Activation of astroglial calcium signaling by endogenous metabolites succinate and gamma-hydroxybutyrate in the nucleus accumbens

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INTRODUCTION
Using Ca\(^{2+}\) imaging in combination with immunohistochemistry, we have recently demonstrated significant co-localization of ATP-stimulated Ca\(^{2+}\) bursts with the glial marker protein connexin43 (Cx43) in slices containing the brain reward area (Berridge and Kringelbach, 2008), the nucleus accumbens (NAc). Cells responding to SUC by Ca\(^{2+}\) transient constitute a subset of ATP-responsive astrocytes that are activated in a neuron-independent way. In this study we show that GHB-evoked Ca\(^{2+}\) transients were also found to constitute a subset of ATP-responsive astrocytes in the NAc. Repetitive Ca\(^{2+}\) dynamics evoked by GHB suggested that Ca\(^{2+}\) was released from internal stores. Similarly to SUC, the GHB response was also characterized by an effective concentration of 50 μM. We observed that the number of ATP-responsive cells decreased with increasing concentration of either SUC or GHB. Moreover, the concentration dependence of the number of ATP-responsive cells were highly identical as a function of both [SUC] and [GHB], suggesting a mutual receptor for SUC and GHB, therefore implying the existence of a distinct GHB-recognizing astroglial SUC receptor in the brain. The SUC-evoked Ca\(^{2+}\) signal remained in mice lacking GABAB receptor type 1 subunit in the presence and absence of the N-Methyl-d-Aspartate (NMDA) receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV), indicating action mechanisms independent of the GABAB or NMDA receptor subtypes. By molecular docking calculations we found that residues R99, H103, R252, and R281 of the binding crevice of the kidney SUC-responsive membrane receptor SUCNR1 (GPCR91) also predict interaction with GHB, further implying similar GHB and SUC action mechanisms. We conclude that the astroglial action of SUC and GHB may represent a link between brain energy states and Ca\(^{2+}\) signaling in astrocytic networks.

Keywords: energy metabolites, succinate, gamma-hydroxybutyrate, astroglial calcium signaling, nucleus accumbens

Accumulating evidence suggests that different energy metabolites play a role not only in neuronal but also in glial signaling. Recently, astroglial Ca\(^{2+}\) transients evoked by the major citric acid cycle metabolite succinate (SUC) and gamma-hydroxybutyrate (GHB) that enters the citric acid cycle via SUC have been described in the brain reward area, the nucleus accumbens (NAc). Cells responding to SUC by Ca\(^{2+}\) transient constitute a subset of ATP-responsive astrocytes that are activated in a neuron-independent way. In this study we show that GHB-evoked Ca\(^{2+}\) transients were also found to constitute a subset of ATP-responsive astrocytes in the NAc. Repetitive Ca\(^{2+}\) dynamics evoked by GHB suggested that Ca\(^{2+}\) was released from internal stores. Similarly to SUC, the GHB response was also characterized by an effective concentration of 50 μM. We observed that the number of ATP-responsive cells decreased with increasing concentration of either SUC or GHB. Moreover, the concentration dependence of the number of ATP-responsive cells were highly identical as a function of both [SUC] and [GHB], suggesting a mutual receptor for SUC and GHB, therefore implying the existence of a distinct GHB-recognizing astroglial SUC receptor in the brain. The SUC-evoked Ca\(^{2+}\) signal remained in mice lacking GABAB receptor type 1 subunit in the presence and absence of the N-Methyl-d-Aspartate (NMDA) receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV), indicating action mechanisms independent of the GABAB or NMDA receptor subtypes. By molecular docking calculations we found that residues R99, H103, R252, and R281 of the binding crevice of the kidney SUC-responsive membrane receptor SUCNR1 (GPCR91) also predict interaction with GHB, further implying similar GHB and SUC action mechanisms. We conclude that the astroglial action of SUC and GHB may represent a link between brain energy states and Ca\(^{2+}\) signaling in astrocytic networks.

