell membranes in the pellet were stabilized by shaking for 1 h. For the extracellular analysis, the cells were suspended in blocking buffer (2% FBS in PBS) for 20 min and incubated with the primary antibody rat anti-Gal3 (1:100) for 1 h under constant agitation. The cells were washed and incubated with the secondary antibody, Alexa Fluor 488- conjugated goat anti-rat IgG (1:500) under the same conditions. Finally, cells were fixed in 4% PFA for 20 min, washed, and the pellet was suspended in PBS. Conversely, for intracellular analyses, after membrane stabilization, cells were fixed in 4% PFA for 20 min and permeabilized using a Perm Wash Buffer solution for 1 h (BD Bioscience, San Jose, United States). Concomitantly, the pellet was incubated with the corresponding primary antibody, rat anti-Gal3 (1:100) and/or chicken anti-GFAP (1:1,000), depending on the experiment, for 1 h under constant agitation. Secondary antibodies used were Alexa Fluor 488-conjugated goat anti-rat IgG and Alexa Fluor°647-conjugated goat anti-chicken IgG (1:500). For both experiments, one aliquot of unstained control cells (negative control) was used for evaluating autofluorescence (omission of antibodies) and other to assess the non-specific binding of the secondary antibody (omission of the primary antibody). Non-scratched astrocytes were used as control. Finally, the samples were suspended in PBS 1X and analyzed in a FACS Canto II flow cytometer (BD Biosciences, Mountain View, CA, United States). A minimum of 10.000 events per sample were collected, and data were analyzed using Cyflogic™ software (CyFlo Ltd., Finland).

Statistical Analysis

Statistical analysis of data and graphical representations were performed using GraphPad Prism (version 5.0)[3] and Microsoft Excel (version 2016)[4]. The graphs presented show mean ± standard error. The difference among groups was assessed using unpaired Student's *t*-test unless stated otherwise. The results were reported in absolute and relative values and the level of statistical significance adopted was 5% ($p < 0.05$). Differences among groups are indicated in the graphs with asterisks: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

RESULTS

Notch1 Signaling Is Activated in Reactive Astrocytes *in vitro*

To investigate the Notch signaling role in astrocyte reactivation, we initially established a scratch-assay model to promote astrocyte reactivity *in vitro*. Confluent astrocyte cultures were scratch-activated, and their activation status was analyzed 3 days post-lesion (3 dpl, **Figure 1A**). Under control conditions,

[3]https://www.graphpad.com/scientific-software/prism/

[4]https://www.microsoft.com/pt-br/microsoft-365/excel

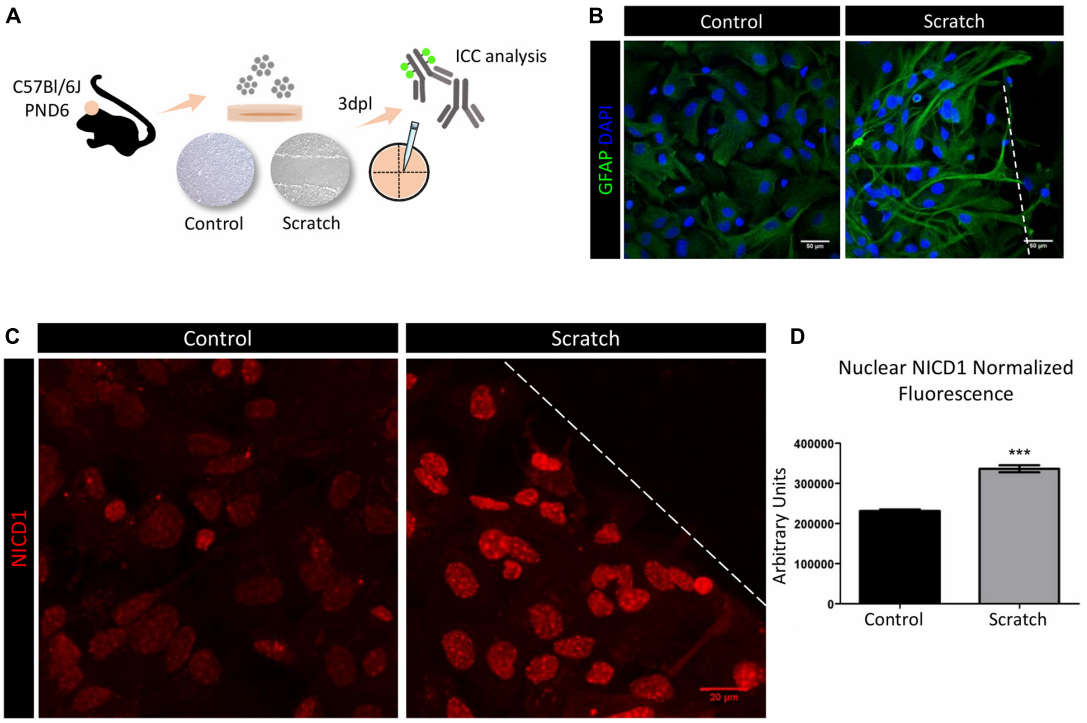

FIGURE 1 | Notch1 signaling is activated in reactive astrocytes *in vitro*. **(A)** Experimental design: astrocytes were isolated from the cortex of postnatal day six (PND6) C57BL/6J mice and cultured until confluency in 13 mm coverslips. Astrocyte reactivity was induced by scratch assay, and expression of the reactivity marker GFAP was analyzed 3 days post lesion (3dpl) by immunocytochemistry and confocal imaging. **(B)** Scratch-induced astrocytes are GFAP$^+$ and show reactive morphology. Scale bar: 50 µm. **(C)** Representative confocal images of NICD1 staining in control and reactive astrocytes. Dashed line indicates the scratch border. Scale bar: 20 µm. **(D)** Normalized fluorescence analysis of nuclear NICD1 immunostaining revealed increased NICD1 nuclear localization in reactive astrocytes compared to control (***$p \leq 0.001$); unpaired Student's *t*-test, $n = 154$ nuclei in scratch / 217 nuclei in control, three culture replicates. Data are mean ± SEM. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue).

astrocytes showed a polygonal to fusiform and flat morphology, and when reactivated by scratching, we observed morphological changes that included hypertrophic cell bodies and increased secondary processes emerging from primary branches. These processes frequently overlapped in a projection to the lesion core and showed increased expression of GFAP, shown by immunocytochemical analysis (**Figure 1B**). We next evaluated the activation of Notch signaling in primary cultures of reactive astrocytes from C57Bl/6J mice. Using an anti-Notch1 that mainly recognizes NICD1, we observed stronger immunolabeling in the nucleus of reactive astrocytes when compared to control astrocytes (**Figures 1C,D**), as well as in cytoplasmic vesicles (**Supplementary Figure 2**). Even though the antibody we used in this work does not specifically recognize NICD1 generated by γ-secretase cleavage between positions Gly1743 and Val1744 of Notch, our results show nuclear localization of NICD1, suggesting that the Notch1 signaling pathway is activated in reactive astrocytes.

To investigate the molecular mechanisms underlying astrocytes response, we next quantitatively compared the presence of NICD1 and Notch1 receptor ligand, Jagged1, in scratch-reactivated astrocytes in two distinct regions: (i) astrocytes at the lesion core and (ii) astrocytes located away from the lesion (periphery). We observed that reactive astrocytes located at the lesion border showed more NICD1 and Jagged1, with a strong colocalization pattern in the lesion core compared to the periphery (**Supplementary Figure 3**). This result suggests that Notch1 signaling is activated in the astrocytes closer to the injury site, where cell-cell interaction was disrupted by scratching the cell monolayer.

In vitro Astrocyte Activation Increases Intracellular Gal3

Gal3 is ubiquitously distributed in the cerebral cortex, and it has been related to the modulation of diverse intra- and extracellular processes. We observed strong Gal3 immunostaining in reactive astrocytes and sought to investigate the cellular location of Gal3 by using conventional flow cytometry analysis to search for its presence at the cell membrane (non-permeabilized) and in intracellular (permeabilized) compartments (**Figures 2A,B**).

First, we characterized the population of control (non-reactive) and reactive astrocytes cultures depending on the intracellular content of GFAP and Gal3 protein. Scatter plot analysis of both control and reactive astrocytes showed two subpopulations: GFAP$^+$/Gal3$^+$ cells corresponding to the R1 gate (75.70%) and a second population, GFAP$^+$/Gal− cells corresponding to the R2 gate (3.25%), with no statistical

