Sonic hedgehog enhances calcium oscillations in hippocampal astrocytes

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Sonic hedgehog (SHH) is important for organogenesis during development. Recent studies have indicated that SHH is also involved in the proliferation and transformation of astrocytes to the reactive phenotype. However, the mechanisms underlying these are unknown. Involvement of SHH signaling in calcium (Ca) signaling has not been extensively studied. Here, we report that SHH and Smoothened agonist (SAG), an activator of the signaling receptor Smoothened (SMO) in the SHH pathway, activate Ca oscillations in cultured murine hippocampal astrocytes. The response was rapid, on a minute time scale, indicating a noncanonical pathway activity. Pertussis toxin blocked the SAG effect, indicating an involvement of a Gt coupled to SMO. Depletion of extracellular ATP by apyrase, an ATP-degrading enzyme, inhibited the SAG-mediated activation of Ca oscillations. These results indicate that SAG increases extracellular ATP levels by activating ATP release from astrocytes, resulting in Ca oscillation activation. We hypothesize that SHH activates SMO-coupled Gi in astrocytes, causing ATP release and activation of Gt coupled to P2 receptors on the same cell or surrounding astrocytes. Transcription factor activities are often modulated by Ca patterns; therefore, SHH signaling may trigger changes in astrocytes by activating Ca oscillations. This enhancement of Ca oscillations by SHH signaling may occur in astrocytes in the brain in vivo because we also observed it in hippocampal brain slices. In summary, SHH and SAG enhance Ca oscillations in hippocampal astrocytes, Gi mediates SAG-induced Ca oscillations downstream of SMO, and ATP-permeable channels may promote the ATP release that activates Ca oscillations in astrocytes.

Astrocytes are a major glial cell population in the central nervous system (CNS) with important roles in brain homeostasis, such as clearance of glutamate and GABA from the extracellular space, provision of nutrients from blood vessels to neurons, and control of extracellular pH. Astrocytes are transformed into reactive astrocytes in response to brain injury and inflammation. Reactive astrocytes have altered gene expression patterns and morphology and play roles in scar formation and in preventing the spread of inflammation. Astrocytes also modulate neural excitability and synaptic connectivity by releasing so-called gliotransmitters, among which glutamate and ATP are major components.

Sonic hedgehog

The Hedgehog gene was identified in the 1970s as a gene involved in Drosophila larval segmentation. There are three Hedgehog homologs in vertebrates, Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh). Shh is involved in organogenesis and development of the CNS and is expressed throughout the body. In the absence of SHH, an SHH receptor, Patched, keeps a 7-transmembrane receptor, Smoothened (SMO), from activating a transcription factor, GLI. Binding of SHH to Patched releases SMO to activate GLI, which translocates into the nucleus and activates transcription, thereby promoting cell proliferation and differentiation. Aside from this well-known canonical pathway, noncanonical pathways triggered by Patched activation have also been reported. These pathways are not linked to GLI activation but regulate cell death, axon guidance, and cytoskeleton with or without SMO activation.

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This article contains Figs. S1–S5.

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**SHH in the CNS**

In early CNS development, SHH is secreted from the notochord and floor plate as a morphogen to direct dorso-ventral patterning of the CNS. During late CNS development, SHH is found in the cerebral cortex, optic tectum, and cerebellar cortex (15). SHH is also expressed in the adult CNS (16); SHH and Patched are expressed in the forebrain, cerebellar Purkinje cells, and spinal cord motor neurons. SMO is expressed in circumventricular organs, granular cells in the hippocampal dentate gyrus, and neurons in the reticular thalamic nuclei (17). The expression of SHH is particularly strong in the hippocampal dentate gyrus and the subventricular zone where adult neurogenesis takes place and retention, proliferation, and differentiation of neural stem cells occurs (16). In hippocampal neurons, SHH is present presynaptically and postsynaptically (18), and Patched and SMO are localized not only in cell bodies but also in dendrites and postsynapses (19). Involvement of SHH in synaptic plasticity was also reported (20). SHH expression is activated upon traumatic injury in the brain (21, 22) including in astrocytes (21, 23). During injury, released SHH increases the expression of glial fibrillary acidic protein in astrocytes and induces transformation of astrocytes to reactive astrocytes. SHH administration also induces transformation to reactive astrocytes (24, 25) and gliotransmitter release from astrocytes. All of these observations indicate important roles of SHH in the regulation of astrocytes. However, detailed mechanisms for these actions have not been elucidated.

**Ca oscillation in glia**

Although neurons communicate with each other by electrical activity, astrocytes transmit information by changing intracellular Ca\(^{2+}\). Ca oscillation is frequently observed in astrocytes, in which Ca transients repeatedly occur in individual cells. These changing Ca patterns sometimes display wave-like propagation among astrocytes, which is called the Ca wave (26, 27). Various extracellular stimuli evoke Ca oscillations in astrocytes through various plasma membrane receptors, whereas intracellular Ca release occurs from the endoplasmic reticulum (ER) through inositol trisphosphate receptors (IP3Rs) downstream of G\(_{q/11}\)-coupled receptors.

ATP is a well-known stimulant that causes Ca oscillations in astrocytes. A class of ATP receptor, P2 receptors, namely P2X1/2/3/4/5/7 and P2Y1/2/4/6/12/13/14, is expressed in astrocytes (28). ATP-evoked Ca oscillation in astrocytes is not prevented by extracellular Ca\(^{2+}\) removal; therefore, involvement of intracellular Ca release from IP3R downstream of G\(_{q/11}\)-coupled P2Y receptors is postulated (28).

ATP is released from astrocytes as a gliotransmitter and influences neuronal excitability (3, 4) and regulates Ca dynamics in astrocytes (29). Okuda et al. (30) reported that SHH-stimulated astrocytes release ATP. Two mechanisms for releasing ATP from astrocytes are known: vesicular release and release through channels. Supporting vesicular release, a vesicular nucleotide transporter is expressed in astrocytes. For release through channels, ATP-permeable channels, maxichannel, connexin hemichannels, pannexin hemichannels, and the P2X7 receptor are postulated to be involved. Several studies have shown that deprivation of oxygen and glucose, osmotic stimulation, and stretch stimulation induce ATP release from astrocytes through channels on the plasma membrane.

**Aims of this study**

The high levels of SHH and related proteins in the adult hippocampus together with the inferred roles of SHH signaling in adult neurogenesis and brain injury led us to characterize cellular responses to SHH in cultured hippocampal cells. We found the enhancement of Ca oscillations in astrocytes within several minutes after application of SHH pathway agonists, namely SHH and Smooyed agonist (SAG). This enhancement was blocked by inhibition of G\(_{i}\) and removal of extracellular ATP. Together with other lines of evidence, we propose that the enhancement of Ca oscillations in astrocytes is initiated by the activation of SMO-coupled G\(_{i}\), which leads to ATP release through ATP-permeable channels. This released ATP then enhances Ca oscillations in nearby astrocytes. We also observed enhanced Ca oscillations in astrocytes in brain slices; therefore, this mechanism may be functional in the in vivo brain as well.

