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Review Article

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Optogenetic and Chemogenetic Approaches for Studying Astrocytes and Gliotransmitters

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The brain consists of heterogeneous populations of neuronal and non-neuronal cells. The revelation of their connections and interactions is fundamental to understanding normal brain functions as well as abnormal changes in pathological conditions. Optogenetics and chemogenetics have been developed to allow functional manipulations both *in vitro* and *in vivo* to examine causal relationships between cellular changes and functional outcomes. These techniques are based on genetically encoded effector molecules that respond exclusively to exogenous stimuli, such as a certain wavelength of light or a synthetic ligand. Activation of effector molecules provokes diverse intracellular changes, such as an influx or efflux of ions, depolarization or hyperpolarization of membranes, and activation of intracellular signaling cascades. Optogenetics and chemogenetics have been applied mainly to the study of neuronal circuits, but their use in studying non-neuronal cells has been gradually increasing. Here we introduce recent studies that have employed optogenetics and chemogenetics to reveal the function of astrocytes and gliotransmitters.

Key words: optogenetics, chemogenetics, astrocytes, channelrhodopsin, DREADD, gliotransmitter

INTRODUCTION

Astrocytes are the most abundant population of non-neuronal cells in the brain. It is well known that they provide structural and metabolic support for neuronal networks, but a growing body of evidence indicates that they also play an active role in modulating neuronal activity. Astrocytes make close contact with perisynaptic regions, forming a functional structure called the "tripartite synapse," together with presynaptic and postsynaptic nerve terminals [1-3]. Indeed, one astrocyte in the hippocampus makes contact with tens of thousands of synapses [4].

It is well established that astrocytes clear away excessive neurotransmitters and ions released from synaptic clefts through uptake via specific transporters and channels. For example, astrocytes remove excess extracellular glutamate using sodium-dependent glutamate transporters, such as the glutamate aspartate transporter and glutamate type 1 transporter [5-9]. Excessive glutamate is cytotoxic to neurons, causing an influx of calcium that far exceeds physiological levels and triggering the activation of enzymes and signaling proteins that are detrimental to neurons [10-12].

Evidence from a number of studies indicates that astrocytes release signaling molecules, the so-called "gliotransmitters," such as glutamate, GABA, D-serine, and ATP, into the extracellular milieu in response to extracellular stimuli (Fig. 1) [13-18]. Gliotransmitter release often results from the activation of G protein-coupled receptors (GPCRs) that trigger downstream signaling cascades in astrocytes, including phospholipase C, adenylate cyclase, inositol 1,4,5-trisphosphate (IP_3), and cause an intracellular calcium increase [19-21]. Gliotransmitters facilitate or inhibit the

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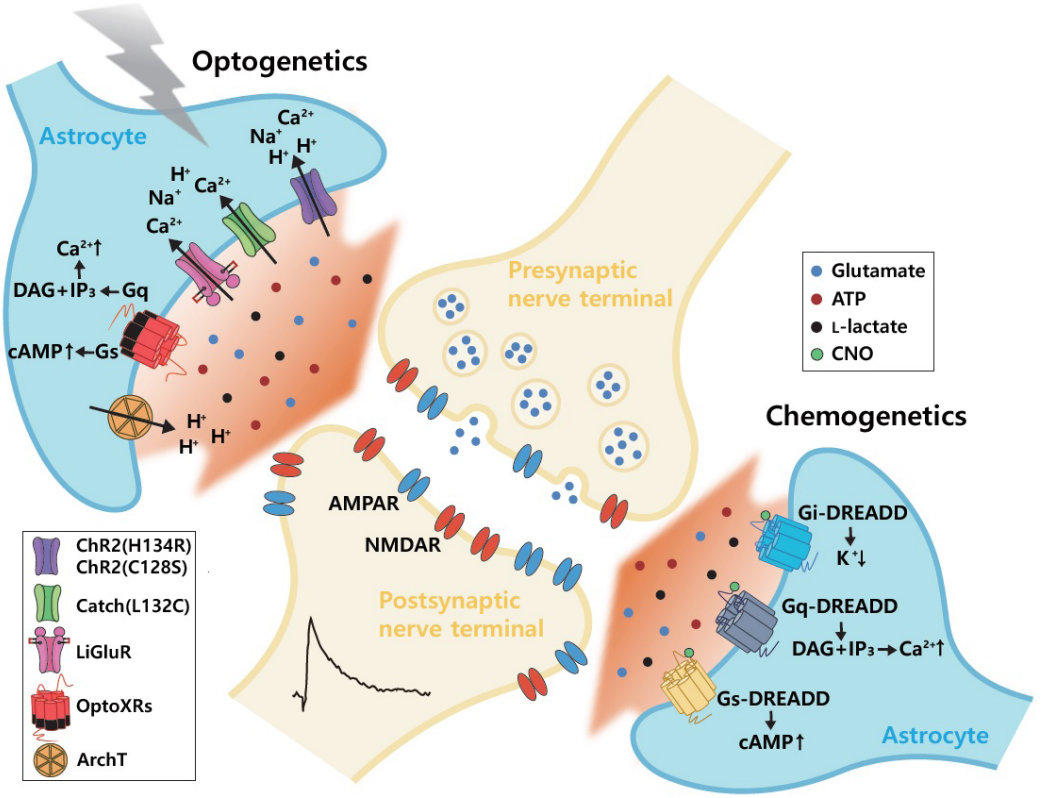

Fig. 1. Optogenetic and chemogenetic stimulation of astrocytes. A variety of genetically encoded effector molecules for optogenetics (left) and chemogenetics (right) have been employed to manipulate intracellular ionic concentrations (H^+, Na^+, Ca^{2+}, K^+) and signaling cascades (Gq, Gs, DAG, IP_3, cAMP) in astrocytes. Intracellular changes such as cytosolic calcium increase and acidification, in turn, evoke release of signaling molecules, so-called gliotransmitters (glutamate, ATP, L-lactate), from astrocytes, which modulate excitability as well as synaptic transmission of neighboring neurons. Optogenetic effectors can be activated by specific wavelengths of photostimulation, and chemogenetic effectors can be activated by synthetic ligands, such as CNO. ChR2, channelrhodopsin-2; CatCh, calcium translocating channelrhodopsin; LiGluR, light-gated ionotropic glutamate receptor 6; ArchT, archaerhodopsin; OptoXRs, light-driven chimeric G protein-coupled receptors; NMDAR, N-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Gi-DREADD, Gi-coupled designer receptors exclusively activated by designer drugs; Gq-DREADD, Gq-coupled DREADD; Gs-DREADD, Gs-coupled DREADD; CNO, clozapine-N-oxide; ATP, adenosine triphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; cAMP, cyclic adenosine monophosphate.