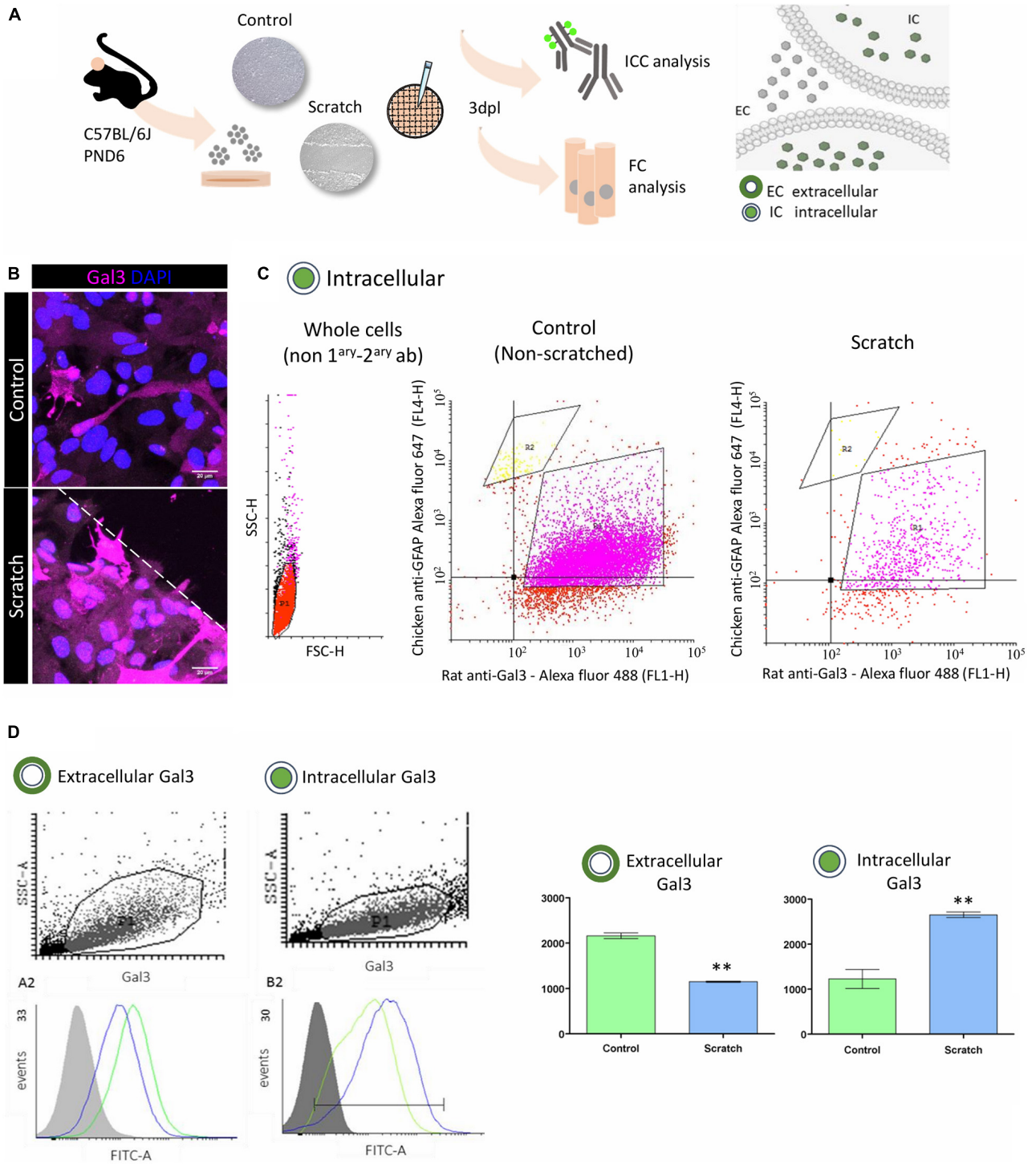

FIGURE 2 | *In vitro* astrocyte activation increases intracellular Gal3. **(A)** Experimental design: Gal3 localization in control (non-scratched) and reactive (scratched) astrocytes was analyzed by immunocytochemistry and flow cytometry. **(B)** Representative Z-stack confocal images of Gal3 immunolocalization in control and reactive astrocytes *in vitro*. Dashed line indicates scratch border. Scale bar: 20 μm. **(C)** Flow cytometry cell distribution analysis of control and reactive astrocytes showed two subpopulations: R1, $GFAP^+/Gal3^+$ (75.70% of total number of cells) and R2, $GFAP^+/Gal3^-$ (3.25% of total number of cells). Data correspond to geometric mean. **(D)** Analysis of Gal3 distribution between the extracellular and intracellular compartments in control and reactive astrocytes showed that at 3dpl, control astrocytes presented more extracellular Gal3 compared to reactive astrocytes (**$p \leq 0.01$, unpaired Student's *t*-test, $n = 3$). Conversely, intracellular Gal3 was increased in reactive astrocytes compared to control (**$p \leq 0.01$, unpaired Student's *t*-test, $n = 3$). Data correspond to mean ± SD. A minimum of 10,000 events per sample per experiment were analyzed, and data was processed using Cyflogic™ software (CyFlo Ltd., Finland).

differences between control and reactive astrocytes in R1 and R2 gates (**Figure 2C**). Next, in a second experiment, and given that GFAP$^+$/Gal3$^+$ (R1 gate) corresponded to 75.70% of the cell population, we sought to evaluate the location of Gal3 protein between the extracellular (non-permeabilized) and intracellular (permeabilized) compartments of control and reactive astrocytes. The Gal3 histogram of the non-permeabilized assay showed a higher amount of the protein at the surface of control (non-reactive) astrocytes (2,163 ± 62.69) when compared with reactive astrocytes (1,151 ± 13.04) (**Figure 2D**). Conversely, the histogram of the permeabilized assay showed that Gal3 was predominantly present in the intracellular compartment of reactive astrocytes (2,655 ± 60.58) when compared to control (non-reactive) astrocytes (1,229 ± 211.7). Altogether, these results suggest that there were two main populations of GFAP$^+$ cells (R1 and R2 gates) and that at 3dpl, Gal3 is preferentially localized in the intracellular compartment of reactive astrocytes.

NICD1 and Gal3 Colocalize in Vesicles in Reactive Astrocytes

Since we showed NICD1 nuclear localization in reactive astrocytes and Gal3 is distributed intracellularly, we asked whether NICD1 and Gal3 colocalized in reactive astrocytes. Initially, we analyzed and found two distinct Gal3 immunostaining patterns: diffuse and vesicular. We were able to distinguish granules with intense staining for the vesicular pattern, and in the diffuse pattern, we observed a weaker Gal3 signal. Similarly, the distribution patterns could also be attributed to NICD1 (**Figures 3A–C** and **Supplementary Figure 4**). Of note, Gal3 was predominantly distributed in a diffuse pattern in non-reactive astrocytes (76.5% of astrocytes, **Figure 3D**), and in contrast, 72.7% of reactive astrocytes presented vesicular Gal3. Interestingly, NICD1 distribution pattern shift was more pronounced in control astrocytes, in which NICD1 was distributed in vesicular (41.2%) and diffuse (58.8%) patterns. However, in 100% of reactive astrocytes analyzed, NICD1 was found in vesicular pattern (**Figure 3D**). When classifying astrocytes according to both NICD1 and Gal3 distribution patterns, we noticed that 52.9% of control astrocytes had diffuse Gal3 and NICD1, while none of the reactive astrocytes presented the same pattern (**Figure 3D**). In contrast, Gal3 vesicular/NICD1 vesicular pattern was found in 72.7% of the reactive astrocytes. It is important to note that both Gal3 and NICD1 could be mostly found in a diffuse pattern in control astrocytes. Upon lesion and astrocyte reactivation, the vesicular pattern became prevalent for both NICD1 and Gal3. This observation supports the hypothesis that NICD1 and Gal3 interact in reactive astrocytes.

To address this hypothesis, we analyzed if Gal3 and NICD1 vesicles colocalized in reactive astrocytes. In line with the previous observation on the distribution pattern of NICD1, we showed that reactive astrocytes have more NICD1$^+$ vesicles than control astrocytes (**Figure 3E**). The colocalization analysis revealed that there are more NICD1$^+$/Gal3$^+$ vesicles in reactive astrocytes, both in the cytoplasm and nucleus (**Figure 3E**). By quantifying the mean number of vesicles per cell, we observed that the higher number of vesicles in reactive astrocytes were both from NICD1 vesicles and NICD1/Gal3 vesicles (**Figure 3F**). Also, there was a 7.12-fold increase of NICD1/Gal3 cytoplasmic vesicles in contrast to 2.15-fold increase of NICD vesicles in reactive astrocytes when compared to control astrocytes (**Figure 3F**).

Notch1 Signaling Is Impaired in Gal3$^{-/-}$ Astrocytes

Since our results showed colocalization of NICD1 and Gal3 in reactive astrocytes, we asked whether Notch1 signaling would be activated upon scratch-induced reactivation of astrocytes lacking Gal3. We evaluated astrocyte reactivation status by GFAP expression and investigated the presence of NICD1 in primary cultures of astrocytes obtained from Gal3 knockout mice (**Figure 4A**). We noted that Gal3$^{-/-}$ astrocytes acquired a reactive morphology upon scratch-reactivation *in vitro*; however, there were no significant changes in GFAP expression (**Figures 4B,D**). GFAP upregulation is one of the hallmarks of astrocyte reactivation, and our results showed this response only in C57Bl/6J reactive astrocytes (**Figures 4B,D**). Next, NICD1 immunostaining analysis revealed the total absence of nuclear NICD1 in Gal3$^{-/-}$ control and scratch-reactivated astrocytes at 3dpl. In contrast, wild type (C57BL/6J) astrocytes immunomarked for nuclear NICD1 (**Figure 4C**). These results suggested that the absence of Gal3 impairs GFAP overexpression, therefore altering astrocyte reactive response, and impairs activation of Notch1 receptor and NICD1 translocation to the nucleus (**Figures 4B–D**).

Next, we asked if the lack of activation was due to a lack of Notch1 expression or a dysfunction in the signaling pathway. Further analysis of *Notch1* mRNA expression revealed that in C57BL/6J astrocytes there was a 14.1 ± 2.4 fold increase in *Notch1* expression after astrocyte reactivation (**Figure 4E**). In agreement with NICD1 immunostaining, *Notch1* mRNA expression did not change upon scratch-induced activation of Gal3$^{-/-}$ astrocytes (**Figure 4E**). We next asked whether the mRNA expression level of Notch1 ligands, Delta-like1 and Jagged1, would be altered in C57BL/6J or Gal3$^{-/-}$ astrocytes upon scratch-induced activation. In reactive C57BL/6J astrocytes, *Jagged1* expression increased by 10.5 ± 0.9-fold (**Figure 4E**), whereas the expression of *Delta-like1* increased by 2.7 ± 0.5-fold (**Figure 4E**). However, there were no significant changes in the expression of both ligands between Gal3$^{-/-}$ control and reactive astrocytes (**Figure 4E**). To address gene expression regulation of Notch1 effectors, we analyzed *Hes5*, *Hes1*, and *Mash1* mRNA expression. Scratch-stimulus positively regulated *Hes5* transcription by 1.9 ± 0.3-fold change and negatively regulated *Mash1* (0.7 ± 0.1-fold change) in C57Bl/6J reactive astrocytes. *Hes1* mRNA expression remained unchanged (**Figure 4F**). In Gal3$^{-/-}$ astrocytes, reactivation stimulus did not modify *Hes1* and *Mash1* mRNA expression level, but downregulated Hes5 expression by 0.6 ± 0.1 fold change (**Figure 4F**).

Collectively, these findings indicate that both ligands Jagged1 and Delta-like might promote Notch1 activation and consequent Hes5 expression in C57BL/6J reactive astrocytes. Moreover, our results indicate that Gal3 ablation impairs Notch1 signaling