Keywords: energy metabolites, succinate, gamma-hydroxybutyrate, astroglial calcium signaling, nucleus accumbens
Additionally, intracellular Ca$^{2+}$ store-reliant astroglial Ca$^{2+}$ transients evoked by GHB have been described in the NAc (Molnár et al., 2009). We have shown that this action of GHB is GABA$\beta$ receptor independent, since it remains in mice lacking functional GABA$\beta$ receptors unlike the majority of GHB-mediated physiological and pharmacological actions including "rape drug effects" that were demonstrated to be dependent on GABA$\beta$ receptors (Kaufmann et al., 2003; Wong et al., 2004). Only a few study addressed GHB receptor dependent GHB actions (Molnár et al., 2006, 2009), although high-affinity GHB binding sites were demonstrated in mice lacking GABA$\beta$ receptors (Kaufmann et al., 2003) and the function of endogenous GHB has never been clearly defined. A number of studies indicate that in addition to its neurotransmitter/neuromodulatory role, GHB may function in the control of physiological states, like sleep and hibernation. Moreover, it is an endogenous protective agent when tissue energy supplies are limited (MacMillan, 1980a,b; Mamelak, 1989; references cited). The molecular identity of the GHB receptor has not been identified yet satisfactorily, the putative GHB receptor mRNA showed different brain distribution than the native GHB receptors (Andriamampandry et al., 2003, 2007). A binding site recognizing GHB and SUC has been disclosed in the NAc (Molnár et al., 2006, 2008a,b), its relationship with the G-protein–coupled heptahelical kidney SUC receptor (SUCCN1:GPCR91, He et al., 2004) has not previously been conjectured.

GHB-induced functional coupling of Cx43 hemichannels is known to evoke store-mediated repetitive Ca$^{2+}$ transients triggered by activation of purinergic GPCRs in vitro (Stout et al., 2001; Anderson et al., 2004; Beierlein and Regehr, 2006; Piet and Jahr, 2007) and in vivo (Hirase et al., 2004; Takata and Hirase, 2008; Hoogland and Kuhn, 2010; Hoogland et al., 2009; Nimnerjahn, 2009; Nimnerjahn et al., 2009). Different in vitro paradigms, including locally administered ATP stimuli (100 μM) were found effective (Barry and Cheek, 1994; Li et al., 2001; Zur Nieden and Deitmer, 2006; D’Ascenzo et al., 2007; Fischer et al., 2009). In awake, behaving animal, three types of glial Ca$^{2+}$-excitation were distinguishable according to the number of networking cells and the dependence on neuronal stimulation (Nimnerjahn et al., 2009): flares (involving large networks of astrocyte fibers), sparks (restricted to individual fibers), and bursts (expanding radial waves). Spontaneous bursting can be contrasted to sparks and flares as being independent of neuronal activity (Nimnerjahn et al., 2009). Thus, the question may also come up, how ATP-responsive glial Ca$^{2+}$-bursting proceeds and what functions it perform in cellular communication?

In order to better understand these issues, we used different models and/or approaches to compare the effects of GHB with those of SUC and/or ATP: (i) rat brain slice containing the NAc studied by combined application of confocal Ca$^{2+}$ imaging and Cx43 immunohistochemistry to explore and compare GHB-responsive and ATP puff-evoked astroglial Ca$^{2+}$ bursting; (ii) evaluation of SUC/GHB- and ATP-evoked Ca$^{2+}$ transients to disclose mechanistic clues; (iii) homology model of SUCCN1 (He et al., 2004) to simulate binding interactions between SUC/GHB and SUCCN1; (iv) slices containing the NAc from mice lacking GABA$\beta$ receptor type 1 subunit in combination with confocal Ca$^{2+}$ imaging to distinguish SUC-responsive Ca$^{2+}$ transients and to compare with GHB-evoked Ca$^{2+}$ signals (Molnár et al., 2009). We report on astroglial Ca$^{2+}$ bursting linked to endogenous metabolites GHB and SUC performing similar function that might possibly be related to cellular energy states.