**Results**

**SHH and SAG evoke Ca oscillations in mouse hippocampal cultures**

Calcium imaging was performed by loading cultured mouse hippocampal cells with Fura-2 (Fig. 1A). Primary cultures of mouse hippocampal cells were exposed to SHH (500 pm, 10 ng/ml) in the presence of 1 \(\mu M\) tetrodotoxin (TTX), and some of the cells exhibited spontaneous Ca oscillations before the agonist application. Addition of SHH evoked Ca oscillations in quiescent cells and enhanced the frequency of Ca oscillations in cells that had already shown spontaneous Ca oscillations before the agonist application (Fig. 1B): a cumulative histogram during the agonist application periods was right-shifted from that during the baseline period (Fig. 1B, b, \(p < 0.001\)), indicating an increase in Ca oscillation frequency by SHH. A SMO agonist, SAG (5 \(\mu M\)), induced a similar increase in Ca oscillation frequency (Fig. 1C, \(p < 0.001\)). A series of different SHH and SAG concentrations resulted in increased or decreased Ca oscillation frequencies, but did not follow simple dose-response relationships (Fig. 1, B and C, b panels, and Fig. S1). Agonist-induced Ca frequency increase was evaluated by subtracting the baseline Ca frequency from that after the drug application in each cell (\(\Delta\)Frequency). \(\Delta\)Frequencies of cells applied with concentrations of SHH or SAG were compared with that of vehicle-applied control cells (0.1% DMSO; Fig. 1, D and E). SHH increased Ca oscillation frequency significantly at 500 pm (\(p < 0.001\)) but less at 50 pm (\(p < 0.01\)) and not at 5 nm (\(p = 0.82\)). SAG increased Ca event frequency significantly at 5 \(\mu M\) (\(p < 0.01\)) and 50 nm (\(p < 0.01\)), and decreased the Ca event frequency at 500 nm (\(p < 0.05\)) and 100 nm (\(p < 0.001\)). SAG at 1 \(\mu M\) did not show a difference from the control (\(p = 0.31\)). We used SHH at 500 pm hereafter. For SAG, we considered that 5 \(\mu M\) would produce the most reliable results because 100 \(\mu M\) SAG showed a large decrease in Ca oscillation frequency (Fig. S1F). The SAG concentration used hereafter was, therefore,
5 μM unless otherwise indicated. Some cells that did not show Ca transients during the initial 10-min baseline period started showing Ca transients after SHH or SAG stimulation; therefore, the stimuli increased the proportion of cells showing Ca transients as well as the frequency of Ca oscillations in each cell. About half of the cells did not show Ca transients throughout the recording period.

We tested cyclopamine (CPN), which is widely used as a SMO antagonist (31), on the assumption that it would block the effect of SAG. However, administration of CPN per se caused an increase in Ca oscillation frequency to a similar extent as SAG, and the addition of SAG to CPN further enhanced Ca oscillation frequency (Fig. 2A, a and b, p < 0.001 for base versus CPN and base versus CPN + SAG, respectively), which concealed the effect of subsequently applied SAG. ΔFrequencies of Ca oscillation both in the CPN and CPN + SAG periods were significantly larger than that of a control cell group (Fig. 2A, c, p < 0.001, respectively). CPN seemed to act as an agonist on the Ca oscillation enhancement mechanism originating from SMO. Such an agonistic action of CPN on a noncanonical SHH pathway has been previously reported (32, 33); therefore, we consider that the enhancement of Ca oscillations observed in this study was derived from the activation of a noncanonical SHH signaling pathway.

The SAG induced Ca oscillations are mediated by extracellular ATP

We next investigated whether extracellular ATP is involved in the enhancement of Ca oscillations by SHH signaling because ATP is a well-known activator of Ca oscillations in astrocytes (34, 35). Administration of apyrase, an ATP-degrading enzyme, to the extracellular space abolished the enhanced Ca oscillations evoked by SAG (Fig. 2B, b, p < 0.001 for base versus SAG and p < 0.05 for SAG versus apyrase), which was also shown in the ΔFrequency analysis (Fig. 2B, c, p < 0.001). This result indicates that SMO activation induced the increase
in extracellular ATP, which then enhanced Ca oscillations in the hippocampal cells. In a control experiment, a vehicle solution, HEPES-buffered saline (HBS) containing TTX, was added instead of apyrase, which produced an enhancement of Ca oscillation frequency (Fig. 2C, a, p < 0.001). We have no clear explanation for this, but the dilution of SAG could be a cause because a relatively low concentration of SAG (50 nM) was as effective as 5 μM (Fig. 1E).

**Astrocytes are responsible for the enhancement of Ca oscillations**

Primary hippocampal cell cultures are heterogeneous, containing neurons, astrocytes, microglia, oligodendrocytes, and other cell types. We, therefore, identified the cell types relevant to SAG-enhanced Ca oscillation. We assessed cell types in the hippocampal culture by immunohistochemistry with anti-MAP2, anti-S100, anti-Iba1, and anti-Olig2 antibodies, which are markers for neurons, astrocytes, microglia, and oligodendrocytes, respectively (Fig. S2). MAP2- and S100β-positive cells were observed throughout cultures (Fig. S2, A and B). Iba1-positive cells were only found in one batch (10–20 cells in each coverslip culture) of three culture batches (Fig. S2C). Olig2-positive cells were only found where neuron density was very high, but not in fields of view where neuron density was modest; we used such fields of view with modest neuron density for Ca imaging (Fig. S2D). Next, we performed Ca imaging followed by immunohistochemistry with anti-MAP2 and S100β antibodies (Fig. 3A). All the Fura-2–loaded cells were stained with either MAP2 or S100β in three fields of view from different cultures. S100β-positive cells were 58.9 ± 9.8% of all cells and the remainder were all MAP2-positive (n = 3 cultures). Ca transients were observed in 69.6 ± 14.1% of the S100β-positive cells and 32.5 ± 16.0% of the MAP2-positive cells. In our cultures, neurons showed frequent Ca transients in a synchronized fashion unless TTX was included in the bath solution (data not shown), but neurons showed far fewer spontaneous Ca transients than astrocytes when action potential generation was
SHH enhances Ca oscillations in astrocytes

From these results, we consider that the major cell population that showed Ca oscillations was astrocytes. We tested whether the enhancement of Ca oscillations by SHH or SAG could be evoked in astrocyte cultures as well as in the primary heterogeneous culture, and this was indeed the case: the enhancing effects of SHH or SAG on Ca oscillation frequency were observed in the astrocyte culture (Fig. 3B, c, p < 0.001; and Fig. 3C, b, p < 0.001, respectively). ΔFrequency of SHH and SAG compared with a DMSO (0.1%) applied control cell group (n = 642 cells from 5 cultures).

Figure 3. The SAG-induced Ca oscillation enhancement takes place in astrocytes. A, cell types showing Ca oscillations were characterized by immunohistochemistry. Time lapse Ca imaging of a 10-min baseline (HBS-TTX) and 10 min in SAG was performed in hippocampal cultures (A, a), which were then fixed and stained with anti-MAP2 (red) and anti-S100β (green) antibodies (A, b). Filled arrowheads indicate S100β-positive cells, and open arrowheads indicate MAP2-positive cells. Scale bar, 100 μm. B, a, Fura-2–loaded astrocyte culture. The enhancing effects of SHH (B, 500 pM, n = 465 cells from 5 cultures) or SAG (C, 5 μM, n = 1731 cells from 12 cultures) on Ca oscillation frequency were observed in the astrocyte culture. D, cumulative histograms of ΔFrequency of SHH and SAG together with a DMSO (0.1%) applied control cell group (n = 642 cells from 5 cultures).

