Dopamine controls neuronal spontaneous calcium oscillations via astrocytic signal

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Abstract

Dopamine is a neuromodulator and neurotransmitter responsible for a number of physiological processes. Dysfunctions of the dopamine metabolism and signalling are associated with neurological and psychiatric diseases. Here we report that in primary co-culture of neurons and astrocytes dopamine-induces calcium signal in astrocytes and suppress spontaneous synchronous calcium oscillations (SSCO) in neurons. Effect of dopamine on SSCO in neurons was dependent on calcium signal in astrocytes and could be modified by inhibition of dopamine-induced calcium signal or by stimulation of astrocytic calcium rise with ATP. Ability of dopamine to suppress SSCO in neurons was independent on D1- or D2- like receptors but dependent on GABA and alpha-adrenoreceptors. Inhibitor of monoaminoxidase bifemelane blocked effect of dopamine on astrocytes but also inhibited the effect dopamine on SSCO in neurons. These findings suggest that dopamine-induced calcium signal may stimulate release of neuromodulators such as GABA and adrenaline and thus suppress spontaneous calcium oscillations in neurons.

Introduction

Dopamine (DA) is neurotransmitter and neuromodulator, which releases by midbrain neurons. DA crucial for fundamental and complex brain functions, such as voluntary movement and goal-directed behaviour, as well as cognition, emotion, reward, motivation, working memory, associative learning and decision making [1–3]. Dysfunctions of the DA metabolism and signalling are associated with neurological and psychiatric diseases, such as schizophrenia, addiction, attention deficit, hyperactivity disorder and Parkinson's disease (PD) [3–5].

The actions of the neurotransmitter dopamine are mediated by specific G-protein coupled receptors, which are divided into two major subfamilies based on their ability to stimulate (D1-like) or inhibit adenylate cyclase (D2-like). Three human D2-like receptors have been cloned: D2, D3 and D4 [6]. Dopaminergic receptors are mostly distributed in the striatum, and to a lesser degree in other parts of the brain. DA is catabolized by monoamine oxidase (MAO), a mitochondrial-located enzyme, which breaks down monoamines using FAD and producing aldehydes. The FAD-$FADH_2$ cycle generates hydrogen peroxide. There are two forms of MAO – MAO-A (expressed in neurons and glia) and MAO-B (glia) [7].

Dopamine can induce calcium signalling in neurons from the different brain areas via various, receptor dependent and independent mechanisms [8–12] and also in neighbouring astrocytes [13,14]. However, pre-incubation of neurons with DA limit glutamate-induced calcium signal glutamate excitotoxicity [15] by modulation of the current in NMDA-receptor [16,17]. Considering multiple effects of dopamine on signals of neurons and astrocytes we can suggest that neurons and astrocytes could affect to each other using this monoamine.

Dopamine and other catecholamines including tyramine and adrenaline can activate calcium signal astrocytes via production of hydrogen peroxide in MAO that lead to redox changes and stimulation of phospholipase C [10,18,19]. However, activation of dopamine receptors also could have an effect on calcium signalling in astrocytes. Thus, D2 receptor-specific agonist, quinpirole, can induce Ca^{2+} elevation in cortical astrocytes [20].

Neuroglial interaction is important for maintenance of cellular homeostasis of both cell type but also for signal transduction in the brain [21]. Astrocytes can be

stimulated by gliotransmitters and release compounds, which activate or modify neuronal signal – neurotransmitters [22]. Recently it was demonstrated that astrocytes also could release GABA in response to calcium stimulation through specific GABA-transporter [23,24]. Neuroglial interaction is very important process and changes in this intercommunication may lead to pathology [25].

Considering the ability of DA to increase $[Ca^{2+}]_c$ in astrocytes and modulate (increase or decrease) calcium signal in neurons we suggested that dopamine can be a regulator of spontaneous calcium oscillations in neurons and astrocytes and that this regulation can be affected by interaction between neurons and astrocytes in co-culture.

Here, we used primary rat co-culture of hippocampal neurons and astrocytes to study the effect of dopamine on spontaneous calcium activity and attempted to dissect the mechanisms of the action of dopamine on calcium signalling in neurons and astrocytes.

2. Results

2.1 Dopamine induce calcium signal in astrocytes and reduce spontaneous oscillations in neurons

Neurons and astrocytes in co-culture were identified by short (20 sec) application of 10 μM NMDA in Mg^{2+}-free medium that induce rise of $[Ca^{2+}]_c$ in neurons but not in astrocytes as it can be seen from the images of mixed co-culture of neurons and astrocytes loaded with a Fura-2 indicator (Figure 1A, B). For neurons (N) exposed to NMDA or at the peak of SSCO, the calcium level increases, that is corresponded with a red-orange colour changes of neurons in Figure 1A. With the stimulation of a calcium signal in glial cells (G) under the action of dopamine, their colour turns to the red-orange, while in neurons the level of calcium is low and they show a yellow-green colour.

Although NMDA receptors shown for astrocytes as well [26,27] in our experiments astrocytes which also were detected by morphology had no calcium responses. In agreement with previous publications, subsequent addition of 10 μM dopamine reduced $[Ca^{2+}]_c$ in neurons (n = 110 cells) and induced calcium signal in the majority of neighbouring astrocytes (84 ± 1%, n = 287 cells; Figure 1C) [13,14]. Here and below, we show representative data from one of 3-4 similar experiments.

