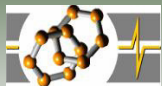


Effects of succinate on ATP-induced Ca²⁺ waves in the *nucleus accumbens*



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INTRODUCTION

Succinate (SUC) is an essential compound of all living cells as an intermediate of the TCA cycle. In addition, SUC is the product of gamma-aminobutyric acid (GABA) catabolism in the brain via gamma-hydroxybutyric acid (GHB) (GABA shunt; Schousboe és Waagepetersen, 2006). GHB is also known as a drug of abuse with dose-dependent behavioural effects (Wong et al., 2003) and can activate its own receptor and also GABA_B receptors (Wellendorph et al., 2005). Surprisingly, a GABA_B-independent GHB and SUC binding protein interacting the gap junction blocker carbenoxolone (CBX) has been found in synaptosomal membrane fractions of the *nucleus accumbens* (NA) (Molnár et al., 2006; Molnár et al., 2008), the brain area responsible for addictive properties of different drugs (Berridge and Robinson, 2003).

In order to explore the possible functional interaction of SUC and GHB with gap junction proteins, a novel test assaying functional gap junctions in NA tissue slices by ATP-induced Ca²⁺ waves has been worked out and applied to follow the effects of SUC and GHB.

CONCLUSION

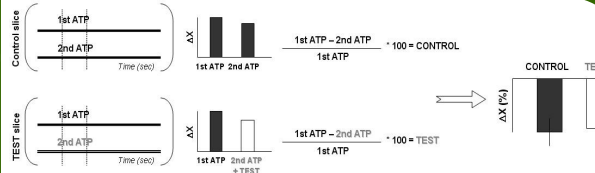
- Spreading Ca²⁺ waves can be evoked in the NA and in the *striatum* by extracellular ATP.

- These Ca²⁺ waves of the NA are blocked by gap junction blockers and can be influenced by SUC and GHB.

- Astrocytic Ca²⁺ wave propagation exerted by low concentration of external SUC may indicate a role for SUC as a signalling metabolite

METHOD

Coronal brain slices (300 μm) containing the NA or the *striatum* were prepared from young (9-12 days old) Wistar rats. Slices were incubated with the cell permeable form of the Ca²⁺-sensitive indicator Fluo-4 AM (5 μM for 1 h). Changes in the intracellular Ca²⁺ ion level were measured by a confocal laser scanning microscope (Olympus BX61WI, FluoView300) at a 488 nm excitation wavelengths and emitted green fluorescence was collected through a 515 nm long-pass barrier filter through a 20x water immersion objective. During recording slices were perfused with carbonated (5%CO₂/95%O₂) ACSF. Ca²⁺ waves were induced by application of 100 μM ATP dissolved in ACSF and puffed from a glass micropipette (10-15 μM O) on the slice surface for 1 min. Fluorescence intensity changes within a 300 x 300 μm field of view, containing ~100 Fluo-4 AM loaded cells, were followed over time (2 seconds/image). For cell identification, a specific glia marker, sulforhodamine 101 (SR101; Matthias et al., 2003; Kalfitz et al., 2008) dissolved in ACSF (100 μM), was used and applied together with ATP. SR101-labelled cells were visualized between 610-630 nm using a 543 nm laser for excitation.



Scheme 1.

From each slice two 10 min-long recordings were performed. ATP was applied 4 minutes after the beginning of each 10 minutes-long recording period. All tested drugs were applied during the second 10 minute-long recording period and were present at the time of the second ATP application. The cells showing Ca²⁺ increase after each ATP application were counted (N) and the altered fluorescence activity of cells (F) in response to both ATP applications have been measured. The difference between these two recordings (ΔN and ΔF) was expressed as the percentage of the effects elicited by the first ATP application.

Fig. 1.

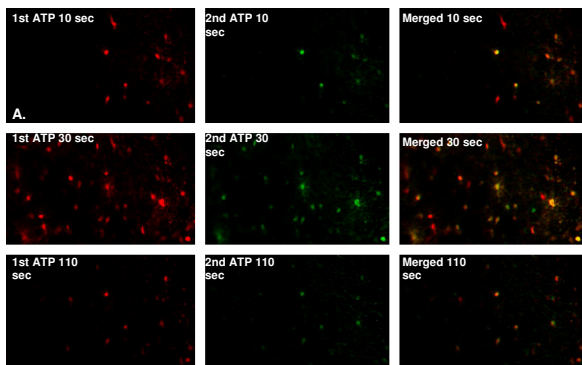


Fig. 2.

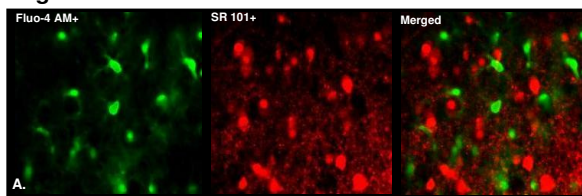


Fig. 2. Identification of cells participating in the ATP-induced Ca²⁺ wave in the NA.

A: Parallel labelling of cells by intracellular Ca²⁺ ion indicator Fluo-4 AM (5 μM) and the glutamate transporter-expressing astrocyte marker, sulforhodamine 101 (SR101, 100 μM) in a 100x80 μm field of view (400x magnification).

B: High resolution image of a Fluo-4 AM positive cell that participated in ATP-induced Ca²⁺ waves (2000x magnification). The cell shows typical astrocytic morphology: small, <10 μm perikarya and fine-, heavily branched processes (N=36).

Fig. 3.

