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Inorganic polyphosphate regulates AMPA and NMDA receptors and protects against glutamate excitotoxicity via activation of P2Y receptors

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Inorganic polyphosphate regulates AMPA and NMDA receptors and protects against glutamate excitotoxicity via activation of P2Y receptors

Running title: Regulation of glutamate signal in neurons by inorganic polyphosphate

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Abstract

Glutamate is one of the most important neurotransmitters in the process of signal transduction in the central nervous system. Excessive amounts of this neurotransmitter lead to glutamate excitotoxicity which is accountable for neuronal death in acute neurological disorders including stroke, trauma, and in neurodegenerative diseases. Inorganic polyphosphate (PolyP) plays multiple roles in the mammalian brain, including function as a calcium-dependent gliotransmitter mediating communication between astrocytes, while its role in the regulation of neuronal activity is unknown. Here we studied the effect of polyP on glutamate-induced calcium signal in primary rat neurons in both physiological and pathological conditions. We found that pre-incubation of primary neurons with polyP reduced glutamate- and AMPA- but not the NMDA-induced calcium signal. However, in rat hippocampal acute slices polyP reduced ion flux through NMDA and AMPA receptors in native neurons. The effect of polyP on glutamate and specifically on the AMPA receptors was dependent on the presence of P2Y1 but not of P2X receptor inhibitors and also could be mimicked by P2Y1 agonist 2MeSADP. Pre-incubation of cortical neurons with polyP significantly reduced the initial calcium peak as well as the number of neurons with delayed calcium deregulation in response to high concentrations of glutamate and resulted in protection of neurons against glutamate-induced cell death. As a result, activation of P2Y1 receptors by polyP reduced calcium signal acting through AMPA receptors, thus protecting neurons against glutamate excitotoxicity by reduction of the calcium overload and restoration of mitochondrial function.

Keywords: inorganic polyphosphate, glutamate excitotoxicity, AMPA, NMDA, calcium signaling

Significance Statement

One of the oldest polymers in the evolution of living matter is the inorganic polyphosphate. It is shown to play a role of gliotransmitter in the brain; however, the role of polyphosphate in neuronal signalling is not clear. Here we demonstrate that inorganic polyphosphate is able to reduce calcium signal, induced by physiological or high concentrations of glutamate. The effect of polyphosphate on glutamate-induced calcium signal in neurons is due to the effect of this polymer on the AMPA receptors.

The effect of polyP on glutamate- and AMPA-induced calcium signal is dependent on P2Y receptor antagonist. The ability of polyphosphate to restrict glutamate-induced calcium signal lies in the basis of its protection of neurons against glutamate excitotoxicity.

Introduction

Signal transduction in the central nervous system is a well-balanced process which includes multiple players and factors. In astrocytes signals propagate predominantly through calcium signalling, in neurons it is mostly a change in plasma membrane potential and calcium signal (Fellin, 2009; Bazargani and Attwell, 2016). Neurons are capable of the release of multiple and very diverse neurotransmitters and neuromodulators. Astrocytes release gliotransmitters which can also have an effect on neurons (Gundersen et al., 2015). One of the major neurotransmitters is glutamate, which can act as an excitatory transmitter in vast majority of the neurons and play a role of a signaling molecule for astrocytes (Hertz and Zielke, 2004). Synaptically released glutamate activates ionotropic or metabotropic glutamate receptors which changes electrical activity of neurons and triggers Ca^{2+} signal. Prolonged stimulation of neurons with high concentration of glutamate, as seen in many pathologies, leads to excitotoxicity, and is believed to be one of the major reasons behind neuronal loss in stroke, epilepsy and some neurodegenerative diseases (Olney, 1969; Choi, 1985). Both excitatory and inhibitory types of neurotransmission are mediated in large part by glutamate and gamma-aminobutyric acid (GABA), however, some other neurotransmitters and neuromodulators, including dopamine and those of the purinergic system could modify the glutamate signal (Krnjevic, 1970; Vaarmann et al., 2013).

One of the oldest polymers in the evolution of the living matter is the inorganic polyphosphate (polyP). It plays an essential role in bacteria, yeast and protozoa, but is also presented in relatively high concentration in the mammalian brain – up to 50 μM, where it acts as a gliotransmitter (Lorenz et al., 1997; Holmstrom et al., 2013);(Angelova et al., 2014) (Angelova et al., 2018). As seen by the relatively small amount of publications in this field up-to-date, the function of this polymer is largely underestimated, especially for the brain. Potentially, polyP can play multiple roles in the CNS (Kumble and Kornberg, 1995; Angelova et al., 2016) (Cremers et al., 2016; Muller et al., 2017). For example, in astrocytes it is localised in mitochondria, lysosomes and VNUT-containing vesicles (Abramov et al., 2007; Pavlov et al., 2010; Holmstrom et al., 2013; Angelova et al., 2018). PolyP is released from lysosomes or VNUT vesicles by exocytosis which leads to the development of calcium waves (Holmstrom et al., 2013; Angelova et al., 2018). Structurally, PolyP consists of long chains of only orthophosphate residues, but despite such a simple composition it is able to activate P2Y1 receptors, stimulating the activation of phospholipase C and inducing the release of Ca^{2+} from the endoplasmic reticulum via the IP3 receptors (Holmstrom et al., 2013; Dinarvand et al., 2014). Moreover, PolyP directly induces calcium signal in only ~3% of the neurons and it has been found positioned to the synapses (Holmstrom et al., 2013; Stotz et al., 2014). Furthermore, it has been shown to modulate the activity of ion channels, i.e. TRPM8 channels (Zakharian et al., 2009). However, polyP significantly changes neuronal activity *in vivo* (Holmstrom et al., 2013) and this effect can be induced by both – a direct effect of polyP on neurons or indirectly through activation of astrocytes. Here, we study whether polyP is as well able to reduce glutamate-induced calcium signal and in particular the signal mediated by AMPA and NMDA receptors. We then tested whether the effect of polyP on the calcium signal stimulated by glutamate is involving the purinergic system. Finally, we tested the ability of polyP to influence the glutamate-induced calcium signal and effectively protect neurons from the delayed calcium deregulation (DCD) and consequently from glutamate-induced cell death.