Considering the ability of dopamine to reduce calcium level in activated neurons [15] we suggested that it also can have effect on spontaneous synchronous calcium oscillations (SSCO). Application of 10 µM dopamine to hippocampal cell co-culture with SSCO in neurons induced typical calcium signal in astrocytes and suppression of frequency of calcium oscillations in neurons (n = 118 neurons and 283 astrocytes; Figure 1 B, C, D).

Omitting Mg^{2+} from the medium induced seizure like activity in the neurons [28] in the form of calcium oscillations of higher frequency than SSCO (n = 157 neurons; Figure 1D). Application of 10 µM dopamine followed by sequential 50 µM dopamine adding induced minor changes in Mg^{2+}-induced oscillations in neurons but produced profound increase in $[Ca^{2+}]_c$ in astrocytes from the same co-culture (n = 268 cells; Figure 1E, F, G).

2.2 Effect of dopamine on SSCO of neurons and calcium signal in astrocytes is independent of D1- and D2-like receptors

Pre-incubation of the co-culture of neurons and astrocytes with D1-like antagonist SKF83566 (0.5 µM for 7 min) had no effect on dopamine-induced calcium signal in astrocytes and almost not altered effect of dopamine on SSCO in neurons (n = 134 neurons and 249 astrocytes; Figure 2A-B). D2-like antagonist L-741,626 (2 µM) did not change effect of dopamine on SSCO of neurons and calcium signal in astrocytes (n = 27 neurons and 173 astrocytes; Figure 2C). Although antagonists did not change effect of dopamine of SSCO frequency, L-741,626 (2 µM) reduced amplitude of SSCO in neurons (Figure 2D). Thus, effect of dopamine on SSCO in neurons is not induced by the action of this neuromodulator on D1- or D2-like receptors.

2.3 Effect of dopamine on SSCO is dependent on GABA receptor antagonists

Inhibitor of GABAA receptor bicuculline (10 µM) reduced ability of dopamine to block SSCO in neurons (n = 113 neurons; Figure 3A). Another inhibitor of $GABA_A$ receptor picrotoxin completely and irreversibly blocked the effect of 10 µM dopamine on spontaneous oscillations (n = 62 neurons; Figure 3C). It should be noted that both inhibitors did not block dopamine-induced calcium rise in astrocytes but changed the shape of this signal (n = 130 and 129 astrocytes; Figure 3B and D respectively).

Thus, inhibitory effect of dopamine on SSCO can be explained by activation of GABA$_A$ receptors.

2.4 Effect of dopamine on SSCO is dependent on adrenoreceptors and ATP

Effect of dopamine can also be affected by adrenoreceptors [29–31]. Nonselective βAR blocker propranolol (2 µM) had no effect on astrocytic dopamine-induced calcium signal (n = 48 astrocytes; Figure 4A) and on the effect of dopamine on SSCO in neurons (n = 69 cells; Figure 4A, D). In contrast to βAR blocker, application of nonselective alpha-AR antagonist phentolamine (2 µM) significantly changed action of dopamine on $[Ca^{2+}]_c$ of astrocytes and completely prevent suppressive effect of dopamine on SSCO in neurons (Figure 4B, D). Specific antagonist of α2AR rauwolscine (0.5 µM) not only effectively suppressed the action of the dopamine on SSCO of neurons (n = 105 cells; Figure 4C, D) but also reduced dopamine-induced calcium signal in astrocytes (n = 230 cells; Figure 4C). It should be noted that rauwolscine also inhibited SSCO even before dopamine application (Figure 4C). Thus, dopamine regulates SSCO in neurons through α2-AR receptors. Considering the absence of the direct binding of dopamine with this receptor we can suggest indirect effect of dopamine on the release of different vesicles by induction of calcium signal.

2.5 SSCO in neurons dependent on calcium signal in astrocytes

It can be also confirmed by application of 10 µM ATP, which induced P2Y receptor activation and transient Ca^{2+} signal in astrocytes (n = 221 cells; Figure 5B). It also suppressed SSCO in neurons (n = 95 neurons; Figure 5A, G). We suggested that ability of dopamine and ATP to reduce SSCO in neurons is dependent on the calcium signal in neighboring astrocytes. However, we cannot exclude direct effect of ATP on neurons because it also can activate purinoreceptors on neurons and reduce glutamate induced calcium signal [32].

Inhibitor of vacuolar H^+-ATPase Bafilomycin A1 (5 µM, 1 hour of pre-incubation) is used to block vesicular release in astrocytes [33]. In our experiments it did not block dopamine-induced calcium signal in astrocytes (n = 141 cells; Figure 5D). However, bafilomycin A1 suppressed SSCO in majority of the neurons and further application of dopamine had no notable effect on spontaneous oscillation in the rest

of the neurons (n = 52 neurons; Figure 5C, G). These suggest that SSCO dependent on release of neuroglia transmitters from the vesicles and that suppressive effect of dopamine on SSCO dependent on release of GABA and possibly adrenaline induced by calcium signal in astrocytes.

In agreement with previous publication [14,34] inhibitor of monoamine oxidase A and B bifemelane (10 µM), completely inhibited dopamine-induced calcium signal in astrocytes (n = 178 cells; Figure 5F, G). Thus, bifelamane completely suppressed SSCO in neurons and subsequent application of dopamine (10 µM) to these neurons had no effect on $[Ca^{2+}]_c$ (n = 66 neurons; Figure 5E).

2.6 Dopamine decrease inducible synchronous calcium oscillations

Application of 7 mM NH4Cl induce synchronous calcium oscillations [35] (Figure 6A). Subsequent adding of PD-168,077, a D2-like receptor agonist had an effect on frequency of NH4Cl-induced calcium oscillations only in higher concentrations (12 µM; Figure 6A, B). In order to prove the involvement of D2-receptors in this process, we used selective D2-receptor antagonist L-741,626 which blocked effect of PD-168,077 on NH4Cl-induced calcium oscillations (n = 78 neurons; Figure 6A, B).

