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Tripartite synaptomics: Cell-surface proximity labeling *in vivo*

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Abstract

The astrocyte is a central glial cell and plays a critical role in the architecture and activity of neuronal circuits and brain functions through forming a tripartite synapse with neurons. Emerging evidence suggests that dysfunction of tripartite synaptic connections contributes to a variety of psychiatric and neurodevelopmental disorders. Furthermore, recent advancements with transcriptome profiling, cell biological and physiological approaches have provided new insights into the molecular mechanisms into how astrocytes control synaptogenesis in the brain. In addition to these findings, we have recently developed *in vivo* cell-surface proximity-dependent biotinylation (BioID) approaches, TurboID-surface and Split-TurboID, to comprehensively understand the molecular composition between astrocytes and neuronal synapses. These proteomic approaches have discovered a novel molecular framework for understanding the tripartite synaptic cleft that arbitrates neuronal circuit formation and function. Here, this short review highlights novel *in vivo* cell-surface BioID approaches and recent advances in this rapidly evolving field, emphasizing how astrocytes regulate excitatory and inhibitory synapse formation *in vitro* and *in vivo.*

Keywords

Split-TurboID; BioID; Astrocyte; Synapse; Neuron; Tripartite synapse; Synaptomics

1. Introduction

Astrocytes are the most abundant glial cells in the central nervous system (CNS) and extend thousands of fine processes that structurally and functionally associate with neuronal synapses to form “tripartite synapses”. Most neuronal synapses (approximately 50-75%) in the cortex and hippocampus are contacted by astrocytes and form tripartite synapses (Lanjakornsiripan et al., 2018; Ventura and Harris, 1999). Astrocytes have traditionally been considered to have key roles in metabolic homeostasis and synaptic transmission through ionic balance and neurotransmitter clearance as supporting cells (Araque et al.,

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Conflict of Interest Statement

The authors declare no competing financial interests.

2014). Interestingly, in addition to this traditional concept of astrocytes, recent evidence has discovered that astrocytes also tightly control individual local synaptic development and circuit connectivity in the brain (Allen and Eroglu, 2017; Baldwin and Eroglu, 2017; Stogsdill et al., 2017; Takano et al., 2020). This new conceptual framing of the tripartite synapse has emerged as a rapidly expanding field. It is one of the most exciting topics in cellular neuroscience that is changing our understandings of brain circuitry formation and function. Furthermore, new evidence from genomic and physiological studies reveals that dysfunction of astrocyte-synaptic interactions may contribute to psychiatric and neurodevelopmental disorders (Baldwin and Eroglu, 2017; Stogsdill and Eroglu, 2017; Yu et al., 2020). Despite the vital role of astrocytes in synaptic development and physiology, deciphering the molecular composition of tripartite synaptic connections that drive these processes remains a significant challenge.

In recent years several synapse-specific proteomics profiling techniques, such as a cell sorting of growth cones and synaptosomes, imaging-based approaches, and affinity purification, have been established for analyzing synaptic molecular networks *in vivo* (Apostolo et al., 2020; Cizeron et al., 2020; Li et al., 2020b; Loh et al., 2016; Micheva et al., 2010; Poulopoulos et al., 2019; Zhu et al., 2018). However, despite these advances, it has been technically difficult to profile the molecular composition at the cell-type-specific cell-cell contacts such as tripartite synaptic sites *in vivo*. Recently, we developed novel *in vivo* cell-surface proximity-dependent biotinylation (BioID) approaches, TurboID-surface, and Split-TurboID, and demonstrated they are highly successful for discovering the molecular network of tripartite synaptic connections (Takano et al., 2020). Here, we highlight the application of these novel proteomic approaches and the current understating of how astrocytes control synapse formation and function *in vivo*.

Cell-surface TurboID-based proteome *in vivo*

2-1. Proximity-based labeling via BioID and APEX

Spatially-restricted, proximity-dependent biotinylation (BioID and APEX) is a powerful chemico-genetic approach that enables the identification of specific intracellular and extracellular proteomes as they exist in situ (Branon et al., 2018; Kim et al., 2016; Roux et al., 2012; Spence et al., 2019; Uezu et al., 2016). The BioID approach first utilized an *Escherichia coli*-derived mutant biotin ligase (BirA*-R118G), which generates reactive biotin (biotinoyl-5'- AMP) and has an enhanced off-rate so that biotin covalently attaches to exposed lysine residues of any neighboring protein (approximately 10 nm) (Kim et al., 2016; Roux et al., 2012). More recently, TurboID, a directed-evolution mutant of BirA, was developed that exhibits higher enzymatic activity for proximity-dependent labeling than BirA*-R118G (Branon et al., 2018). For BioID, expression constructs encoding a bait protein fused to BirA*-R118G or TurboID are delivered into the cells *in vitro* or *in vivo*, and they are treated with biotin, which is taken up into cells, to label nearby proteins (Fig. 1). The biotinylated proteins are purified by affinity-isolation using streptavidin-coupled beads and then subjected to liquid chromatography-tandem mass spectrometry (LC/MS/MS) to discover the local proteomes (Fig. 1). BioID has high spatial resolution and can label and identify insoluble proteins, membrane-associated proteins, and weak and/or transient

protein-protein interactions (Kim et al., 2016; Roux et al., 2012). Indeed, two examples of this approach from our laboratory, gephyrin-BirA that is an inhibitory synaptic organizer, and Wrp-BirA that is a Rac-GAP localized to nascent dendritic spines, both identified a large number of proteins from these synaptic sites in the brain that were previously difficult to dissect by traditional biochemistry methods such as affinity purification and subcellular fractionation method (Spence et al., 2019; Uezu et al., 2016).