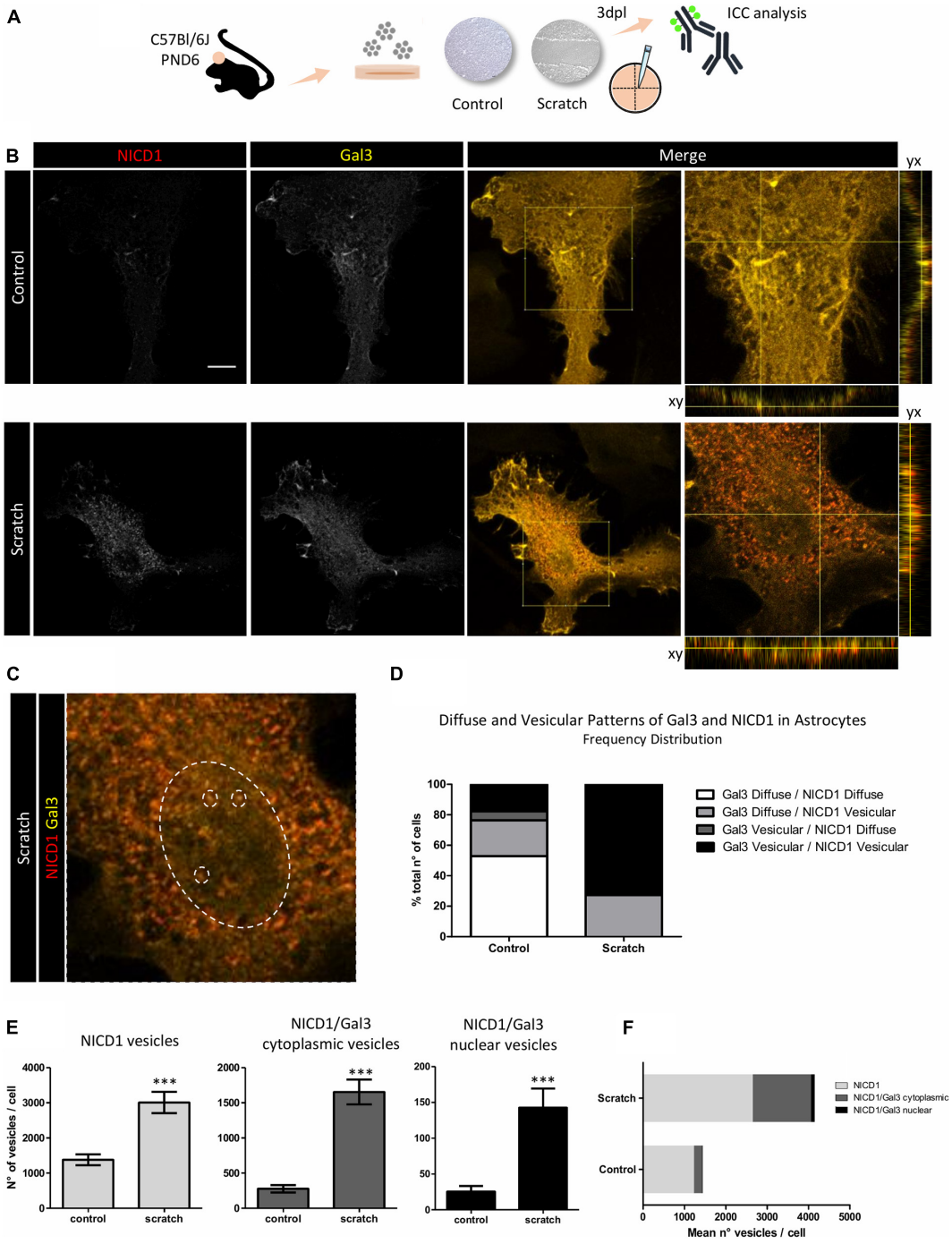

FIGURE 3 | NICD1 and Gal3 colocalize in vesicles in reactive astrocytes. **(A)** Experimental design: NICD1 and Gal3 were immunolabeled in scratch-reactivated and control (non-reactivated) astrocytes and analyzed by confocal microscopy. **(B,left)** Representative confocal Z-stack gray scale images of NICD1 and Gal3 in control and reactive astrocytes. **(B,right)** Merged images of NICD1 and Gal3 and detailed images with the orthogonal view of the immunostaining. Scale bar: 20 μm. **(C)** Zoom image from one reactive astrocyte: large dashed circle indicates the nucleus and the three small dashed circles inside the large circle indicate representative vesicles, highlighting NICD1 and Gal3 intranuclear colocalization. **(D)** Frequency analysis of NICD1 and Gal3 immunolabeling patterns (diffuse and vesicular) in control and reactive astrocytes. Values are plotted as percentage of total number of cells analyzed: 52.9% control astrocytes showed Gal3/NICD1 diffuse pattern; 23.5% Gal3 diffuse/NICD1 vesicular; 5.9% Gal3 vesicular/NICD1 diffuse, and 17.6% Gal3 vesicular/NICD1 vesicular. Reactive astrocytes showed 27.3% Gal3 diffuse/NICD1 vesicular pattern and 72.7% Gal3 vesicular/NICD1 vesicular (control $n = 17$ cells, scratch $n = 22$ cells). **(E)** Quantification analysis of $NICD1^+$ and $NICD1^+Gal3^+$ vesicles revealed a higher number of $NICD1^+$ vesicles, $NICD1^+Gal3^+$ cytoplasmic vesicles and $NICD1^+Gal3^+$ nuclear vesicles in reactive astrocytes compared to control. (***$p \leq 0.001$; unpaired Student's t-test; control $n = 18$ cells, scratch $n = 27$ cells). **(F)** Graphical representation of mean number of $NICD1^+$, $NICD1^+Gal3^+$ cytoplasmic and nuclear vesicles per cell in control and reactive astrocytes. (control $n = 18$ cells, scratch $n = 27$ cells).

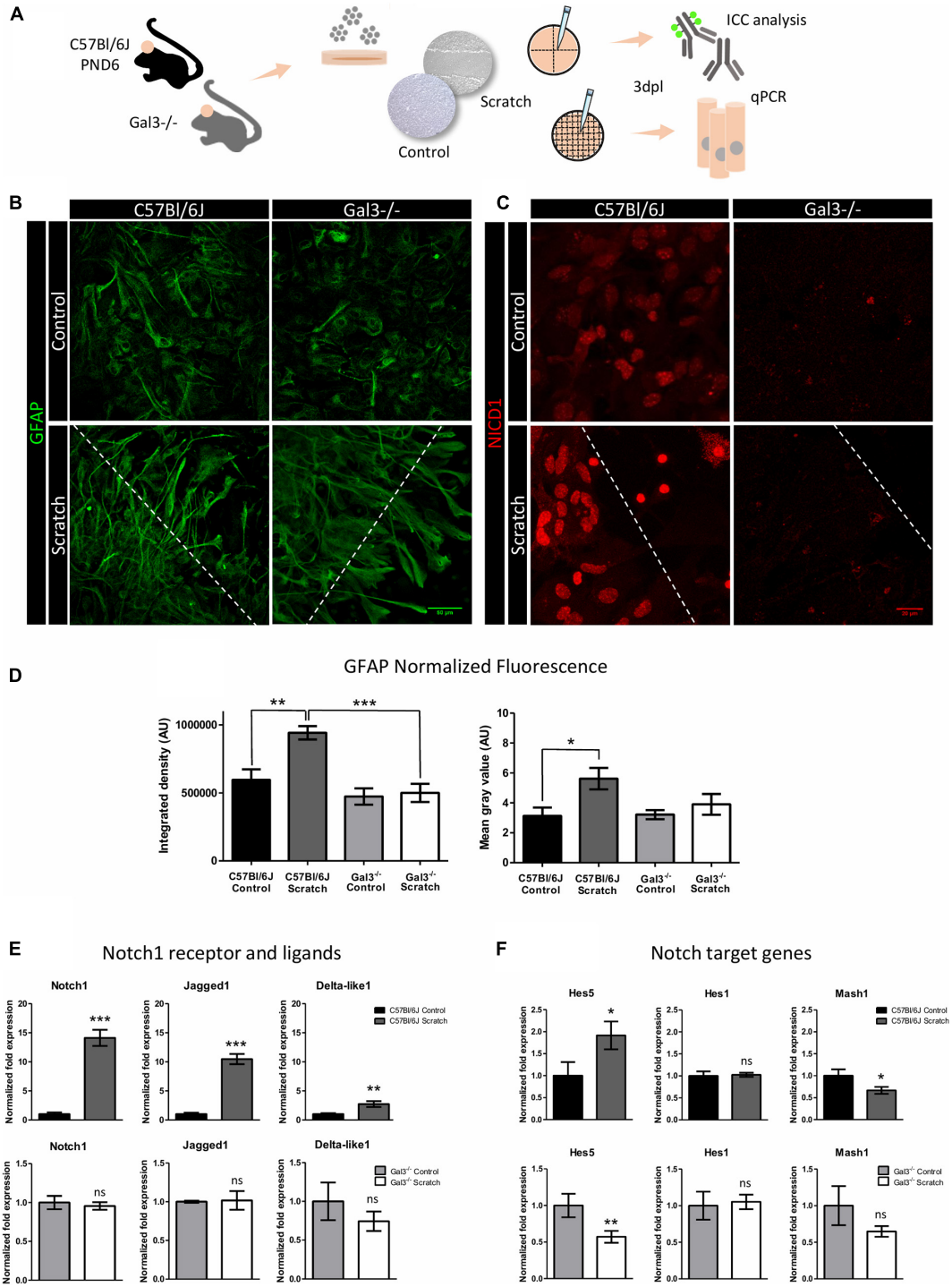

FIGURE 4 | Notch1 signaling is impaired in Gal3$^{-/-}$ astrocytes. **(A)** Experimental design: C57Bl/6J and Gal3$^{-/-}$ astrocytes were scratch-reactivated. Protein and gene expression were analyzed by immunocytochemistry and qPCR. **(B)** Representative Z-stack confocal images of GFAP staining in C57Bl/6J and Gal3$^{-/-}$ reactive and non-reactive astrocytes. C57Bl/6J and Gal3$^{-/-}$ reactive astrocytes project cellular processes to the lesion border. Scale bars: 50 μm. **(C)** Gal3$^{-/-}$ astrocytes do not activate Notch1 signaling, as shown by total absence of nuclear NICD1 immunostaining. In contrast, C57Bl/6J reactive astrocytes display strong nuclear NICD1 immunostaining, suggesting activation of Notch1 signaling. Dashed lines indicate scratch border. Scale bar: 20 μm. **(D)** GFAP normalized fluorescence analysis of C57Bl/6J and Gal3$^{-/-}$ control and scratched-astrocytes. Values are reported in integrated density **(D,left)** and mean gray value **(D,right)**. AU = arbitrary units (***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$). qPCR analysis of **(E)** *Notch1* receptor and ligands and **(F)** mRNA expression levels of Notch1 target genes in C57Bl/6J and Gal3$^{-/-}$ astrocytes. Values are relative to control group and are expressed in fold change. mRNA expression level was normalized to *Gapdh*. (***$p \leq 0.001$; **$p \leq 0.01$; ns = not significant; unpaired Student's *t*-test; $n = 3$ biological and technical replicates).

activation in reactive astrocytes *in vitro*, which was corroborated by mRNA expression of Notch1 target genes.

Gal3$^{-/-}$ Reactive Astrocytes Show Incomplete Response and Dysfunctional Notch1 Signaling Activation Following Traumatic Brain Injury

We gathered evidence of Gal3 modulatory role on Notch1 signaling by investigation of the signaling dynamics of Notch1 in scratch-reactivated astrocytes. To confirm these findings and considering that the reactive response is a progressive and active process that follows brain injury, we aimed to study astrocyte reactivity and the participation of Gal3 in C57BL/6 and Gal3$^{-/-}$ mice submitted to a model of TBI (**Figure 5A**).

A general evaluation of GFAP reactive astrocytes in C57BL/6J mice showed that cells were distributed, forming net-like structures in the edge of the lesion; however, in Gal3$^{-/-}$ mice, reactive astrocytes did not display these arrangements. Quantification of the total number of cells, the proportion of GFAP cells, and cell body perimeter as indirect parameters of reactivity response showed that Gal3$^{-/-}$ mice had an incomplete reactive astrocyte response compared with C57BL/6J mice (**Figures 5B–F**).

Notch1 protein analysis showed that activation of the signaling pathway in the TBI model had a close/far dependent distribution, being higher in areas close to the lesion core and sequentially decreasing in the C57BL/6J mice (**Figures 5G,I**), a pattern not reproduced in Gal3$^{-/-}$ mice (**Figures 5G,J**). In C57BL/6J, Notch1 expression extended around 500 μm of distance from the lesion core and just around 250 μm in Gal3$^{-/-}$ mice. Additionally, overall Notch1 expression was higher in C57BL/6J mice than Gal3$^{-/-}$ mice (**Figures 5G,H**). These results suggest that in the absence of Gal3, there was an incomplete activation response and low Notch signaling pathway activation after brain injury.

DISCUSSION

Reactive, mature cortical astrocytes are the first line of response to a brain injury. These cells comprise a large heterogeneous population of glial cells, which display a set of dynamical and complex changes at the molecular, biochemical, and cellular levels. The combination of those changes allows astrocytes to generate a reparative response to cell death and tissue dysfunction. Here we successfully evaluated the changes generated after a TBI at the cellular and molecular level, with the *in vitro* model of astrocyte reactivation, and at the tissular level, using a mice model of TBI.

Given the complexity of astrocyte reactivity, we focused on determining isolated cortical astrocytes response to a mechanical lesion. *In vitro* culture systems for accessing astroglial biology have been used for decades. In face of astrocyte cellular complexity, *in vitro* cultures benefit from offering a simplified microenvironment and a way of studying astrocyte behavior and molecular signaling without the interference of other cell types. On the other hand, it should be mentioned that two-dimensional culture systems for astrocytes limit their morphological complexity and promote an undesired baseline reactivity (Zamanian et al., 2012; Pekny and Pekna, 2014). Noteworthy, protoplasmic astrocytes from the healthy cortex do not express GFAP, but isolated cortical astrocytes display a basal expression of GFAP *in vitro*. However, astrocytes acquire a much higher degree of reactivity upon scratch-activation and can be used as a model of astrocyte reactivation response to traumatic injury (**Figures 1B**, **4D** and **Supplementary Figure 5**). In this work, we took advantage of a simplified *in vitro* scenario to understand the role of a highly conserved intercellular signaling pathway, Notch1, and a multifunctional lectin binding protein (Gal3) in modulating astrocyte reactivation to trauma. Next, we based our findings on the *in vivo* model of TBI, which offers a higher level of cellular complexity.