**MATERIALS AND METHODS**

**BUFFERS AND TEST COMPOUNDS**

Slice preparing buffer contained in mM: 250 sucrose, 2 KCl, 1.25 KH$_2$PO$_4$, 10 MgSO$_4$, 2 CaCl$_2$, 16 Na$_2$CO$_3$, and 10 glucose. Artificial cerebrospinal fluid (ACSF) contained in mM: 129 NaCl, 2 KCl, 1.25 KH$_2$PO$_4$, 1 MgSO$_4$, 2 CaCl$_2$, 16 NaHCO$_3$, and 10 glucose.

The following drugs, applied via the ACSF perfusion including SUC, GHB, and adenosine 5’-triphosphate disodium salt (ATP) were obtained from Sigma-Aldrich (Budapest, Hungary). Fluorescence indicators Fluo-4 acetoxymethyl ester (Fluo-4 AM) was purchased from Molecular Probes (Eugene, OR, USA). Stock solutions of ester fluorescence indicators prepared in DMSO were diluted to 0.2% DMSO in the staining solution.

**ANIMALS**

Animal care and preparation were in accordance with the Helsinki declaration, European Council Directive of 24 November 1986 (86/609/EEC), Hungarian Animal Act 1998 and associated institutional guidelines, as approved by the local authority. BALB/c GABAB1 (−/−) mice exhibit spontaneous seizures (Prosser et al., 2001; Schuler et al., 2001) thus BALB/c GABAB1 (+/−) mice were bred, their offspring genotyped at the seventh day and used by the tenth day before seizure could develop in (−/−) mice. All efforts were made to minimize animal suffering and the number of animals used.

**ACUTE SLICE PREPARATION AND DYE-LOADING**

Coronal slices from the forebrain containing the NAc and the caudate putamen (CP) were prepared for the imaging experiments. Ten to 14-day-old male Wistar rats or 10–15-lb mice were decapitated and the forebrain blocks were sliced into 300–μm thin coronal slices by a vibratome (Vibratome, Technical Products International Inc., St. Louis, MO, USA) in ice-cold preparation buffer. Slices were incubated for 1 h under humidified gas-mixture carbogen (5% CO$_2$ + 95% O$_2$) atmosphere in an interface-type holding chamber containing warmed (35°C) ACSF. After preincubation in 2% pluronic acid containing ACSF for 2 min, slices were incubated with 5 μM Fluo-4 AM in ACSF for 1 h at 35°C in the dark under humidified carbogen (5% CO$_2$ + 95% O$_2$) atmosphere (Molnár et al., 2009). In order to monitor cell death, several slices were exposed to double dye-loading protocol, performed by adding 7.5 μM propidium iodide (PI excitation: 534 nm, emission: 570–600 nm) to the Fluo-4 AM containing ACSF. In order to allow for cleavage of the AM ester group of Fluo-4 AM, slices were transferred to dye-free ACSF at least 30 min before the start of the experiment (Porter and McCarthy, 1996).

**CONFOCAL IMAGING AND DRUG TESTING PROTOCOL**

Fluorescence recordings of changes in the intracellular Ca$^{2+}$ ion level in cells loaded with Fluo-4 AM were performed as described (Molnár et al., 2009) with an upright epifluorescent microscope.
microscope (Olympus BX61WI, Olympus, Budapest, Hungary) equipped with the FluoView300 confocal laser-scanning system (Olympus, Budapest, Hungary) using 20× (0.5 numerical aperture) or 60× (0.9 numerical aperture) water immersion objectives. Image acquisition rate was controlled by a computer running FluoView 5.0 software (Olympus, Budapest, Hungary).