APEX is an ascorbate peroxidase that catalyzes the oxidation of biotin-phenol to the biotin-phenoxyl radical in the presence of H_2O_2. APEX-based labeling has some advantages to BirA-based approaches, including the ability to label the proteins by biotinylation within 1 minute in living cells (Loh et al., 2016; Martell et al., 2016). Furthermore, a recent study demonstrates that APEX can be applied to transcriptome profiling through direct proximity labeling of endogenous RNA within specific cellular compartments of the living cells (Fazal et al., 2019; Han et al., 2020). The APEX peroxidase also catalyzes the polymerization and local deposition of diaminobenzidine (DAB) and enables to recruit of electron-dense osmium that is easily visualized by electron microscopy. Thus, APEX is useful for proteomic analysis and spatiotemporally high-resolution transcriptome profiling and imaging *in vitro.* However, the application of APEX *in vivo* has limited because it requires H_2O_2, which can be toxic and is less amenable to labeling reactions in tissue.

2-2. Astrocyte-specific cell-surface TurboID *in vivo*

Barres and colleagues initially discovered that treatment with astrocytic-conditioned media (ACM) or neuronal and astrocyte co-cultures promote the maturation and functional formation of excitatory synapses (Pfrieger and Barres, 1997; Ullian et al., 2001). In addition to the excitatory synapse, these conditions also induce inhibitory synapse formation *in vitro*, suggesting that astrocytes control both excitatory and inhibitory synapse formation and function through the astrocytic-secreted and/or cell-adhesion molecules (Allen and Eroglu, 2017; Baldwin and Eroglu, 2017; Elmariah et al., 2005; Stogsdill and Eroglu, 2017; Takano et al., 2020). Interestingly, astrocytic morphogenesis is also tightly linked to synaptogenesis during brain development (Sakers and Eroglu, 2019; Stogsdill et al., 2017). These findings imply that bidirectional signalings between astrocytes and neurons play a critical role in proper brain development and function. However, because cell-surface proteins are typically low abundance with high hydrophobicity and heterogeneity (Kuhlmann et al., 2018; Li et al., 2019), the identification of the molecular composition at astrocytic cell-surface and tripartite synaptic contacts *in vivo* has been significantly limited.

Proximity-dependent cell-surface labeling of cells can reveal their extracellular molecular landscape and also provide a roadmap to investigate mechanisms of how astrocytes control synaptic connectivity and function in the brain. Recently, horseradish peroxidase (HRP)-based cell-surface proteomic profiling has been achieved for neuronal synapse *in vitro* and *ex vivo* (Cijsouw et al., 2018; Li et al., 2020b; Loh et al., 2016). For example, HRP-fused with the known synaptic cleft proteins such as LRRTM1, LRRTM2, Siltrk3, or Neuroligin2 (NL2) has been used to specifically dissect the excitatory and inhibitory synaptic cleft proteomes in cultured neurons (Cijsouw et al., 2018; Loh et al., 2016). These HRP-based cell-surface proteomics at excitatory and inhibitory synaptic clefts identified

199 glutamatergic and 42 GABAergic synaptic cleft proteins *in vitro* and found that a novel synaptic cleft protein Mdga2 controls inhibitory synapse formation through the postsynaptic recruitment of NL2 (Loh et al., 2016). In addition, HRP-fused with the known excitatory cell adhesion molecule SynCAM1 identified several excitatory synaptic cleft proteins including, Receptor-type tyrosine-protein phosphatase zeta (R-PTP-zeta) (Cijsouw et al., 2018). Furthermore, cell-type-specific expression of HRP fused to the N-terminal extracellular domain CD2 (HRP-CD2) was recently used to identify novel cell surface molecules of Drosophila olfactory projection neurons *ex vivo* (Li et al., 2020b).