excitability and synaptic transmission of neighboring neurons, and the outcome of their release is dependent on the site of action and types of activated receptors [3, 13, 14, 22-24]. Through the use of volume-sensitive organic anion channels, gap junction hemichannels, P2X7, Bestrophin-1, and reverse-orientation plasma membrane glutamate transporters, diverse mechanisms for gliotransmitter release have been identified in astrocytes, including calcium-dependent exocytotic vesicular release as well as non-exocytotic mechanisms [25-39]. Although accumulating evidence suggests a coupling between various intracellular changes in astrocytes, such as intracellular calcium increase and gliotransmitter release, there is also evidence against this view; thus, the mechanisms underlying astrocyte-neuronal communication are still debated [40-45]. In this review, we present recent studies that have using optogenetic and chemogenetic approaches to explore the function of astrocytes and gliotransmitters.

OPTOGENETICS AND CHEMOGENETICS

Optogenetics is a novel biological technique based on a variety of light-sensitive proteins called opsins, which include microbial ion channels and ion pumps as well as engineered GPCRs (Fig. 1) [46, 47]. Following absorption of a specific wavelength of light, an opsin undergoes a conformational change that triggers diverse cellular changes in opsin-expressing cells. Some of the opsins induce the translocation of ions, and others activate intracellular signaling cascades, such as G protein-mediated signaling. Since most of these opsins do not exist in experimental-model organisms, and photostimulation itself has a negligible effect on cells and tissues, optogenetics has instead been used as a powerful

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experimental tool to manipulate specific populations of cells both *in vitro* and *in vivo* by means of a combinatorial approach of cell type-specific promoters and additional genetic tricks. This technique has also enabled the manipulation of cellular activity with millisecond-scale temporal precision [46]. The time-resolved stimulation has made possible the revelation of causal relationships between manipulated cellular activity and functional outcomes, particularly in the study of neuronal circuits mediating specific behaviors.

Opsins have been modified to generate mutants and chimeric proteins with diverse features, including their intracellular effects, optimal wavelengths of light for activation, and temporal dynamics in activation and inactivation; thus, they offer great flexibility in designing experiments and conducting more refined manipulations [47, 48].

Channelrhodopsin-2 (ChR2), originally identified in green algae, is a cation channel that becomes permeable to positively charged ions such as proton and sodium when it is stimulated with blue light [49-51]. When it is expressed in neurons, photostimulation elicits an influx of cations, which causes depolarization and the firing of action potentials in the stimulated cells [46]. An influx of protons though ChR2 can also acidify the cytosolic compartment of photostimulated cells [51, 52]. In the study of neurons, the frequency and duration of neuronal spiking can be easily controlled using variants of ChR2, such as ChR2(H134R), ChR2(C128S), ChETA, and step function opsin (SFO) [53-57]. For example, ChETA can drive ultrafast spiking up to 200 Hz, and SFO can elicit prolonged, bi-stable, sub-threshold depolarization of membranes [55, 56]. Some light-gated cation channels, such as calcium-translocating channelrhodopsin (CatCh) and LiGluR, are more permeable to calcium than ChR2, and therefore they have been preferentially used in studies exploring the role of intracellular calcium [58-61]. LiGluR is a mutated ionotropic glutamate receptor 6 containing its ligand attached to an optically switchable tether called maleimide-azobenzene-glutamate [61, 62].

Halorhodopsin is an opsin identified from Archaea which, when stimulated with yellow light, pumps chloride ions into cells [63-69]. When halorhodopsin is expressed in neurons, photostimulation promotes an influx of chloride ions that results in hyperpolarization and the inhibition of the firing of action potentials in the stimulated cells. Archaerhodopsins, such as Arch and ArchT, are light-driven outward proton pumps that inhibit the firing of action potentials during photostimulation when they are expressed in neurons; the efflux of protons can also cause alkalization of the cytosol [70-72]. Finally, optoXRs, such as optoα1AR and optoβ2AR, are chimeric GPCRs in which the intracellular loops of rhodopsin are replaced with those of other GPCRs, such as adrenergic receptors and dopamine receptors [73, 74]. Photostimulation can initiate diverse intracellular signaling cascades in target cells, depending on the type of G protein replacing the intracellular loops of rhodopsin. Thus, these opsins enable the acute activation of different GPCR signaling pathways in cultured cells and animals.

Chemogenetics is based on engineered proteins, such as GPCRs and ligand-gated ion channels, that are no longer responsive or only very weakly responsive to their endogenous ligands but strongly respond to synthetic chemical ligands that are otherwise biologically inert [75-77]. For example, hM3Dq, one of the designer receptors exclusively activated by designer drugs (DREADDs), is generated by multiple cycles of randomized mutagenesis of the human M3 muscarinic receptor, which is linked to the Gq protein [78]. It is neither sensitive to the endogenous muscarinic acetylcholine receptor ligand acetylcholine nor is it constitutively active, but it is strongly activated in response to a synthetic ligand, clozapine-N-oxide (CNO), with nanomolar potency [75, 76]. In response to CNO, hM3Dq can induce an enhancement of neuronal excitability that can lead to burst-like firing [79-83]. Thus, it is one of the most frequently used chemogenetic tools to activate neurons.

Another DREADD molecule, hM4Di, is a mutant of the Gi-coupled human M4 muscarinic receptor that responds to CNO [75, 80, 84]. Upon an application of the chemical agonist, hM4Di activates the Gβγ subunit of the Gi protein, which then stimulates G protein inwardly rectifying potassium channels (GIRK), causing an efflux of potassium and a resulting robust hyperpolarization when it is expressed in neurons [85, 86]. Thus, hM4Di has been used to silence spontaneous and depolarization-evoked neuronal firing [75].