Freshly isolated slices were transferred to the submerge-type recording chamber mounted on the stage of the microscope and were superfused with carbogenated (5% CO2 + 95% O2) ACSF (3 ml/min, room temperature). Serial scanning of slices were made at 488 nm excitation wavelength and emitted green fluorescence was collected through a 510–530 nm bandpass filter. Ca2+ transients were initiated by application of SUC or GHB in the perfusion or by pressure-ejection of 100 μM ATP in ACSF through a glass micropipette (5–10 μM diameter) on the slice surface. Fluorescence intensity changes within a 355 μm × 355 μm field containing approximately 100 Fluo-4 AM loaded cells around the area of the ATP puff, were followed over a 10-min interval (2 s/image).

Two 10-min-long recordings were made from each slice with a 20-min resting time between the two recordings. ATP was applied 3 min after the beginning of each 10-min-long recording period. The cells showing Ca2+ increase after ATP applications at approximately 15–30 μm below the surface were counted (approximately 45 cells in response to the ATP stimulus) and fluorescence changes of cells that responded to ATP applications were measured. The observation threshold was 5× of the baseline fluorescence.

N denotes the number of slices in a given experimental condition. Statistical analysis was performed using the non-parametric Mann–Whitney test with Bonferroni post hoc test (OriginLab Corporation, Northampton, UK) and p < 0.05 was considered statistically significant. Unless otherwise stated, the effects of different treatments were compared to the control. Images recorded by the FluoView300 software were processed using the free ImageJ 1.41 image analysis software (http://rsbweb.nih.gov/ij/).

**POST-CALCIUM IMAGING IMMUNOHISTOCHEMISTRY PROTOCOL FOR CX43 AND GFAP**

In order to identify the cell types involved in Ca2+ bursts, we followed our recently described protocol (Molnár et al., 2011). The brain slices were immunostained with antibodies for astrocyte marker proteins (Cx43 and GFAP). However, Fluo-4 signal could not be preserved through fixation with either 0.4% paraformaldehyde or 40 mg/ml EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Sigma-Aldrich). Therefore co-localization between SUC/ATP-responsive cells and cell type markers could not be resolved. Hence we opted to perform immunostaining in non-fixed slices directly after the Ca2+ imaging protocol. Slices used previously to measure Ca2+ changes in response to SUC/ATP application were treated as follows: upon completion of the calcium-imaging experiments, each slice was kept in its original position in the recording chamber (using a ballast) and incubated with Cx43 (1:300) and GFAP (1:200) primary antibodies for 30 min, at room temperature. After 3 min × 10 min washing in ACSF, the slice was incubated with Chromo 546 goat anti-rabbit (1:100 Abcam, Cambridge, UK, catalog number: ab60317) secondary antibody and Alexa-488 donkey anti-mouse (1:100, Molecular Probes) secondary antibody in ACSF for 30 min at room temperature. This was followed by 3 × 10 min washing in ACSF. Serial Z-scans of Cx43 – (excitation: 543 nm, emission: 570–600 nm) and GFAP – (excitation: 488 nm, emission: 510–530 nm) labeled slices were acquired between the slice surface and the maximal penetration depth of the antibodies (approximately 60–70 μm from the surface) through a 20× objective (1 μm/step). Since the fluorescence emission of both Fluo-4 and Alexa-488 dyes are collected in the 510–530 nm range, GFAP-specific staining was obtained by subtracting the Fluo-4 fluorescence from the GFAP immunolabeling signal at each Z depth. Optical sections from identical depths of Fluo-4 and Cx43 images were merged along the Z axis. Single cells, glia filaments, or blood vessels that showed double immunolabeling were recorded through a 60× objective and Z-scans were performed by alternating the excitation wavelengths between 543 nm (Cx43) and 488 nm (GFAP) using FluoView 5.0 software at each depth (0.1 μm/step). Images were processed using ImageJ 1.41 and Adobe Photoshop 8.0 image analysis softwares.