More recently, TurboID-based cell surface proteomic profiling (TurboID-surface) has been engineered for deciphering the molecular composition of the cell-type-specific surface proteomes *in vivo* (Fig. 1 left) (Takano et al., 2020). TurboID-conjugated with a glycosylphosphatidylinositol (GPI) *a*nchored *r*econstitution-*a*ctivated *p*roteins *hi*ghlight *i*ntercellular *c*onnections (GRAPHIC) was delivered to cortical astrocytes using cell-type selective adeno-associated virus (AAV) to label and purify proteins for LC/MS/MS of the astrocytic surface proteome *in vivo* (Kinoshita et al., 2019; Takano et al., 2020). Super-resolution Stimulated Emission Depletion (STED) microscopy showed that almost half of the proteins labeled by astrocytic TurboID-surface are localized at the peri-synaptic sites *in vivo* (Takano et al., 2020). Using label-free quantitative LC/MS/MS analysis after affinity isolation by streptavidin-coupled beads (Fig. 1 left), a large number of proteins (~3, 000 proteins) are identified. These proteins are further analyzed for those with significant enrichment in astrocytic TurboID-surface the over control group (soluble TurboID) as described previously (Courtland et al., 2021; Spence et al., 2019; Uezu et al., 2016). Astrocytic TurboID-surface revealed 178 extracellular proteins (fold-change>1.5 and $p<0.05$), including 58 known synaptic proteins based on the synaptic SYNGO database (Koopmans et al., 2019; Takano et al., 2020). These findings suggest that cell-surface proximity-dependent labeling using TurboID and HRP are both robust approaches to interrogate the cell-surface proteomes of genetically defined cell types *in vitro* and *in vivo*. This genetic access to cell-type derived surface compartments now enables the proteomic interrogation of sites previously not readily accessible to biochemical studies (Li et al., 2020a, b; Takano et al., 2020).

Split-TurboID enables the labeling and molecular profiling of specific cell-type interfaces

Deciphering the extracellular adhesion codes of cell-type-specific connections in the brain remains a major challenge. Recently, surface HRP or TurboID fragment complementation approaches, split HRP (sHRP) and Split-TurboID, have been engineered to enable the visualization of synapses (sHRP) or the proteomic mapping of the specific cell-type interfaces such as synaptic clefts and astrocyte-neuron contacts (Split-TurboID) (Takano et al., 2020; Martell et al., 2016). These techniques, sHRP and Split-TurboID, divide the proximity-based enzyme into N- and C-terminal fragments such that they can reconstitute the functional enzyme by fragment complementation at specific cell-cell contact sites when brought into proximity of each other (Fig. 1 right). For example, biotin-conjugated synaptic cleft proteins based on sHRP reconstitution at synapses can be used to visualize the specific

synapses between amacrine cells and retinal ganglion cells (RGCs) *in vivo*, indicating that this technique is helpful for mapping neuronal connections in the brain (Martell et al., 2016). Split-TurboID (also Split-BirA) has also been utilized for investigating protein-protein complexes and intracellular membrane-membrane contacts such as ER-mitochondria interactions (Cho et al., 2020; Martell et al., 2016; Schopp et al., 2017).

Split-TurboID at the surface of cells is a newly developed and valuable tool for discovering the molecules at the specific cell-type interfaces such as astrocyte-synapse contacts *in vivo* (Takano et al., 2020). Split-TurboID splits TurboID into N-terminal (N TurboID) and C-terminal (C TurboID) halves and expresses them on the surface of cells using GPI-anchor (Fig. 1 right). Each Split-TurboID fragment is expressed *in vivo* in either neurons or astrocytes using AAVs with cell-type-specific promoters. Where astrocytes ensheath neuronal synapses N-TurboID and C-TurboID reconstitute functional TurboID at the tripartite synaptic clefts (Fig. 1 right). Thus, Split-TurboID enables molecular profiling at specific cell-type interfaces *in vivo*, which is a significant advance over other approaches (Fig. 1). Indeed, STED microscopy reveals that Split-TurboID highly labels the proteins between astrocytes at excitatory or inhibitory synapses in the cerebral cortex (Takano et al., 2020). Using LC/MS/MS analysis after affinity isolation by streptavidin (Fig. 1 right), a large number of proteins (~3, 000 proteins) are identified. These proteins are further analyzed for those with significant enrichment in Split-TurboID over the control group (soluble TurboID) as described previously (Courtland et al., 2021; Spence et al., 2019; Uezu et al., 2016). Split-TurboID discovered 173 proteins (fold-change> 1.5 and $p<0.05$), including 63 known synaptic proteins identified in the synaptic biology SYNGO database. These included synaptic proteins such as Neuroligin/Neurexin (*Neuroligin-3*, *Neurexin I),* calcium channel auxiliary subunits that also regulate glutamate receptor trafficking (*Cacna2d3*, *Ccicng2-3),* AMPA receptors (*Gria2-3*), and known inhibitory synaptic proteins such as $GABA_A$ receptors (*Gabra1, Gabra4, Gabrb2, Gabrg2)* (Koopmans et al., 2019; Takano et al., 2020). Interestingly, over half of the proteins identified by Split-TurboID are unique and have not been previously reported (Takano et al., 2020). Thus, Split-TurboID provided a new molecular framework for understanding tripartite synaptic connections and how these contacts control synapse formation and function in the brain.