Optogenetics and chemogenetics require the expression of genetically encoded effectors in target cells. This goal is often attained by injecting a virus (e.g., adeno-associated virus (AAV) or lentivirus) that encodes an effector into a target region in the brain or other tissue. Alternatively, the effector can be expressed as a transgene in a genetically engineered mouse line. By using cell type-specific promoters, such as the astrocyte-specific glial fibrillary acid protein (GFAP) promoter, the effector's expression can be restricted to a specific population (or more than one population) of cells [87-89]. An intersectional strategy based on a combination of specific promoters and genetic tools, such as Cre- and flippase-mediated recombination, can further restrict the effector expression to specific subpopulations [90, 91]. In addition, other genetic tricks, such as the use of tetracycline-dependent transcriptional regulation, have been used to achieve temporal

control as well as amplification of effector expression [42, 92, 93].

Optogenetics and chemogenetics have different features that make them well suited for different applications. Optogenetics can deliver photostimulation directly to target cells and manipulate cellular activity acutely and reversibly. In contrast, chemogenetics is ideal for a prolonged manipulation of cellular activity in the range of minutes to days, depending on the route of ligand delivery and the pharmacokinetic properties of the synthetic ligand(s) used. Optogenetics is excellent in generating spiking patterns that mimic the endogenous firing responses of neurons by using a pulse generator that produces lights with different frequencies and pulse durations. In addition, photostimulation can be delivered to different subcellular locations such as the soma and nerve terminals, a useful feature for studying neuronal circuits in the brain [47]. On the other hand, chemogenetics is less invasive in experimental animals and hampers animal behaviors only marginally, if at all, because it requires neither the installation of a fiber-optic cable within the brain nor a connection of the cable to a light source, such as a laser or a light-emitting diode (LED). Furthermore, some synthetic ligands for chemogenetics, such as CNO, can be delivered via the animal's water and/or food as well as by systemic injection, permitting the delivery of the ligand with minimal disturbance of the animals, particularly in the case of chronic manipulation [94-96]. This minimal invasiveness can be a strong advantage in the clinical application of chemogenetics.

Optogenetic and chemogenetic techniques have been most frequently used to investigate neuronal circuits, but they also have been used to study non-neuronal cells in the brain and peripheral tissues. In the following section, we will summarize the approaches and findings of recent studies that have employed these techniques to reveal the function of astrocytes (Table 1 and 2).

OPTOGENETIC STIMULATION OF CULTURED ASTROCYTES

Studies using primary astrocytes and immortal astrocyte cell lines have shown that optogenetic stimulation can elicit an elevation of intracellular calcium and subsequent release of gliotransmitters that can activate adjacent astrocytes as well as neurons. For example, Berlinguer-Palamini et al. have reported that photostimulation of ChR2-expressing primary astrocytes using a LED can elicit an intracellular calcium increase and electrophysiological responses not only in the stimulated cells but also in co-cultured astrocytes and neurons that do not express ChR2 [97]. The calcium response in ChR2-negative cells is suppressed by the application of antagonists of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the bath solution, suggesting that the response in ChR2-negative cells is mediated by glutamate released from ChR2-expressing astrocytes.

Similar results have been obtained in another study, in which the coupling has been demonstrated between an intracellular calcium increase in astrocytes and glutamate release [98]. Li et al. have performed fluorescent calcium imaging in primary cultures of mouse cortical astrocytes and shown that photostimulation of astrocytes expressing LiGluR can elicit calcium transients not only in stimulated LiGluR-expressing cells but also in neighboring astrocytes that do not express the opsin. The calcium response in LiGluR-negative astrocytes is affected by antagonists of glutamate receptors, but not by a gap junction blocker or an antagonist of extracellular ATP signaling, suggesting the involvement of glutamate in the communication between astrocytes. Further experiments have shown that the calcium transients in LiGluR-negative astrocytes are inhibited by an anion channel blocker but are unaffected by an inhibitor of V-ATPase, which blocks exocytosis, suggesting that LiGluR-evoked glutamate release is mediated by anion channels [42, 99-105].

In an attempt to investigate whether intracellular ionic alteration in astrocytes triggers gliotransmitter release, Ono and coworkers have co-cultured an astrocytic cell line and a neuronal cell line; in response to photostimulation, the ChR2-expressing astrocytes exhibited diverse cellular changes, including an increase in intracellular sodium and calcium, intracellular acidification, glutamate release, and inhibition of proliferation [106]. A short period of photostimulation (for minutes) elicited calcium transients in the co-cultured ChR2-negative neurons, whereas a long period of stimulation for several days resulted in apoptotic responses in the neurons. Both responses were blocked by an AMPA receptor antagonist. Thus, this study has demonstrated that activation of astrocytes releases glutamate which, in turn, provokes an intracellular calcium increase and cytotoxic cell death.

To mimic GPCR-mediated signaling events occurring in astrocytes in response to extracellular neurotransmitters and neuromodulators, Figueiredo et al. have expressed GPCR-based opsins, such as optoα1AR and optoβ2AR, in astrocytes to activate Gq- and Gs-mediated signaling cascades, respectively [107]. Photostimulation elicited an intracellular calcium increase in astrocytes expressing either opsin, which was blocked by apyrase, an enzyme hydrolyzing extracellular ATP, as well as by pharmacological blockers for the corresponding intracellular signaling cascade, such as inhibitors of phospholipase C and adenylate cyclase; these data indicate that a large portion of the calcium rise that was evoked by the activation of either opsin was a result of the autocrine action of extracellular ATP [108]. This study has demonstrated that GPCR-based opsins can be effectively used

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Table 1. A list of previous studies using optogenetics to manipulate astrocytes