First, we show that at 3 days post lesion (3 dpl), Notch1 intracellular domain (NICD1) localizes in the nucleus (**Figures 1C,D**) and colocalizes with Jagged1 ligand in astrocytes located at the border of the *in vitro* lesion (**Supplementary Figure 3**). We also observed upregulation of the *Notch1* receptor, *Delta-like1*/*Jagged1* ligands, *Hes5* target gene, and downregulation of the proneural gene factor *Mash1* at the mRNA level in reactive astrocytes (**Figures 4E,F**), which complements our immunostaining results and strongly indicates Notch1-Hes5 signaling activation upon astrocyte reactivation *in vitro*. Notch signaling is essential during neurodevelopment, adult neurogenesis, and has been implicated in astrocyte reactivity. Previous studies described Notch1 signaling activation in astrocytes after brain injury *in vitro* and *in vivo*. In one study, the number of proliferating astrocytes decreased after treatment with a specific inhibitor of y-secretase, the enzyme responsible for Notch cleavage and release of NICD, in the peri-infarct area of mice submitted to a model of stroke (Shimada et al., 2011). Also, previous studies have shown Jagged1 localization in endosomes (Heng et al., 2020) and its intracellular fragment in the nucleus (LaVoie and Selkoe, 2003). Accordingly, other study demonstrated Jagged1 upregulation on the ischemic ipsilateral side of mice brain, where Notch1/Jagged1 signaling influenced indirectly reactive astrocyte proliferation through induced expression of endothelin receptor type B (LeComte et al., 2015). A later study in a rat model of intracerebral hemorrhage, in which the specific inhibitor of the y-secretase enzyme DAPT was used, showed that Notch1 signaling was upregulated. DAPT blockade of y-secretase suppressed astrocyte proliferation and GFAP expression 14 days post lesion and improved neurological signs (Zhong et al., 2018). Finally, *in vitro* reactive astrocytes showed increased NICD 12 h after being submitted to hypoxia (Zhang et al., 2015).

Conversely, it was shown in an *in vitro* astrogliosis model that LPS induces reactive astrocyte morphology by Notch signaling inhibition (Acaz-Fonseca et al., 2019). Interestingly, Jagged1 upregulation mediated NICD downregulation in those cells. Astrocyte reactivity was also correlated with Notch signaling downregulation in an entorhinal cortex lesion model in mice (Lebkuechner et al., 2015). Moreover, it was sequentially reported that striatal astrocytes of Rbpj deleted transgenic

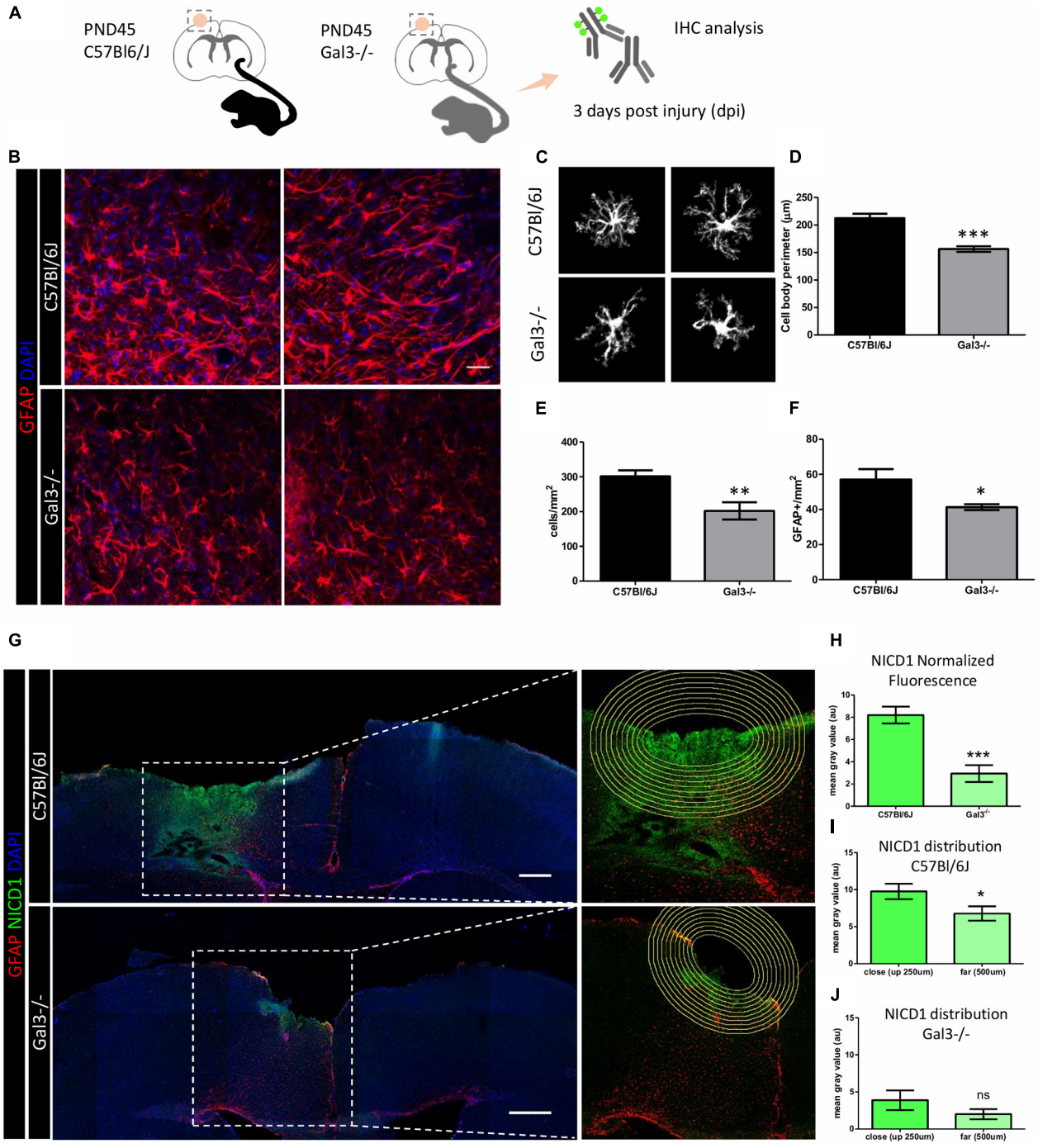

FIGURE 5 | Gal3$^{-/-}$ mice reactive astrocytes show incomplete response and dysfunctional Notch1 signaling activation following TBI. **(A)** Experimental design: Adult mice were submitted to a penetrating stab wound injury at the somatosensorial cortex and tissue sections were processed 3dpl for GFAP and NICD1 immunolocalization. **(B)** Representative confocal images of GFAP staining at TBI core. Scale bar: 50 µm. C57BL/6J reactive astrocytes extend primary branches to the lesion core and acquire a bipolar morphology. In contrast, Gal3$^{-/-}$ reactive astrocytes do not polarize to the lesion core. **(C,D)** Gal3$^{-/-}$ reactive astrocytes show less complex morphology and smaller cell bodies compared to C57BL/6J reactive astrocytes (***$p \leq 0.0001$, unpaired *t*-test, $n = 20$ cells C57BL/6J / 20 Gal3$^{-/-}$ cells). Quantification of the number of cells around the lesion core revealed **(E)** fewer cells per mm^2 (**$p \leq 0.01$, unpaired *t*-test, $n = 11$ C57BL/6J cells / 10 Gal3$^{-/-}$ cells) and **(F)** fewer GFAP$^+$ cells per mm^2 (*$p \leq 0.05$, unpaired *t*-test, $n = 11$ C57BL/6J cells / 10 Gal3$^{-/-}$ cells). **(G,left)** Mosaic composition of TBI confocal images showing NICD1 distribution at the lesion core. Scale bar: 500 µm. **(G,right)** Zoom image of the section. Concentric circles with 50 µm distance between each one were drawn around the lesion core and used to quantify NICD1 labeling intensity **(H–J)**. **(H)** NICD1 immunolabeling is more intense up to 500 µm from the lesion border in C57BL/6J mice than in Gal3$^{-/-}$ mice (***$p \leq 0.001$, unpaired *t*-test). **(I)** In C57BL/6J mice, NICD1 fluorescence intensity is stronger in cells closer to the lesion border (up to 250 µm) compared to the cells that are farther away from the border of the lesion (250 µm to 500 µm) (*$p \leq 0.05$, unpaired *t*-test). **(J)** NICD1 close/far distribution is not seen in Gal3$^{-/-}$ mice (ns = not significant).

mice lacked nuclear NICD protein 2 weeks after stroke. The same study showed that NICD negative striatal astrocytes generated DCX/Ascl1 neuroblasts from 2 to 7 weeks after stroke, suggesting that Rbpj deletion alone was sufficient to activate their neurogenic program (Magnusson, 2014; Santopolo et al., 2020). The differences in Notch1 signaling outcome in reactive astrocytes is not surprising, as astrocyte reactivation is heterogeneous and dependent upon injury type and severity (Burda and Sofroniew, 2014).

In our scratch-induced astrocyte activation model, we also observed Gal3 overexpression (**Figure 2**), a finding previously reported in inflammation and injury. Gal3 was increased in white matter reactive astrocytes in a stab wound injury model in adult mouse cerebral cortex, and that was later correlated with reactive astrocyte proliferation *in vitro* (Sirko et al., 2015). Upregulation of Gal3 was also described in cells of the striatum and subventricular zone of patients with perinatal hypoxia/ischaemia (Al-Dalahmah et al., 2020). Furthermore, here we show a $GFAP^{+}/Gal3^{+}$ cell population in control and reactive astrocytes, and additionally, that Gal3 is preferentially located intracellularly and in a vesicular pattern in reactive astrocytes (**Figures 2**, **3**). It is important to mention that Gal3 vesicular pattern is correlated to its role in phagocytosis, endocytosis, and lysosome repair (Rotshenker, 2009; Lakshminarayan et al., 2014; Jia et al., 2020). Gal3 is released by activated microglia and acts as a phagocytosis ligand, opsonizing apoptotic cells, myelin, and debris for phagocytosis via Mer tyrosine kinase receptor, which is expressed by microglia and macrophages (Venkatesan et al., 2010; Caberoy et al., 2012; Nomura et al., 2017; Puigdellívol et al., 2020). Interestingly, optic nerve head astrocytes constitutively display a phagocytic phenotype, by internalizing axonal evulsions and upregulating Gal3 upon injury (Nguyen et al., 2011). In any case, there is no evidence, to our knowledge, that Gal3 upregulation in cortical reactive astrocytes drives phagocytic activity. Moreover, another Gal3 role is to mediate clathrin-independent endocytosis, through binding with membrane glycosphingolipids and glycosylated proteins (Lakshminarayan et al., 2014; Stanley, 2014). Gal3 interaction with surface glycoproteins induce membrane deformation and clathrin independent carrier (CLIC) formation. Upon internalization, Gal3 can modulate intracellular signaling pathways involved in apoptosis, cell migration, proliferation, and angiogenesis (Rabinovich et al., 2007; Boscher et al., 2011; Elola et al., 2015). Lakshminarayan and collaborators reported several Gal3 cell-surface binders in mouse mammary tumor epithelial cells, including Notch2 (Lakshminarayan et al., 2014).