2.7 Dopamine induce activation of chloride current in cultured neurons

In order to answer the question whether dopamine can exert its action through the activation of chlorine currents, we studied effect of dopamine on primary cell co-culture using the patch-clamp method. Studies were carried out at holding potential − 30 mV in the presence of 1 µM TTX, low-Ca^{2+} medium. We have found that dopamine induces an increase in frequency and amplitude of single peaks (Figure 7). Thus, 10 µM dopamine stimulated changes in slow outward chloride currents (SOCs) in a single neuron shown by whole-cell patch clamp recording (Figure 7 A). Note that individual peaks were different before (Figure 7 B, black curve) and after (Figure 7 B, red curve) 10 µM dopamine addition. Average SOC amplitude was different in different experiments and ranged from 19.7±3.1 pA to 45.1±11.7 pA in control (n = 9 peaks, N = 3 experiments) and from 29.7±7.3 to 45.2±11.7 (n = 17 peaks, N = 3 experiments) in the presence of 10 µM dopamine. The relative SOC amplitude was 159.4±8,2% in the presence of 10 µM dopamine of control (Figure 7C). Also, dopamine increased the frequency of the SOCs (Figure 7A, D). Thus,

effect of dopamine on neurons can be mediated by the activation of chloride currents and the corresponding suppression of the activity of the neural network.

3. Discussion

Spontaneous synchronous calcium oscillations in neurons can be induced by release of various types of neurotransmitters [36,37] but predominantly glutamate. Ability of dopamine to suppress glutamate-induced calcium signal and current in NMDA receptors was shown in details [16,17,34]. Here we demonstrated that dopamine is able to block spontaneous activity in co-cultures of primary neurons and astrocytes. Importantly, dopamine had only minor effect on calcium oscillations in neurons induced by omitting Mg^{2+} from the medium [28]. It could be explained by previously published results that dopamine is limiting but not blocking glutamate-induced calcium signal completely [34].

Although D2-like antagonist reduced amplitude SSCO, action of dopamine on SSCO in neurons had a different mechanism compared to effect of DA on glutamate induced calcium signal and oscillations – in contrast to effect on glutamate receptors dopamine suppress SSCO mainly independently of dopamine receptor without effect on frequency [16,17,34].

Effect of dopamine on SSCO in neurons was dependent on the calcium signal in neighbouring astrocytes. It was confirmed by experiment with stimulation of the astrocytes with ATP. This ATP or dopamine-induced calcium signal induce vesicular release [23,29,30,38]. This is a key process in the mechanism of dopamine-induced suppression of SSCO because it can be blocked by inhibition of exocytosis by bafilomycin.

Calcium dependent exocytosis from astrocytes releases GABA or alpha adrenaline. Stimulation of the alpha-adrenoreceptors can also release GABA [39]. In our experiments both GABA and inhibitors of alpha-adrenoreceptors blocked the effect of dopamine on SSCO. Dopamine-induced boost of inhibitory GABAergic activity could be explained by activation of dopamine receptors in neurons, but this mechanism could be excluded by experiments with D1- and D2- like receptor antagonists.

Considering our results with Cl current (Figure 7) the most probable mechanism of dopamine-induced GABA release from astrocytes is Ca^{2+} -dependent Cl-channels [40].

Effects of inhibitors of MAO on SSCO of neurons under exposure of dopamine can be explained by effective inhibition of the calcium signal in astrocytes [10,18]. Inhibition of SSCO in neurons under application of bifemelane by itself can be explained by decrease of production of ROS in MAO of neurons and reduction of activity of phospholipase C [40].

4. Materials and methods.

Materials. Neurobasal-A Medium, B-27 Supplement, GlutaMAX, HEPES, HBSS and Versene solution were purchased from Gibco, Gentamicin solution was from Sigma-Aldrich. Fura-2AM from Molecular Probes. Other reagents were from Sigma and Tocris.

Cell culture. Mixed neuroglial hippocampal culture was obtained from P1-3 newborn male rat pups. After decapitation, whole brain was removed and placed in Petri dish filled with Versene solution on ice under sterile conditions. Then meninges were removed, hippocampi were extracted, cut into small (1 mm) pieces with scissors and placed in 0.25% Trypsin solution for 10 minutes on shaker (600 rpm). After trypsinization, material was washed twice with Neurobasal A medium and dissociated using fire polished pipette. Then suspension was left for 1 min to let large pieces to settle on the bottom of the microtube. The cell suspension was placed into another tube and centrifugated for 2 minutes at 500 g. Supernatant was removed and the pellet was resuspended in culture medium. Cells were placed on round (25 mm in diameter) coverslips covered with polyethyleneimine in 35-mm Petri dishes (1 brain – 12 dishes) for 1 hour to allow cells to attach. Then coverslips were washed to remove unattached cells and left in incubator. Cell culture was grown in humidified atmosphere with 5% CO_2, t = 37°C. Twice a week half of the culture medium was replaced with fresh one. Cells were used in experiment between 10 and 14 days in culture.

Intracellular Ca^{2+} measurements. Before the experiment culture medium was removed, cells were washed twice with HBSS (pH 7.4 adjusted with NaOH, t =

28°C). Then cells were loaded with fluorescent Ca^{2+}-sensitive ratiometric indicator 5 µM of Fura-2AM for 40 minutes. Then cell culture was washed, left for 10 minutes to allow membrane-bound due to wash out, washed once again and used in experiment.