Effector	Expression	Promoter	Genetic tool	Delivery	Photostimulation		Released gliotransmitter	Response (nearby neurons)	Reference
					Response (opsin$^+$ cells)	Response (animals)			
CatCh or ChR2(H134R)	Rat cortical astrocytes	GFAP	None	Txn.	Inward current, $[Ca^{2+}]_i\uparrow$	n.d.	Glutamate	sEPSC, $[Ca^{2+}]_i\uparrow$	Ref. 97
CatCh or LiGluR	Mouse neocortical astrocytes	CAG CMV	None	Txn.	$[Ca^{2+}]_i\uparrow$	n.d.	Glutamate	n.d.	Ref. 98
ChR2	GL261 cell line (murine astrocytes)	CMV	None	Txn.	Na^+ & Ca^{2+} influx, $[H^+]_i\uparrow$	n.d.	Glutamate	$[Ca^{2+}]_i\uparrow$, apoptosis	Ref. 106
ChR2, ChR2(H134R), CatCh, optoα1AR or optoβ2AR	Rat cortical astrocytes	GFAP	None	AAV	$[Ca^{2+}]_i\uparrow$	n.d.	ATP (autocrine)	n.d.	Ref. 107
ChR2(C128S)	Cerebellar slices	Mlc1	tTA/tetO	TG mice	Inward current, c-fos ↑	n.d.	n.d.	n.d.	Ref. 110
ChR2(C128S)	Cerebellar slices	Mlc1	tTA/tetO	TG mice	Inward current		Glutamate	Inward current in PC c-fos in PC, SC/BC ↑	Ref. 111
	Cerebellum (*in vivo*)	Mlc1	tTA/tetO	TG mice	n.d.	Perturbation of motor behavior, pupil dilation ↑	n.d.		Ref. 111
ChR2(C128S)	Cerebellar slices	Mlc1	tTA/tetO	TG mice	Inward current, $[H^+]_i\uparrow$	n.d.	Glutamate	Inward current in PC	Ref. 70
ArchT	Cerebellar slices	Mlc1	tTA/tetO	TG mice	Outward current, $[H^+]_i\downarrow$		n.d.	Ischemia-induced current in PC ↓	Ref. 70
	Cerebellum (*in vivo*)	Mlc1	tTA/tetO	TG mice		Ischemic infarction ↓			
ChR2(C128S)	Somatomotor cortex (*in vivo*)	Mlc1	tTA/tetO	TG mice	n.d.	CBF ↑	K^+	n.d.	Ref. 112
ChR2(C128S)	V1 cortical slices, V1 (*in vivo*)	GFAP	None	AAV	$[Ca^{2+}]_i\uparrow$	n.d.	Glutamate	mEPSC ↑ (PV^+ neuron) mixed resp. (SOM^+, excitatory neuron)	Ref. 117
ChR2	Brainstem slices, primary culture	GFAP	None	AAV	$[Ca^{2+}]_i\uparrow$		ATP	Depolarization of RTN neurons	Ref. 16
	VS (*in vivo*)	GFAP	None	AAV	n.d.	Respiratory activity ↑	ATP	n.d.	
ChR2(H134R) or optoβ2AR	Brainstem slices	GFAP	None	AAV	$[H^+]_i\uparrow$	n.d.	L-lactate	Depolarization of LC neurons, NE release↑	Ref. 120
ChR2	STN (*in vivo*)	GFAP	None	lentivirus	n.d.	n.d.	n.d.	Firing of STN neurons ↓	Ref. 124
ChR2(H134R)	Hypothalamus (*in vivo*)	GFAP	None	AAV	n.d.	NREM, REM sleep ↑	n.d.	n.d.	Ref. 125
ChR2(H134R)	ACC (*in vivo*)	GFAP	Cre	AAV	n.d.	Wakefulnees ↑, NREM sleep ↓	n.d.	n.d.	Ref. 126
ArchT	V1 cortical slices V1 (*in vivo*)	GFAP	Cre	AAV	$[Ca^{2+}]_i\uparrow$		Glutamate	Slow oscillation state in V1 neurons ↑, firing rate of V1 neurons ↑	Ref. 127

AAV, adeno-associated virus; ACC, anterior cingulate cortex; ArchT, light-gated outward proton pump; BC, basket cells; ChR2, channelrhodopsin-2; CatCh, calcium translocating channelrhodopsin; CBF, cerebral blood flow; CMV, cytomegalovirus; sEPSC, spontaneous excitatory synaptic transmission; GFAP, glial acidic fibrillary acidic protein; GC, granule cell; GL261, murine astrocyte cell lines; LC, locus coeruleus; LiGluR, light-gated ionotropic glutamate receptor 6; mEPSC, miniature excitatory synaptic transmission; Mlc1, megalencephalic leukoencephalopathy with subcortical cysts 1; NE, norepinephrine; NREM, non-rapid eye movement; optoα1AR, a chimeric receptor combined rhodopsin with α1-adrenoreceptor; optoβ2AR, a chimeric receptor combined rhodopsin with β2-adrenoreceptor; PC, purkinje cells; PV, parvalbumin; REM, rapid eye movement; Resp, response; RTN, retrotrapezoid nucleus; SC, stellate cells; SOM, somatostatin; STN, subthalamic nucleus; tTA, tetracycline-controlled transcriptional activator; tetO, tTA-responsive promoter sequence; TG, transgenic; Txn, transfection; V1, primary visual cortex; VS, ventral surface of the brainstem; n.d., not determined.

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Table 2. A list of previous studies using chemogenetics to manipulate astrocytes

Effector	Expression	Promoter	Genetic tool	Delivery	Stimulation		Released gliotransmitter	Response (nearby neurons)	Reference
					Response (effector$^+$ cells)	Response (animals)			
MrgprA1	Hippocampal slices	GFAP	tTA/tetO	TG mice	$[Ca^{2+}]_i$↑	n.d.	n.d.	No response in CA1 pyramidal neurons	Ref. 42
MrgprA1	Hippocampal slices	GFAP	tTA/tetO	TG mice	sEPSC, $[Ca^{2+}]_i$↑	n.d.	n.d.	No response in CA1 pyramidal neurons	Ref. 40
hM3Dq	V1 (*in vivo*)	GFAP	Cre	AAV	$[Ca^{2+}]_i$↑	No change in CBF	n.d.	n.d.	Ref. 41
MrgprA1	Primary astrocytes	GFAP	tTA/tetO	TG mice	$[Ca^{2+}]_i$↑		ATP	n.d.	Ref. 138
	Right cerebral ventricle, mPFC, hippocampus (*in vivo*)	GFAP	tTA/tetO	TG mice			ATP		
Ro1	Whole animal (GFAP$^+$ cells)	GFAP	tTA/tetO	TG mice	pERK↑	Hydrocephalus	n.d.	n.d.	Ref. 139
hM3Dq	Hippocampal slices	GFAP	None	TG mice	$[Ca^{2+}]_i$↑				
	Whole animal (GFAP$^+$ cells)	GFAP	None	TG mice		Change in ANS-mediated responses and motor behaviors	n.d.	n.d.	Ref. 142
hM3Dq	Intestine	GFAP	tTA/tetO	TG mice	$[Ca^{2+}]_i$↑	Gut contraction ↑			
hM3Dq	Whole animal	GFAP	creERT2	TG mice	n.d.	Hypothermia	n.d.	n.d.	Ref. 143
Rs1	Whole animal, primary astrocytes	GFAP	tTA/tetO	TG mice, lentivirus	cAMP↑, pERK↑, pCREB↑	Memory function ↓	n.d.	n.d.	Ref. 144
							n.d.	n.d.	Ref. 145
hM3Dq	Arcuate nucleus (*in vivo*)	GFAP	None	AAV	c-fos↑	Ghrelin-evoked feeding ↓	Adenosine		
						Leptin-induced anorexia↑		n.d.	Ref. 147
	Hypothalamic slices	GFAP	None	AAV	n.d.		Adenosine	Firing of AgRP$^+$ neurons ↓	Ref. 147
hM4Di	Arcuate nucleus (*in vivo*)	GFAP	None	AAV	no change in c-fos	Ghrelin-evoked feeding ↑	n.d.	n.d.	Ref. 147
						Leptin-induced anorexia ↓			
hM3Dq	Primary astrocytes	GFAP	None	AAV	$[Ca^{2+}]_i$↑		n.d.	n.d.	Ref. 154
	NAcore (*in vivo*)	GFAP	None	AAV	$[Ca^{2+}]_i$↑	Ethanol seeking ↓			
						Intracranial self-stimulation ↑			
hM3Dq	NAcore (*in vivo*)	GFAP	None	AAV	n.d.	Cue-induced reinstatement of cocaine seeking ↓	Glutamate	n.d.	Ref. 155