Ca release from ER is necessary for Ca oscillations

To characterize the mechanism of SAG-enhanced Ca oscillations, the source of Ca2+ was investigated. Removal of Ca2+ from the extracellular solution did not alter the SAG- or SHH-induced enhancement of Ca oscillations in astrocytes (Figs. 4A and Fig. S3A, respectively): SAG enhanced Ca oscillation frequency with Ca-free medium (Fig. 4A, b, p < 0.05), and ΔFrequency(SAG − base) in Ca-free medium showed apparent difference from that of a control cell group (Fig. 4A, c, p <
In contrast, disruption of intracellular Ca release mechanisms resulted in drastic changes: 2-aminoethyl diphenylborinate (2-APB, 50 μM), an IP3R inhibitor, and thapsigargin (Tg, 100 nM), an inhibitor of the ER Ca-ATPase (36), blocked the SAG-induced enhancement of Ca oscillations (Fig. 4, B and C). SAG did not take effect under 2-APB (Fig. 4B, b, base versus 2-APB:

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Figure 4. Ca release through IP3R is involved in the SAG-enhanced Ca oscillations. A, a, the SAG-induced Ca oscillation frequency enhancement was tested in Ca-free media, in which Ca-free HBS supplemented with EGTA (1 μM) was used as an extracellular medium throughout the recording period. b, cumulative histograms of Ca oscillation frequency with Ca-free medium during the baseline period and after the application of SAG (n = 431 cells from 5 cultures). c, a cumulative histogram of ΔFrequency(SAG - base) in Ca-free medium and ΔFrequency(DMSO - base) obtained from a control group with DMSO (0.1% in Ca-free medium) in place of SAG (n = 377 cells from 4 cultures). B–D, 2-APB (B, 50 μM, n = 417 cells from 5 cultures), an IP3R inhibitor, Tg (C, 100 nM, n = 416 cells from 5 cultures), an inhibitor to the endoplasmic reticulum Ca-ATPase, or dantrolene (D, 10 μM, n = 505 cells from 5 cultures), a ryanodine receptor antagonist, was applied and addition of SAG followed. B–D, b panels, cumulative histograms of Ca oscillation frequency during the baseline period, after antagonist application and after SAG addition. B–D, c panels, cumulative histograms of ΔFrequency together with those obtained from a control cell group with DMSO in place of the antagonists (n = 384 cells from 4 cultures).
SHH enhances Ca oscillations in astrocytes

Figure 5. The SAG-induced enhancement of Ca oscillations requires Gi activation. A, astrocyte cultures were treated with 100 ng/ml of PTX for 24 h prior to Ca imaging. A, SAG increased the Ca oscillation frequency compared with baseline. b and c panels, cumulative histograms of Ca oscillation frequency during the baseline period and after SAG (A, b, n = 714 cells from 7 cultures) or control DMSO (A, c, n = 537 cells from 4 cultures) application. d, cumulative histograms of ∆Frequency together with those from the control cell group shown in Fig. 3D. B, CAMP imaging revealed an increase in cAMP. a, a cAMP indicator, Fluminido-2 (green), was expressed in astrocytes and fluids were loaded for simultaneous measurement. Scale bar, 100 μm. b, time course of cAMP and [Ca], were monitored before and after SAG application. c, averaged time course of Fluminido-2 signal from astrocytes to which SAG (n = 97 cells from 8 experiments) or vehicle (0.1% DMSO; n = 58 cells from 4 experiments) was applied.

SMO is coupled to G\textsubscript{i}

SMO is a 7-transmembrane receptor that couples with a trimeric G protein, G\textsubscript{i} (38, 39). We tested if the SMO enhancement of Ca oscillations requires G\textsubscript{i} activation by incubating astrocytes with pertussis toxin (PTX), a G\textsubscript{i} inhibitor. SAG induced an enhancement of Ca oscillations in PTX-treated astrocytes (Fig. 5A, b, p < 0.001), but it was significantly smaller than the SAG-induced enhancement without PTX treatment. Furthermore, the addition of DMSO (0.1%) in place of SAG in PTX-treated astrocytes induced an even larger increase in Ca oscillation frequency than that produced by SAG in PTX-treated astrocytes (Fig. 5A, c, p < 0.001). ∆Frequency analysis shows that the SAG-induced increase in Ca event frequency in the PTX-treated cells was smaller than that by DMSO in PTX-treated cells (Fig. 5A, d, ∆Frequency(SAG - base in PTX) versus ∆Frequency(DMSO - base in PTX); p < 0.001) and much smaller than that by SAG without PTX treatment (ΔFrequency(SAG - base in PTX) versus ∆Frequency(SAG - base without PTX); p < 0.001). Thus, we concluded that the enhancement of Ca oscillations by SAG requires G\textsubscript{i} activity. G\textsubscript{i} suppresses cAMP production from ATP by inhibiting adenylate cyclase. We confirmed that G\textsubscript{i} was actually activated by SAG stimulation by intracellular cAMP imaging using a fluorescent protein-based cAMP indicator, Fluminido2 (40). SAG stimulation increased the fluorescence intensity of Fluminido2 in astrocytes (Fig. 5B, c) compared with a control cell group to which vehicle (0.1% DMSO) was applied in place of SAG (p < 0.001, two-way analysis of variance), indicating that cAMP concentration was decreased. These results showed that SAG stimulation activated G\textsubscript{i}, presumably via SMO, which is necessary for the downstream SAG-induced enhancement of Ca oscillations.
We hypothesized that the SMO and G\textsubscript{i} activation by SAG induced ATP release, which led to the increased extracellular ATP concentration. To test this, carbenoxolone (CBX; 100 \textmu M) and 1-octanol (2 mM), blockers of connexin hemichannels (41, 42), gadolinium (Gd\textsuperscript{3+}; 50 \textmu M), a blocker of maxianion channels (43), and Brilliant Blue G (BBG; 1 \textmu M), a blocker of P2X7 receptors (44), were applied to astrocytes. The SAG-induced enhancement of Ca oscillation frequency was completely suppressed by CBX, 1-octanol, and Gd\textsuperscript{3+} (Fig. 6, A–C). CBX and 1-octanol induced slow Ca transients lasting 5–10 min. CBX induced Ca event frequency enhancement, which was then inhibited by the addition of SAG (Fig. 6A, b, base versus CBX: b, cumulative histograms of Ca oscillation frequency before application of the blockers, under the blockers, and after SAG addition. c, cumulative histograms of \Delta Frequency together with those from a control cell group in which 0.1% DMSO was applied in place of the blockers (n = 368 cells from 5 cultures).
SHH enhances Ca oscillations in astrocytes

$p < 0.001; \text{CBX versus CBX + SAG: } p < 0.001)$. ΔFrequency analysis together with a vehicle-applied control cell group (Fig. 6A, c) shows an apparent inhibition of the CBX-induced Ca oscillation enhancement by SAG (Fig. 6A, c; ΔFrequency[CBX + SAG] – base] versus ΔFrequency[(DMSO + SAG) – base]; $p < 0.001$). Under BBG, SAG increased Ca oscillation frequency (Fig. 6D, b, BBG versus BBG + SAG; $p < 0.001$). There was no statistically significant difference between ΔFrequency[(BBG + SAG) – base] and a control, ΔFrequency[(DMSO + SAG) – base] ($p = 0.16$). A concentration of 1 μM BBG blocks most P2X7 activity (44); therefore, P2X7 activity may not play a key role in the SAG-induced enhancement of Ca frequency. These results raise the possibility that astrocytes release ATP in response to SAG stimulation through maxinian channels and/or connexin hemichannels.