Because Gal3 displayed vesicular pattern distribution similar to NICD1 immunostaining (**Figures 3B–D** and **Supplementary Figure 4**), we sought to determine if Gal3 and NICD1 colocalized in reactive astrocytes. Our results revealed strong Gal3/NICD1 colocalization in reactive astrocytes in comparison to control (**Figures 3E,F**). NICD1/Gal3 interaction was previously described in ovarian cancer stem-cells and was related to stemness maintenance. In this study, the authors showed that Gal3 silencing in SKOV3 ovarian cancer stem line decreased the levels of cleaved NICD1 without changing Notch1 receptor expression. Furthermore, it was found that Gal3 interacted with NICD1 through its carbohydrate recognition domain (CRD) (Kang et al., 2016). Our results point to a possible interaction of Gal3 with NICD1 in reactive astrocytes, but whether this interaction occurs and if it involves Gal3 CRD is subject to future studies.

It is well-known that Gal3 interacts with glycosylated proteins through CRD, forming a dynamic and complex structure at the cell membrane which regulates protein diffusion, compartmentalization, and endocytosis (Johannes et al., 2018). Noteworthy, tumor secreted Gal3 was shown to increase Jagged1 half-life at endothelial cell surface, promoting Notch1/Jagged1 signaling (Nascimento dos Santos et al., 2017). The authors showed that Notch1/Jagged1 signaling activation occurs through Gal3 modulation, and in turn, promoted HUVEC spheroid sprouting in an *in vitro* model of tumor angiogenesis. Gal3 modulatory role in Notch1 signaling was also described in preventing osteoblast (Nakajima et al., 2014) and B cell differentiation (De Oliveira et al., 2018). Although we cannot discard Gal3 interaction with Notch receptor and ligands at the cell surface, our analysis suggests Gal3 is mainly distributed in the intracellular compartment of reactive astrocytes, where it might interact with NICD in the cytoplasm and nucleus (**Figures 3E,F**). Besides, not all NICD1 positive vesicles were Gal3 positive, indicating that we might be facing two different signaling mechanisms, in which one of them, Gal3 could be facilitating Notch signaling, or triggering a distinct signaling response, as to canonical Notch signaling alone.

Finally, at a functional level, and taking advantage of the $Gal3^{-/-}$ transgenic mice, we examined the overall effects of Gal3 ablation on astrocyte reactive response and Notch signaling activation. Normalized fluorescence analysis revealed that GFAP protein level did not rise after $Gal3^{-/-}$ astrocyte reactivation (**Figure 4D**). This data is in agreement with our *in vivo* results, in which we show fewer $GFAP^{+}$ cells around the TBI border of $Gal3^{-/-}$ mice (**Figure 5F**, discussed below). *In vitro*, we observed NICD1 absence in the nucleus of reactive and control astrocytes (**Figure 4C**). Consistent with that, scratch stimuli did not induce changes in *Notch1* receptor, *Jagged1*, and *Delta-like1* ligands and target genes *Hes1* and *Mash1* gene expression (**Figures 4E,F**). Hes5 downregulation at $Gal3^{-/-}$ reactive astrocytes further indicates that Notch1 signaling is inactive. The scenario was the opposite in C57BL/6J astrocytes: *Notch1* and *Jagged1* were highly upregulated upon lesion stimuli (**Figure 4E**). It is known that NICD1 induces the expression of *Jagged1* (Manderfield et al., 2012) leading to a positive biochemical feedback in which both cells have high Notch1 and high Jagged1 levels. This mechanism, named lateral induction, was observed during inner ear (Petrovic et al., 2014) and inner heart development (Manderfield et al., 2012). Of note, *Delta-like1* was also upregulated in reactive astrocytes (**Figure 4E**), although not to the same level as *Jagged1*. Thus, the functional implication of Jagged vs. Delta-like signaling in reactive astrocytes is yet to be determined. We further investigated and determined *Hes5* as the Notch1 effector gene upregulated in C57Bl/6J reactive astrocytes. Hes1 protein negatively regulates *Mash1* proneural gene expression (Kobayashi and Kageyama, 2014), and although *Hes1* mRNA

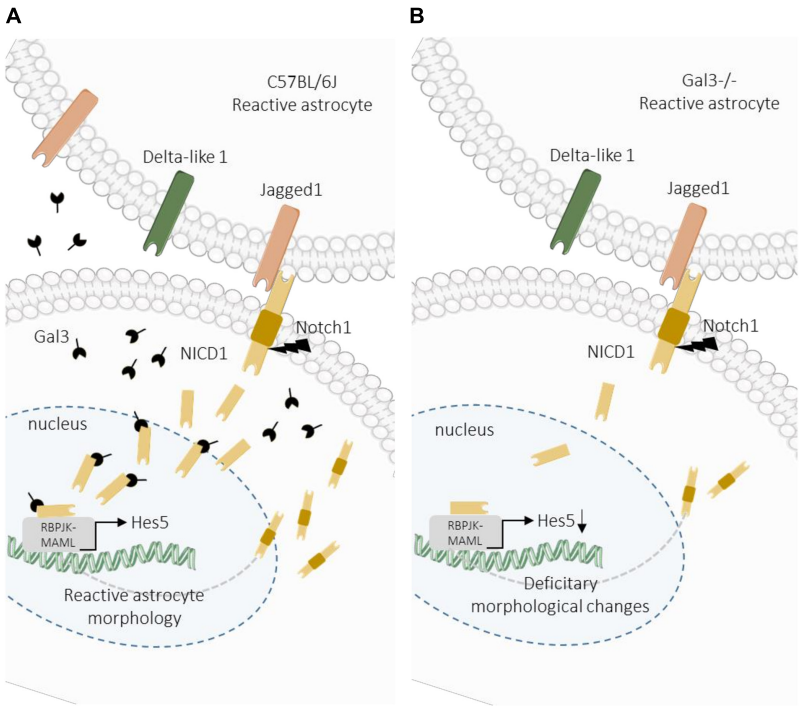

FIGURE 6 | Proposed model for Gal3 modulation of Notch1/Jagged1 signaling in reactive astrocytes. **(A)** After TBI in wild type mice, Notch1/Jagged1 signaling is activated in astrocytes, and NICD1 nuclear translocation and Hes5 transcription activation are Gal3-dependent. Activation of Notch1 signaling promotes astrocyte morphological response to TBI. **(B)** In Gal3$^{-/-}$ mice, after TBI Notch1 signaling is activated at the lesion core, possibly through Jagged1, however activation is less intense compared to wild type mice. We hypothesize that Gal3 modulates NICD1 signaling, which is necessary for astrocyte activation in response to TBI. In the absence of Gal3, NICD1 signaling is disrupted and astrocyte activation is incomplete.

expression level did not change, *Mash1* was downregulated in reactive astrocytes, which is indicative of Notch signaling activation (**Figure 4F**).

In vivo, the absence of Gal3 generates an incomplete response in reactive astrocytes as evidenced by the decrease in the number of GFAP reactive astrocytes and the morphological changes describing atrophy and loss of function in the TBI model. We assessed astrocyte perimeter as an indirect measure of cell domain and reactive response (**Figures 5C,D**). Gal3$^{-/-}$ GFAP astrocytes have smaller cell body perimeter when compared to C57BL/6J GFAP astrocytes, indicating a loss of complexity, which at the functional level suggests impaired reactive response to TBI. In addition, we showed a decreased number of GFAP reactive astrocytes around the lesion border in Gal3$^{-/-}$ mice. This phenomenon was also described after stab wound injury in gray matter astrocytes of the cortex of Gal1$^{-/-}$ and Gal3$^{-/-}$ mice; however, the authors showed that it reflected a reduction in *Gfap* expression level and in GFAP^{+} cells rather than in astrocyte number (Sirko et al., 2015). Interestingly, a significantly smaller percentage of total GFAP and Olig2 glial cells was found in the striatum and subventricular zone of electroporated mice with Gal3 knockdown constructs and Gal3fl/fl mice. Conversely, in this same study, Gal3 overexpression in wildtype mice increased the proportion of glial cells. Authors suggest that endogenous Gal3 is necessary for striatal gliogenesis from the subventricular zone (Al-Dalahmah et al., 2020).

Mature astrocytes occupy specific cellular domains, which are respected upon mild astrocyte reactivation. When there is a violation of astrocyte territorial domain, such as in traumatic injury, astrocyte reactivity involves territorial overlap and ultimately glial scar formation (Verkhratsky et al., 2017). It was previously shown that reactive astrocytes from the cerebral cortex do not overlap domains, but rather show hypertrophy after electrical lesion (Wilhelmsson et al., 2006; Pekny et al., 2019). Hypertrophic reactive astrocytes are morphologically thicker in their soma and primary and secondary branches, as a consequence of GFAP and vimentin upregulation (Pekny et al., 2019). In contrast, astrocyte atrophy, characterizing domain loss, was correlated to neurological diseases and aging (Verkhratsky and Parpura, 2016; Verkhratsky et al., 2020).

Moreover, in the border of the lesion of Gal3$^{-/-}$ mice there was a poor Notch signaling activation response (**Figure 5G**). This result suggests a lack of the lateral induction mechanism, where atrophic astrocytes do not communicate properly, affecting the collective cell-fate decision and function. Gal3 alone or Gal3/NICD1 signaling could be involved in astrocyte morphological dysfunction after TBI. Gal3 loss leads to deficient morphological reactive response, which in turn affects cell-cell communication and, consequently, prevents Notch1 signaling to be propagated to the adjacent tissue. In C57Bl/6J mice, we hypothesize that Notch1/Jagged1 signaling is upregulated in astrocytes in the lesion border and propagate to the periphery

by lateral induction. The diminished morphological response in Gal3$^{-/-}$ astrocytes could be a consequence of impaired Notch signaling. We hypothesize that in the absence of Gal3, NICD1 does not signal efficiently, which could lead and/or worsen the morphological defects observed. Noteworthy, we did not address cause and consequence, but the data we presented here suggest that these events are linked and operate proper reactive astrocyte responses to TBI.

CONCLUSION

Efforts have been made to unravel the molecular factors and signaling pathways involved in astrocyte reactivation with the final aim to direct astrocytes response for regenerative medicine. Here we provide new evidence of the response of reactive astrocytes and the participation of Notch signaling pathway and Gal3 in the modulation of this response. Our results indicate that Gal3 is essential for proper activation of the Notch signaling pathway, facilitating the cleavage and nuclear translocation of NICD to the nucleus of reactive cortical astrocytes. Additionally, we hypothesize that the reactive astrocyte response is dependent on Notch1/Jagged1/Hes5 signaling activation during a brain injury (**Figure 6**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Ethics in the Use of Animals from Universidade Federal de São Paulo (Comitê de Ética no Uso de Animais; CEUA numbers 7740290318 and 2451111116).