AAV, adeno-associated virus; ANS, autonomic nervous system; AgRP, agouti-related protein; CA1, cornu ammonis area 1; CBF, cerebral blood flow; cAMP, cyclic adenosine monophosphate; pCREB, phosphorylated cAMP response element binding protein; creERT2, tamoxifen-inducible cre; sEPSC, spontaneous excitatory synaptic transmission; pERK, phosphorylated extracellular signal-regulated kinase; GFAP, glial acidic fibrillary acidic protein; mPFC, medial prefrontal cortex; MrgprA1, mas-related G protein-coupled receptor A1; NAcore, nucleus accumbens core; tTA, tetracycline-controlled transcriptional activator; tetO, tTA-responsive promoter sequence; TG, transgenic; V1, primary visual cortex; n.d., not determined.

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in the study of astrocytic GPCR-mediated signaling.

OPTOGENETIC STIMULATION OF ASTROCYTES USING TRANSGENIC MICE

Tetracycline-dependent expression, using the so-called Tet-Off system, has been employed in several studies to generate transgenic mouse models expressing an opsin. In the Tet-Off system, the tetracycline-controlled transcriptional activator (tTA) binds to a tTA-responsive promoter sequence (tetO) to induce the expression of a downstream gene [109]. When bound to the tetracycline derivative doxycycline, tTA undergoes a conformational change that prevents tTA from binding to tetO, inhibiting the transcription of a target gene. Thus, this system enables a reversible control of gene expression produced by treatment with doxycycline. Tanaka and coworkers have generated a mouse line by knocking in a transgene cassette encoding tetO-driven ChR2(C128S) downstream of a housekeeping gene, β-actin, to obtain a high level expression [110]. The knockin mice have been crossed to tTA driver lines in which tTA is driven by cell type-specific promoters, the Mlc1, PLP, and Iba-1 promoters, to induce ChR2(C128S) expression in astrocytes, oligodendrocytes, and microglia, respectively.

The double-transgenic mice containing Mlc1-driven tTA and tetO-driven ChR2(C128S) have been used to reveal the role of Bergman glia (BG), a specialized subtype of astrocytes in the cerebellum, in modulating the activity of Purkinje neurons. First, photostimulation of acute brain slices prepared from the transgenic mice was found to elicit current responses from ChR2-expressing BG, suggesting that ChR2 is expressed in glial cells to a level sufficient for electrophysiological responses [70, 110, 111]. Second, photostimulation of the cerebellum using a fiber-optic cable installed above the skull, to avoid the generation of injury-induced reactive gliosis, was found to be sufficient to evoke an induction of a surrogate marker for cellular activation, c-fos, in ChR2-expressing BG [110]. Third, photostimulation of ChR2-expressing BG in acute cerebellar slices was shown to be sufficient to trigger glutamate release and firing of nearby Purkinje cells (PCs), resulting in long-term plasticity between parallel fibers and PCs [111]. Finally, *in vivo* photostimulation of glia cells using fiber-optic cable inserted into the cerebellar flocculus was found to cause pupil dilation as well as perturbation of smooth eye pursuit of visual stimuli in head-fixed mice [111].

Beppu et al. have recently demonstrated using the same mouse line that neuronal damage in the mouse model of ischemia can be exacerbated by optogenetically induced acidosis and attenuated by alkalization of the cytosolic compartment of astrocytes [11, 70]. Under ischemic conditions, such as deprivation of oxygen and glucose, cerebellar BG exhibited intracellular acidosis and glutamate release, followed by an inward excitatory current in the surrounding PCs. The response in PCs was inhibited by a cocktail of glutamate receptor and transporter blockers, suggesting the involvement of glutamate in the interaction between BG and PCs. Acidosis induced in BG by optogenetic stimulation of ChR2(C128S) was sufficient to evoke an inward excitatory current in the adjacent PCs. The response in PCs was inhibited by a non-competitive AMPA and kainate receptor antagonist, confirming the involvement of glutamate in the signaling between BG and PC. In contrast, an efflux of proton from BG produced by optogenetic stimulation of a light-gated outward proton pump, ArchT, led to a reduction in the inward currents in the PCs elicited by the deprivation of oxygen and glucose. Furthermore, *in vivo* photostimulation of ArchT-expressing BG caused a substantial reduction in cerebellar infarction following a local thrombosis-caused ischemic stroke, whereas control mice without ArchT activation exhibited severe neuronal death under the same conditions. Taken together, the results of this study have demonstrated that ischemic injury causes glial acidosis, which, in turn, releases glutamate into the extracellular space and causes ischemic neuronal death. However, the mechanism underlying the glutamate release remains to be determined.