**SIMULATIONS**

The sequence of the human kidney SUC receptor1 (SUCNR1; accession code: Q9BXA5) was downloaded from the UniprotKB database. The SUCNR1 receptor model was built based on the bovine rhodopsin (RHO) structure (PDB code 1R88; Palczewski et al., 2000). The alignment was adopted from He et al. (2004), with one manual adjustment: Asn253 of SUCNR1 was aligned to Ala269 of RHO by reducing the five residue gap of He et al. (2004). The alignment continued according to He et al. (2004), from and Ser275 of SUCNR1, and Ile286 of RHO. The model was built by Modeller9v3 (Sali and Blundell, 1993), and prepared in SYBYL8.0 (Tripos Inc., St. Louis, MO, USA). Hydrogen atoms were added to the protein, and chains were terminated by N-methyl and acetyl groups by the Analyze Protein menu of the Biopolymer module. His103 was protonated at both N atoms, corresponding to an acidic environment. SUC and GHB ligands were prepared from the PDB database (SUC ligand of 2WBP; Helmetag et al., 2009) in their protonated form according to the acidic pH of the receptor binding (Molnár et al., 2006, 2008a,b). Ligands were minimized in SYBYL until the RMS gradient reached 0.01 kcal/mol Å. Docking was performed by GOLD5.1 (Cambridge Crystallographic Data Center). A cavity of 10 Å was searched in the docking studies containing the predicted binding crevice residues of SUCNR1 (Arg99, His103, Arg252, Arg281), see Figure 4 GoldScore was used for evaluating the results. Figures were prepared by Pymol.

**RESULTS**

**GHBR-RESPONSIVE CELLS CONSTITUTE A SUB-POPULATION OF ASTROCYTES RESPONDING TO ATP**

As outlined in the Section “Introduction,” we tested the hypothesis that similarly to SUC (Molnár et al., 2011), GHB also acts on a sub-population of ATP-responsive cells. Indeed, the presence of GHB (50 μM) in the superfusion evoked Ca2+ transients (Figure 1A), covering some astroglial Ca2+ bursts occurred in response to the ATP puff (100 μM, Figure 1B, see also Movie S1 in Supplementary Material). The ATP-evoked Ca2+ signal propagated circularly...
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**FIGURE 1 | GHB and ATP evoke overlaying Ca\(^{2+}\) transients in the rat NAc slice.**

Representative confocal image of GHB-responsive cells ([A] circled) participated in ATP-stimulated concerted Ca\(^{2+}\) burst ([B] circled). Arrow indicates the direction of the superfusion buffer. Arrowhead indicates the location and direction of the ATP puff. Scale bar is 50 μm. (C) Pseudo-color depiction of the spatiotemporal characteristics of the astroglial Ca\(^{2+}\) transients evoked by GHB and ATP shown in (B) (see also Movie S1 in Supplementary Material).

**D** GHB

**E** ATP

50% 50 s

| Number of GHB-responsive cells |
|-------------------------------|
| control | 0.05 | 0.2 | 2 |
| ![Graph](image)

Material. (C) Representative dF/F\(_0\)-time plots showing effects of 50 μM GHB alone ([A]) and with 100 μM ATP puff ([B]). (D) Color traces correspond to the GHB-responsive cells circled in (A) and (B) while gray traces show the fluorescence changes of ATP-responsive cells. (E) Summary plot of the effects of increasing (GHB) on the number of cells showing GHB-responsive Ca\(^{2+}\) transients. N = 32 (control), N = 13 (0.05 mM), N = 9 (0.2 mM), N = 9 (2 mM). *p < 0.05 and **p < 0.01 compared to control.

from the point of application, independent of the perfusion direction (Figure 1C) with the speed of approximately 10 μm/s, 1.5 times slower than the speed of ATP diffusion as monitored by addition of the fluorescent dye sulforhodamine 101 in the ATP-containing pipette (Molnár et al., 2011). The waveform and speed of the signal propagation conclusively exclude the possibility that Ca\(^{2+}\) bursts were triggered by merely the exogenously applied ATP. Comparing the GHB- and ATP-evoked signals, the responses in the presence of GHB and ATP appeared relatively more robust than responses evoked by GHB only (Figure 1D). Summary plot of the effects of increasing (GHB) on the number of cells showing GHB-responsive Ca\(^{2+}\) transients indicated that similarly to SUC (Molnár et al., 2011) GHB had an EC\(_{50}\) value of approximately 50 μM, however smaller number of cells responded to GHB than to SUC, indicating lower efficacy (Figure 1E).