Methods

Cell culture

Co-cultures of cortical neurons and astrocytes were prepared from Sprague-Dawley rat pups either sex 2-4 days post-partum (UCL breeding colony) as described (Angelova et al., 2019). Brain cortices were removed into ice-cold HBSS (Ca^{2+}, Mg^{2+}-free, Invitrogen, Paisley, UK). The tissue was minced and trypsinised (0.1% for 15 min at 37^{o}C), triturated and plated on poly-D-lysine-coated coverslips and cultured in Neurobasal medium (Gibco-Invitrogen, Paisley, UK) supplemented with B-27 (Gibco-

Invitrogen, Paisley, UK) and 2 mM L-glutamine. The cultures were maintained at 37 °C (5% CO_2) and the media changed twice a week. To avoid the age dependence on the DCD, cells were used after 12–15 days in vitro in all experiments.

$[Ca^{2+}]_c$ and [Δψm] imaging

Hippocampal or cortical neurons were loaded for 30 min at room temperature with 5μM fura-ff AM (Molecular probes) or 5μM fura-2 AM (Molecular probes) and 0.005% Pluronic acid in a HEPES-buffered salt solution (HBSS): 156 NaCl, 3 KCl, 2 $MgSO_4$, 1.25 KH_2PO_4, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.35. For simultaneous measurement of $[Ca^{2+}]_c$ and mitochondrial membrane potential ($\Delta\psi_m$), Rh123 (1 μM, Molecular Probes) was added into the cultures during the last 15 min of the fura-2 or fura-ff loading period, and the cells were then washed 3-5 times before experiment. Fluorescence measurements were obtained on an epifluorescence inverted Olympus microscope equipped with a × 20 fluorite objective. $[Ca^{2+}]_c$ and $\Delta\psi_m$ were monitored in single cells using excitation light provided by a Xenon arc lamp, the beam passing sequentially through 10 nm band pass filters centred at 340, 380 and 490 nm housed in computer-controlled filter wheel (Cairn Research, Kent, UK). Emitted fluorescence light was reflected through a 515 nm long-pass filter to a cooled CCD camera (Retiga, QImaging, Canada). The fura–2 or fura-ff data have not been calibrated in terms of $[Ca^{2+}]_c$ because of the uncertainty arising from the use of different calibration techniques. For ΔFura ratio calculation, we used the maximal value of fluorescence obtained during glutamate exposure and, as the baseline, the mean of fluorescence recorded during the time preceding the stimulation. For $\Delta\psi_m$ measurements, we have normalised the fluorescence signals between resting level (set to 0) and a maximal signal (which correspond to full mitochondrial depolarisation) generated in response to the protonophore FCCP (1μM; set to 100%). Changes in Rh123 fluorescence were then expressed as the difference between peak values (at 15 min) attained during glutamate stimulation and basal values. All imaging data were collected and analysed using software from Andor (Andor IQ3, Belfast, UK).

PolyP standards (sodium salt): Short (S-PolyP) 14 ortophosphates, Medium (M-PolyP) 60 ortophospahtes and long (L-PolyP) 130 ortophosphates were provided by Dr T Shiba (Regenetiss, Inc., Japan).

Neuronal toxicity experiment

For toxicity assay, cortical neurons were sequentially exposed to 20 μM PolyP for 10 minutes and to 100 μM glutamate for further 30 minutes. When P2Y and P2X inhibitors were tested, compounds

were added 20 min before polyP exposure. Then, cells were cultured with fresh media without polyp and glutamate and cell viability was assessed 24 hours after treating neurons with 20 μM propidium iodide (PI) and 4.5 μM Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 30 min before imaging. The PI is excluded from viable cells and exhibits a red fluorescence (excitation/ emission 530 nm/ >560 nm) following a loss of membrane integrity, while the Hoechst 33342 labels all nuclei blue (excitation 405 nm, emission 460 nm). This allows subtraction of the number of dead (red stained) cells from the total number of cells counted (blue stained nuclei). Using phase contrast optics, a bright field image allowed identification of neurons, which are morphologically different to the flatter glial component and lie in a different focal plane, above the glial layer. A total number of 100–300 neurons or glial cells were counted in 5-6 fields of each coverslip. Each experiment was repeated three or more times, using different neuronal preparations.

Electrophysiology:

Transverse hippocampal slices (300-400 μm thick) were used for *in vitro* electrophysiological recordings. Slices were prepared from 3- to 5-week old Sprague-Dawley rats. Animals were killed by cervical dislocation after being anesthetized by an overdose of isoflurane according to the United Kingdom Animals (Scientific Procedures) Act of 1986. After decapitation, brains were rapidly removed and dissected, and whole-brain sagittal slices were prepared with a Leica VT1200S vibratome in ice-cold sucrose-based solution containing the following (in mM): 70 sucrose, 80 NaCl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 22 glucose, equilibrated with 95% O_2 plus 5% CO_2, pH 7.35, 315–330 mOsm. Slices were equilibrated to a room temperature and then placed to recover in continuously oxygenated sucrose-ACSF chamber for at least 1 h before recording. After recovering, individual slices were transferred into recording chamber. The perfusion solution contained the following (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 22 glucose and was continuously gassed with 95%O_2 and 5%CO_2, pH 7.35; 290–298 mOsm. For the whole-cell patch-clamp recordings, we used borosilicate glass electrodes of 4-6 MΩ impedance. Recordings were performed with Multiclamp 700B amplifier running under pClamp 11x software (Molecular Devices). The intracellular pipette solution contained the following (mM): 120.5 CsCl, 10 KOH-HEPES, 2 EGTA, 8 NaCl, 5 QX-314 Br^- salt, 2 Mg-ATP, 0.3 Na-GTP. Voltage-clamp recordings of NMDA receptors (NMDARs) activity in hippocampal dentate gyrus granule cells (DGCs) were performed at +30 mV holding potential. To isolate NMDAR response pharmacologically we added to perfusion solution the following antagonists (μM): picrotoxin 50, GGP-55645 1, strychnine 1, NBQX 20, S-MCPG 200. Voltage-clamp recordings of AMPA receptors (AMPARs) activity in DGCs were performed at -70 mV holding potential. To isolate AMPAR response pharmacologically we added to perfusion solution the following antagonists (μM):