Ca oscillations in brain slice astrocytes are enhanced by SAG

Astrocytes in culture have different features to those in the brain, e.g., in morphology and proliferation state (45). To test if the enhancement of Ca oscillations by SHH pathway activation is an atypical phenomenon in cultured astrocytes or a general feature of astrocytes, we performed Ca imaging in astrocytes in hippocampal slices. [Ca$^{2+}$]i in astrocytes labeled with SR101 (46) was monitored with a Ca dye, Fluo-4 (Fig. 7). In dentate gyrus of hippocampus, intensively stained cells with SR101 were found in the molecular layer and weakly stained cells in the granule cell layer. Cell bodies of some interneuron species are known to exist in the inner molecular layer (47–49), and we indeed observed NeuN-positive, a general neuron marker, cell bodies in the inner molecular layer: some in proximity to the granule cell layer and much fewer distant from granule cell layer (Fig. S4). Therefore, ROIs were placed on SR101 and Fluo-4 double positive cells in the molecular layer excluding cells within 30 μm from the granule cell layer, where most, not to say all, SR101-positive cells should be astrocytes. During the baseline period, some astrocytes showed Ca oscillations, and SAG (50 nM) enhanced Ca oscillation frequency (Fig. 7C, $p < 0.01$), whereas vehicle (0.1% DMSO) did not (Fig. 7B, $p = 0.45$). ΔFrequency analysis also showed a significant difference between SAG and vehicle (Fig. 7D, $p < 0.01$). This result indicates that the enhancement of Ca oscillations by SHH signaling operates not only in cultured astrocytes but also in brain slice astrocytes.

Discussion

Astrocytes are known to respond to stimuli by evoking or altering their Ca oscillation patterns (29, 50, 51), in which $G_{q/11}$-coupled receptors, namely metabotropic glutamate receptors and P2Y receptors are often involved (52–54). In this study, the enhancement of Ca oscillations by SAG was inhibited by the degradation of extracellular ATP with apyrase or inhibition of $G_i$ with PTX. Therefore, the enhancement of Ca oscillations did not result from direct activation of $G_{q/11}$ but from $G_i$ activation by SMO, which in turn evoked ATP release. ATP release from astrocytes by SHH has been previously reported (30). The increased extracellular ATP may have activated P2Y receptors in adjacent astrocytes, and possibly in the astrocytes that released ATP, thereby enhancing the Ca oscillations in these cells. P2Y1/2/4/6/12/13/14 receptors are expressed in astrocytes, and P2Y1/2/4/6 receptors are coupled with $G_{q/11}$ (52, 55).

The finding that CBX, 1-octanol, and Gd$^{3+}$ inhibited the enhancement of Ca oscillations by SAG raises the possibility that the ATP release downstream of activated SMO was not mediated by a vesicular release mechanism but through ATP-permeable channels, namely connexin hemichannels and maxianion channels, which are sensitive to these antagonists. SLCO2A1 is a core component of the maxianion channel (56), although the precise molecular composition of the maxianion channel is still unclear (57). $G$ protein–coupled receptor activation (58) and dephosphorylation of maxianion channels (59) are involved in the opening mechanisms. The presumed suppression of cAMP-dependent protein kinase (PKA) downstream of $G_i$ activation by SAG could lead to dephosphorylation of maxianion channels. The opening mechanism of connexin hemichannels has not been clarified, and thus the mechanisms underlyng ATP release through them are unknown (60). However, because both CBX and Gd$^{3+}$ block a wide range of channels (61), they could directly inhibit the Ca oscillation mechanism downstream of ATP release rather than affecting the ATP release channels. Furthermore, 2-APB inhibits connexin channels with varying potencies depending on the subunit (62). Thus, an alteration of connexin-mediated ATP release by 2-APB could also be involved in the 2-APB inhibition of the SAG-induced Ca oscillation enhancement (Fig. 4) in addition to the inhibition of IP3R by 2-APB. Characterization of the ATP-release mechanisms downstream of SMO in astrocytes remains to be elucidated.

The lag in the onset of changes in the frequency of Ca transient on application of SHH or SAG varied considerably among cells. In some cells the increase started within 1 min and in others responses started after delays of 2–5 min. This variability indicates that some of the steps from SMO activation to the increase in extracellular ATP concentration are slow with variable speeds among cells. Extracellular ATP accumulation is one such variable step. The not-so-simple dose-response in Ca oscillation frequency in response to SHH or SAG (Fig. 1, D and E) may reflect such a complicated mechanism, together with a possibility that SHH and SAG have multiple sites of action on their receptors.

Although we consider that the direct ATP release and subsequent Ca oscillations was via a noncanonical SHH pathway through $G_i$ activation, the canonical SHH pathway, in which GLI is activated and transported to the nucleus, may be influenced by the noncanonical series of events. When the SHH signal is turned off, GLI is ubiquitinated by $\beta$-TrCP, which is induced by the activities of PKA, glycogen synthase kinase 3β,
and CK1. This removes the GLI active domain and keeps GLI inactive (9). When the SHH signal is turned on, GLI escapes from ubiquitination, and full-length GLI dissociates from the negative regulator, Suppressor of Fused (SuFu), and migrates into the nucleus (9, 63, 64). Although the complex interactions between SMO and GLI are not understood in detail, several lines of evidence indicate that PKA is a key negative regulator of the canonical SHH signal downstream of SMO (65–69). Thus, the presumed PKA deactivation following Gq activation and the decrease in cAMP concentration, which were confirmed in this study, may reduce the negative effect of PKA and push the balance of inactive/active GLI molecules to the active side.

Whether this deactivation of PKA by activation of Gq downstream of SMO is peculiar to astrocytes or a more general feature needs to be clarified.

Adenylyl cyclase 5 and 6 (AC5/6) localize to primary cilia (70) and are sensitive to $[\text{Ca}^{2+}]_i$ (71). SHH and SAG raise $[\text{Ca}^{2+}]_i$, in primary cilia, possibly via Trp channels and Gd$^{3+}$-sensitive plasma membrane channels (72–74). Moore et al. (74) reported that the cAMP concentration in primary cilia is 5-fold higher than that of whole cells, and SHH stimulation increased ciliary $[\text{Ca}^{2+}]_i$ and decreased ciliary cAMP concentration in mouse embryonic fibroblasts. They suggested that inhibition of AC5/6 by Ca$^{2+}$ is the mechanism for the reduction of cAMP in cilia by SHH. Primary cilia may behave as isolated compartments where concentration and dynamics of signaling molecules, including Ca$^{2+}$, are separate from global cytoplasmic Ca$^{2+}$ (73, 75). Therefore, the Ca increase and cAMP reduction observed in this study may have occurred in SAG-stimulated primary cilia of astrocytes in parallel with the global enhancement of cytoplasmic Ca oscillations and reduced concentrations of cAMP.

In the adult brain, SHH is secreted upon brain injury and makes astrocytes reactive (21, 25). In severe cases, the gathered...
astrocytes then form a characteristic astrocyte scar around the injured site (76, 77). Although the intercellular and intracellular signaling mechanisms that make astrocytes reactive and form scars are not well-characterized, an increase in extracellular ATP has been implicated in the initial microglial activation (78), leading to transformation of astrocytes to the reactive state (79). Although it is proposed that ATP is released from injured cells as “find me” (80) or “eat me” (81) signals, intact astrocytes could be involved in the increase in extracellular ATP in the early phase of the response to injury by responding to SHH release because SHH levels are raised after traumatic brain injury (21, 22). SHH is also expressed after brain ischemia (82), and administration of SHH to rats just after stroke partially relieves neurological damage with improved angiogenesis and neuron survival (83). The release of ATP from astrocytes and enhancement of Ca oscillations in astrocytes may also be involved in recovery from brain ischemia.