AUTHOR CONTRIBUTIONS

TR performed experiments, wrote the original draft, reviewed and edited the final document and figures, and funded by grant foundations. LD-G performed experiments, wrote the original draft, reviewed and edited the final document and figures, and received fellowship from grant foundations. MP conceptualized the study, advised TR and LD-G during execution of experiments, reviewed and edited the final document, and received funding from grant foundations. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.649854/full#supplementary-material

Supplementary Figure 1 | Gal3$^{-/-}$ mice genotyping. Adult male mice (45 days) isogenic, Lgals3$^{-/-}$ (Gal3$^{-/-}$) (Hsu et al., 2000) and wild type (C57BL/6J, Lgals3$^{+/+}$) were genotyped using the "*quick-dirty HotSHOT*" (Truett et al., 2000) method. Tail samples from Gal3$^{-/-}$ (n = 3) and wild-type (n = 1) animals were incubated with lysing agent (25 mM NaOH and 0.2 mM EDTA) (95°C, 60 min; hold 4–15°C) and neutralized with 75 μl 40 mM TrisHCl. The samples were centrifuged (4,000 rpm, 3 min, −20°C) and analyzed by conventional PCR. Gal3$^{-/-}$ (n = 3) and C57BL/6J (n = 1). Agarose gel (1.5%) electrophoresis of PCR products. The 300 bp band corresponds to Gal3 in Gal3$^{-/-}$ mice and 490 bp to Gal3$^{+/+}$ in C57BL/6J mice.

Supplementary Figure 2 | NICD1 immunostaining pattern in C57Bl/6J reactive astrocytes. Representative confocal Z-stack images of NICD1 immunostaining in astrocytes at 3 days post scratch-reactivation stimuli. NICD1 is found in the nucleus and in cytoplasmic vesicles. Arrowheads indicate astrocytes with nuclear and vesicular NICD1. Arrows indicate astrocytes with nuclear NICD1. Scale bar: 20 μm.

Supplementary Figure 3 | NICD1 and Jagged1 colocalize in reactive astrocytes at the border of the *in vitro* lesion. **(A)** Colocalization images for NICD1/Jagged1 reveal strong colocalization at the lesion core compared to the periphery. White dots indicate NICD1/Jagged1 colocalization. **(B)** Representative confocal image of GFAP/NICD1/Jagged1 stained reactive astrocytes. The dashed line represents the two comparative regions, lesion core and periphery. The core region extends 100 μm from the border of the scratch. Scale bar: 50 μm. **(C)** Colocalization coefficient was used for statistical analysis (***$p \leq 0.001$; unpaired Student's t-test, n = 19 cells at lesion core / 21 cells in periphery; 9 images were analyzed from three culture replicates). Data are mean ± SEM.

Supplementary Figure 4 | NICD1 and Gal3 distribution patterns. **(A)** Representative confocal Z-stack images of the four labeling patterns for NICD1 and Gal3 in cortical astrocytes *in vitro*. Reactive astrocytes display NICD1 vesicular pattern, and control astrocytes present NICD1 diffuse pattern distribution. Arrowheads indicate vesicles. Scale bar: 20 μm. **(B)** Schematic representation of NICD1 vesicle distribution patterns in control and reactive astrocytes.

Supplementary Figure 5 | GFAP in C57Bl/6J and Gal3$^{-/-}$ astrocytes *in vitro*. Representative confocal images of GFAP immunostaining in C57Bl/6J and Gal3$^{-/-}$ astrocytes used for normalized fluorescence analysis. Dashed lines indicate scratch border. Scale bar: 50 μm.

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BRIEF RESEARCH REPORT
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Transcriptome Profiling of Mouse Corpus Callosum After Cerebral Hypoperfusion

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White matter damage caused by cerebral hypoperfusion is a major hallmark of subcortical ischemic vascular dementia (SIVD), which is the most common subtype of vascular cognitive impairment and dementia (VCID) syndrome. In an aging society, the number of SIVD patients is expected to increase; however, effective therapies have yet to be developed. To understand the pathological mechanisms, we analyzed the profiles of the cells of the corpus callosum after cerebral hypoperfusion in a preclinical SIVD model. We prepared cerebral hypoperfused mice by subjecting 2-month old male C57BL/6J mice to bilateral carotid artery stenosis (BCAS) operation. BCAS-hypoperfusion mice exhibited cognitive deficits at 4 weeks after cerebral hypoperfusion, assessed by novel object recognition test. RNA samples from the corpus callosum region of sham- or BCAS-operated mice were then processed using RNA sequencing. A gene set enrichment analysis using differentially expressed genes between sham and BCAS-operated mice showed activation of oligodendrogenesis pathways along with angiogenic responses. This database of transcriptomic profiles of BCAS-hypoperfusion mice will be useful for future studies to find a therapeutic target for SIVD.

Keywords: cerebral hypoperfusion, corpus callosum, dementia, white matter, RNAseq

INTRODUCTION

Vascular cognitive impairment and dementia (VCID) syndrome is clinically defined as cognitive decline with evidence of subcortical brain infarction (Erkinjuntti et al., 2000a,b). Subcortical ischemic vascular dementia (SIVD) is the most common subtype of VCID, and patients with SIVD suffer from a vast amount of white matter degeneration due to prolonged cerebral hypoperfusion. White matter damage is a clinically important parameter, as the severity of white matter lesions correlates strongly with the degree of cognitive dysfunction (Pantoni and Garcia, 1997; Medana and Esiri, 2003; Esiri, 2007; Lampe et al., 2019). In SIVD, white matter dysfunction is progressive and is often associated with poor neurological outcome (Roman et al., 2002; Longstreth et al., 2005). Although the number of patients with SIVD is predicted to increase with the aging population, to

date there are no established treatments for this pathological condition, partly because of a lack of understanding of the gene expression changes under the conditions of SIVD.

To advance the understanding of SIVD pathology and to find effective approaches for this disease, several animal models have been developed (Tsuchiya et al., 1992; Kurumatani et al., 1998; Lin et al., 2001; Shibata et al., 2004; Yang et al., 2018). Because prolonged cerebral hypoperfusion is a major characteristic that leads to white matter dysfunction in SIVD, the mouse model of prolonged cerebral hypoperfusion by bilateral carotid artery stenosis (BCAS) is considered a well-accepted model for SIVD. Hypoperfused-BCAS mice replicate the pathophysiology of SIVD patients, such as oligodendrocyte/myelin damage and cognitive decline (Ihara and Tomimoto, 2011; Ihara et al., 2014). This model has also been used in various pharmacological studies investigating the treatment of SIVD, which led to the discovery of the potential efficacy of some drugs that are currently approved for other clinical indications (Watanabe et al., 2006; Ueno et al., 2009; Miyamoto et al., 2013a, 2014). Therefore, in this study, we utilized the mouse cerebral hypoperfusion model of SIVD to examine the gene expression changes in the corpus callosum region after cerebral hypoperfusion.

MATERIALS AND METHODS

Animals

All experimental procedures followed NIH guidelines and were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Male C57BL/6J mice were purchased from The Jackson Laboratory and were housed in a specific pathogen-free conditioned 12-h light/dark cycle room with free access to food and water throughout the experiment. A total of 12 male mice (8 weeks old) were used in this study.

Prolonged Cerebral Hypoperfusion Model by Bilateral Carotid Artery Stenosis (BCAS)

After a week-long habituation period in our animal facility, 12 mice were randomly divided into two groups, for sham operation and for BCAS operation. For the mice in the BCAS group, a microcoil (0.18 mm diameter; Samini, Japan) was applied to the bilateral common carotid arteries for the induction of chronic cerebral hypoperfusion as previously described (Shibata et al., 2004). The sham group received a cervical incision followed by exposure of the bilateral common carotid arteries without microcoil application. Body weight of the mice was measured before operation and 4, 7, 14, 21, and 28 days after operation. No intra-operative or post-surgical complications were observed in this study.

Novel Object Recognition Test (NORT)

Four weeks after sham or BCAS operation, mice were tested for short-term recognition memory by NORT between 8 and 10 am, as previously described with slight modifications (Wang et al., 2004; Kleschevnikov et al., 2012; Ohtomo et al., 2020). Briefly, mice were placed in a clean empty cage for 10 min. Mice were then exposed to two identical objects in the same cage for 5 min (acquisition period). After an interval of 30 min, mice were then presented with two different objects (one original and one novel object, which were placed in the same position as the objects in the acquisition period) in the same cage for 5 min (retention period). Object recognition was videotaped and scored by the total investigation time either sniffing or touching the object. The performance of short-term recognition memory was described by the ratio of the time spent on the new object to the total time spent on both objects minus 0.5 (e.g., Discrimination index: ranged from −0.5 to 0.5). Experiments and analyses were conducted by an investigator who was blinded to the group allocation.

Corpus Callosum Sampling

One day after NORT, mice were transcardially perfused with ice-cold 0.9% physiological saline followed by decapitation. Brains were then removed and cooled in ice-cold Hanks' Balanced Salt Solution for 1 min. After removal of meninges and the choroid plexus, the cerebrum was sliced into five coronal sections using a brain matrix. To minimize inclusion of tissue outside of the corpus callosum., the thicker parts of the corpus callosum from the 2nd and 3rd slices were isolated with direct visualization using a light microscope. Samples of the corpus callosum were put into an RNA free tube and then quickly frozen using liquid nitrogen.

RNA Extraction

RNA extraction from the corpus callosum samples was performed using QIAzol® (QIAGEN, Germany) following manufacturer's instructions. Briefly, sonicated tissue was resuspended in 1 ml of ice-cold QIAzol, and 0.2 ml of chloroform was added to the lysate. After mixing by Vortex Mixer, the tube was centrifuged for 15 min at 12,000 *g*. The supernatant was then transferred to another tube, and the same amount of propanol was added. After centrifugation for 10 min at 12,000 *g*, the supernatant was aspirated, and 1 ml of 75% ethanol was added. Finally, the tube was centrifuged for 5 min at 7,500 *g*, followed by suspension with nucleus-free water. The amount and purity of purified RNA was measured by NanoDrop Spectrophotometers. The RNA sample was stored at −80°C before use.

RNA Sequencing (RNAseq)

Three RNA samples from each group were randomly selected for RNAseq experiments. Library preparation and RNAseq was performed by Genewiz, Inc. (NJ, United States). Libraries for RNAseq were prepared based on the PolyA selection method, and RNAseq was performed by Illumina HiSeq 2 × 150 bp sequencing (single index). The raw data was obtained in FASTQ format, and Kallisto (ver. 0.46.2) was used for quantifying the abundance of transcripts, expressed as transcript per kilobase million (TPM). The bioinformatics analysis was conducted using the R software (ver. 4.0.0). DESeq2 was used for differential expression analysis, and the level with adjusted p-value <0.1 was set to filter differential expression genes (DEGs). Metascape was used for the gene set enrichment analysis (Tripathi et al., 2015). The sequence data (FASTQ files) were deposited under the accession #PRJNA727284.

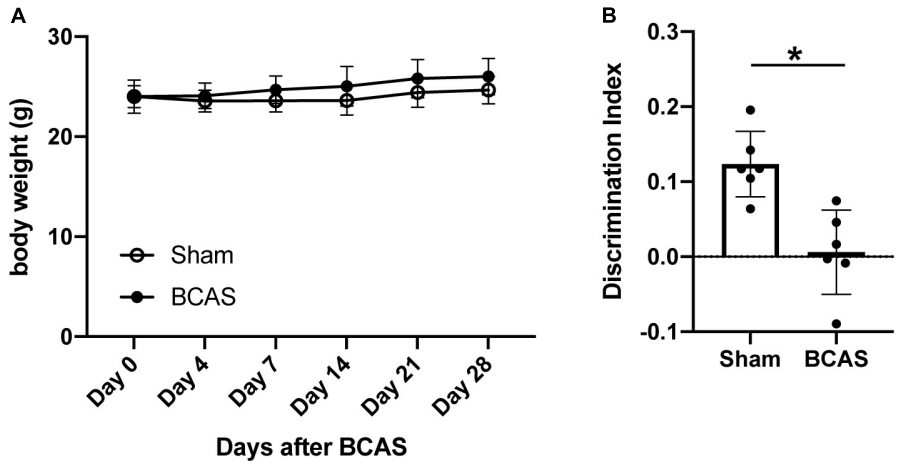

FIGURE 1 | Body weight changes and cognitive function after cerebral hypoperfusion. **(A)** Body weight changes after cerebral hypoperfusion. There was no significant difference between sham-operated and BCAS-hypoperfusion mice. Data are expressed as mean ± SD. $N = 6$ each. **(B)** Cognitive function was assessed by NORT at 4 weeks after sham or BCAS operation. While sham-operated mice accessed the novel object, BCAS-hypoperfusion mice did not show any preference between the novel and familiar objects. Data are expressed as mean ± SD. $N = 6$ each. $^*p < 0.05$.

