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Amines, astrocytes and arousal

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Amine neurotransmitters such as noradrenaline mediate arousal, attention and reward in the CNS. New data suggest that, from flies to mammals, a major mechanism for amine transmitter action is to raise astrocyte $[Ca^{2+}]_i$ and release gliotransmitters that modulate neuronal activity and behaviour.

Classically, the amino acid neurotransmitters glutamate, GABA and glycine mediate rapid spatially-localised point-to-point neurotransmission. In contrast, the action of the amine transmitters noradrenaline, dopamine and serotonin can be more widespread, slower and modulatory, resulting from the amine transmitter diffusing through the extracellular space and acting on a spatially broader pool of cells (Fuxe et al., 2015; Moss & Bolam, 2008). In mammals noradrenaline release generates arousal, attention, wakefulness and fight or flight responses, while dopamine modulates motor function and signals some aspects of reward. Similarly, lower down the evolutionary tree in invertebrates, the adrenaline/noradrenaline analogues octopamine and tyramine promote arousal and wakefulness, mediate fight or flight responses, and regulate metabolism, while dopamine modulates behaviours from locomotion and sleep to courtship and learning. Amine transmitters have been thought to produce these effects by acting directly on neurons to regulate their excitability and synaptic strength. Surprisingly, however, recent experiments have suggested that a major contributor to the CNS actions of octopamine/tyramine (Oct/Tyr) in flies and of noradrenaline in mammals is a rise of $[Ca^{2+}]_i$ in astrocytes, which leads to a release of factors that modulate neuronal function and behaviour.

Neurotransmitter release from neurons is one cause of astrocyte $[Ca^{2+}]_i$ transients (reviewed by Bazargani & Attwell, 2016). In line with this, a new study found that, in Drosophila larvae, one half to three quarters of the $[Ca^{2+}]_i$ transients in ventral nerve cord astrocyte cell bodies were driven by Oct/Tyr signalling from neurons which receive olfactory input (Ma et al., 2016). Synaptic release of Oct/Tyr led to activation of the Ca^{2+}-permeable TRP channel 'Water witch' (Wtrw) in the astrocytes, and knockdown with RNAi of Wtrw, or the presence of the null allele *Wtrw*^{ex}, led to a halving of the astrocyte $[Ca^{2+}]_i$ transient rate (with no change in the firing of Oct/Tyr neurons). Knockdown of Wtrw, or of the Oct/Tyr

receptor, in astrocytes also inhibited larval chemotaxis to an olfactory stimulus and startle-induced escape responses (Ma et al., 2016). This behavioural inhibition resulted from the block of a pathway (Fig. 1A) in which astrocyte $[Ca^{2+}]_i$ rises trigger a release of the "gliotransmitter" ATP which is converted extracellularly to adenosine and thus (probably by activating K^+ currents or blocking Ca^{2+} currents) inhibits dopaminergic neurons. Since dopamine release suppresses movement responses by motoneurons, the overall effect of the Oct/Tyr - astrocyte Ca^{2+} pathway is to facilitate movement. Thus, with the astrocyte pathway blocked by deletion of Wtrw or Oct/Tyr signalling, dopaminergic neurons were found to fire more than normal, and the behavioural effect of this was confirmed by showing that the deficits in chemotaxis evoked by Wtrw knockdown could be rescued by administering a dopamine (D1) receptor antagonist. These data, along with previous experiments showing that astrocyte $[Ca^{2+}]_i$ transients regulate synaptic gain earlier in the olfactory pathway (Liu et al., 2014), indicate a role for astrocyte $[Ca^{2+}]_i$ transients in regulating fly behaviour.