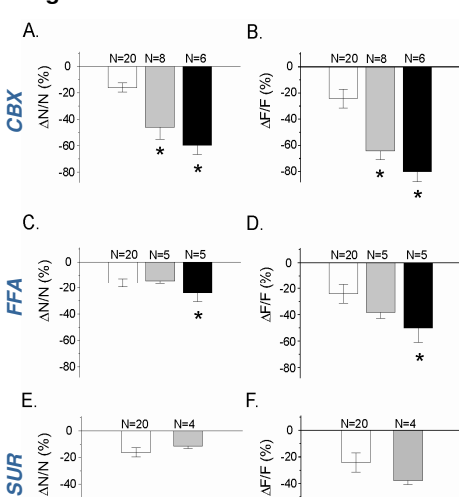


Fig. 3. Effects of gap junction blockers (CBX; flufenamic acid, FFA) and ATP channel blocker (suramin, SUR) on Ca²⁺ wave propagation.

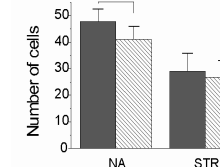
Significance is compared to the control values (empty column) at **p*<0.05 (independent Student's *t*-test) on all panes.

A-B: The effects of CBX (gray column: 0.1 mM and black column: 1 mM) on the cell number (ΔN/N) and fluorescence (ΔF/F) of ATP-induced Ca²⁺ waves in the NA.

C-D: The effects of FFA (gray column: 0.1 mM and black column: 1 mM) on the cell number (ΔN/N) and fluorescence (ΔF/F) of ATP-induced Ca²⁺ waves in the NA.

E-F: The effects of SUR (gray column: 0.1 mM) on the cell number (ΔN/N) and fluorescence (ΔF/F) of ATP-induced Ca²⁺ waves in the NA.

B.



C.

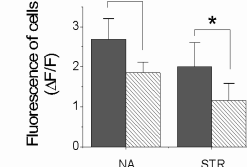


Fig. 1. Comparison of the effects of the 1st and the 2nd ATP (100 μM) applications on the percent change of the number (ΔN/N) and the fluorescence intensity change (ΔF/F) of cells in the NA and the *striatum*.

A: Serial exposures of repeated ATP-induced Ca²⁺ waves in the NA. Images in the first column indicate the Ca²⁺ wave induced by the first ATP application (1st ATP), all participating cells are coloured red. Images in the second column indicate the Ca²⁺ wave induced by the second ATP application (2nd ATP), all participating cells are shown in green colour. "Time" refers to the time passed from the beginning of the ATP applications. The third column (Merged) contains the merged colour images of the 1st and 2nd ATP-induced Ca²⁺ waves.

B: Changes in the number of cells participating in the first (grey columns) and in the second (striped columns) ATP-induced Ca²⁺ waves in the NA (N=24) or in the *striatum* (STR, N=18). Data represent means ± SEM. (**p*<0.05 paired Student's *t*-test).

C: Changes in the fluorescence of cells participating in both ATP-induced Ca²⁺ waves in the NA (N=24) and in the *striatum* (STR, N=18). Data represent the mean fluorescence ± SEM of cells of the first (grey columns) and the second (striped columns) ATP-induced Ca²⁺ waves. (**p*<0.05 paired Student's *t*-test).

Fig. 4.

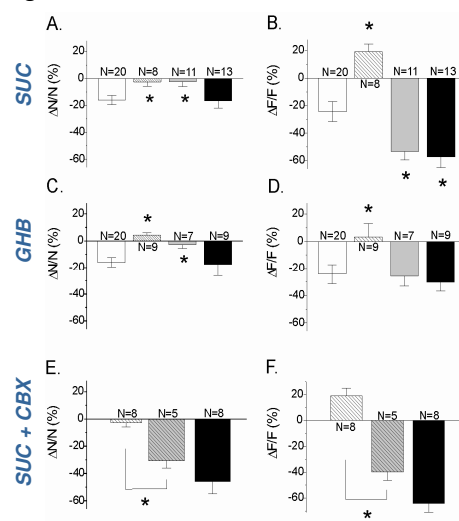


Fig. 4. Effects of SUC and GHB on ATP-induced Ca²⁺ waves and modulation of the effect of SUC (0.05 mM) in the NA effects by CBX (0.1 mM).

Significance is compared to the control values (empty column, A-D) or to the effects of SUC (E-F) at **p*<0.05 (independent Student's *t*-test).

A-B: The effects of SUC (striped column: 0.05 mM, gray column: 0.2 mM and black column: 2 mM) on the cell number (ΔN/N) and fluorescence (ΔF/F) of ATP-induced Ca²⁺ waves in the NA.

C-D: The effects of GHB (striped column: 0.05 mM, gray column: 0.2 mM and black column: 2 mM) on the cell number (ΔN/N) and fluorescence (ΔF/F) of ATP-induced Ca²⁺ waves in the NA.

E-F: The additive effects (gray striped column) of CBX (0.1 mM, black column) and SUC (0.05 mM, striped column) on the cell number (ΔN/N) and cells fluorescence (ΔF/F) of ATP-induced Ca²⁺ waves in the NA.

Fig. 5.

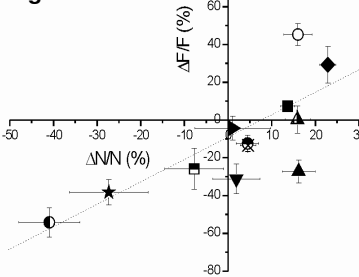


Fig. 5. Correlation of cell number changes (ΔN/N) and fluorescence changes (ΔF/F).

The axis of ΔN/N and ΔF/F are intersected at the control values. The *R* value of fitted linear is 0.74 (OriginPro 7.5). All symbols represent a tested ligand in a given concentration and data expressed in mean ± SEM.

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