Fluorescence measurements. Measurements were performed using Cell observer imaging system (Carl Zeiss, Germany), equipped with excitation filter fast switching system Ludl MAC 5000, AxioCam HSm CCD-camera and 10× PlanApochromat lens. The fluorescent signal was collected simultaneously from 120-200 neurons and approximately 100-200 astrocytes every. The Fura-2 fluorescence was excited in 340 ± 15 and 387 ± 8 nm, beam splitter FT 409 (HE) was used to split excitation and emission beams. Two wavelength Fura-2 fluorescence signal was collected in 510 ± 45 nm every 2-3 seconds. Medium change was performed using self-made perfusion system, which was switched on during cell bathing solution change. This system allows performing 10× volume change in 8 seconds.

Patch-clamp recording and analysis. Whole-cell patch-clamp recordings were obtained from visually identified cells using Axio Observer Z1 imaging system (Carl Zeiss, Germany) with Axopatch 200B amplifier (Molecular Devices, San Jose, CA, USA). Data were digitized by Low-noise Data Acquisition System (Axon DigiData 1440A digitizer) with pCLAMP 10 software (Molecular Devices, San Jose, CA, USA). All electrophysiological recordings were performed at 28°C. The experiments were performed using a patch-pipettes (4–7 MΩ tip resistance) fabricated from borosilicate glass (WPI) using a puller P-97 (Sutter instrument). Intracellular solution containing (in mM): 5 KCl, 130 K-gluconate, 1 $MgCl_2×6\ H_2O$, 0.25 EGTA, 4 HEPES, 2 Na_2-ATP, 0.3 Mg-ATP, 0.3 Na-GTP, 10 Na_2-phosphocreatine (305–310 mOsm, pH 7.2). As an extracellular solution we used low-Ca^{2+} HBSS, pH 7.35. We registered slow outward currents (SOCs). For these purposes, the membrane potential of the patched neurons was held at −30 mV. The gradient of Cl^- inverts under these conditions, and chloride currents become outward. To exclude the contribution of action potentials in trace, we performed all measurements in the presence of a blocker of voltage-gated sodium channels, tetrodotoxin (TTX). The SOCs were analyzed using Clampfit 10.2.

In our experiments some chemicals were dissolved in DMSO. In these cases, the final concentration of DMSO never exceeded 0.3% (v/V) and DMSO alone did not alter the cytosolic calcium level in cells.

Statistical analysis

Results are expressed as means ± SEM (standard error of the mean); one-way ANOVA with post-hoc Tukey's HSD correction for multiple comparisons and Student's t-test were used, where appropriate. Statistical analysis was performed using Origin 2019 (Microcal Software Inc., Northampton, MA, USA) software. Differences were considered to be significantly different if $p < 0.05$.

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Figure Legends

Figure 1. Dopamine suppresses the spontaneous synchronous calcium oscillations (SSCO) in neurons.

A Representative images of rat primary neurolgial culture loaded with fura-2 ratiometric calcium indicator in four different conditions (from left to right): resting cells between SSCO peaks; at the maximum of $[Ca^{2+}]_c$ in neurons (*N*) under the action of 10 μM NMDA; at the maximum of $[Ca^{2+}]_c$ in glial cells (*G*) under the action of 10 μM Dopamine; and at the peak of single SSCO event. The fura-2 signal excited at 340 nm is coloured in green and when excited at 380 nm – in red. N – neurons, G – glial cells. Scalebar – 20 μm. **B** Dopamine (10 μM) inhibit SSCO in neurons and activates calcium signal in astrocytes from the same co-culture (**C**). **D** Dopamine reduced frequency of SSCO to 60 % of control. 40 sec adding of 10 μM NMDA (in Mg^{2+} free medium) was applied to distinguish neurons from glial cells in the mixed hippocampal primary culture. Mg^{2+}free medium induce calcium oscillations (E) which partially could be reduced by 10 or 50 μM dopamine, which induce Ca^{2+} signal in astrocytes from the same co-culture (F). Increase in dopamine concentration elevate dopamine induced calcium signal but reduced amplitude of SSCO in neurons (G).

Figure 2. Suppressive effect of dopamine on SSCO is independent of D1- and D2-like receptors. A- Dopamine (10 μM) inhibit SSCO in neurons and induce calcium signal in astrocytes. B- D1-like dopamine receptor antagonist SKF83566 (0.5 μM) has not changed effect of dopamine on SSCO of neurons and calcium signal in astrocytes. C- D2-like dopamine receptor antagonist L-741,626 (2 μM) also did not change effect of dopamine on SSCO of neurons and calcium signal in astrocytes. D- Effect of D1- and D2-like dopamine antagonists on the amplitude of SSCO in neurons. *p<0.05

Figure 3. Effect of dopamine on SSCO of neurons is dependent on GABAR. Inhibitors of GABA receptors bicuculline (10 μM), reversibly (A) and picrotoxin (10 μM, irreversibly (C) abolish dopamine action on SSCO of neurons but had only minor effect on dopamine-induced calcium signal in astrocytes (B-D).

Figure 4. Role of adrenoreceptors in the effect of dopamine of SSCO. A- Preincubation of primary co-cultures with nonselective beta-AR blocker propranolol

(2 µM) did not change effect of 10 M dopamine on neurons and astrocytes. B- Nonselective alpha-AR blocker phentolamine (2 µM) abolishes effect of dopamine (10 and 50 µM) on in neurons and partially on astrocytes. D- Specific alpha2-AR antagonist rouwolscine (0.5 µM) inhibited effect of 10 and 50 µM dopamine on SSCO neurons and reduced dopamine-induced calcium signal in astrocytes. E-effect of adrenoreceptor antagonist and dopamine on frequency of SSCO in neurons (% to the basal SSCO).