Using the same mouse line, Masamoto et al. have demonstrated that *in vivo* optogenetic stimulation of cortical astrocytes elicits a rapid, robust, and widespread increase in cerebral blood flow (CBF) [112]. The increased CBF was abolished by an application of the inward rectifier potassium channel blocker, $BaCl_2$, on the exposed cortex, indicating the importance of potassium signaling in astrocytic modulation of CBF [113-115]. In contrast, the study found that neither astrocytic intracellular calcium signaling nor glutamate release was involved in the increase in CBF.

OPTOGENETIC STIMULATION OF ASTROCYTES EXPRESSING VIRALLY DELIVERED OPSINS

A number of studies have employed virally mediated expression of opsins to manipulate astrocytes, despite the possibility of inducing reactive gliosis as a result of viral infection [116]. This approach appears to be as successful as using transgenic mouse lines. For example, an AAV encoding GFAP promoter-driven ChR2 has been used to reveal a causal relationship between the activity of astrocytes and visual processing in the primary visual cortex (V1) [117]. Although a previous study had shown that astrocytes in the visual cortex respond to visual sensory stimuli, their roles had not been clearly determined because of

the difficulty in selectively manipulating astrocytes among the heterogeneous populations of cells in the region. Perea et al. reported that *in vivo* optogenetic stimulation of astrocytes in the V1 enhanced the spontaneous firing of a population of inhibitory neurons expressing parvalbumin, and this firing was suppressed by treatment with an antagonist of type 1a metabotropic glutamate receptors, suggesting the involvement of glutamate in astrocyte-mediated visual processing. In contrast, optical stimulation of astrocytes had mixed effects in terms of activation and inhibition on excitatory neurons and another population of inhibitory neurons expressing somatostatin. Finally, *in vivo* optogenetic stimulation of astrocytes in the V1 strongly affected the responses of neuronal populations to visual stimuli.

Optogenetic manipulations have revealed the involvement of other signaling molecules released by astrocytes, such as extracellular ATP and L-lactate, in modulating the activity of neurons in the brainstem. Gourine et al. have reported that astrocytes in the ventral surface of the medulla oblongata (VS) are exquisitely pH-sensitive [16]. In response to a decrease in pH in anesthetized rats, astrocytes residing near the VS exhibited an intracellular calcium increase. Furthermore, a decrease in pH in brainstem slices elicited a sustained ATP release in the VS region, as well as extracellular ATP-dependent calcium responses in VS astrocytes. To mimic pH-elicited calcium excitation in astrocytes, an AAV encoding enhanced GFAP promoter-driven ChR2(H134R) was injected into the brainstem. In organotypic brainstem slices, photostimulation elicited not only calcium transients in ChR2-expressing astrocytes but also long-lasting depolarization in adjacent chemo-sensitive neurons in the retrotrapezoid nucleus (RTN). RTN neurons have been found to play an important role in monitoring glucose concentrations, pH, and partial pressure of CO_2 [118, 119]. Either apyrase or an antagonist of extracellular ATP receptor blocked the response of the RTN neurons, suggesting that extracellular ATP mediates the interaction between astrocytes and adjacent neurons. Finally, *in vivo* unilateral optogenetic stimulation of astrocytes in anesthetized, vagotomized, and artificially ventilated rats elicited a robust respiratory activity from hypocapnic apnea and an increase in phrenic nerve amplitude, which was suppressed by an antagonist of the extracellular ATP receptor; these results indicate that astrocytes are critical components of the central respiratory and chemosensory functions, and extracellular ATP is a key molecule in the signaling between astrocytes and neighboring neurons in the RTN.

The same group of researchers has investigated the astrocytic modulation of norepinephrine (NE) release in the locus coeruleus (LC) using an AAV encoding GFAP promoter-driven ChR2(H134R) [120]. Evidence existed to suggest that L-lactate is involved in the process, but the exact mechanism was unclear [121-123]. Photostimulation of ChR2-expressing astrocytes in organotypic cultured brain slices elicited delayed depolarization and increased firing rates in norepinephrine (NE)-ergic neurons. Pharmacological interventions that reduce the level of L-lactate suppressed light-induced depolarization of NEergic neurons, suggesting that astrocytes activate NEergic neurons via L-lactate. Indeed, the application of L-lactate to brain slices provoked similar electrophysiological responses in NEergic neurons and caused NE release from the activated neurons. Finally, optogenetic activation of astrocytes using either optoβ2AR or ChR2(H134R) was sufficient to trigger NE release. Thus, this study clearly demonstrated that activated astrocytes in the LC release L-lactate, which then triggers NE release from NEergic neurons.

A similar viral approach was used by Gradinaru et al. to examine whether local astrocytes contribute to the therapeutic effect of deep-brain stimulation (DBS) delivered to the subthalamic nucleus (STN) to relieve tremor in Parkinson's disease [124]. To deliver photostimulation and measure neuronal activity from a parkinsonian rodent model, optrode recordings were performed in anesthetized rats, in which 6-hydroxydopamine (6-OHDA) had been unilaterally injected into the right medial forebrain bundle to cause a loss of nigral dopaminergic cells. 6-OHDA-treated animals displayed rotations ipsilateral to the lesion as a result of specific deficits in contralateral limb function, which became more obvious when amphetamine was administered to the subjects to increase locomotion. This study revealed that photostimulation of ChR2-expressing astrocytes in the STN can reversibly inhibit firing of STN neurons in 6-OHDA-treated animals; this treatment, however, failed to cause any changes in pathological motor behavior in parkinsonian rats, suggesting that astrocytes are unlikely to be critical players in the DBS-elicited effects.

Optogenetic manipulation has been used in two recent studies to examine the role of astrocytes in sleep. Pelluru et al. have reported that optogenetic activation of the posterior hypothalamus using ChR2(H134R) promotes both rapid and non-rapid eye movement sleep [125]. On the other hand, Yamashita et al. have reported that *in vivo* optogenetic stimulation of ChR2-expressing astrocytes in the anterior cingulate cortex results in a significant increase in wakefulness as well as disturbance of non-rapid eye movement sleep [126].