Ca oscillations are observed in a wide range of cell types (51), and controls various vital functions of cells, e.g. egg activation in fertilization (84) and differentiation of osteoclasts (85). However, outcomes of many Ca oscillation events are not known, including for the Ca oscillations in astrocytes. A possible scenario is that Ca oscillations activate Ca-dependent transcription factors and alters gene expression patterns, which may modulate cellular proliferation, differentiation, and programmed cell death. It was proposed that different sets of transcription factors are activated according to the frequency and amplitude of Ca oscillations following experiments in which intracellular Ca patterns were artificially controlled (86, 87). NFAT activity is controlled by Ca oscillations (86–88), and dephosphorylation of NFAT by calcineurin, a Ca-dependent phosphatase, is postulated as a mechanism for NFAT activation by Ca oscillations (89). It is conceivable that the activity of GLI via the canonical SHH pathway is regulated by Ca oscillations evoked through a noncanonical pathway, because the activity of GLI is controlled by dephosphorylation (68). The expression of SHH is increased during development and upon traumatic injury; therefore, the Ca oscillations in astrocytes observed in this study may play roles in differentiation and activation of astrocytes and may affect nearby neurons by releasing gliotransmitters. In future research, it will be of great interest to determine the outcome of Ca oscillations induced by SHH in slice preparations or in vivo.

In summary, we found that: 1) Ca oscillations in the hippocampus were enhanced in astrocytes in response to SHH or SAG; 2) the enhancement of Ca oscillations by SAG required IP3R-dependent Ca release; 3) Gp plays a role downstream of SMO in the enhancement of Ca oscillations by SAG; 4) ATP-permeable channels may be responsible for the ATP release that activates Ca oscillations in surrounding astrocytes; and 5) the enhancement of Ca oscillations by SHH signaling was not peculiar to cultured astrocytes and was also observed in slice preparations.

Experimental procedures

Animal care

Animal care was in accordance with guidelines outlined by the Institutional Animal Care and Use Committee of Waseda University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Waseda University. All efforts were made to minimize the number of animals used and their suffering during experiments.

Cell culture

Primary hippocampal cultures were prepared from E17 ICR mice as described previously (90) with modifications: hippocampi were dissociated with 0.25% papain (38N18758, Worthington, Lakewood, NJ) containing 0.25% DNase in Glucose mix (PBS containing 0.4% glucose, 0.04% BSA, and 0.04% L-cysteine) at 37 °C for 5 min. Dissociated cells were seeded on poly-ethyleneimine (PEI)-coated round glass coverslips (12 mm in diameter, 1 × 10⁶ cells/slip) with Neurobasal medium (12349-015, Thermo Fisher Scientific, Tokyo, Japan) containing 2% B-27 supplement (Thermo Fisher Scientific), 1% L-glutamine, and 0.05% penicillin-streptomycin. Cells kept in vitro for 11–17 days were used. The culture confluence was 80–90%.

Hippocampal astrocyte cultures were prepared from E17 ICR mice as described previously (91) with modifications: dissociated cells were plated in PEI-coated 75-cm² culture flasks (15 × 10⁶ cells/10 ml) in Dulbecco’s modified Eagle’s medium/F-12 medium (Sigma) containing 5% horse serum and 5% fetal bovine serum, 0.5% L-glutamine, and 0.36% penicillin-streptomycin. After 10 days in culture, the cells were suspended with 0.025% trypsin in Hanks’ balanced salt solution, Ca²⁺ and Mg²⁺ free, and plated on PEI-coated round glass coverslips (12 mm in diameter, 5 × 10⁴ cells/slip) with the same culture medium. Cultures at 80–90% confluence were used for imaging.

Immunohistochemistry

Cells were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.25% Triton X-100 in PBS for 10 min, and blocked with 1% BSA for 1 h at room temperature. Cells were incubated overnight at 4 °C with the primary antibody and for 1 h at room temperature with the secondary antibody (1:1000). The primary antibodies used were: anti-MAP2 (1:500) (sc-212905, Santa Cruz Biotechnology, or S0224, KT Laboratories, St. Paul, MN) was dissolved in DMSO at 50 mM and stored at −80 °C in 100 μg/ml of aliquots. 2-APB (D0281, Tokyo Chemical Industry, Tokyo, Japan) was dissolved in DMso at 50 μM and stored at −20 °C. PTX (516560, Calbiochem (Sigma)) was dissolved in H₂O at 100 μg/ml, stored at 4 °C, and used within 6 months. CBX (C4790, Sigma) was dis-
solved in DMSO at 100 mM and stored at −20 °C. Gadolinium chloride (16506-71, Nacalai Tesque, Kyoto, Japan) was dissolved in H₂O at 50 mM, stored at −20 °C, and used within 10 days. 1-Octanol (25506-62, Nacalai Tesque) was dissolved in DMSO at 2 mM and stored at −20 °C. BBG (B1146, Tokyo Chemical Industry) was dissolved in DMSO at 1 mM and stored at −20 °C.

**DNA transfection**

Cultured astrocytes were electroporated with plasmid DNA encoding Flamindo 2 (40) using the Amaza Basic Nucleofector Kit for Primary Mammalian Glial Cells (Lonzza Japan, Tokyo, Japan) according to the manufacturer’s protocol. Nucleofected astrocytes were placed on PEI-coated round glass coverslips (12 mm in diameter, 1 × 10⁵ cells/slip). After 1–2 h, Dulbecco’s modified Eagle’s/F-12 medium (Sigma) containing 5% horse serum, 5% fetal bovine serum, 0.5% L-glutamine, and 0.36% penicillin-streptomycin was added. Two-5 days after nucleofection 50–60% confluent cells were used.

**Cell culture imaging**

Coverslips holding cultured cells were maintained in a stainless steel chamber containing HBS (in mM, 20 HEPES, 115 NaCl, 5.4 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, pH 7.4). A Ca indicator, Fura-2 was loaded into cells by incubation with 2.5 mM Fura-2-AM/HBS (Dojindo, Kumamoto, Japan) at 37 °C for 10 min followed by three washes with HBS. TTX (1 μM) was added to HBS throughout the imaging procedures (HBS-TTX) when primary culture preparations were used to avoid Ca events evoked by neuronal activities. Time-lapse imaging was performed with an inverted microscope (IX71, Olympus, Tokyo, Japan) with a ×20 objective (U-Apo/340, N.A., 0.75, Olympus). Fura-2 was excited at 340 and 380 nm wavelength light alternating every 3 s and fluorescence was detected with a cooled CCD camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan) through a 495–540 or 575–630–nm band-pass filter for the Fura-2 dual wavelength acquisition described above. There was no detectable cross-talk between the Fura-2 and Fluo-4 optical paths. For time course analysis of Flamindo 2 results, the fluorescence signal of each frame was divided by that of the first image frame (F/F₀) after background subtraction. Image acquisition and data analysis were performed with custom-made T1 Workbench software written by T. I. (92).