In mammals, neurotransmitter-evoked $[Ca^{2+}]_i$ rises in astrocytes have been suggested to release the gliotransmitters ATP (generating adenosine via ectonucleotidase activity), glutamate, GABA and D-serine, which act on neuronal receptors to regulate synaptic gain and neuronal excitability (Fig. 1B; reviewed by Bazargani & Attwell, 2016). Noradrenaline released from locus coeruleus axons evokes α_1 receptor mediated $[Ca^{2+}]_i$ rises in astrocytes (Duffy & MacVicar, 1995), which are larger than the $[Ca^{2+}]_i$ rises evoked by glutamate and GABA released by local neuronal activity (Ding et al., 2013). Intriguingly, in visual cortex, when visually-evoked local neuronal activity and movement-evoked noradrenaline release occur together, the $[Ca^{2+}]_i$ responses evoked are larger than with either alone (Paukert et al., 2014). It is possible that this synergy occurs at the level of the locus coeruleus, with the combination of movement and visual stimuli evoking more firing of the locus coeruleus neurons that release noradrenaline in visual cortex. Alternatively, this synergy might be generated locally in cortical astrocytes: when noradrenaline is being released from locus coeruleus axons, astrocyte $[Ca^{2+}]_i$ rises evoked by local neuronal activity

may be potentiated, along with the downstream effects on neuronal function that they produce (see below). The spatially extensive nature of the locus coeruleus axons, together with the large number of synapses that each astrocyte is associated with, thus confers a mechanism by which information processing throughout a large region of the brain can be modulated - a phenomenon that could underlie context-dependent gating of attention and arousal by noradrenaline.

Examination of how $[Ca^{2+}]_i$ is elevated in fly and rodent astrocytes reveals similarities between the signalling mechanisms in the different species, but also raises questions that it will be important to resolve in order to understand fully the function of amine transmitter signalling via astrocytes. In mammalian astrocytes, $[Ca^{2+}]_i$ can be raised by neurotransmitters such as noradrenaline or glutamate acting on G protein coupled receptors that activate phospholipase C (PLC) to generate IP_3 and release Ca^{2+} from internal stores (Fig. 1B). Alternatively, Ca^{2+} can enter across the cell membrane through ion channels such as ATP-gated $P2X_1$ receptors (Mishra et al., 2017) or spontaneously active TRPA1 channels in astrocyte processes (Shigetomi et al., 2011). TRPA1-mediated Ca^{2+} entry into mammalian astrocytes suggests a similarity with the mechanism by which the TRP channel Wtrw contributes to the $[Ca^{2+}]_i$ transients in fly astrocytes. Indeed, a BLAST homology search on Wtrw indicates that its closest related rodent protein is TRPA1.

This might seem to indicate a satisfying preservation across species, by evolution, of an important signalling mechanism. However, there is controversy over the dependence of the resulting TRP-mediated $[Ca^{2+}]_i$ transients on neuronal activity. The Drosophila larva work indicated that Wtrw is activated by amine neurotransmitters acting on the Oct/Tyr receptor on astrocytes (Ma et al., 2016), which couples to G_i and G_q proteins to inhibit adenylate cyclase and activate PLC (Robb et al., 1994). Activation of PLC may open Wtrw by lowering PIP_2 level or raising $[Ca^{2+}]_i$, as previously reported for TRPA1 (Bellono et al., 2014; Zurborg et al., 2007), although Wtrw lacks the clear EF hand Ca^{2+}-binding domain predicted (by http://prosite.expasy.org) to exist in TRPA1. In contrast, in young rodents TRPA1 was reported to be spontaneously active rather than transmitter-gated (Shigetomi et al., 2011).

Furthermore, two other groups have reported that in older mammalian astrocytes TRPA1 does not contribute at all to transient $[Ca^{2+}]_i$ signals. Rungta et al. (2016) suggested that Ca^{2+} entry occurs via a non-TRPA1 channel. Alternatively Agarwal et al. (2017) reported an intracellular source for many spontaneous $[Ca^{2+}]_i$ transients, with Ca^{2+} being released by opening of the mitochondrial permeability transition pore (mPTP) in mitochondria located near internal store sites that release Ca^{2+} in response to noradrenaline. The release mechanism might involve Ca^{2+} leaving mitochondria through the mPTP itself, and perhaps also activation of ryanodine receptor and IP3 receptor mediated release from the adjacent Ca^{2+} stores by reactive oxygen species that are released in association with mPTP opening (Angelova et al., 2015; Zorov et al., 2014). Since neuronal activity localises mitochondria at positions in astrocyte processes that are near synapses (Stephen et al., 2015), this mechanism will tend to produce astrocyte $[Ca^{2+}]_i$ transients near active synapses, i.e. suitable for releasing gliotransmitters onto those synapses. The different reported contributions of TRPA1 to mammalian astrocyte $[Ca^{2+}]_i$ transients in these three studies might reflect the different ages of animal studied, difficulty in dissolving the TRPA1 blocker used (HC030031, which is rather insoluble), or some yet-to-be-determined difference in the state of the preparations.