Overview of the molecular mechanisms by which astrocytes control synaptic connectivity

4-1. Astrocytic molecules control excitatory synapse *in vivo*

Several recent studies have highlighted new molecular mechanisms of astrocyte-neuron communication to generate excitatory synapses (Allen and Eroglu, 2017; Baldwin and Eroglu, 2017; Stogsdill and Eroglu, 2017; Takano et al., 2020). Remarkably, some of these studies have described a role for astrocytes in directing the formation of specific types of synaptic connections to build different circuits. Based on our tripartite synaptic cleft proteomes, using astrocytic TurboID-surface and Split-TurboID, several extracellular proteins likely play synaptogenic roles at astrocyte contact sites were identified. These included Hevin (SPARC-like 1), Cacna2d3 (α2δ-3), Semaphorin 7A (Sema 7A), Plexin A4, Erythropoietin producing hepatocellular receptor tyrosine kinase B2 (EphB2), Neurexin

I (NRX1), and Neuroligin 3 (NL3); all crucial regulators of astrocyte-neuron signaling communication for excitatory synapse formation and function *in vitro* and *in vivo* (Allen and Eroglu, 2017; Baldwin and Eroglu, 2017; Stogsdill and Eroglu, 2017; Takano et al., 2020). For example, secreted protein Hevin, and its homolog protein SPARC, are highly expressed in astrocytes of the cortex during the synaptogenesis (Kucukdereli et al., 2011; Risher et al., 2014). Purified Hevin from astrocyte conditioned media (ACM) promotes synapse formation, resulting in structurally normal but postsynaptically silent excitatory synapses in cultured retinal ganglion cells (RGCs) (Kucukdereli et al., 2011). Mechanistically, the astrocytic secreted Hevin binds to presynaptic NRX1α and postsynaptic NL1B (Fig. 2) (Kucukdereli et al., 2011; Singh et al., 2016). In contrast, SPARC doesn't enhance synapse formation but antagonizes Hevin-induced synapse formation (Kucukdereli et al., 2011). Consistently, Hevin knockout in mouse impairs synapse formation in the thalamocortical circuit that results from the morphological immature dendritic spine (Kucukdereli et al., 2011; Risher et al., 2014).

Cacna2d3/α2δ-3 (Calcium channel, voltage-dependent, alpha 2/delta subunit 3) is an L-type calcium channel voltage-dependent subunit. Aberrant α2δ subunit expression has been implicated in the pathogenesis of several syndromes and diseases, such as chronic neuropathic pain, autism spectrum disorder, and epilepsy (Geisler et al., 2015; Vergult et al., 2015; Zamponi et al., 2015). Among of α2δ subunits, α2δ-1 and α2δ-3 are highly expressed in the cerebral cortex and hippocampus (Cole et al., 2005; Klugbauer et al., 1999; Schlick et al., 2010). α2δ-1 promotes excitatory synapse formation *in vivo* through the interaction with astrocytic secreted factor thrombospondin (TSP) (Fig. 2) (Eroglu et al., 2009). α2δ-1 is also a receptor for Gabapentin, which is a drug used to treat epilepsy and neuropathic pain. Gabapentin antagonizes the interaction of α2δ-1 with TSP and inhibits excitatory synapse formation (Eroglu et al., 2009). It has recently been reported that the expression of α2δ-3 increases the excitatory and inhibitory synaptic density and facilitates spontaneous GABA release in cultured neurons (Bikbaev et al., 2020). Interestingly, α2δ-3 knockout mice exhibit anxiety-like behavior and auditory deficits (Landmann et al., 2019). These findings demonstrated that α2δ-1 and α2δ-3 might have distinct roles in the formation of specific synaptic connections *in vivo*, and further studies are needed to elucidate that how astrocytes regulate α2δ-3 for generating excitatory and inhibitory synapses *in vivo*.

Semaphorins (Semas) and their receptors, Plexins, form a protein complex with Neuroplins (Nrps) that are known to regulate many different developmental steps, including axon and dendrite outgrowth, neuronal migration, and synapse formation (Takano et al., 2019; Takano et al., 2015). In the spinal cord, Sema3A is highly expressed in astrocytes, and this astrocytic Sema3A controls proper motor neuron and sensory neurocircuit organization (Molofsky et al., 2014). The deletion of astrocytic Sema3A decreases excitatory and increases inhibitory inputs *in vivo*, indicating that astrocytic Sema3A regulates the motor neuron firing properties (Molofsky et al., 2014). Consistent with these data, the Sema3A receptor Plexin A4 was detected by our tripartite synaptic cleft proteome from the cerebral cortex (Takano et al., 2020). Sema3A-Plexin A4 signaling between astrocytes and neurons may be involved in excitatory synaptic connection and function in the brain (Fig. 2). In addition to Sema3A, Sema7A is highly expressed in astrocytes and is involved in the formation of glial scars after spinal cord injury (Eixarch et al., 2013; Kopp et al., 2010).