Figure 5. Calcium signal in astrocytes reduces SSCO in neurons.

10 µM ATP induce calcium signal in astrocytes (B) and suppressed SSCO in neurons (A). 1 hour preincubation of the co-culture with 5 µM bafilomycine A1 blocked SSCO in neurons (C) but not the effect of 10 µM dopamine on astrocytes (D). Bifemelane (10 µM), suppressed SSCO in neurons (E) and the dopamine-induced calcium response in astrocytes (F). G- Effects of 10µM ATP, 5 µM bafilomicine A1 and 10 µM bifemelane on amplitude of SSCO in neurons

Figure 6. Effect of dopamine and dopamine agonist on inducible calcium oscillations in neurons.

A - Effect of dopamine D2-like agonist PD-168,077 and D2- antagonists L-741, 626 on calcium oscillations induced by 7mM NH_4Cl. **B**- Effect of D2-like agonist PD-168,077 and D2- antagonists L-741, 626 on frequency of ammonium-induced calcium oscillations (taken as 100%). MEAN±SEM, *p < 0.05 (ANOVA)

Figure 7. Activation of chloride current by dopamine in cultured neurons.

A. Representative recording at holding potential − 30 mV in the presence of 1 µM TTX, low-Ca^{2+} medium showing the effects of dopamine on slow outward chloride currents (SOCs). **B.** The amplitude of single SOC before (black line) and after (red line) application of 10 µM dopamine. **C.** Statistical analysis shows that dopamine increases the amplitude of SOC up to 159.4±8.2% compared to control (n = total 9 events in control and 17 events in the presence of dopamine. N = 3 experiments). **D.** Dopamine significantly increases frequency of SOCs (N = 3 experiments, MEAN±SEM, p < 0.05 (ANOVA)).

Figure 1

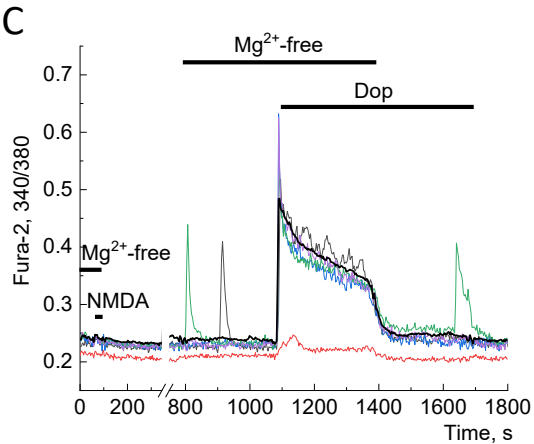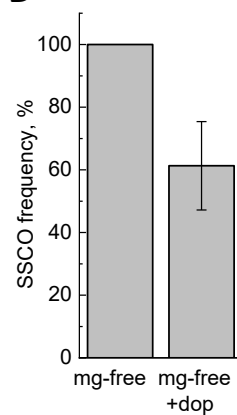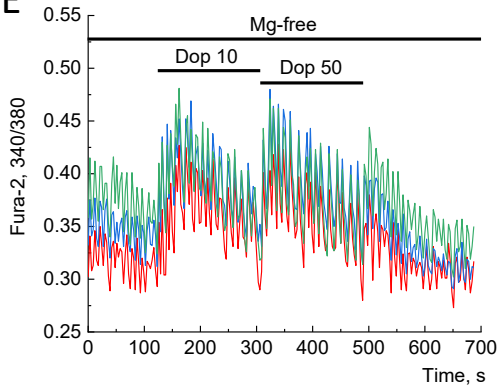**A**

| Resting culture | NMDA | Dopamine | SSCO peak |

B — Fura-2, 340/380 vs Time, s

Mg²⁺-free
NMDA
Mg²⁺-free
Dop

C — Fura-2, 340/380 vs Time, s

Mg²⁺-free
Dop
Mg²⁺-free
NMDA

D — SSCO frequency, %

mg-free   mg-free +dop

E — Fura-2, 340/380 vs Time, s

Mg-free
Dop 10   Dop 50

F — Fura-2, 340/380 vs Time, s

Mg-free
Dop 10   Dop 50

G — R.U.