The controversy over the source of most astrocyte $[Ca^{2+}]_i$ transients raises the important question of the main mechanism by which amine neurotransmitters raise $[Ca^{2+}]_i$. While, conventionally, this was thought to reflect release of Ca^{2+} from internal stores, to this we must now add Ca^{2+} entry mediated by TRP-like channels (Wtrw and TRPA1). Ca^{2+} release from mitochondria may also be increased by noradrenaline, since noradrenaline increases the rate of "spontaneous" $[Ca^{2+}]_i$ transients even when the main astrocyte IP3 receptor ($IP3R_2$) mediating release from internal stores is knocked out (Agarwal et al., 2017). This increase in $[Ca^{2+}]_i$ transients could reflect increased mPTP opening, produced when noradrenaline increases the rate of oxidative phosphorylation by stimulating breakdown of astrocyte glycogen, providing a mechanism coupling metabolism to astrocyte signalling.

A key issue, for understanding how amine neurotransmitters might gate arousal, as outlined above, is how local release of fast transmitters such as glutamate and more global release of noradrenaline (and perhaps other amine transmitters) might synergistically evoke a $[Ca^{2+}]_i$ signal in astrocytes that is larger than for either stimulus alone (Paukert et al., 2014). Fast transmitters can increase astrocyte $[Ca^{2+}]_i$ by acting on G_q-coupled receptors, or by activating ionotropic receptors or neurotransmitter transporters to raise $[Na^+]_i$ which slows the action of Na^+/Ca^{2+} exchangers (Bazargani & Attwell, 2016). In addition, Agarwal et al. (2017) found that an increase in neuronal activity increased the rate of mPTP opening and associated $[Ca^{2+}]_i$ transients in astrocytes. More opening of the mPTP may be caused by an increase in oxidative phosphorylation occurring to power the Na^+/K^+ pump when neuronal activity evokes a Na^+ influx through glutamate-gated receptors. Since Ca^{2+} release from stores by both IP3 receptors and ryanodine receptors depends on activation by cytoplasmic Ca^{2+} (Berridge, 2016), a rise in $[Ca^{2+}]_i$ produced by any of the mechanisms above could synergise supralinearly with Ca^{2+} released from internal stores by noradrenaline, to generate more Ca^{2+} release from the stores than would occur if only noradrenaline release or only local release of fast neurotransmitters occurred (Fig. 1B). Alternatively, a rise in cyclic AMP level produced by local neuronal activity can potentiate IP3 receptor mediated Ca^{2+} release (Taylor, 2016) and thus potentiate the astrocyte $[Ca^{2+}]_i$ rise evoked by noradrenaline activating α_1 receptors. Metabotropic glutamate and $GABA_B$ receptors linked to adenylate cyclase inhibit rather than promote cAMP formation, but neuronal activity does release adenosine which could raise the cAMP level by activating astrocyte A2a receptors, and thus potentiate the response to noradrenaline. Future experiments will need to delineate in detail how the effects on astrocyte $[Ca^{2+}]_i$ of activating α_1 receptors are modulated by the activity of A2a, mGluR and $GABA_B$ receptors.