177 picrotoxin 50, GGP-55645 1, strychnine 1, APV 50, S-MCPG 200. For both NMDAR and AMPAR
178 recordings response amplitude after polyphosphate application was normalized to that of control
179 recording from the same cell. Synaptic responses were evoked by bipolar tungsten electrode placed in
180 perforant path and connected to DS2A constant-voltage stimulator (Digitimer). Electrical stimuli were
181 applied with 200 ms time interval, once per 15 seconds.

182

183 **Statistical and data analysis**

184 Statistical analysis and data analysis were performed using Origin 9 (Microcal Software Inc.,
185 Northampton, MA) software. Statistical analysis was performed using unpaired or paired two tailed
186 Student's *t* test, as indicated. A test for outliers was not conducted on the data. Results are expressed
187 as means ± standard error of the mean (S.E.M.). Differences were considered to be significantly
188 different if $p < 0.05$.

189

190 **Results**

191 ***PolyP decreases glutamate-induced calcium signal***

192 The application of physiological concentration of glutamate (5μM) to primary cortical co- culture of
193 neurons and astrocytes induced a fast and transient increase in $[Ca^{2+}]_c$ of neurons (by 1.57±0.04 fura-2
194 ratio; n=120; Figure 1A, C). Stimulation of neurons with short length polyP (S-PolyP; 5-15
195 orthophosphates; 20μM,) induced only minor changes in $[Ca^{2+}]_c$ of neurons (Figure 1B). However,
196 consequent addition of 5 μM glutamate results in significantly lower calcium signal in these neurons
197 compared to control (from 1.56±0.04, n=120, to 0.98±0.04 fura-2 ratio; n=63; p>0.0001; figure 1 B,
198 C). The ability of polyP to reduce glutamate-induced calcium signal was dependent on the length of
199 polymer: it was less effective with elongation of the chain (Figure 1 C).

200 High and toxic concentrations of glutamate induce initial calcium influx followed by DCD which
201 triggers excitotoxicity (Figure 2A). Pre-incubation of neurons with 20 μM short (p=0.0157, Figure
202 2B), medium (p=0.0250, Figure 2C), both significantly reduced the number of cells with delayed
203 calcium deregulation. Importantly, short polyP was more effective in the overall protection of the
204 neurons against occurrence of DCD compared to medium and long polyP (Figure 2E) but
205 interestingly all types of PolyP significantly decreased the amplitude of glutamate-induced (100 μM)
206 calcium signal (from 0.64±0.03, n=178 in CTRL to 0.32±0.04, n=64, p>0.0001 for S-PolyP; to
207 0.45±0.03, n=146 , p>0.0001 for M-PolyP and to 0.45±0.03, n=97, p>0.0001 by L-PolyP,
208 respectively; Figure 2F). Thus both short and medium polyP are more effective in suppressing the

glutamate-induced calcium signal that results in protection of the neurons against excitotoxic DCD, compared to L-PolyP.

PolyP reduces glutamate-induced mitochondrial depolarisation

Glutamate-induced DCD is associated with profound mitochondrial depolarisation (Vergun et al., 1999; Abramov and Duchen, 2008). In our experiments exposure of neurons to 100 µM glutamate induced simultaneous DCD and mitochondrial depolarisation (Rh123 signal rose by 38±12% in 7 min after glutamate addition, n=81; Figure 3A, D). Pre-incubation of cortical neurons with 20 µM S-PolyP did not change basal mitochondrial potential but significantly reduced the effect of high glutamate exposure on $\Delta\psi m$ (Figure 3 B, C, D). Thus, Rh123 fluorescence rose only to 17±5%, n=55, p=0.0279 that correlates with the decrease of the number of cells with DCD in cultures treated with short PolyP.

PolyP reduces glutamate-stimulated calcium signal via P2Y receptors

PolyP can stimulate P2Y receptors and induce the release of vesicles with transmitters that can have potentially an effect on glutamate signal (Holmstrom et al., 2013; Angelova et al., 2018). To assess the potential effect of the purinergic system in the polyP-induced reduction of glutamate response we used inhibitors of P2Y and P2X receptors, Suramin and TNP-ATP, respectively. In agreement with the data presented in Figure 1 short polyP induced significant reduction of glutamate-induced calcium signal compared to untreated cells (from 1.57±0.05, n=120 in CTRL to 0.98±0.05 with S-PolyP, n=91; p>0.0001; Figure 4A, B, F). Pre-incubation of the cortical co-culture with 20 µM Suramin significantly mitigated the effects of S-PolyP on calcium signal induced by 5 µM glutamate (from in 0.98±0.05 with S-PolyP, n=91to 1.27±0.07, n=63; p=0.0002; Figure 4 C, F). Importantly, pre-incubation of the cortical co-culture with the more specific inhibitor for P2X receptors (20 µM TNP-ATP) had no significant effect on the action of polyP on glutamate-induced rise in $[Ca^{2+}]_c$ of cortical neurons (n=76; Figure 4 D, F). Another inhibitor of P2 (more specific for P2X) receptors PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt) also had as well no effect on the ability of PolyP to reduce calcium signal under application of 5µM glutamate (n=44; Figure 4 E, F).