In olfactory sensory neurons, the deletion of Sema7A perturbs activity-dependent synapse formation (Inoue et al., 2018). However, the role of astrocytic Sema7A in modulating synaptic connections and their functions remains to be determined.

Eph receptors (EphA and EphB) and their ligand ephrin have a critical role in contact-dependent astrocyte-neuron communication to generate synapses (Carmona et al., 2009; Nguyen et al., 2020; Nishida and Okabe, 2007). Ephrin-A3 is expressed in hippocampal neurons and ephrin-A3 is particularly enriched on fine astrocytic processes (Carmona et al., 2009). Ephrin-A3 knockout mice have abnormal dendritic spines and impaired the acquisition of contextual fear memory, suggesting astrocytic ephrin-A3 controls proper spine formation important for behavior *in vivo* (Carmona et al., 2009). Interestingly, ephrin-A3 reverse signaling to astrocytes from neuronal synapses regulates the glutamate uptake through the glutamate transporters GLAST (EAAT1) and GLT1 (EAAT2) (Carmona et al., 2009). These results indicate that astrocytes and neurons utilize bidirectional signaling through EphA4 and ephrin-A3 at the tripartite synaptic sites. More recently, it was reported that astrocyte-specific ephrin-B1 knockout mice exhibit an increase of excitatory synapses and elevated excitation in CA1 pyramidal neurons that is responsible for imbalanced excitatory and inhibitory synaptic activity and associated with impaired sociability. These data indicate that astrocytic ephrin-B1 negatively regulates excitatory synapse formation and function *in vivo* (Koeppen et al., 2018; Nguyen et al., 2020).

Recent cell-type-specific RNAseq reveals that the NL1-3 family is highly expressed in astrocytes in addition to neurons (Sakers and Eroglu, 2019; Stogsdill et al., 2017). The individual knockdown of astrocytic NL1-3 impaired astrocytic morphogenesis *in vitro* and *in vivo* (Stogsdill et al., 2017). Interestingly, the knockdown of neuronal NRX1/2 also prevents astrocytic morphogenesis, indicating that astrocyte-neuron communication through the trans-synaptic interactions of astrocytic NLs with neuronal NRXs is essential for astrocytic development *in vivo* (Scheiffele et al., 2000; Stogsdill et al., 2017). Notably, each astrocytic NL isoform might have different roles for brain development. For example, the loss of astrocytic NL2 prevented astrocytic morphogenesis at both early and late developmental stages, while the loss of astrocytic NL1 and NL3 impaired the astrocytic complexity of only early or late developmental stages, respectively (Stogsdill et al., 2017). Furthermore, the astrocyte-specific NL2 knockout mice display an impairment of excitatory synapse formation and function in the cortex (Stogsdill et al., 2017). Thus, astrocytic NL and neuronal NRX complexes play a critical role in bidirectional signaling between astrocyte and neuronal synapses that are responsible for the astrocytic morphogenesis and excitatory synapse formation during development.

4-2. Astrocyte control inhibitory synapse *in vivo*

In addition to excitatory synapse formation and function, the astrocyte has a critical role in establishing GABAergic inhibitory synapses (Elmariah et al., 2005). However, the molecular mechanism by which astrocytes control inhibitory synapse formation and function has remained less understood than their excitatory counterparts and it is an ongoing research field. It has been reported that astrocytic-derived transforming growth factor beta 1 (TGF-β1) is involved in inhibitory synapse formation and function (Diniz et al., 2014). TGF-β1

induces inhibitory synapse formation through the Ca^{2+}/calmodulin-dependent kinase II (CaMKII)-mediated clustering of NL2 at the inhibitory postsynaptic terminal *in vitro* and *in vivo* (Diniz et al., 2014). Interestingly, astrocytic TGF-β1 also induces excitatory synapse formation that involves neuronal activity and secretion of the NMDA co-agonist D-serine (Diniz et al., 2014). However, the molecular mechanisms that modulate or signal astrocytic TGF-β1 release remain unclear.

Both synaptic proteomes, using astrocyte-neuron Split-TurboID and inhibitory postsynaptic proteome using gephyrin-BirA, identified the neuronal cell adhesion molecular (NRCAM), and our later Split-TurboID study demonstrated it is a key regulator of astrocytic morphogenesis and inhibitory synaptogenesis (Takano et al., 2020; Uezu et al., 2016). Multiple human genetic analyses indicate that the *NRCAM* gene may be associated with autism spectrum disorders (Bonora et al., 2005; Marui et al., 2009; Sakurai et al., 2006), yet most studies have focused on its role in neurons and have reported that NRCAM controls spine formation and axon guidance (Demyanenko et al., 2014; Mohan et al., 2019). Despite this, the expression level of NRCAM in astrocytes is higher than that of neurons, and the endogenous NRCAM is highly enriched at the astrocytic plasma membrane *in vivo* (Takano et al., 2020; Zhang et al., 2014; Zhang et al., 2016). Interestingly, the deletion of astrocytic NRCAM stimulates astrocytic expansion of territory and infiltration during brain development, indicating that NRCAM is a unique negative regulator of astrocytic outgrowth (Takano et al., 2020). Notably, the regulation of astrocytic morphogenesis by NRCAM appears to require homophilic binding to neuronal NRCAM. What might be the astrocyte signaling pathway downstream of NRCAM? It is reported that neuronal NRCAM limits spine development through the interaction with the Npn-2/PlexinA3 complex that stimulates Semaphorin signaling pathway (Demyanenko et al., 2014; Mohan et al., 2019). This signaling pathway suppresses small GTPase Rap1 and Rac1 activity that is required for the clustering of a cell adhesion molecule β1 integrin and cytoskeletal reorganization (Mohan et al., 2019; Takano et al., 2019; Takano et al., 2015). Thus, the astrocytic NRCAM may activate the Npn-2/Rap1/Rac1 pathway through neuronal NRCAM to establish a large number of astrocytic peri-synaptic processes. Further exploration of the extracellular and intracellular signaling pathways regulating astrocytic morphogenesis is a crucial issue in the brain developmental research field.