**GHB AND SUC SHAPED ATP-EVOKED CA\(^{2+}\) SIGNALS IN THE SAME WAY**

We observed that the number of ATP-responsive cells decreased with increasing concentration of either SUC or GHB (Figure 2A). Moreover, the concentration dependence of the number of ATP-responsive cells were highly identical as a function of both (SUC) and (GHB; Figure 2A), suggesting a mutual target for SUC and GHB actions. Increasing [SUC] may activate this mutual target, a presumable GHB-responsive astroglial SUC receptor and consequently reduce ATP-responsiveness of astrocytes (Figures 2A,B). Furthermore, increasing [SUC] resulted in a rise of the relative abundance of SUC- and shared SUC/ATP-responsive astroglial Ca\(^{2+}\) transients (Figure 2B) as well as in an increase in the number of cells showing repetitive Ca\(^{2+}\) dynamics (Figure 2C). The SUC-evoked repetitive Ca\(^{2+}\) transients (Figure 3A; Molnár et al., 2011) mimicked the GHB response dynamics described above (Figure 1D; Movie S1 in Supplementary Material), further suggesting the existence of a GHB-responsive astroglial SUC receptor. In a previous work we provided evidence on store-dependent astroglial Ca\(^{2+}\) transients evoked by ATP acting through the G protein-coupled purinergic P2Y\(_1\) receptor subtypes in the NAc slice (Molnár et al., 2011). Therefore, SUC- and shared SUC/ATP-responsive astroglial Ca\(^{2+}\) transients may suggest co-localization of GHB-responsive SUC and P2Y\(_1\) receptors on astrocytes.

**THE PRESUMED GHB-RESPONSIVE ASTROGLIAL SUC RECEPTOR WAS CLASSIFIED AS BEING INDEPENDENT OF BOTH GABA\(_B\) AND N-METHYL-D-ASPARTATE RECEPTORS**

We supposed that similarly to GHB, its metabolite SUC also acts independently from GABA\(_B\) receptors in the NAc. To verify this hypothesis we tested the effect of SUC (2 mM) on astroglial Ca\(^{2+}\) transients in the NAc slice prepared from mice lacking the GABA\(_B\) receptor functional subunit 1 (GABA\(_B\) KO). We found that similarly to GHB (Molnár et al., 2009), SUC-responsive Ca\(^{2+}\) transients were preserved in NAc slices isolated from GABA\(_B\) KO mice (Figure 3A). Next we asked, if NMDA receptors of the NAc slice were involved in the SUC action mechanisms. We observed that SUC responses remained unaltered in the presence of NMDA...
FIGURE 2 | GHB and SUC shaped ATP-evoked Ca\textsuperscript{2+} signals in the same way. (A) Average number of cells showing Ca\textsuperscript{2+} transients in response to ATP (100 μM) puff-application in the presence of different concentrations of SUC or GHB. (B) Average number of cells showing Ca\textsuperscript{2+} transients during SUC or SUC plus ATP (100 μM) application as a percent of the total number of cells showing Ca\textsuperscript{2+} transients during SUC or ATP application. (C) Average number of cells showing multiple Ca\textsuperscript{2+} transients during application of different SUC concentrations. \(N = 32\) (control), \(N = 13\) and 9 (0.05 mM), \(N = 9\) and 16 (2 mM) for GHB and SUC, respectively, \(* p < 0.05\).