Stimulation of P2 receptors by polyP reduce number of cells with DCD

Ability of polyP to reduce DCD in neurons under exposure of the 100 µM glutamate was also dependent on the presence of the inhibitor of P2Y receptor Suramin (n=48, p=0.0007; Figure 5 C, F), PPADS (n=32, p=0.0208; Figure 5 E, F) but not TNP-ATP (n=36, p=0.0676; Figure 5 D, F). Thus,

PolyP acts more likely through P2Y receptors to reduce calcium responses to physiological (5μM) and excitotoxic (100 μM) concentrations of glutamate.

PolyP stimulates P2Y1 receptor and reduces calcium signal through AMPA receptors

In order to identify which type of glutamate receptors is affected by polyP, we used application of receptor agonist AMPA and NMDA to fura-2 loaded cortical neurons. Addition of 20 μM NMDA induced rise in $[Ca^{2+}]_c$ of cortical neurons (n=88; Figure 6 A, J). Exposure of the neurons to 20 μM short polyP did not significantly change the amplitude or the shape of the calcium signal in response to NMDA (n=76; Figure 6 B, J). However, short polyP significantly reduced AMPA-induced calcium signal in neurons compared to control cells (; from 2.3±0.142, n=208 to 0.3045±0.0469, n=161; p>0.0001; Figure 6 C, D, J). Importantly, Suramin suppressed the action of S-PolyP on AMPA-induced calcium signal in the same way as it did for glutamate (from 0.3045±0.0469, n=161, to 2.459±0.1806, n=132; p=0.0004, Figure 6 E, J). However, considering that none of the following P2 receptor antagonists used is purely selective for P2X or P2Y (Suramin, PPADS or TNP-ATP), we used selective antagonist for P2Y1 receptor- MRS2179 (2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt). Pre-incubation of primary cortical neurons with 50 μM MRS2179 completely prevented the effect of short polyP on AMPA-induced calcium signal (from 0.3045±0.0469, n=161, to 2.409±0.1765, n=157; p=0.0016, Figure 6 F, J). Thus, polyP activates P2Y1 receptors which reduce calcium signal through AMPA receptors in response to AMPA or glutamate. Application of another classical agonist of P2 receptors - ATP, at 100μM, induced typical calcium signal as expected only in astrocytes (not shown), but not in neurons (Figure 6 G). Interestingly, pre-stimulation of neurons with 100μM ATP did not reach statistical significance in the change of AMPA-induced calcium signal in these neurons (n=82; p=0.0977, Figure 6 G, J). However, when reduced to a concentration of 10μM ATP induced primary response in cortical neurons and significantly reduced the AMPA-evoked calcium signal from 2.3±0.142, n=208 to 0.7±0.055, n=54, p>0.0001, Figure 6 H, J. Additionally, application of another P2Y agonist 2MeSADP (2-Methylthio-adenosine-5'-diphosphate, 1 μM, a P2Y1, P2Y12 and P2Y13 receptors, moderately potent at P2Y11 receptors), was able to reduce the AMPA-induced calcium signal in neurons similar to the level of reduction by 20 μM short-chain PolyP (0.3045±0.0469, n=161, for AMPA+S PolyP compared to 0.796±0.095, n=77, for AMPA+2MeSADP, p>0.0001; Figure 6 I, J) .

PolyP downregulates electrophysiological neural response in living tissue

Considering the role that has been given to the P2Y receptors in the literature for activation of electrical activity of glutamate receptors and in order to test effects of PolyP in a system where intercellular interaction is more pronounced, we studied the effect of stimulation with polyP in *ex vivo* hippocampal slices. To test whether PolyP affects electrical charge transfer through NMDARs in native dentate gyrus granule cells (DGCs), we performed electrophysiological recording of

pharmacologically isolated NMDAR and AMPAR response evoked by electrical stimulation (Figure 7A, B). We found, that 20 μM of both long and short chain polyP, when added to perfusion solution, decreased significantly NMDAR response amplitude. L-PolyP: to 0.66±0.09 of control value, $p<0.05$, n=5, paired Student's t-test; S-polyP: to 0.67±0.08 of control, $p<0.01$, n=8, paired Student's t-test. However, neither L-PolyP, nor S-PolyP affected significantly the paired-pulse ratio (PPR) of paired responses: for L-PolyP 0.97±0.07, $p>0.05$, n=5, paired Student's t-test; for S-PolyP 0.94±0.38, $p>0.05$, n=8, paired Student's t-test.

Similarly, PolyP species affected the response amplitude, but not PPR of AMPARs (Figure 7C, D). 20 μM of both long and short PolyP downregulated amplitude of pharmacologically isolated AMPAR response to 0.72±0.068 and 0.58±0.092 of control, respectively (n=8, $p<0.01$ for both cases, paired Student's t-test). PPR of AMPAR response was calculated to be 1.12±0.11 of control with L-PolyP and 0.97±0.083 of control with S-PolyP (n=8, $p>0.05$ for both cases, paired Student's t-test).

PolyP protects neurons against glutamate excitotoxicity via P2Y receptors

The application of 100 μM glutamate to cortical co-culture neurons and astrocytes induced significant cell death measured 24 hours after stimulation (36.6±5.4, n=250, Figure 8B). High concentrations of long PolyP can be also toxic for neurons (Angelova et al., 2016). However, pre-incubation (20 μM, 10 min) of the cortical co-cultures with long, medium or short PolyP significantly decreased the number of glutamate-induced dead cells (to 10.47±0.95; 12.54±1.75 and to 12.62±2.33, respectively, for both n=250; $p>0.0001$; Figure 8 A, B). Importantly, inhibitor of P2Y receptors Suramin (20 μM added 20 min before S-PolyP and glutamate) significantly reduced protective effect of 20 μM short PolyP against glutamate-induced excitotoxicity (from 26.48±2.38 to 10.18±0.89, for both n=250; $p>0.0001$; Figure 8 C) while more selective inhibitor of P2X receptor TNP-ATP (20 μM, 20 min before PolyP and glutamate) had no effect on neuronal viability. This strongly suggests that PolyP protects neurons against glutamate-induced toxicity via stimulation of P2Y receptors.