glial Ca²⁺ response   SSCO frequency

Dop10 Dop50 Dop10 Dop50
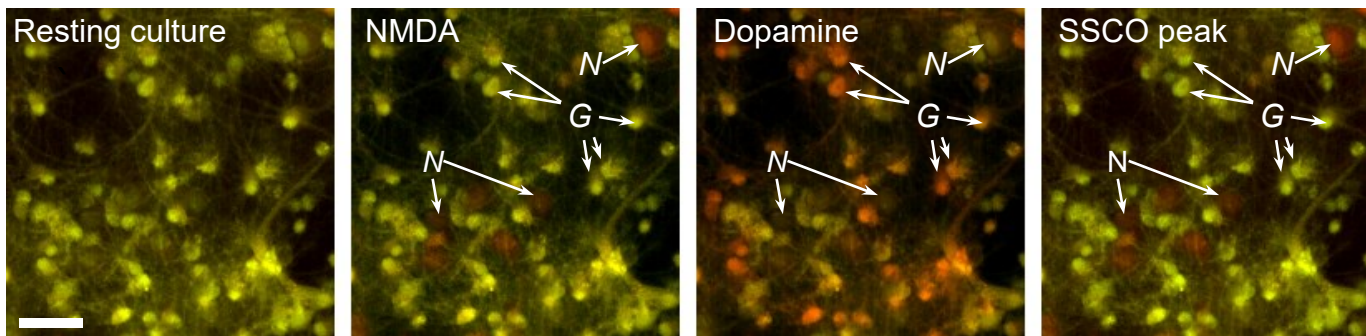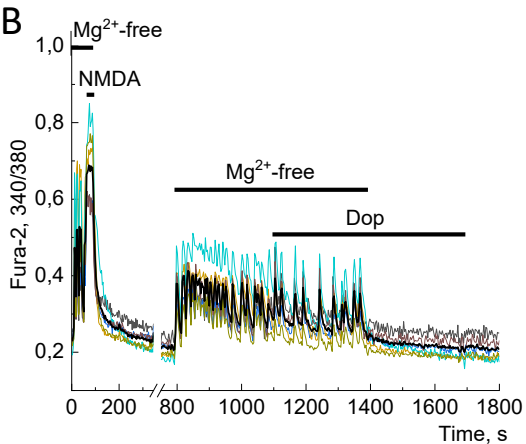