**Brain slice**

Hippocampal slices, 400 μm thick, were prepared from 4–8-week-old male C57BL/6 mice using a standard method (93) and used within 1 day of preparation. A Ca indicator, Fluo-4 AM (Thermo Fisher Scientific), and an astrocyte marker, sulforhodamine 101 (SR101, sc-215929, Santa Cruz), were loaded onto the slices by incubating with 5 μM Fluo-4 AM in artificial cerebrospinal fluid (ACSF; in mM, 1 NaCl 125; KCl 2.5; NaH₂PO₄ 1.25; NaHCO₃ 26; MgCl₂ 1; CaCl₂ 2; and glucose 20, 310–315 mosmol and bubbled with a 95% O₂ and 5% CO₂ gas mixture) at 34 °C for 40 min. SR101 (1 μM) was then added and the slices were stained for another 20 min. After staining, slices were transferred to ACSF and kept at room temperature for 30 min before the time-lapse imaging. Time-lapse imaging was performed with an in-house two-photon microscope (92) mounted on an upright microscope (BX51, Olympus) with a ×20 water immersion objective (XLUMPlanFL, N.A., 0.95, Olympus). Brain slices were continuously superfused with ACSF containing 1 μM TTX at 34 °C. An excitation laser of wavelength at 920 nm (titanium-sapphire pulse laser, Mai Tai DeepSee, Spectra-Physics, Santa Clara, CA) was used and the emission was divided with a 580-nm beam splitter and passed through a 495–540 or 575–630–nm band-pass filter for the Fluo-4 and the SR101 signals, respectively. Data analysis was performed as described for the cell culture experiments except that the ROIs were placed on SR101 and Fluo-4 double positive
SHH enhances Ca oscillations in astrocytes

cells in the molecular layer and the Fluo-4 fluorescence intensity of each ROI was normalized by dividing by that of the first image frame (F/F₀). A baseline value for each data point was calculated as described above by averaging the preceding 20 frames, and fluorescence intensity of the data point of the ROI was subtracted with the baseline value. We used 0.3 as the threshold to detect Ca transients. Although SR101 is widely used to stain astrocytes, hyperexcitation of neurons is a known side effect (94, 95). We consider that if this side effect occurred in this study it was minute because the concentration of SR101 used was much lower than the suggested threshold (between 50 and 250 μM) (95).

Statistics

Mann-Whitney U test included in the ALGLIB library (www.alglib.net)³ implemented in TI Workbench was used to compare two groups unless otherwise indicated. All indicated data are given as the average ± S.D.

Author contributions—C. A. and T. Inoue formal analysis; C. A., N. K., S. J., T. Ishii, Y. A., S. T., and T. Inoue investigation; C. A. and T. Inoue visualization; C. A. and T. Inoue writing-original draft; S. J. writing-review and editing; S. A., T. K., and T. Inoue resources; S. T. and T. Inoue conceptualization; T. Inoue software; T. Inoue supervision; T. Inoue funding acquisition; T. Inoue validation; T. Inoue methodology; T. Inoue project administration.

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References

1. Kimelberg, H. K., and Nedergaard, M. (2010) Functions of astrocytes and their potential as therapeutic targets. *Neurother. J. Am. Soc. Exp. Neurorother. 7, 338–353* CrossRef
2. Voutsinos-Porche, B., Bonvento, G., Tanaka, K., Steiner, P., Welker, E., Chatton, J.-Y., Magistretti, P. J., and Pellerin, L. (2003) Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. *Neuron 37*, 275–286 CrossRef Medline
3. Butt, A. M. (2011) ATP: a ubiquitous gliotransmitter integrating neuronal-glial networks. *Semin. Cell Dev. Biol. 22*, 205–213 CrossRef Medline
4. Parpura, V., and Zorc, R. (2010) Gliotransmission: Exocytotic release from astrocytes. *Brain Res. Rev. 63*, 83–92 CrossRef Medline
5. Nüsslein-Volhard, C., and Wieschaus, E. (1980) Mutations affecting segment number and polarity in *Drosophila*. *Nature 287*, 795–801 CrossRef Medline
6. Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell 75*, 1417–1430 CrossRef Medline
7. Briscoe, J., and Thérond, P. P. (2013) The mechanisms of hedgehog signalling and its roles in development and disease. *Nat. Rev. Mol. Cell Biol. 14*, 416–429 CrossRef Medline
8. Choudhry, Z., Rikani, A. A., Choudhry, A. M., Tariq, S., Zakaria, F., Asghar, M. W., Sarraf, M. K., Haider, K., Shaﬁq, A. A., and Mobassarah, N. J. (2014) Sonic hedgehog signalling pathway: a complex network. *Ann. Neurosci. 21*, 28–31 Medline
9. Sasai, N., and Briscoe, J. (2012) Primary cilia and graded Sonic Hedgehog signaling. *Wiley Interdiscip. Rev. Dev. Biol. 1*, 753–772 CrossRef Medline
10. Brennan, D., Chen, X., Cheng, L., Mahoney, M., and Riobo, N. A. (2012) Noncanonical Hedgehog signaling. *Vitam. Horm. 88*, 55–72 CrossRef Medline
11. Jenkins, D. (2009) Hedgehog signalling: emerging evidence for non-canonical pathways. *Cell Signal. 21*, 1023–1034 CrossRef Medline
12. Chang, H., Li, Q., Moraes, R. C., Lewis, M. T., and Hamel, P. A. (2010) Activation of Erk by sonic hedgehog independent of canonical hedgehog signalling. *Int. J. Biochem. Cell Biol. 42*, 1462–1471 CrossRef Medline
13. Yann, P. T., Langlois, S. D., Morin, S., and Charron, F. (2009) Sonic hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. *Neuron 62*, 349–362 CrossRef Medline
14. Chinchilla, P., Xiao, L., Kazanietz, M. G., and Riobo, N. A. (2010) Hedgehog proteins activate pro-angiogenic responses in endothelial cells through non-canonical signaling pathways. *Cell Cycle 9*, 570–579 CrossRef
15. Marti, E., and Bovolenta, P. (2002) Sonic hedgehog in CNS development: one signal, multiple outputs. *Trends Neurosci. 25*, 89–96 CrossRef Medline
16. Álvarez-Buylla, A., and Ihrie, R. A. (2014) Sonic hedgehog signaling in the postnatal brain. *Semin. Cell Dev. Biol. 33*, 105–111 CrossRef Medline
17. Traiffort, E., Charonytis D., Watroba, L., Faure, H., Sales, N., and Raut, M. (1999) Discrete localizations of hedgehog signalling components in the developing and adult rat nervous system. *Eur. J. Neurosci. 11*, 3199–3214 CrossRef Medline
18. Petralia, R. S., Wang, Y.-X., Mattson, M. P., and Yao, P. J. (2011) Sonic hedgehog distribution within mature hippocampal neurons. *Commun. Integr. Biol. 4*, 775–777 CrossRef Medline
19. Petralia, R. S., Schwartz, C. M., Wang, Y.-X., Mattson, M. P., and Yao, P. J. (2011) Subcellular localization of Patched and Smoothened, the receptors for Sonic Hedgehog signaling, in the hippocampal neuron. *J. Comp. Neurol. 519*, 3684–3699 CrossRef Medline
20. Yao, P. J., Petralia, R. S., and Mattson, M. P. (2016) Sonic Hedgehog signaling and hippocampal neuroplasticity. *Trends Neurosci. 39*, 840–850 CrossRef Medline
21. Amankulor, N. M., Hambardzumyan, D., Pyontek, S. M., Becher, O. J., Joyce, J. A., and Holland, E. C. (2009) Sonic hedgehog pathway activation is induced by acute brain injury and regulated by injury-related inflammation. *J. Neurosci. 29*, 10299–10308 CrossRef Medline
22. Lee, J. H., Chung, Y. C., Bok, E., Lee, H., Huh, S. H., Lee, J. E., Jin, B. K., and Ko, H. W. (2017) Injury-stimulated Sonic hedgehog expression in microglia contributes to neuroinflammatory response in the MPTP model of Parkinson’s disease. *Biochem. Biophys. Res. Commun. 482*, 980–988 CrossRef Medline
23. Pitter, K. L., Tamagno, I., Feng, X., Ghosal, K., Amankulor, N., Holland, E. C., and Hambardzumyan, D. (2014) The SHH/Gli pathway is reactivated in reactive glia and drives proliferation in response to neurodegeneration-induced lesions. *GLIA 62*, 1595–1607 CrossRef Medline
24. Garcia, A. D., Petrova, R., Eng, L., and Joyner, A. L. (2010) Sonic hedgehog regulates discrete populations of astrocytes in the adult mouse forebrain. *J. Neurosci. 30*, 13597–13608 CrossRef Medline
25. Sirko, S., Behrendt, G., Johansson, P. A., Tripathi, P., Costa, M., Bek, S., Heinrich, C., Tiedt, S., Colak, D., Reich, M., Fischer, I. R., Plesnila, N., Stamenbier, M., Haass, C., Snapyan, M., et al. (2013) Reactive glia in the injured brain acquire stem cell properties in response to sonic hedgehog. *Cell Stem Cell 12*, 426–439 CrossRef Medline
26. Scemes, E., and Giaume, C. (2006) Astrocyte calcium waves: What they are and what they do. *GLIA 54*, 716–725 CrossRef Medline
27. Pasti, L., Volterra, A., Pozzan, T., and Carmignoto, G. (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. *J. Neurosci. 17*, 7817–7830 CrossRef Medline
28. Verkhratsky, A., Verkhrasky, A., Kristol, O. A., and Burnstock, G. (2009) Purinoceptors on neuroglia. *Mol. Neurobiol. 39*, 190–208 CrossRef Medline
29. Hamilton, N., Vayro, S., Kirchhoff, F., Verkhratsky, A., Robbins, J., Gorecki, D. C., and Butt, A. M. (2008) Mechanisms of ATP- and glutamate-mediated calcium signaling in white matter astrocytes. *GLIA 56*, 734–749 CrossRef Medline