A further question for the future is whether $[Ca^{2+}]_i$ elevations in astrocytes regulate neuronal function by releasing gliotransmitters that act on neuronal receptors to alter synapse strength or neuronal excitability. A possible alternative scenario is that astrocytes

alter neurotransmitter action on surrounding neurons cell-autonomously, as a result of $[Ca^{2+}]_i$ changes altering the surface expression of neurotransmitter transporters (which regulate transmitter action in flies as in mammals: MacNamee et al., 2016), or perhaps of surface membrane enzymes that hydrolyse ATP, thus altering the level of neurotransmitters or adenosine reaching the surrounding neurons (Shigetomi et al., 2011; discussed in Bazargani & Attwell, 2016). Additionally, the reported increase of astrocyte volume evoked during arousal by noradrenaline activating β receptors (Sherpa et al., 2016), which may contribute to the decrease in extracellular volume seen during wake periods (Xie et al., 2013), may alter the concentration of neurotransmitters in the extracellular space. Establishing the mechanism of this swelling, which could reflect an increased ion influx into astrocytes or a decrease of Na/K pump activity, is an important issue for the future.

We have focused here on the actions of noradrenaline on astrocytes, but dopamine and 5-HT also evoke astrocyte $[Ca^{2+}]_i$ rises that may similarly alter neuronal function. These rises can occur by the transmitters acting on receptors or, in a non-receptor manner, by intracellular oxidation and generation of reactive oxygen species that activate PLC to release Ca^{2+} from internal stores (Vaarmann et al., 2010; Jennings et al., 2016; Schipke et al., 2011). Intriguingly, in the mammalian brain, astrocytes have also been suggested to regulate the release of noradrenaline from locus coeruleus neurons, using lactate as a gliotransmitter (Tang et al. 2013).

To sum up, wide-ranging aminergic axons may be able to gate arousal and other behavioural changes by raising astrocyte $[Ca^{2+}]_i$ levels. A key question raised by this work is the relative importance of amine signalling that is mediated by astrocytes, as opposed to mediated by direct effects of amine transmitters on neurons. The demonstration of amine-regulated behaviour produced by astrocytes raises the possibility of new pharmacological targets for therapy, once the underlying mechanisms are completely understood.

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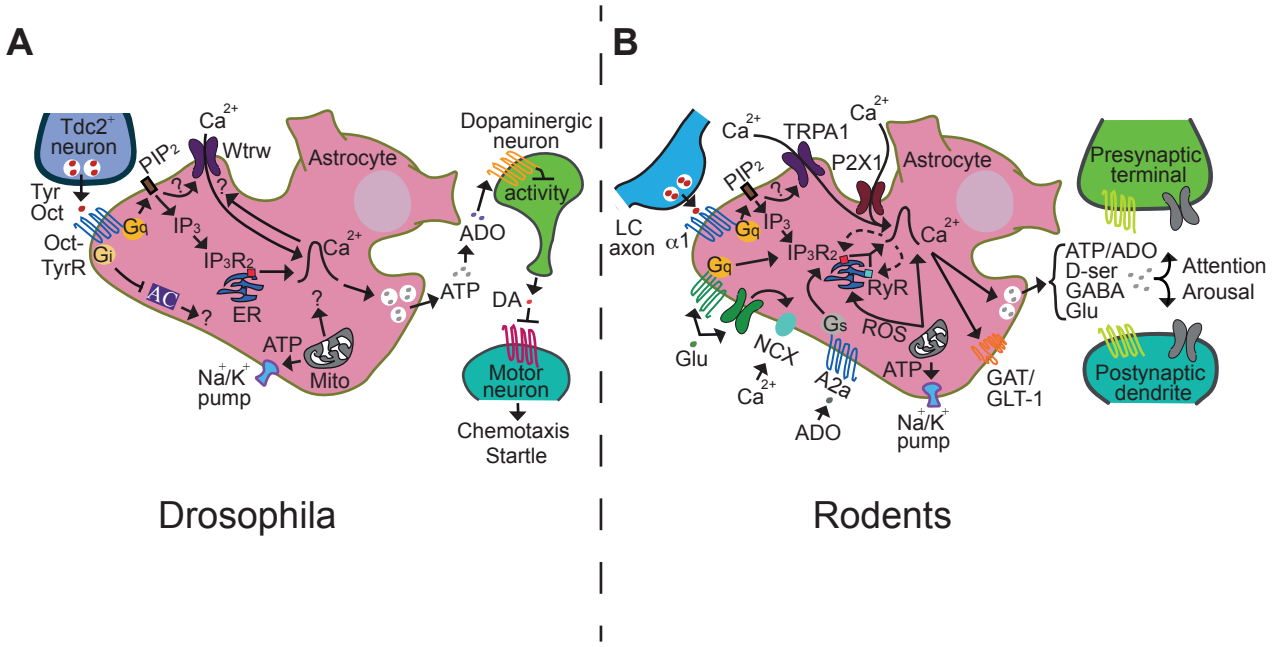
Figure 1. Mechanisms and consequences of elevation of $[Ca^{2+}]_i$ in astrocytes.