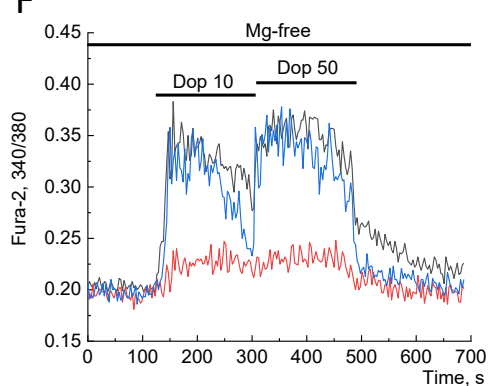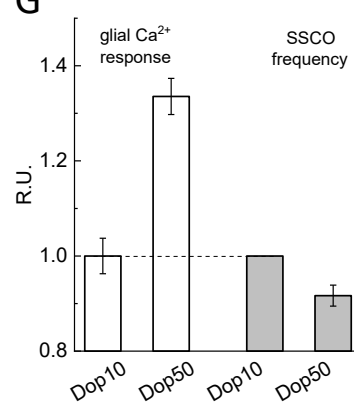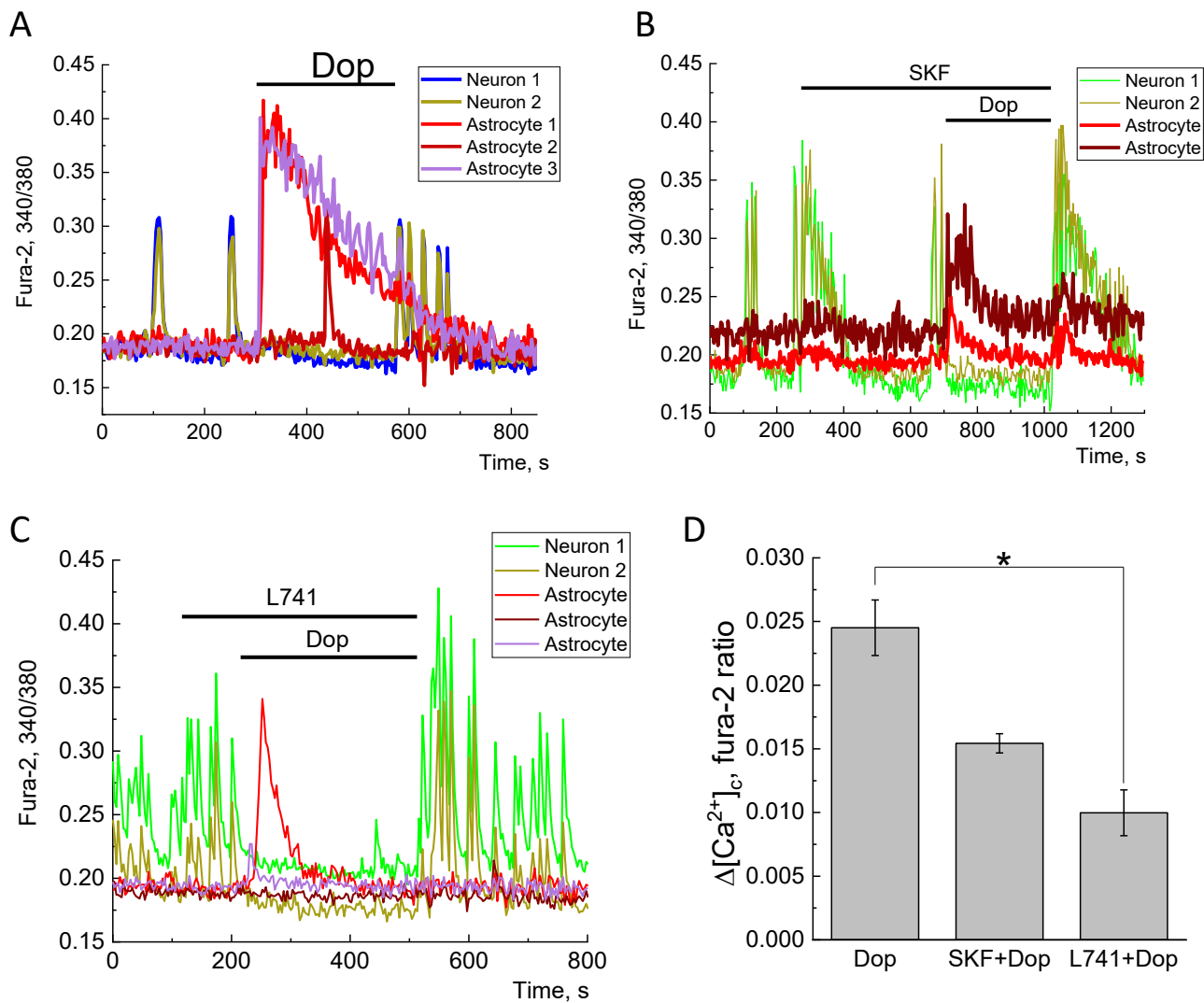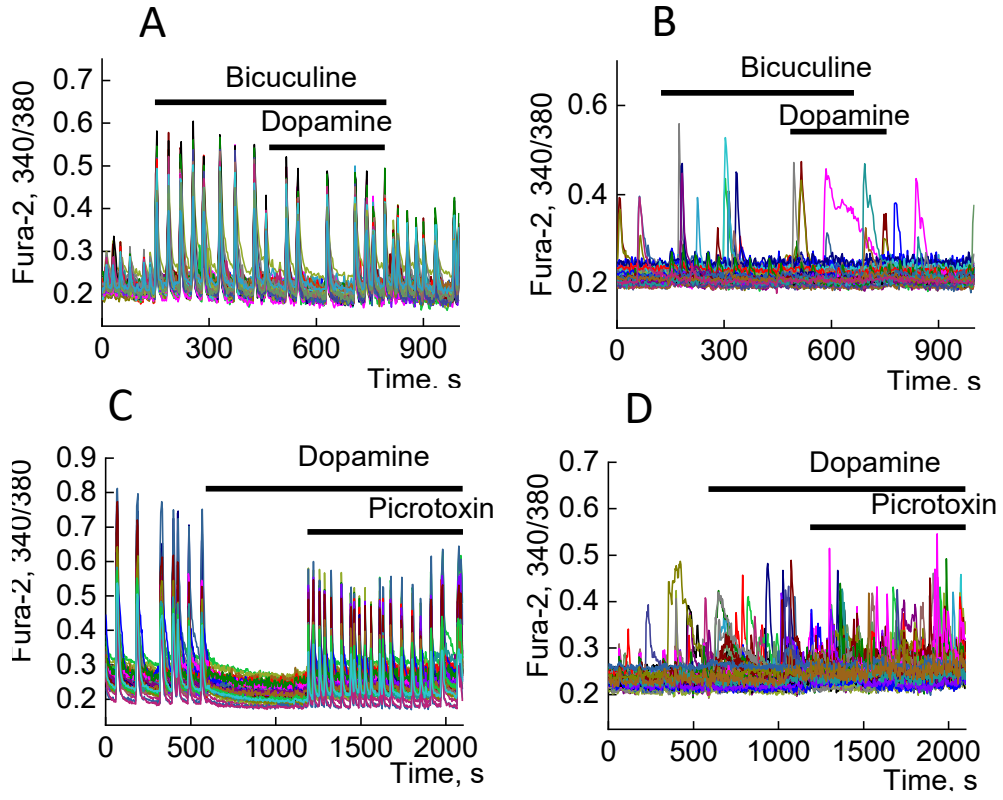
Figure 2