NRCAM plays a critical role in inhibitory synapse formation and function in the cerebral cortex (Fig. 2) (Takano et al., 2020). NRCAM-expressed in HEK293 cells induce GABAergic synapses but not glutamatergic synapses of the cultured neurons (Takano et al., 2020). In the cerebral cortex, the depletion of astrocytic NRCAM significantly impairs astrocyte interactions with inhibitory synapses and thereby leads to a decrease in the number of inhibitory synapses, but not excitatory synapses. This loss of NRCAM also results in significant inhibitory synaptic transmission deficits, with minor effects on glutamatergic responses. Mechanistically, astrocytic NRCAM transcellular binds to neuronal NRCAM that is coupled to gephyrin at inhibitory postsynapses (Fig. 2). Thus, astrocytes directly control inhibitory synapse formation and function through the NRCAM-dependent astrocyte-neuron contact (Fig. 2) (Takano et al., 2020). Together, astrocytes control both excitatory and inhibitory synaptic connectivity and establish proper neurocircuit formation and synaptic function in the brain. Furthermore, our novel TurboID-surface- and Split-TurboID-based

proteomic profiling establishes a framework to fully elucidate the molecular mechanisms underlying tripartite synaptic formation and function *in vivo* (Takano et al., 2020).

Perspectives

Since the cell-surface proteins are typically present at low abundance and have high hydrophobicity and heterogeneity (Kuhlmann et al., 2018; Li et al., 2019), dissecting the molecular composition of specific cell-cell connections from the brain remains a major challenge in the neuroscience field. The development of novel *in vivo* cell-surface BioID approaches, TurboID-surface and Split-TurboID (Fig. 1), has provided the first insights into the specific extracellular molecular landscape and cell-surface interactome of the tripartite synapse *in vivo* (Fig. 2) (Takano et al., 2020). However, TurboID-surface and Split-TurboID may have a few potential limitations. The enzyme activity of all BirA variants requires ATP, which is only found at low amounts extracellularly *in vivo.* In addition, these approaches need the administration of exogenous biotin for 7 days to obtain enough biotinylation labeling (Spence et al., 2019; Uezu et al., 2016). Thus, a further challenge is the need to improve the temporal resolution of these approaches. Nevertheless, these approaches revealed a large number of uncharacterized molecules at the tripartite synaptic cleft in the brain. Interestingly, emerging evidence shows that astrocytes are highly heterogeneous in the brain and may control specific types of synaptic connections to establish different neurocircuits (Allen and Eroglu, 2017; Baldwin and Eroglu, 2017; Sakers and Eroglu, 2019; Stogsdill and Eroglu, 2017). Furthermore, recent genome-wide and imaging studies demonstrate that many proteins that are tightly linked to neurological diseases such as autism and schizophrenia are highly expressed in astrocytes in addition to neurons (Foo et al., 2011; Srinivasan et al., 2016; Zhang et al., 2014; Zhang et al., 2016). However, the majority of studies on these proteins view their neuronal loss of function as the primary cause of synaptic abnormalities seen in neurological diseases. Yet clearly, astrocytes play key roles in organizing synaptogenesis and physiology. An important issue for the field is how defects in the astrocytic compartment of tripartite synapses may cause neurological diseases that result from synaptic disassembly and dysfunction. Our tripartite synaptic cleft proteome provides insight into how astrocytes control neurocircuit connectivity and brain functions and how astrocyte-neuron signaling could be altered in neurological diseases. Future challenges in astrocyte biology will entail exploring these issues using advances in imaging technology, genetic models, and innovative experimental approaches *in vivo* (Yu et al., 2020). It can be expected that these cutting-edge approaches to study tripartite synaptic connectivity will provide new paradigms for how neurocircuits develop in normal and maladaptive states, and shed light on therapeutic strategies for neurological diseases.