Discussion

In the present study, we found that PolyP is able to reduce physiological calcium signal induced by glutamate. This effect was dependent on the length of the polymer and activity was reduced with elongation of polyphosphate chain. The ability of PolyP to reduce Ca^{2+} influx through glutamate receptors is more likely to be a basis for significant reduction of the number of neurons with DCD under exposure of the cells with toxic concentration of glutamate (100 μM). DCD is induced by

multiple factors (Vergun et al., 1999; Khodorov, 2004; Abramov and Duchen, 2010) but initiated by massive ion flux through glutamate receptors. Reduction of the calcium influx or activation of Ca^{2+}-ATPases protects neurons against DCD induced by high concentrations of glutamate in the same way as PolyP (Suwanjang et al., 2013; Vaarmann et al., 2013). Although PolyP is shown to play an important role in mitochondria (Abramov et al., 2007; Pavlov et al., 2010) and in our experiments PolyP significantly reduced glutamate-induced depolarisation (Figure 4), the protective effect of PolyP is less likely due to direct effect on mitochondria. The effect of this polymer on $\Delta\psi m$ under glutamate exposure is more likely due to reduction of $[Ca^{2+}]_c$ that lead to lower calcium uptake into mitochondria. Interestingly, inhibition or sequestration of mitochondrial calcium uptake by mitochondria leads to protection against glutamate-induced depolarisation (Abramov and Duchen, 2008; Angelova et al., 2019).

Most of the effects which we observed here: reduction of the calcium signal upon stimulation with low or high concentrations of glutamate by PolyP and protection of the cells against glutamate toxicity were dependent of the presence of P2Y (but not P2X) antagonist suramin. Previously, we and others have shown that PolyP activates calcium signal in astrocytes (and in 3% neurons) via stimulation of P2Y1 receptor (Holmstrom et al., 2013; Dinarvand et al., 2014). Purinergic receptors (P2X and P2Y) can be activated by purines (Burnstock, 2013) and induced calcium signal in astrocytes which depends on the activation of phospholipase C (Domijan et al., 2014). Activation of both ionotropic (P2X) and metabotropic (P2Y) receptors suppresses AMPA and NMDA and protects against glutamate excitotoxicity (Ortega et al., 2011; Pougnet et al., 2016). PolyP suppresses glutamate-induced calcium signal and glutamate toxicity via P2Y1 that can be proven by experiments with the highly selective inhibitor MRS2179 and less likely through P2X due to weaker effects of TNP-ATP and PPADS in our experiments.

Clear downregulation of NMDA receptor response amplitude without significant changes in paired-pulse ratio suggests suppression of NMDA receptor conductance due to postsynaptic mechanisms (i.e. impact on biochemical regulatory networks) rather than due to presynaptic (i.e. modulation of glutamate release from synaptic bouton).

Absence of significant PolyP -generated suppression of NMDA-induced Ca^{2+} waves (Figure 7B) and presence of such a suppression of NMDAR electrophysiological response (Fig. 7A, B) looks to some extent counter-intuitive. However, this apparent discrepancy has two potential explanations. First, NMDA-induced Ca^{2+} elevation may not be only due to Ca^{2+} transport through NMDA receptor channels: Ca^{2+}-induced Ca^{2+} release from endoplasmic reticulum in response to NMDA application, which has long been established for DGCs (Lazarewicz et al., 1998) provides a good alternative source. Hence, Ca^{2+} wave after glutamate receptors activation may be generated mostly by Ca^{2+} release from endoplasmic reticulum, whereas, to start such a wave, NMDARs at cytoplasmic

membrane should provide just an small initial amount of Ca^{2+} which works as a trigger; therefore, reduction of Ca^{2+} ion transfer through NMDARs by PolyP leaves enough Ca^{2+} to initiate release from endoplasmic reticulum, and thus does not cause a significant impact on the overall Ca^{2+} rise in cytoplasm.

Second, permeability of NMDARs to Ca^{2+} depends on NMDAR subunit composition (Evans et al., 2012). Therefore, PolyP may suppress selectively NMDARs of subunit composition with low permeability to Ca^{2+}: this would reduce the overall charge transfer (Fig. 6), but not the overall Ca^{2+} influx (as in Figure 6).

On other side, PolyP can directly act on P2Y1 receptors in neurons producing (or not producing) much smaller calcium signal due to much smaller calcium ER pool compared to astrocytes. This effect results in suppression of glutamate receptors. However, we cannot exclude the fact that PolyP induces activation of astrocytic P2Y1 receptors (Holmstrom et al., 2013) and stimulates the release of neuromodulators which in turn reduces the conductance of glutamate receptors for Ca^{2+}, one example of which could be underlying neuron-glia interaction (Angelova and Abramov, 2014).