FIGURE 3 | The presumed GHB-responsive astroglial SUC receptor was classified as being independent of both GABA\textsubscript{B} and \(N\)-methyl-D-Aspartate (NMDA) receptors. (A) Pseudo-color images showing fluorescence in NAc astrocytes during control and SUC (2 mM) application in wild type and GABA\textsubscript{B} R type 1 subunit KO mice. Astrocytes showing SUC-evoked Ca\textsuperscript{2+} transients are circled and color-coded and the change in fluorescence is shown in \(dF/F_{0}\)-time plots below the images. (B) Summary plots show that SUC-evoked Ca\textsuperscript{2+} transients, similarly to GHB-evoked Ca\textsuperscript{2+} transients were preserved in GABA\textsubscript{B} R1 KO mice. (C) Summary plots indicate that SUC/GHB-evoked Ca\textsuperscript{2+} transients were unaltered by the presence of NMDA receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV, 20 μM).
receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV, 20 μM; Figure 3C). Therefore, both SUC and GHB effect on astroglial Ca^{2+} signals could be classified as being independent of GABA_B and NMDA receptors. These data further strengthen the possibility that SUC and GHB act through a common target protein, the presumed GHB-responsive astroglial SUC receptor in the NAc.

MODELING PREDICTED INTERACTION OF GHB WITH THE PRESUMED ASTROGLIAL SUC RECEPTOR

To explore the possibility of a shared target protein for SUC and GHB actions, we used the kidney-type SUC receptor (SUCNR1:GPCR91) model structure (He et al., 2004) and simulated interactions of SUC and GHB with this protein. Docking of SUC and GHB into SUCNR1 resulted in similar ligand
conformations occupying the same binding site. The predicted binding crevice was formed by residues R99, H103, R252, and R281 (Figure 4) in accordance with previous studies (He et al., 2004). In addition, residues T107, N173, H249, and M251 were also found to be involved in the binding of SUC and GHB (Figure 4B). Agonist SUC participated in more extensive H-bond interactions (Figure 4B) enabling higher docking score value (35 for SUC vs. 31 for GHB). In addition, we also disclosed a previously unrecognized binding site appearing near to the entrance of the receptor, showing preference to GHB over SUC. However, being more distal to the agonist binding crevice, preferential GHB binding into this “vestibular” binding site is expected to be less effective in forming active receptor conformations, explaining the previously experienced lower efficacy of GHB.

**DISCUSSION**

Our previous (Molnár et al., 2006, 2008a,b, 2009, 2011) and recent (this work) findings conclusively suggest the existence of a SUC receptor in brain tissue that also recognizes GHB. Activation of the presumed brain type SUC receptor (as an analogy to SUCNR1 we reference this putative receptor as SUCBR1) by agonists SUC or GHB evokes repetitive Ca^{2+} transients in astrocytes independently of neuronal signaling (Molnár et al., 2009, 2011). A sub-population of SUCBR1-triggered repetitive Ca^{2+} transients can also be activated by ATP making Ca^{2+} signaling more robust. We conjecture physiological significance for the detection of these endogenous metabolites and ATP performing energy supply dependent regulation of astroglial Ca^{2+} bursting.

SUC shaped the astroglial Ca^{2+} bursting activity in different ways. Both the absolute and relative abundance of astrocytes showing ATP-evoked, P2Y_{1} receptor-mediated Ca^{2+} release from internal stores tended to decay with increasing [SUC]. By contrast, the astroglial SUC- and SUC/ATP-responsive Ca^{2+} signals were enhanced with increasing [SUC]. These findings suggest the occurrence of distinct ATP-responsive astrocytic sub-populations that can be distinguished by their responsiveness to SUC. These findings imply several mechanistic clues reasoning reduced responsiveness to ATP: (i) refilling of Ca^{2+} stores may take longer after activation by SUC and/or (ii) SUCBR1 may desensitize slower than P2Y_{1}. These mechanistic clues, however, does not rationalize why the relative abundance of cells responsive to both SUC and ATP increases with [SUC]. Such a unique phenomenon can be explained by supposing that the presence of SUCBR1 may make desensitization of P2Y_{1} slower, also implying co-localization of SUCBR1 and P2Y_{1} receptors in the SUC/ATP-responsive sub-population of astrocytes.