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Highlights

- Cell-type-specific proximity-based labeling TurboID-surface enables to map and identify the cell surface proteins from brain tissue.
- A novel proteomically approach Split-TurboID enables the mapping and molecular profiling at specific cell-type interfaces *in vivo*.
- Astrocytes control excitatory and inhibitory synapse formation and function *in vivo*.

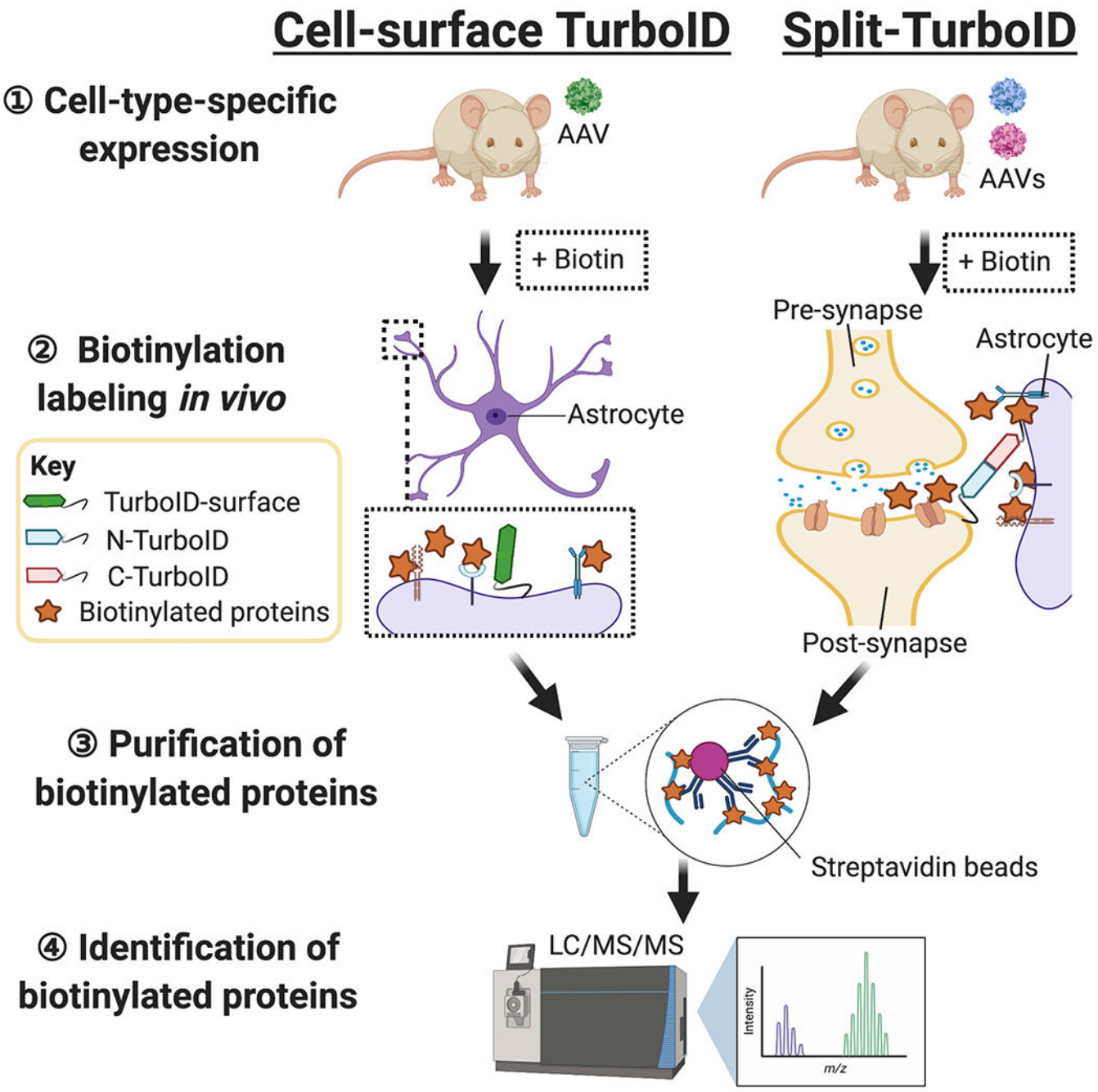

Fig. 1.

Outline of cell-surface TurboID and Split-TurboID-based proteomics *in vivo.* 1) Cell-surface TurboID or Split-TurboID is delivered to the brain using cell-type-specific AAVs. 2) After the expression of TurboID probes, mice are given subcutaneous injections of biotin (24 mg/kg) for 7 days to induce proximity-dependent biotinylation labeling at cell-type-specific cell-surface or cell-cell contact sites *in vivo*. 3) The biotinylated proteins are purified by affinity-isolation using streptavidin-coupled beads. 4) The bound biotinylated proteins are subjected to liquid chromatography-tandem mass spectrometry (LC/MS/MS) to discover the molecular networks.