Despite the fact that the P2 agonist ATP was shown to be able to decrease conductance of glutamate receptors (Ortega et al., 2011; Pougnet et al., 2016) , in our experiments 100 μM ATP had no effect on AMPA-induced calcium signal (Figure 6 G). This could possibly be explained by the higher selectivity of PolyP to P2Y1 receptor compared to 100 μM ATP, which is possibly more selective to other type of P2 receptors. When we tested lower concentration, 10 μM ATP, we obtained a similar effect to those of short PolyP. Importantly, it also can be proven with P2Y1 specific agonist 2MeSADP**.**

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Figure Legends

Figure 1. PolyP supresses the effect of low glutamate concentrations (5μM) on neuronal calcium signal. A, Application of 5 μM glutamate to cortical neurons induced a rise in $[Ca^{2+}]_c$ (Fura-2 ratio). **B,** Pre-treatment of 20μM of S-PolyP significantly reduced glutamate-induced calcium signal. **C**, Histogram representing the effect of different lengths of PolyP on the glutamate-induced calcium

signals in cortical neurons as a ΔFura-2 ratio between basal level of $[Ca^{2+}]_c$ and maximal peak after glutamate (5μM) application. *P<0.05, **P<0.001

Figure 2. Poly-P reduces the number of neurons with delayed calcium deregulation (DCD) induced by high concentration of glutamate. **A,** Kinetic measurements of the changes in $[Ca^{2+}]_c$ (Fura-FF ratio) of single neurons in response to 100 μM glutamate in primary cortical co-cultures. **B, C, D**, Pre-treatment (6 min) of cortical neurons with respectively Short, Medium and Long Poly-P reduced $[Ca^{2+}]_c$ responses to glutamate (100μM). **E**, Changes in the percentage of neurons responding to glutamate (100μM)-induced DCD after pre-treatment with different lengths of PolyP. **F**, Histogram showing the effect of different length of PolyP on total glutamate-induced calcium increase (ΔFura-ff ratio between basal and maximal amplitude) in cortical neurons.*P<0.05, **P<0.001

Figure 3. Poly-P mitigates the glutamate-induced mitochondrial membrane depolarization. A, Application of toxic (100 μM) concentrations of glutamate induced a decrease in $\Delta\psi_m$ (represented as increase in Rhodamine123 signal, maximal depolarisation after 1μM FCCP was taken as 100%). **B**, Pre-treatment of cortical neurons with S-PolyP reduced the effect of high concentration of glutamate on mitochondrial membrane potential. **C**, Average traces from single representative experiments showing kinetic changes in $\Delta\psi_m$ of control and polyP pre-treated neurons in response to 100 μM glutamate **D**, Summary of the effect of S-PolyP on the glutamate-induced mitochondrial depolarization in cortical neurons. *P< 0.05

Figure 4. Modulation of glutamate-induced calcium signal by S-PolyP is P2Y receptor-mediated. Kinetic measurement of the changes in $[Ca^{2+}]_c$ from single neurons in response to 5μM glutamate in cortical cultures with (B, C, D, E) and without (A) S-PolyP, in the presence of P2 receptors antagonists 20 μM suramin (C), 20μM TNP-ATP (D) and 20 μM PPADS (E). **F**, Summary of the effects of P2 receptor antagonists on the S-PolyP action on glutamate-induced calcium signal. *P< 0.05

Figure 5. Effect of polyP on $[Ca^{2+}]_c$ rise in response to toxic concentration of glutamate (100 μM) in the presence of P2 receptor antagonists. 100 μM glutamate induced profound elevation of $[Ca^{2+}]_c$ in primary cortical neurons (A), which could be reduced by pre-treatment of the cells with 20μM PolyP (B). Pre-incubation of primary cortical co-culture with P2 receptors blockers 20 μM Suramin (C), 20μM TNP-ATP (D) and 20 μM PPADS (E) modified the effect of S-polyP on

glutamate-induced calcium signal. F, Summary the effects of P2 antagonists on reduction of glutamate-induced calcium signal by PolyP (ΔFura-ff ratio between basal calcium level and maximal calcium amplitude after application of 100 μM glutamate). *P<0.05, **P<0.001

Figure 6. Effects of PolyP on the AMPA and NMDA-induced calcium signals. A. 20μM NMDA induced calcium signal in cortical neurons (fura-2 ratio). B. Pre-application of short polyP (20 μM) had no significant effect on NMDA-induced increase in fura-2 ratio. Effect of AMPA (20 μM) on $[Ca^{2+}]_c$ of cortical neurons (C) can be reduced by pre-treatment of cells with 20 μM S-PolyP (D). P2 antagonist Suramin (20 μM, E, J) or P2Y1 blocker MRS2179 (50 μM, F) completely abolished the effect of S-PolyP on AMPA-induced calcium signal. Agonist of P2 receptor ATP induced calcium rise in astrocytes did change neuronal $[Ca^{2+}]_c$ in response to 20 μM AMPA significantly when applied at 10 μM (H), but not at 100 μM (G) concentration. I – A more specific agonist of the P2Y1, P2Y12 and P2Y13 receptors -2MeSADP, at 1 μM, mimicked the effect of S-PolyP on the AMPA receptors. J, Summary of the effects of P2 agonists and antagonists on the S-PolyP effect on the AMPA-induced calcium signal. ***P<0.0001

Figure 7. Polyphosphate suppresses glutamate receptor synaptic response. A, representative traces illustrating the impact of 20μM PolyP on the response generated by isolated NMDARs from dentate gyrus cells from the hippocampus (DGCs). Top: L-polyP (L-PP); bottom: S-PolyP (S-PP). **B,** Summary of the effects depicted in A: first response amplitudes and paired-pulse ratio (PPR). **C,** Representative traces illustrating the impact of 20μM PolyP on the response generated by isolated AMPARs in DGCs. Top: L-polyP (L-PP); bottom: S-PolyP (S-PP). **D:** Statistical summary of the effects of PolyP in C on the first response amplitudes and PPR. Data was normalised to values generated under control, asterisks denote significance of difference from unity. * P<0.05, ** P<0.01.