Identity of SUC-sensitive GHB (Molnár et al., 2006) and GHB-sensitive SUC (Molnár et al., 2008a) binding sites in rat forebrain and human NAc membrane homogenates has previously been disclosed (Molnár et al., 2008b). We have shown that drug of abuse (Wong et al., 2003, 2004), dietary supplement for body builders (Camacho et al., 2005) and brain metabolite (Bessman and Fishbein, 1963) GHB also activates store-dependent astrocytic Ca^{2+} transients in the brain reward area NAc (Molnár et al., 2009). Moreover, binding of GHB and SUC can be characterized by interaction with CBX (Molnár et al., 2006, 2008a,b), a blocker of gap junctions that are major players in astroglial Ca^{2+} wave propagation (Finkbeiner, 1992). In spite of their disclosure, molecular and cellular characterization of binding effects (Molnár et al., 2006, 2008a,b, 2009, 2011), the functional significance of astroglial SUC receptor recognizing GHB was less appreciated. Thus metabolic actions of GHB may be explained by the activation of the SUCBR1 signaling route.

Understanding of the role of Ca^{2+} signaling in astrocytes at neuronal and vascular interfaces through specific intercellular communication mechanisms has grown considerably in recent years (Newman and Zahs, 1997; Scemes et al., 2000; Parri et al., 2001; Matthias et al., 2003; Verkrachtsy, 2006; Fiacco et al., 2007; Iadicola and Nedergaard, 2007; Sadaogopan et al., 2007; Barres, 2008; Gordon et al., 2008; Hall and Attwell, 2008; Li et al., 2008; Doengi et al., 2009; Hoogland and Kuhn, 2010; Hoogland et al., 2009; Koehler et al., 2009; Nimmerjahn, 2009; Nimmerjahn et al., 2009; Agulhon et al., 2010; Hamilton and Attwell, 2010; Xi et al., 2010). It may have significance in this respect that we observed SUC/GHB and ATP-responsive cells around NAc vessels at the end feet of astroglial processes. Representative image indicating SUC-evoked Ca^{2+} transient in a sub-population of ATP-responsive astrocytes (Figure 5A circles and arrows, respectively) along with a vessel contoured by Cx43 positive plaques (Figure 5A middle and right panels). GFAP and Cx43 expression in cerebrocortical astrocyte end feet plastered at the vessel wall has previously been characterized (Simard et al., 2003). Accordingly, double immunolabelling for Cx43 and GFAP showed large Cx43-immunoreactive plaques interconnecting astrocytic GFAP-positive end feet along with vessels in the NAc (Figure 5B). Co-localization of SUC-responsive astrocytes and vessel wall may provide a mechanistic clue for cellular energy metabolite-responsive Ca^{2+} signaling activity around vessels, at the end feet of astrocyte processes interconnected by gap junctional plaques. The setting can possibly imply a role for Ca^{2+} signaling at the glio-vascular interface. Regulation of Ca^{2+} bursting through cellular energy metabolites may in turn provide a mechanism for the control of astrocyte activation and will therefore benefit from further study in awake, behaving animal.

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**SUPPLEMENTARY MATERIAL**

The Movie S1 for this article can be found online at http://www.frontiersin.org/neuroenergetics/10.3389/fnene.2011.00007/abstract

**Movie S1** File name: 3-31-29_PM-diff.avi. Ca^{2+} signaling evoked by GHB and ATP in the acute NAc slice from the rat brain. Movie of time measurement showing GHB (50 µM) and ATP (100µM) application onto a Fluo-4 AM loaded acute, 300µm thick NAc slice. GHB was applied between 0 and 2 min in the perfusion buffer. ATP was applied for 60 s starting from 1.5 min through a glass micropipette right above the tissue surface. Image acquisition frequency was 2 s in depth of ~25µm from the slice surface. The background fluorescence (first frame) was subtracted from all subsequent frames in the image stack. The resulting images were false colored (more red pixels represent higher dF/F_{0} values) and converted to avi file by ImageJ 1.44 image processing and analysis software.
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