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43. Liu, H.-T., Toychiev, A. H., Takahashi, N., Sabirov, R. Z., and Okada, Y. (2016) Hedgehog signaling modulates the release of glutamatergic neurotransmitters from cultured cerebellar astrocytes. Neurochem. Res. 41, 278–289 CrossRef Medline

36. Charles, A. C., Dirksen, E. R., Merrill, J. E., and Sanderson, M. J. (1993) Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process. Proc. Natl. Acad. Sci. U.S.A. 99, 14071–14076 CrossRef Medline

37. Bootman, M. D., Collins, T. J., Mackenzie, L., Roderick, H. L., Berridge, M. J., and Peppiatt, C. M. (2002) 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of Inp3-induced Ca2+ release. FASEB J. 16, 1145–1150 CrossRef Medline

40. Odaka, H., Arai, S., Inoue, T., and Kitaguchi, T. (2014) Genetically-enforced expression of the primary cilium to prevent Gli2 activation and ventralization of the mouse neural tube. Proc. Natl. Acad. Sci. U.S.A. 111, 5857–5862 CrossRef Medline

48. Freund, T. F., and Buzsáki, G. (1996) Interneurons of the hippocampus. Hippocampus 6, 347–470 Medline

38. Birchmeier, W., and Birchmeier, C. (1989) Smoothened is an integral membrane protein. Proc. Natl. Acad. Sci. U.S.A. 86, 8491–8495 CrossRef Medline

44. Schratt, G., Inoue, T., Sakurai, T., and Shi, Y. (2004) Synaptic activity regulates hedgehog signaling through cilia. Nature 428, 684–688 CrossRef Medline

50. Kim, S.-J., Kang, M. J., Lee, J. S., Cho, J., Shin, H. J., Lee, J. H., Han, J. R., Hwang, J. H., Lim, K. S., Park, J. H., and Lee, S. J. (2019) Inhibition of linker histidines of SHH by synthetic GLI antagonists. Nat. Chem. Biol. 15, 310–316 CrossRef Medline

41. Mukhopadhyay, S., and Rohatgi, R. (2014) G-protein-coupled receptor, hedgehog partial agonism drives Warburg-like metabolism in muscle and neuronal cells: glutamatergic neurons in the rat dentate gyrus. Genes Dev. 28, 368–379 CrossRef Medline

49. Hajo, H., Yamasaki, K., Ishii, T., Ohta, M., and Hara, K. (2013) Smoothened antagonizes cAMP signaling in the mammalian ventral hindbrain to establish dorsal-ventral patterning. Dev. Biol. 391, 104–115 CrossRef Medline

51. Tsien, R. Y. (1990) Calcium indicators and methods of analysis. J. Neurosci. Methods 30, 251–259 CrossRef Medline

52. Salter, M. W., and Hicks, J. L. (1999) ATP-evoked increases in intracellular calcium in neurons and glia from the dorsal spinal cord. J. Neurosci. 14, 1563–1575 Medline

53. Williams, P. A., Larimer, P., Gao, Y., and Strowbridge, B. W. (2007) Semi-synthetic libraries: a new approach to the discovery of hedgehog inhibitors. J. Med. Chem. 50, 10408–10415 CrossRef Medline

54. Arabshahi, S., and Bhat, M. P. (2006) Selective hedgehog antagonists block Hedgehog-induced glioma proliferation. Biochem. J. 395, 193–199 Medline

55. Burnstock, G. (2008) Purinergic signalling and disorders of the central nervous system. Nat. Rev. Drug Discov. 7, 575–590 CrossRef Medline

56. Sabirov, R. Z., Merzlyak, P. G., Okada, T., Islam, M. R., Uramoto, H., Mori, T., Makino, Y., Matsuura, H., Xie, Y., and Okada, Y. (2017) The organic anion transporter SLCO2A1 constitutes the core component of the Maxi-C1 channel. EMBO J. 36, 3309–3324 CrossRef Medline

57. Sabirov, R. Z., Merzlyak, P. G., Islam, M. R., Okada, T., and Okada, Y. (2016) The properties, functions, and pathophysiology of maxi-anion channels. Pflugers Arch. 468, 405–420 CrossRef Medline

58. Saigusa, A., and Kokubun, S. (1988) Protein kinase c may regulate resting anion conductance in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 155, 882–889 CrossRef Medline

59. Toychiev, A. H., Sabirov, R. Z., Takahashi, N., Ando-Akatsuka, Y., Liu, H., Shintani, T., Noda, M., and Okada, Y. (2009) Activation of maxi-anion channel by protein tyrosine dephosphorylation. Am. J. Physiol. Cell Physiol. 297, C990–C1000 CrossRef Medline

60. Nielsen, B. S., Hansen, D. B., Ransom, B. R., Nielsen, M. S., and MacAulay, N. (2017) Connexin hemichannels in astrocytes: an assessment of controversies regarding their functional characteristics. Neurochem. Res. 42, 2537–2550 CrossRef Medline

61. Connors, B. W. (2012) Tales of a dirty drug: carbenoxolone, gap junctions, and seizures. Epilepsy Curr. 12, 66–68 CrossRef Medline

62. Bai, D., del Corso, C., Srinivas, M., and Spray, D. C. (2006) Block of receptor-operated anion conductance in vascular smooth muscle cells. J. Biol. Chem. 281, 35353–35365 CrossRef Medline

63. Arensdorf, A. M., Marada, S., and Ogden, S. K. (2016) Smoothened regulation: a tale of two signals. Trends Pharmacol. Sci. 37, 62–72 CrossRef Medline

64. Ryan, K. E., and Chiang, C. (2012) Hedgehog secretion and signal transduction in vertebrates. J. Biol. Chem. 287, 17905–17913 CrossRef Medline