Figure 3

Figure 4
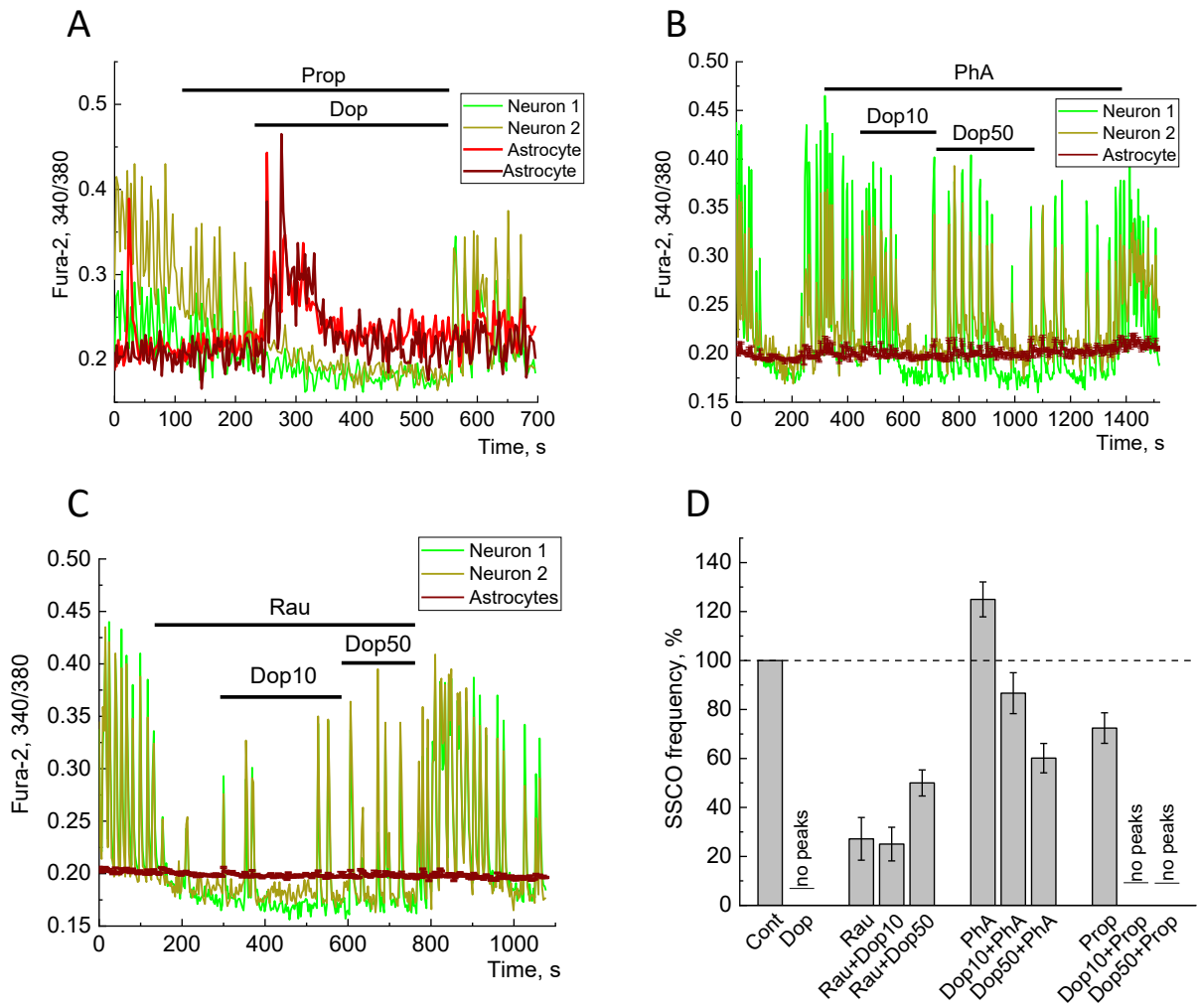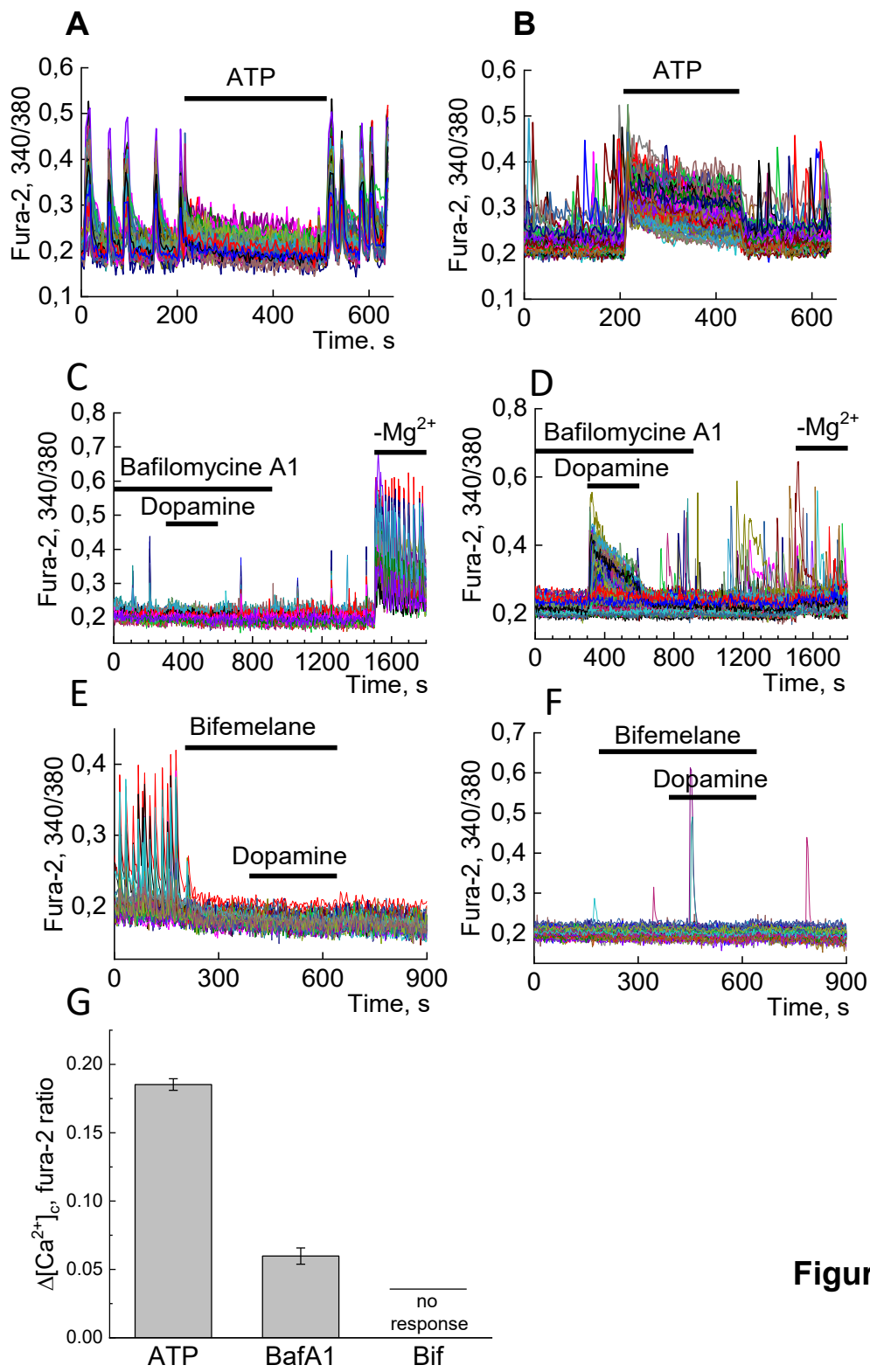
Figure 5.

Figure 5

Figure 6

Figure 7