In a very recent study reported by Poskanzer and Yuste, the role of neocortical astrocytes in the control of cortical circuit functions was examined using *in vivo* two-photon calcium imaging based on the genetic calcium indicator GCaMP6s, together with electrophysiological recording from cortical neurons [127]. To

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examine the causal relationship between the calcium signaling in astrocytes and neuronal activity in the V1, an AAV encoding Cre-dependent Arch was injected into the V1 of transgenic mice expressing GFAP promoter-driven Cre, which resulted in astrocyte-specific expression of the opsin. When expressed in neurons, Arch hyperpolarizes membrane potentials and inhibits neuronal firing by pumping protons out of neurons in response to yellow-light photostimulation. Surprisingly, photostimulation of Arch in the astrocytes triggered calcium transients that were specifically localized to the processes of astrocytes and largely undetected in the soma. In contrast, neighboring Arch-negative cells failed to exhibit a calcium response during photostimulation. The exact mechanism governing the Arch-mediated calcium increase is unknown. A previous study has reported that Arch-mediated stimulation of cerebellar BG increases the intracellular pH as the result of an efflux of protons out of cells under oxygen-glucose-deprived conditions. In contrast, Poskanzer and Yuste found no significant changes in pH in stimulated astrocytes as well as in surrounding cells in the V1. It is not clear whether this discrepancy is a byproduct of cell-type specificity. Finally, local field potential recordings have revealed that *in vivo* optogenetic stimulation of astrocytes in the V1 results in calcium transients, followed by a brief increase in extracellular glutamate and a shift in neuronal firing patterns in V1 from a desynchronized state to the synchronized slow oscillation-dominated state.

CHEMOGENETIC STIMULATION OF ASTROCYTES IN TRANSGENIC MICE

Among diverse chemogenetic effectors, DREADDs, such as hM3Dq, have been used most frequently in studies focusing on astrocytes. As in optogenetic approaches, astrocyte-specific expression of chemogenetic proteins has been achieved by using viral and transgenic delivery in combination with astrocyte-specific promoters such as the GFAP and Mlc1 promoters.

To manipulate Gq-coupled receptor signaling in astrocytes, Fiacco et al. generated a bi-transgenic mouse line encoding GFAP promoter-driven tTA and tetO promoter-driven Mas-related G protein-coupled receptor A1 (MrgprA1) to express GPCR selectively in astrocytes [42, 128]. MrgprA1 can be activated by RF amides, such as a peptide Phe-Leu-Arg-Phe amide (FLRFα) [129]. Since endogenous MrgprA1 is specifically expressed in dorsal root ganglion neurons but not in the brain, this protein is a useful molecular tool for manipulating neurons and glia in the brain when it is exogenously expressed in these cells. An infusion of FLRFα into acute hippocampal slices prepared from the transgenic mice elicited a robust calcium increase in widespread astrocytes, suggesting that MrgprA1 is able to activate the Gq-coupled intracellular signaling pathway. It is particularly interesting that such a widespread calcium rise in astrocytes failed to affect neuronal activity in the same slices. This finding is at odds with other studies, questioning the hypothesis that an astrocytic calcium increase causes the release of gliotransmitters which, in turn, activate nearby neurons [20, 130-136]. In a follow-up study using MargprA1 mice together with mice lacking inositol 1,4,5-trisphosphate receptor 2 (IP_3 R2), the astrocyte-specific IP_3 receptor isoform, the same group of researchers further confirmed that activation of Gq protein-coupled signaling affects neither spontaneous excitatory postsynaptic currents nor the induction and maintenance of long-term potentiation in CA1 hippocampal neurons [40, 137]. In another study performed by the same group of researchers, Bonder and McCarthy reported that hM3Dq can be selectively expressed in astrocytes by injecting AAV incorporating Cre-dependent hM3Dq into the visual cortex of transgenic mice encoding GFAP promoter-driven Cre, and they have used this system to investigate whether the astrocytic calcium elevation triggers vasodilation and a change in local blood flow in the cortex [41]. Activation of hM3Dq with CNO was sufficient to increase the intracellular calcium level in astrocytes but not to alter the basal blood flow in the visual cortex. The study also reported the absence of a temporal correlation between the astrocytic calcium increase and the change in cortical blood flow following either visual stimulation or a startle-evoking air puff. Furthermore, genetic deletion of IP_3 R2 did not affect neurovascular coupling, suggesting that Gq signaling and IP_3-dependent calcium elevation in astrocytes do not mediate vasodilation in the visual cortex.

The GFAP-driven MrgprA1 mouse line was used in a more recent study of Cao and coworkers to investigate the role of astrocytic ATP release in depression-like behaviors [138]. Application of the MrgprA1 agonist FLRFα elicited not only a robust increase in intracellular calcium in MrgprA1-expressing primary astrocytes but also a 2.5-fold increase in the ATP concentration in the culture medium. Furthermore, MrgprA1 mice infused with FLRFα into the cerebral ventricle exhibited a substantial reduction in depression-like behavior elicited in the murine paradigm of chronic social-defeat stress. Together with other results reported in the study, these findings suggest that endogenous ATP released from astrocytes can induce antidepressant-like behavior. Whether intracellular calcium increases in astrocytes can affect the activity of neighboring neurons was untested in the study.

Several other transgenic mouse lines expressing DREADDs have been reported. For instance, Sweger et al. developed a mouse line expressing an engineered k-opioid receptor (Ro1) in GFAP-

expressing astrocytes by crossing transgenic mice encoding GFAP promoter-driven tTA mice with another line encoding tetO promoter-driven Ro1 on the background of a genetic deletion of the endogenous k-opioid receptor (KOR) [139]. Ro1 is a Gi-coupled GPCR that is insensitive to endogenous ligands of KOR, such as dynorphin, but highly sensitive to a synthetic ligand of the k-opioid receptor, spiradoline [140, 141]. Ro1-expressing transgenic mice developed hydrocephalus and accumulation of cerebrospinal fluid in the ventricular system, even in the absence of a synthetic ligand, suggesting that Ro1 is constitutively active in this mouse model; unfortunately, this constitutive activity negates one of main features of chemogenetics, its temporal controllability, and limits the use of this model.

The same group of researchers has described another transgenic mouse line expressing GFAP promoter-driven hM3Dq [142]. Systemic treatment of these mice with CNO elicited a number of physiological changes that are controlled by the autonomic nervous system, including cardiovascular function, saliva formation, and homeostasis of body temperature. Furthermore, hM3Dq-expressing mice receiving CNO exhibited substantial changes in activity-related behaviors and motor coordination. Thus, these findings indicate the critical role of astrocytes in a broad range of basic physiological functions. Interestingly, the physiological and behavioral changes were unaffected by genetic deletion of IP_3 R2, suggesting that astrocytic IP_3-mediated calcium increase is dispensable for hM3Dq-elicited responses.