65. Fan, C. M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M. (1995) Long-range sclerotome induction by sonic hedgehog direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. Cell 81, 457–465 CrossRef Medline

66. Hynes, M., Porter, J. A., Chiang, C., Chang, D., Beachy, P. A., and Tessier-Lavigne, M. (1995) Hedgehog signalling and disorders of the central nervous system. Trends Neurosci. 18, 502–512 CrossRef Medline

67. Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A., and Rosenthal, A. (1995) Induction of midbrain dopaminergic neurons in the vertebrate embryo. Genes Dev. 10, 647–658 CrossRef Medline

68. Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A., and Rosenthal, A. (1995) Induction of midbrain dopaminergic neurons by Sonic hedgehog. Neuron 15, 35–44 CrossRef Medline

69. Niewiadomski, P., Kong, J. H., Ahrends, R., Ma, Y., Humke, E. W., Khan, S., Teruel, M. N., Novitch, B. G., and Rohatgi, R. (2014) Gli protein activity is controlled by multisite phosphorylation in vertebrate Hedgehog signaling. Cell Rep. 6, 168–181 CrossRef Medline

70. Tuson, M., He, M., and Anderson, K. V. (2011) Protein kinase A acts at the basal body of the primary cilium to prevent Gli2 activation and ventralization of the mouse neural tube. Development 138, 4921–4930 CrossRef Medline
**SHH enhances Ca oscillations in astrocytes**

70. Vuolo, L., Herrera, A., Torroba, B., Menendez, A., and Pons, S. (2015) Ciliary adenyl cyclases control the Hedgehog pathway. *J. Cell Sci.* **128**, 2928–2937 CrossRefMedline

71. Cooper, D. M., Karpen, J. W., Fagan, K. A., and Mons, N. E. (1998) Ca^{2+}-sensitive adenyl cyclases. *Adv. Second Messenger Phosphoprotein Res.* **32**, 23–51 Medline

72. DeCaen, P. G., Delling, M., Vien, T. N., and Clapham, D. E. (2013) Direct recording and molecular identification of the calcium channel of primary cilia. *Nature* **504**, 315–318 CrossRefMedline

73. Delling, M., DeCaen, P. G., Doerner, J. F., Fevay, S., and Clapham, D. E. (2013) Primary cilia are specialized calcium signalling organelles. *Nature* **504**, 311–314 CrossRefMedline

74. Moore, B. S., Stepanchick, A. N., Tewson, P. H., Hartle, C. M., Zhang, J., Quinn, A. M., Hughes, T. E., and Mirshahi, T. (2016) Cilia have high cAMP levels that are induced by Sonic Hedgehog-regulated calcium dynamics. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 13069–13074 CrossRefMedline

75. Lin, Y.-C., Niewiadomski, P., Lin, B., Nakamura, H., Phua, S. C., Jiao, J., Levenchenko, A., Inoue, T., Rohatgi, R., and Inoue, T. (2013) Chemically inducible diffusion trap at cilia reveals molecular sieve-like barrier. *Nat. Chem. Biol.* **9**, 437–443 CrossRefMedline

76. Burda, J. E., and Sofroniew, M. V. (2014) Reactive gliosis and the multicellular response to CNS damage and disease. *Neuron* **81**, 229–248 CrossRefMedline

77. Liddelow, S., and Barres, B. A. (2017) Reactive astrocytes: production, function, and therapeutic potential. *Immunity* **46**, 957–967 CrossRefMedline

78. Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., Littman, D. R., Dustin, M. L., and Gan, W.-B. (2005) ATP mediates rapid microglial response to local brain injury in *vivo*. *Nat. Neurosci.* **8**, 752–758 CrossRefMedline

79. Shinozaki, Y., Shibata, K., Yoshida, K., Shigetomi, E., Gachet, C., Ikenaka, K., Tanaka, K., F., and Koizumi, S. (2017) Transformation of astrocytes to a neuroprotective phenotype by microglia via P2Y1 receptor downregulation. *Cell Rep.* **19**, 1151–1164 CrossRefMedline

80. Elliott, M. R., Chekeni, F. B., Trampont, P. C., Lazarowski, E. R., Kald, A., Walk, S. F., Park, D., Woodson, R. L., Ostankovich, M., Sharma, P., Lysiak, J. J., Harden, T. K., Leitinger, N., and Ravichandran, K. S. (2009) Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* **461**, 282–286 CrossRefMedline

81. Koizumi, S., Shigemoto-Mogami, Y., Nasu-Tada, K., Shinozaki, Y., Ohsawa, K., Tsuda, M., Joshi, B. V., Jacobson, K. A., Kohsaka, S., and Inoue, K. (2007) UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature* **446**, 1091–1095 CrossRefMedline

82. Sims, J. R., Lee, S.-W., Topalkara, K., Qiu, J., Xu, J., Zhou, Z., and Moskowitz, M. A. (2009) Sonic hedgehog regulates ischemia/hypoxia-induced neural progenitor proliferation. *Stroke* **40**, 3618–3626 CrossRefMedline

83. Chen, S.-C., Huang, M., He, Q.-W., Zhang, Y., Opoku, E. N., Yang, H., Jin, H.-J., Xia, Y.-P., and Hu, B. (2017) Administration of Sonic Hedgehog protein induces angiogenesis and has therapeutic effects after stroke in rats. *Neuroscience* **352**, 285–295 CrossRefMedline

84. Malcuit, C., Kurokawa, M., and Fissore, R. A. (2006) Calcium oscillations and mammalian egg activation. *J. Cell Physiol.* **206**, 565–573 CrossRefMedline

85. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Ishikawa, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniguchi, T. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell* **3**, 889–901 CrossRefMedline

86. Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392**, 933–936 CrossRefMedline

87. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., and Healy, J. I. (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* **386**, 855–858 CrossRefMedline

88. Colella, M., Grisan, F., Robert, V., Turner, J. D., Thomas, A. P., and Pozzan, T. (2008) Ca^{2+} oscillation frequency decoding in cardiac cell hypertrophy: role of calcineurin/NFAT as Ca^{2+} signal integrators. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 2859–2864 CrossRefMedline

89. Hogan, P. G., Chen, L., Nardone, J., and Rao, A. (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* **17**, 2205–2232 CrossRefMedline

90. Bannai, H., Hirose, M., Niwa, F., and Mikoshiba, K. (2019) Dissection of local Ca^{2+} signals in cultured cells by membrane-targeted Ca^{2+} indicators. *J. Vis. Exp.* 10.3791/59246 CrossRef

91. Ito, J., Kato, T., Yamakawa, Y., Kato, H., Sakazaki, Y., Lim, R., and Tanaka, R. (1982) Interaction of glia maturation factor with the glial cell membrane. *Brain Res.* **243**, 309–314 CrossRefMedline

92. Inoue, T. (2018) TI Workbench, an integrated software package for electrophysiological properties of thalamic relay cells. *Neurosci. Res.* **87**, 16–25 CrossRefMedline

93. Hülsmann, S., Hagos, L., Heuer, H., and Schnell, C. (2017) Limitations of sulforhodamine 101 for brain imaging. *Front. Cell. Neurosci.* **44**, 10.3389/fncel.2017.00044 CrossRef

94. Rasmussen, R., Nedergaard, M., and Petersen, N. C. (2016) Sulforhodamine 101, a widely used astrocyte marker, can induce cortical seizure-like activity at concentrations commonly used. *Sci. Rep.* **6**, 30433 CrossRefMedline