(**A**) In Drosophila ventral nerve cord, $Tdc2^+$ neurons releasing the adrenaline/noradrenaline analogues tyramine and octopamine activate the Oct/Tyr receptor on astrocytes that couples to G_q to activate PLC and generate IP_3 from PIP_2. Either the fall of PIP_2 or the IP_3-evoked release of Ca^{2+} from internal stores (ER) can open the TRP-like channel Water witch (Wtrw), which allows Ca^{2+} into the cell. The Oct/Tyr receptor can also activate G_i and modulate cAMP signalling, but the effect of this on Ca^{2+} signalling is unclear. As in rodents, mitochondria (Mito) might release Ca^{2+} via the mitochondrial permeability transition pore (mPTP). Ca^{2+} transients lead to a release of ATP (shown as vesicular), which is converted to adenosine (ADO) by ecto-ATPases. ADO activates receptors on dopaminergic neurons that stop those neurons firing action potentials, and thus inhibit dopamine release. Dopamine, in turn, inhibits chemotaxis and startle movements. (**B**) In rodents, noradrenaline release from locus coeruleus (LC) axons activates astrocyte α_1 receptors which, via G_q and IP_3, release Ca^{2+} from internal stores. Glutamate can release Ca^{2+} via a G_q-IP_3 pathway, or by evoking a Na^+ influx through ionotropic receptors or transporters thus raising $[Na^+]_i$ and slowing Na/Ca exchange (NCX). Ca^{2+} may enter through ATP-gated $P2X_1$ receptors, and by TRPA1 channels but it is unclear whether TRPA1 can be gated by transmitters. Mitochondria supply ATP to the Na^+/K^+ pump, but also release Ca^{2+} through the mPTP, or release reactive oxygen species (ROS) which act on IP_3 and ryanodine receptors (IP_3R_2 and RyR) to promote Ca^{2+} release from internal stores. Because Ca^{2+} release via IP_3R_2 and RyR requires activation by cytoplasmic Ca^{2+} (dashed lines), the Ca^{2+} release evoked by glutamate may potentiate the release evoked by noradrenaline (and vice versa). Neuronal activity may also potentiate Ca^{2+} release by generating ADO which raises $[cAMP]_i$ via A2a receptors. Ca^{2+} transients may evoke the release of gliotransmitters (ATP, D-ser, GABA, glutamate) which modulate the strength of nearby synapses and the excitability of neurons, or may alter trafficking of neurotransmitter transporters (GAT/GLT-1) or ecto-ATPases to the surface membrane. In this way, noradrenergic signalling can, via astrocyte-released gliotransmitters, alter signal processing over a large set of synapses, thus mediating attention and arousal.


A
Tdc2+ neuron
Tyr
Oct
Oct-TyrR
Gi
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PIP2
Ca2+
Wtrw
IP3
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Astrocyte
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ADO
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Chemotaxis
Startle
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Mito
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Drosophila
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LC axon
α1
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TRPA1
P2X1
PIP2
Gq
IP3
IP3R2
RyR
Ca2+
Astrocyte
Glu
NCX
Ca2+
Gs
A2a
ADO
ROS
ATP
Na+/K+ pump
GAT/ GLT-1
Presynaptic terminal
ATP/ADO
D-ser
GABA
Glu
Attention
Arousal
Postynaptic dendrite
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