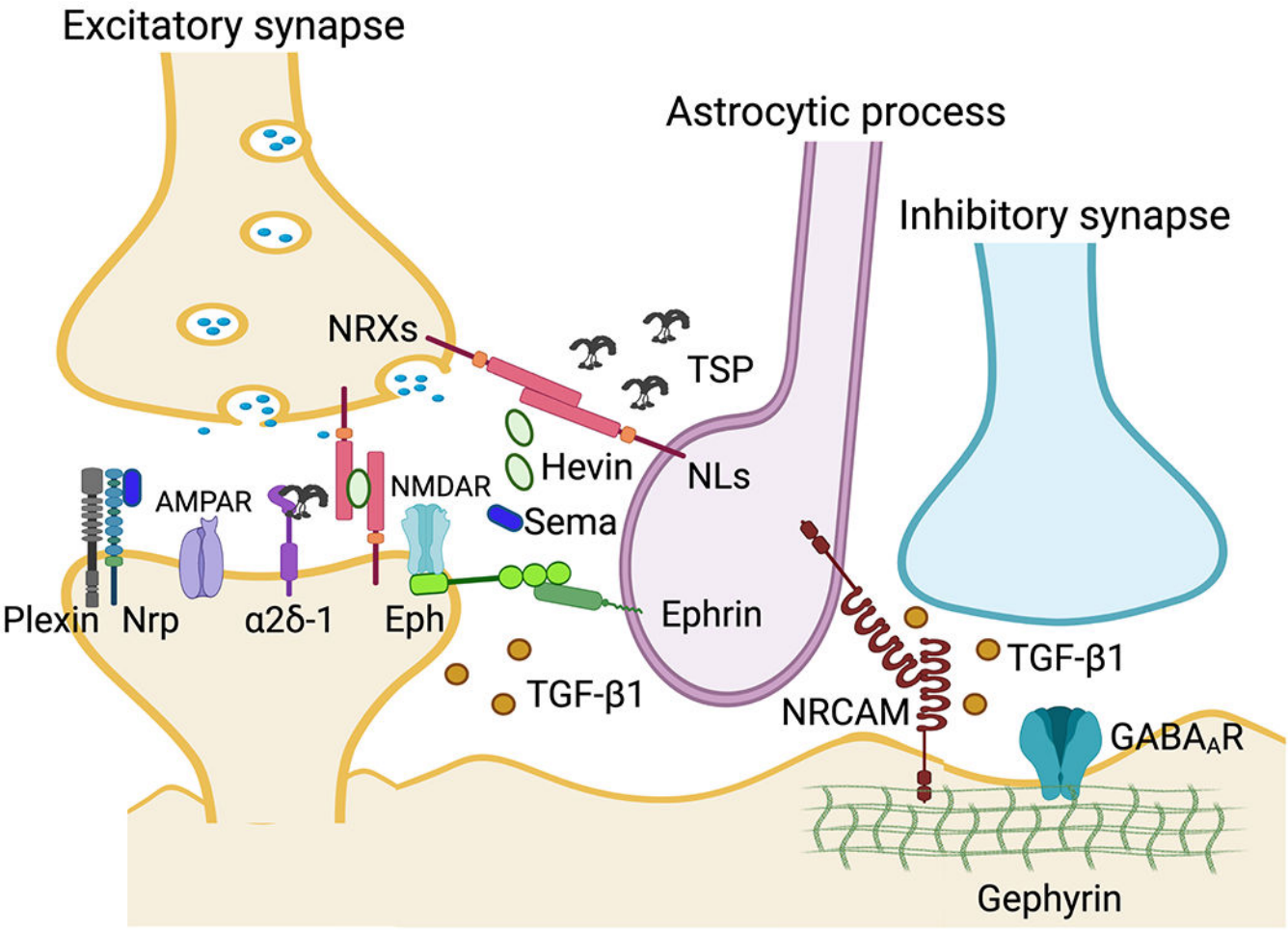

Fig. 2.

Overview of the signal networks that astrocytes-regulated excitatory and inhibitory synapse formation. Astrocytic secreted proteins thrombospondins (TSP) interacts with voltage-dependent calcium channel subunit α2δ-1 (α2δ-1), thereby leads to excitatory synapse formation. Astrocytic Neuroligin 2 (NL2) controls synaptogenesis through neuronal neurexins (NRXs) during development. The NL2-NRXs complex also regulates astrocytic morphogenesis. Astrocytic secreted protein Hevin promotes synapse formation through its interactions with NRX1α and NL1B. Semaphorin 3A (Sema 3A)/Plexin A4/Neuroplins (Nrps) signaling between astrocyte and neuron in the cortex may be involved in excitatory synaptic connection and function. Eph receptors (EphA and EphB) and its ligand ephrin lead to contact-dependent astrocyte-neuron communication to generate synapse. Astrocyte-secreted TGF-β1 induces excitatory synapse formation. Astrocytic cell adhesion protein NRCAM interacts with neuronal NRCAM that is coupled to gephyrin at inhibitory postsynapse, thereby leads to inhibitory synapse formation *in vivo*.