Figure 8. PolyP protects neurons against glutamate-induced cell death A, Representative images depicting the effect of PolyP of different length on cell death induced by 100 μM glutamate using PI fluorescence. **B**, Histogram represents the effect of different length of PolyP on the glutamate-induced cell death in cortical neurons. **C**, Histogram summarising the effect of PolyP on glutamate-induced toxicity in the presence of P2-receptor antagonists. The number of dead cells was counted with respect to the total number of cells identified by staining the nuclei with Hoechst 33342. The percentage of death cells shown in the histogram was calculated as the sum of average number of dead neurons obtained for each field of view. Each experiment was repeated three or more times using 3 or more different neuronal preparations. ** p<0.01, *p<0.05

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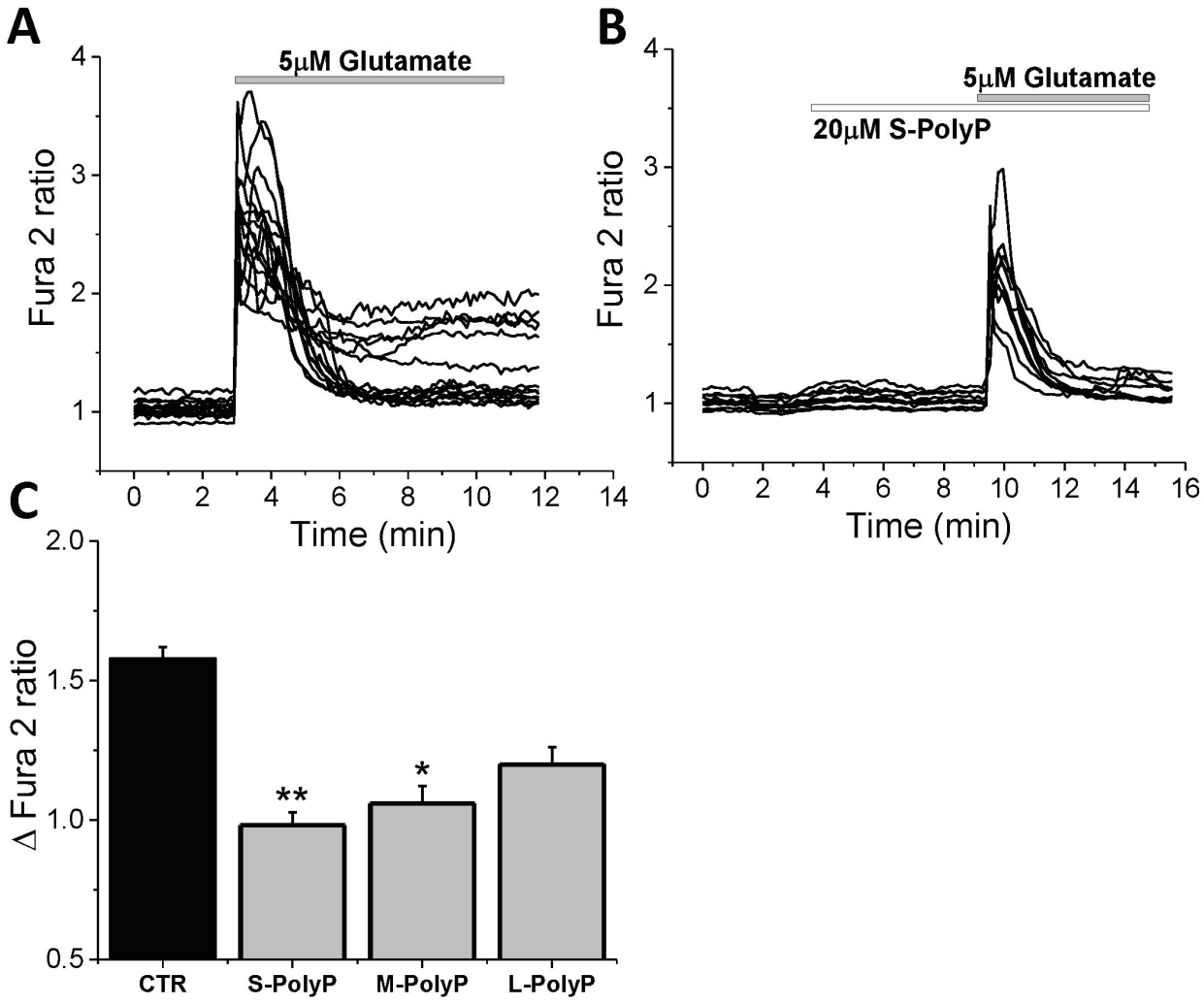
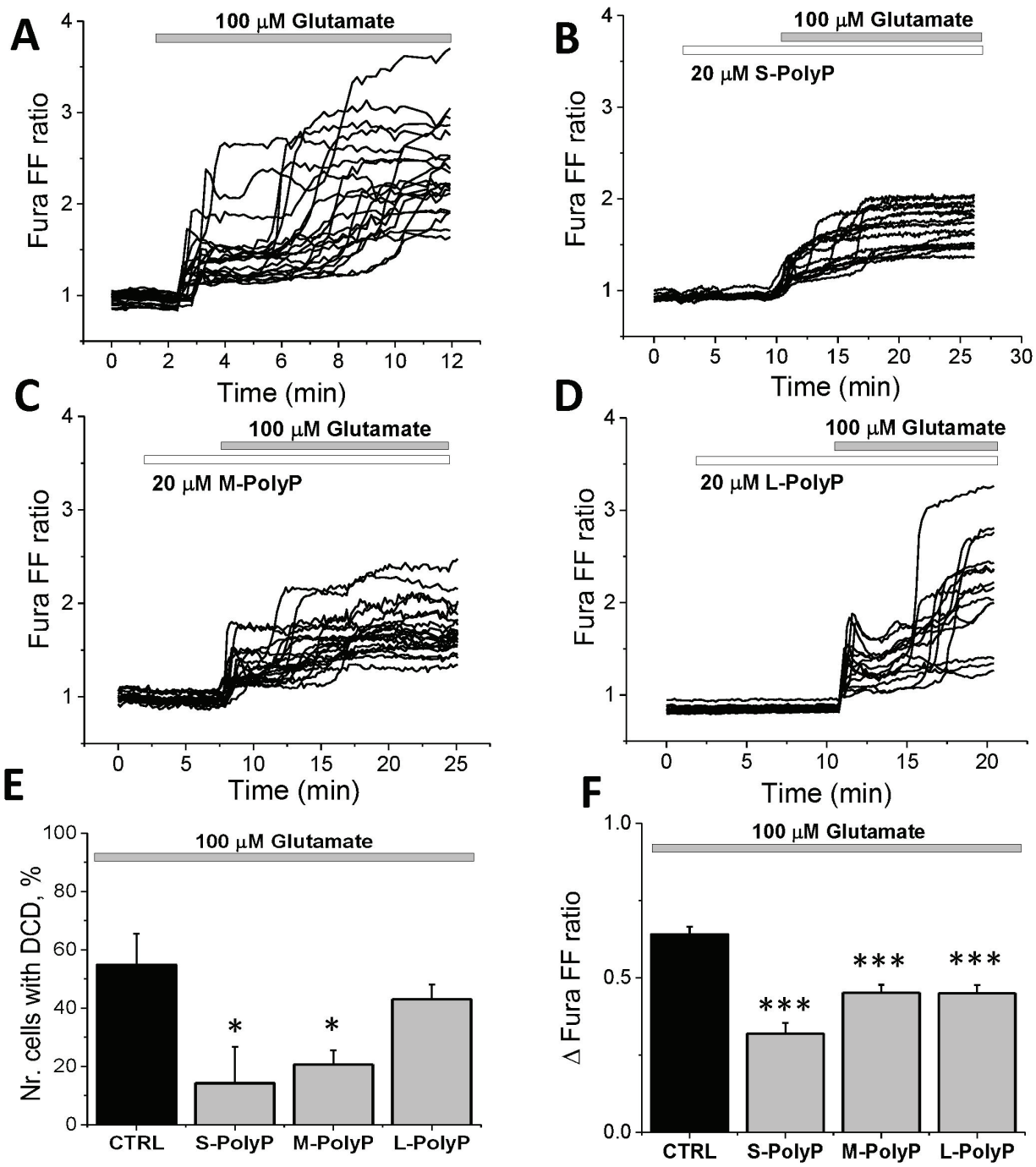