The GFAP promoter-driven hM3Dq mice have been used to study glial cells outside of the brain. McClain et al. examined the potential role of the enteric glia, which are astrocyte-like peripheral glial cells surrounding enteric neurons in the gut [143]. An application of CNO to the ileal and colonic myenteric plexus prepared from transgenic mice not only elicited and intracellular calcium increase in astrocytes but also triggered contraction of the ileum and colon to a degree similar to that elicited by direct stimulation of smooth muscle or electrical stimulation of enteric neurons. The contraction was abolished by the application of tetradotoxin, indicating the involvement of neuronal activation in the process. These findings have demonstrated that astrocytes in the gut play a critical role in the contractions of intestinal smooth muscle. The mechanism by which activation of Gq-coupled receptor in astrocytes leads to activation of enteric neurons remains unknown.

Recently, Sciolino et al. have reported two new transgenic mouse lines expressing hM3Dq, depending on Cre and flippase-mediated recombination [144]. When crossed to Cre or FLP driver lines, the new mouse lines permit the selective expression of hM3Dq in a population of cells that express either Cre or flippase. In addition, the intersectional strategy involving both Cre- and flippase-dependent recombination further restricts hM3Dq expression in a specific subpopulation. This group reported that a systemic application of CNO to mice expressing hM3Dq in GFAP-expressing cells elicits hypothermia, confirming the efficacy of the new mouse line by reproducing the previous finding [142].

Finally, the chemogenetic approach has also been applied to reveal the function of Gs-coupled signaling in longterm memory in normal animals and the Alzheimer animal model. For example, double-transgenic mice encoding GFAP promoter-driven tTA and tetO-driven Rs1 have been generated to acutely modulate Gs-coupled receptor activity [145]. Rs1 is the human Gs-coupled $5\text{-}HT_{4b}$ serotonin receptor with a point mutation that renders this receptor insensitive to serotonin but highly sensitive to a synthetic ligand, GR-125487 [146]. Activation of Gs signaling by systemic delivery of GR-125487 impairs the performance of transgenic mice in the Morris water maze as well as a novel object-recognition task. Orr et al. found that Rs1 is constitutively active in this mouse model, driving the Gs signaling pathway even in the absence of the synthetic ligand.

CHEMOGENETIC STIMULATION OF ASTROCYTES EXPRESSING VIRALLY DELIVERED EFFECTORS

Thus far, only a small number of studies focusing on glia have used a virally mediated method to achieve the expression of chemogenetic proteins. For example, an AAV encoding GFAP promoter-driven hM3Dq or hM4Di has been stereotactically injected into the arcuate nucleus of the mouse brain to investigate the potential role of medial basal hypothalamic astrocytes in regulating food intake [147]. In the feeding assay, hM3Dq-expressing mice receiving CNO exhibited a significant reduction in both baseline feeding and ghrelin-elicited hyperphagia, whereas hM4Di-expressing mice receiving CNO showed substantially enhanced and prolonged ghrelin-evoked feeding [148, 149]. In contrast, following the CNO treatment, leptin-induced anorexia was facilitated in hM3Dq-expressing mice but suppressed in hM4Di-expressing mice [150-153]. Thus, this study employing two different chemogenetic actuators that recruit different downstream signaling molecules clearly demonstrated that astrocytes in the arcuate nucleus exert bi-directional regulation of food consumption.

An AAV virus expressing GFAP promoter-driven hM3Dq was injected into the rat nucleus accumbens core (NAcore) in two recent studies in order to investigate the contribution of glial cells and extracellular glutamate to substance abuse and motivation [154, 155]. Bull et al. reported that an application of CNO

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elicited an elevation of the intracellular calcium level in hM3Dq-expressing primary astrocytes and a decrease in motivation for self-administration of ethanol after 3 weeks of abstinence [154]. Scofield et al. showed that intracranial or systemic administration of CNO triggered an increase in extracellular glutamate in the NAcore [155]. Furthermore, hM3Dq-expressing rats receiving intraperitoneal CNO exhibited a significant reduction in the cue-induced reinstatement of cocaine seeking [155].

CONCLUDING REMARKS

Neurons have always been a main focus of brain research, and non-neuronal cells that make up the majority of brain cells, such as glial cells, have not received much attention until recently. Studies of glia have revealed that they do not merely provide food and support to neurons; rather, they play an important role in brain function. In order to understand astrocytic function, it is critical to be able to control their intracellular activity in a native context. Since glia are intermingled with neurons in the brain and they express receptors and ion channels that are also expressed in neurons, it is difficult to perform such manipulation selectively in glial cells, while leaving neighboring neurons unaffected.

Optogenetics and chemogenetics are novel manipulation techniques based on genetically encoded effector molecules, such as specific ion channels and GPCRs, that respond to exogenously delivered light stimuli or synthetic ligands, but are unresponsive to endogenous molecules. In combination with cell type-specific promoters and other genetic tools, expression of effector molecules can be restricted to specific cell types. In addition, a controlled delivery of exogenous stimuli permits temporal precision of manipulation. Thus, the features of spatial and temporal control make it possible to perform a time-resolved functional manipulation in a specific population of cells. Optogenetics and chemogenetics have been used most extensively in the study of neuronal circuits and behavior, but they have also been employed in a number of studies focusing on glial cells, mainly astrocytes. Such studies have demonstrated that astrocytes play a critical role not only in a variety of basic physiological responses, including visual processing, norepinephrine release, breathing, cerebral blood flow, feeding, memory, and sleep, but also in pathological conditions, including drug addition, depression, and ischemia. Those studies have further revealed the importance of gliotransmitters, such as extracellular glutamate, ATP, and L-lactate, that modulate excitability and synaptic transmission in neighboring neurons. However, it is still debatable whether astrocytic release of gliotransmitters is a calcium-dependent process. In addition, the exact molecular mechanisms governing gliotransmitter release from astrocytes remains to be revealed. A combinatorial approach of advanced functional manipulation techniques such as optogenetics and chemogenetics, together with pharmacological and molecular genetic methods, can further our understanding of glial function in health and disease, including neurodevelopment, neurodegenerative disorders, and neuroinflammatory conditions.

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