Statistical Methods

Statistical analysis was conducted by unpaired *t*-test for the NORT data and two-way repeated-measures analysis of variance followed by *post hoc* multiple comparisons test for the body weight data. Differences with $p < 0.05$ were considered statistically significant, and data were expressed as mean ± SD.

RESULTS

To prepare a mouse model of SIVD, 2-month-old male C57BL/6J mice were subjected to bilateral common carotid artery stenosis (BCAS) by placing microcoils on both common carotid arteries. There was no significant difference in body weight between sham- and BCAS-operated groups up to 4 weeks after hypoperfusion (**Figure 1A**). In the novel object recognition test (NORT), BCAS-operated mice showed no preference between familial and novel objects, whereas sham-operated mice spent more time investigating the novel object (**Figure 1B**), confirming that 4-week cerebral hypoperfusion caused cognitive decline.

We next isolated RNA samples from the corpus callosum region of mice that have been subjected to BCAS-hypoperfusion for 4 weeks (**Figure 2A**). The quality of our RNA samples and RNA sequencing was high (**Supplementary Table 1**), and transcription levels of oligodendrocyte markers (Mbp and Mobp) were significantly higher than the cortical neuron markers (Reln for layer I, Rasgrf2 for layer II/III, Pou3f2 for layers II-V, and Foxp2 for layer IV) (**Figure 2B**), confirming the purity of our corpus callosum samples. In addition, the principal component analysis (PCA) indicated that the cluster of sham mice data were distinct from the cluster of BCAS mice data (**Figure 2C**). The MA plot (**Figure 2D**) and the volcano plot (**Figure 2E**) revealed that while the gene expression changes caused by 4-week cerebral hypoperfusion were relatively mild, there were several upregulated or downregulated genes in the corpus callosum of hypoperfused mice. The list of differentially expressed genes between sham- and BCAS-operated mice is provided in **Supplementary Table 2**.

Finally, we conducted a gene ontology analysis to identify the signaling pathways that were enriched in the upregulated or downregulated genes after cerebral hypoperfusion. For the upregulated genes, pathways that are related to oligodendrocyte/myelin formation and vascular development were highly enriched (**Figure 3** and **Supplementary Table 3**). For the downregulated genes, pathways that are related to the negative regulation of synapse organization and cell-cell adhesion were highly enriched (**Figure 4** and **Supplementary Table 3**).

DISCUSSION

In this study, we used RNA sequencing analyses to examine transcriptomic changes in the mouse corpus callosum after 4 weeks of cerebral hypoperfusion. Our initial findings suggest that (i) transcriptomic changes in the mouse corpus callosum were relatively mild, (ii) upregulated genes were related to pro-oligodendrogenic and pro-angiogenic pathways, and (iii) downregulated genes were related to cell-cell adhesion pathways. These findings have the potential to lay the groundwork for the research identifying and developing effective therapies for SIVD and other white matter-related diseases.

One major innovation of this study is the study of gene expression in the corpus callosum region using RNAseq. White matter dysfunction is a major feature of many CNS diseases; however; basic research of CNS diseases has mostly focused on the pathological mechanisms of gray matter. This is partly because the volume of white matter is much smaller than that of gray matter in rodents (Zhang and Sejnowski, 2000). However, some rodent models of CNS diseases could be used to examine the pathological mechanisms in cerebral

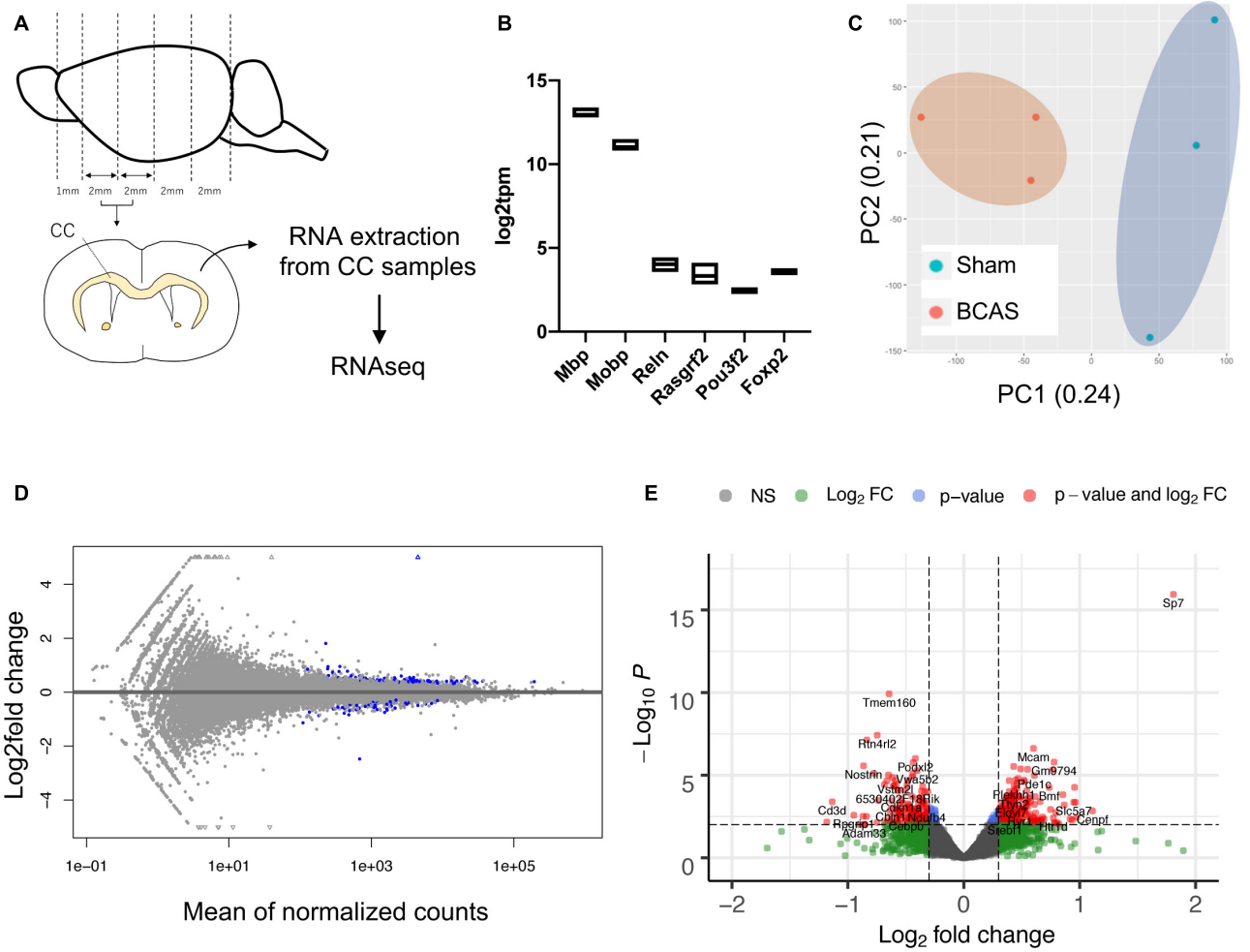

FIGURE 2 | Gene expression changes in mouse corpus callosum after 4-week cerebral hypoperfusion. **(A)** Diagram for corpus callosum preparation. Four weeks after sham or BCAS operation, mice were sacrificed, and the corpus callosum samples were prepared. From each group, three mice were used for the RNAseq studies. CC: corpus callosum. **(B)** Gene expression levels of oligodendrocyte markers (Mbp and Mobp) were much higher than that of cortical neuron markers (Reln for layer I, Rasgrf2 for layer II/III, Pou3f2 for layers II-V, and Foxp2 for layer IV). **(C)** The principal component analysis (PCA) plot. **(D)** The MA plot. Blue dots represent the genes with adjusted *p*-value < 0.1 against the sham group. **(E)** The volcano plot. The red dots represent the genes that showed $|\log2 \text{ fold change}| > 0.3$ with adjusted *p*-value < 0.1 against the sham group. Please see **Supplementary Table 2** for the list of differentially expressed genes between sham- and BCAS-operated mice.

white matter (Arai and Lo, 2009). For example, the BCAS-hypoperfusion model is now well-accepted as a mouse model of SIVD (Ihara and Tomimoto, 2011; Ihara et al., 2014), and our current data support and confirm its utility in the study of cognitive decline along with white matter damage. It is expected that oligodendrogenesis pathways would be activated in the mouse corpus callosum after cerebral hypoperfusion because in young mice, the numbers of oligodendrocyte precursor cells (OPCs) and newly generated oligodendrocytes were reported to be transiently increased as a compensatory response after hypoperfusion (Miyamoto et al., 2013b; Arai, 2020). While no studies have carefully examined the angiogenic responses in the BCAS-hypoperfused mice so far, activation of angiogenic responses had been confirmed in multiple rodent models of white matter damage (Kanazawa et al., 2019; Rust, 2020; Shindo et al., 2021). Interestingly, a microarray study using corpus callosum samples from 3-day cerebral hypoperfusion mice showed an upregulation of angiogenesis-related genes (Reimer et al., 2011). Thus, upregulation of angiogenic pathways would also be expected in the corpus callosum after cerebral hypoperfusion. In addition, our findings that cell-cell adhesion genes were downregulated after cerebral hypoperfusion is consistent with the idea that plasticity of the micro-environment contributes to brain repair/remodeling after injury (Lo, 2008). Furthermore, Rtn4r12 was one of the most significantly downregulated genes in the hypoperfused-BCAS mice. Rtn4r12 encodes Reticulon-4 receptor-like 2 (also known as Nogo-66 Receptor Homolog NgR2), which is a receptor for myelin-associated glycoprotein (MAG) and acts selectively to mediate MAG inhibitory responses (Venkatesh et al., 2005).

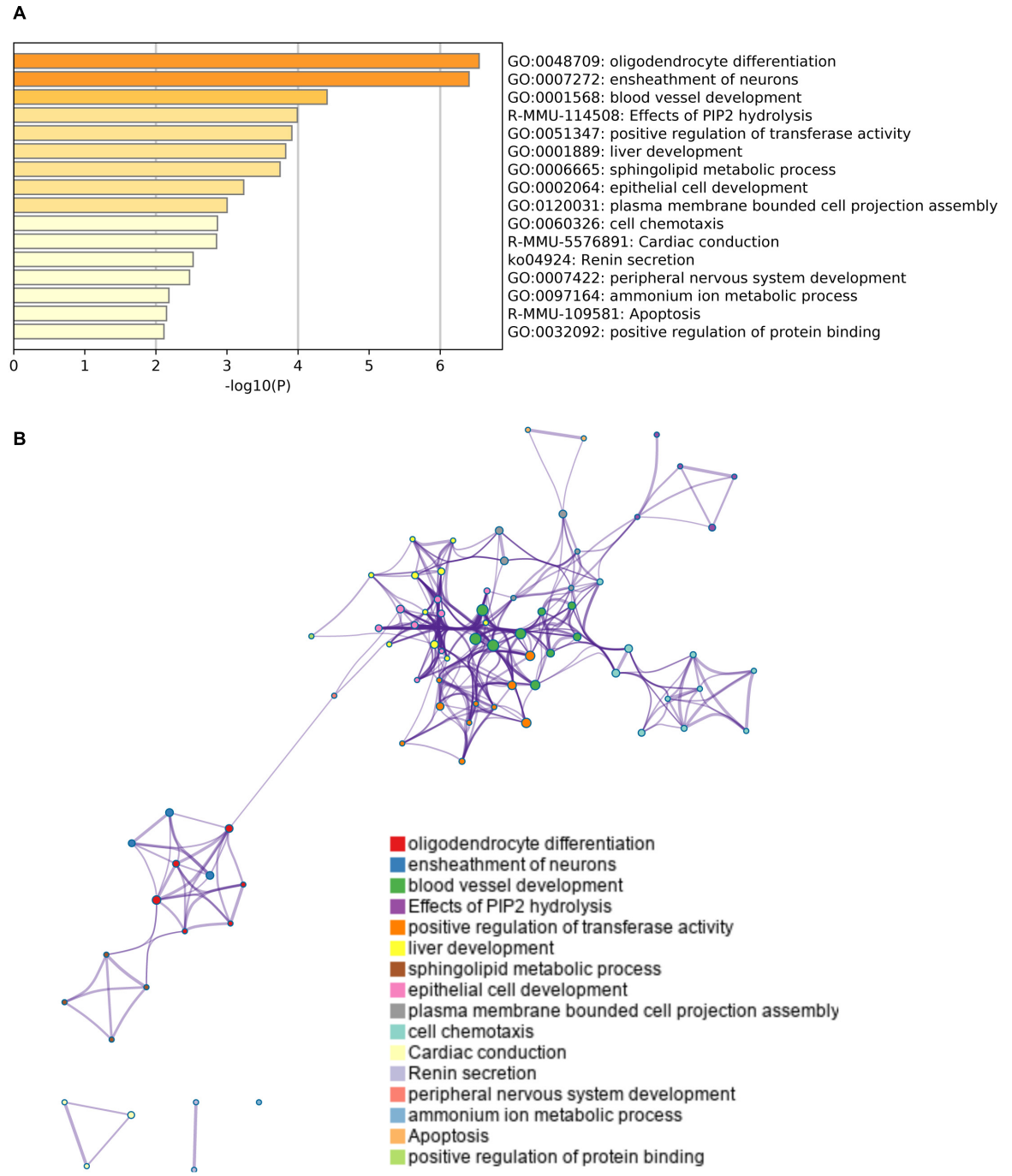

FIGURE 3 | Gene set enrichment analysis of upregulated genes. **(A)** A heatmap of enriched terms across the input genes list. Darker colors indicate smaller *p* values. Upregulated genes were related to the pathways for oligodendrogenesis (GO: 0048709 and GO: 0007272) and angiogenesis (GO: 0001568). Please see **Supplementary Table 3** for the list of enriched terms of upregulated genes. **(B)** Metascape enrichment analysis confirms the close relationship between GO: 0048709 (oligodendrocyte differentiation) and GO: 0007272 (ensheathment of neurons). Clustering was made based on similarity (similarity > 0.3).

Taken together, our database of gene expression profiles in the mouse corpus callosum after 4-week cerebral hypoperfusion will be useful to examine the pathological mechanisms of white matter damage/recovery in SIVD and other white matter-related diseases.

Although our study showed that multiple pathways were affected by cerebral hypoperfusion in mouse corpus callosum, it should be noted that the transcriptomic changes after 4-week cerebral hypoperfusion were mild, with only a few genes exhibiting changes of more than 2-fold or less than 0.5-fold.

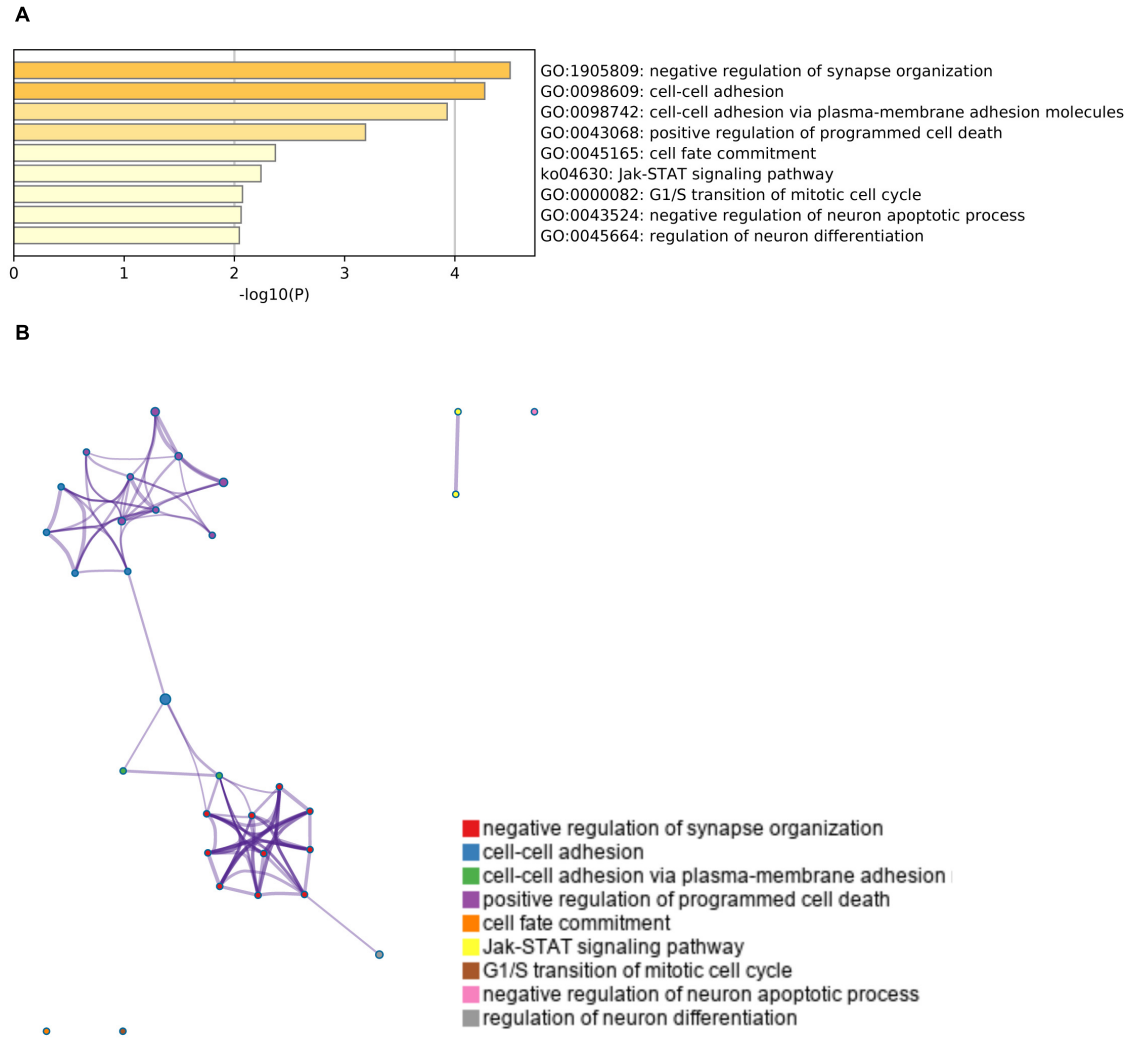

FIGURE 4 | Gene set enrichment analysis of downregulated genes. **(A)** A heatmap of enriched terms across the input genes list. Darker colors indicate smaller *p* values. Downregulated genes were related to the pathways that mediate negative regulation of synapse organization (GO: 1905809) and cell-cell adhesion (GO: 0098609 and GO: 0098742). Please see **Supplementary Table 4** for the list of enriched terms of downregulated genes. **(B)** Metascape enrichment analysis. Clustering was made based on similarity (similarity > 0.3).

This is partly because the stress of cerebral hypoperfusion by BCAS is prolonged and mild but not acute and severe (Shibata et al., 2004), thus causing a gradual detrimental effect on gene expression in the corpus callosum region. This mild change in gene expression pattern after cerebral hypoperfusion in mice is consistent with previous reports. While our study is the first RNAseq experiment for profiling transcriptomic changes in the corpus callosum after cerebral hypoperfusion, one previous study used microarray analyses with corpus callosum samples of BCAS-hypoperfusion mice and showed that the gene expression changes from 2-week to 6-week after hypoperfusion were mild, matching our current findings (Ohtomo et al., 2018). Based on our findings, future studies are warranted to expand the map of gene expression profiles in the corpus callosum of BCAS-hypoperfused mice with different sets of conditions, such as male vs. female, young vs. old, and early vs. late time points after cerebral hypoperfusion. Because OPC functions display some sex-associated differences (Yasuda et al., 2020) and because aging dampens the compensatory response of OPCs and endothelial progenitor cells (Miyamoto et al., 2013b; Pradillo et al., 2019), understanding how these variables affect transcriptomic profiles of the corpus callosum after hypoperfusion may be a key step in finding novel therapeutic approaches for SIVD and other white matter-related diseases.

This study provides a novel dataset of gene expression profiles in the corpus callosum in BCAS-hypoperfused mice. However, there are several caveats to keep in mind. First, our current study is somewhat preliminary and descriptive, and our finding is not directly related to identification of a therapeutic target for SIVD. Within the differentially expressed gene list from our

study, Sp7 showed the most significant difference. Sp7, also called Osterix, is a transcription factor, which plays a role in driving the differentiation of mesenchymal precursor cells into osteoblasts and eventually osteocytes (Sinha and Zhou, 2013). While its role in cerebral white matter is mostly unknown, it was reported that Sp7 is highly expressed in oligodendrocytes (Tabula Muris Consortium, 2018) and would participate in oligodendrocyte maturation (He et al., 2016). Therefore, it is possible that examining the roles of Sp7 in white matter damage and recovery could lead to a novel therapeutic target for SIVD. Second, although we focused on mRNA profiles, there are multiple pseudo-genes in our differentially expressed gene list (**Supplementary Table 4**), and some of the pseudo-genes, such as Gm24270 and Gm23935, are known to function as miRNA. Future research of changes in the expression of pseudo-genes may enable a deeper understanding of the complex mechanisms of white matter pathology in SIVD. Lastly, the use of "bulk" corpus callosum samples in our study leaves open the possibility that significant changes in gene expression in some cell types may have been missed. It will be useful for future studies to examine gene expression profiles with single cell RNA sequencing to further our understanding of transcriptomic profiles of corpus callosum after cerebral hypoperfusion.

In summary, this preliminary study provides the first database of gene expression profiles in the mouse corpus callosum after 4-week cerebral hypoperfusion. This database may be useful as an initial framework for future investigations of effective therapeutic approaches for SIVD and other white matter-related diseases.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI BioProject repository, accession number PRJNA727284.

ETHICS STATEMENT

The animal study was reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

HT, GH, RO, HI, and EM: collection of data. HT, GH, MF, KH, K-HT, EL, and KA: data analysis. KC, JL, MF, KH, K-HT, EL, and KA: manuscript writing. HT, GH, RO, HI, KC, MF, KH, K-HT, EL, and KA: interpretation. MF, KH, K-HT, EL, and KA: conception and design. MF, KH, K-HT, and KA: funding acquisition. HT, GH, RO, HI, KC, EM, JL, MF, KH, K-HT, EL, KA: final approval of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.685261/full#supplementary-material

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