Figure 1



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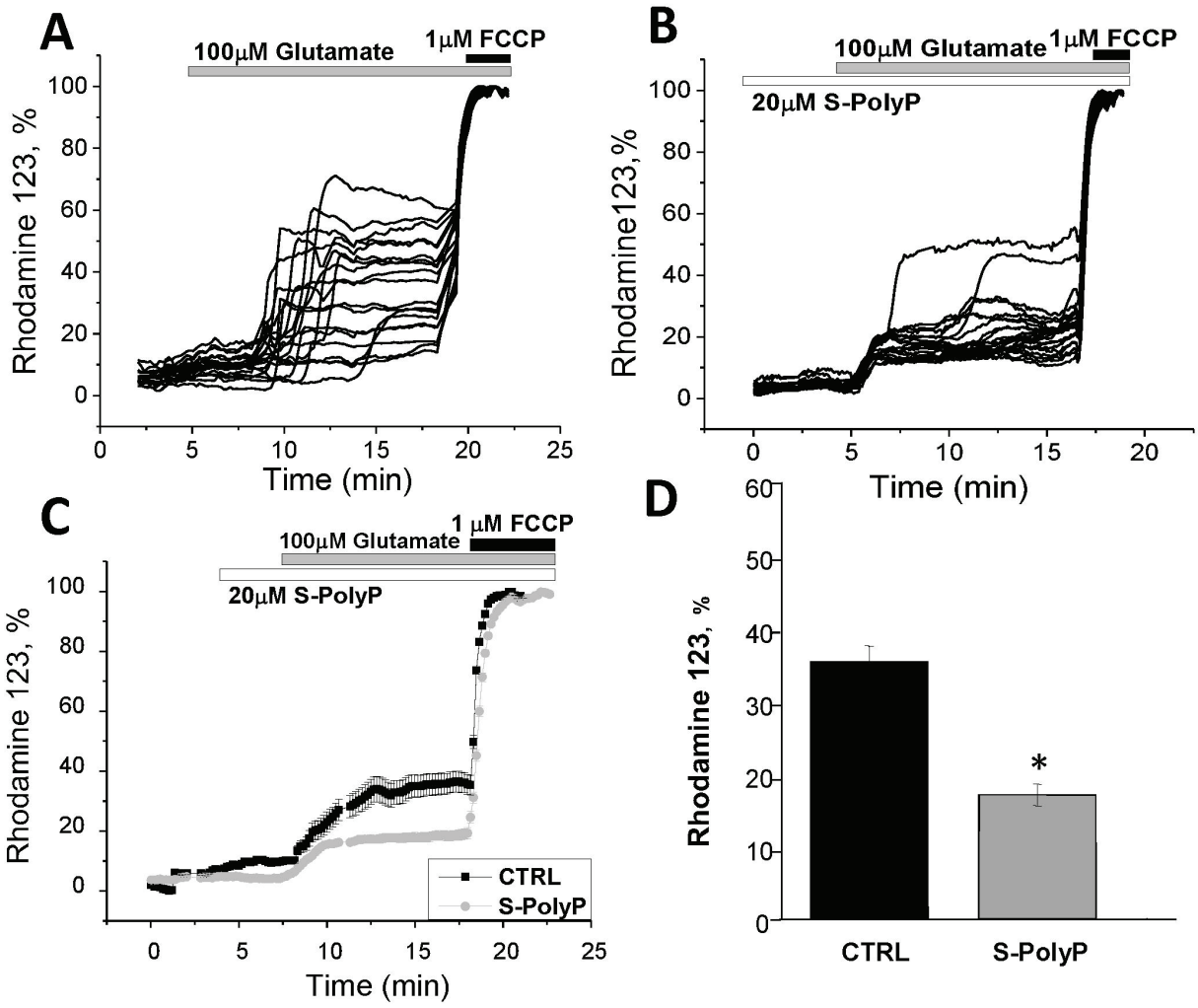

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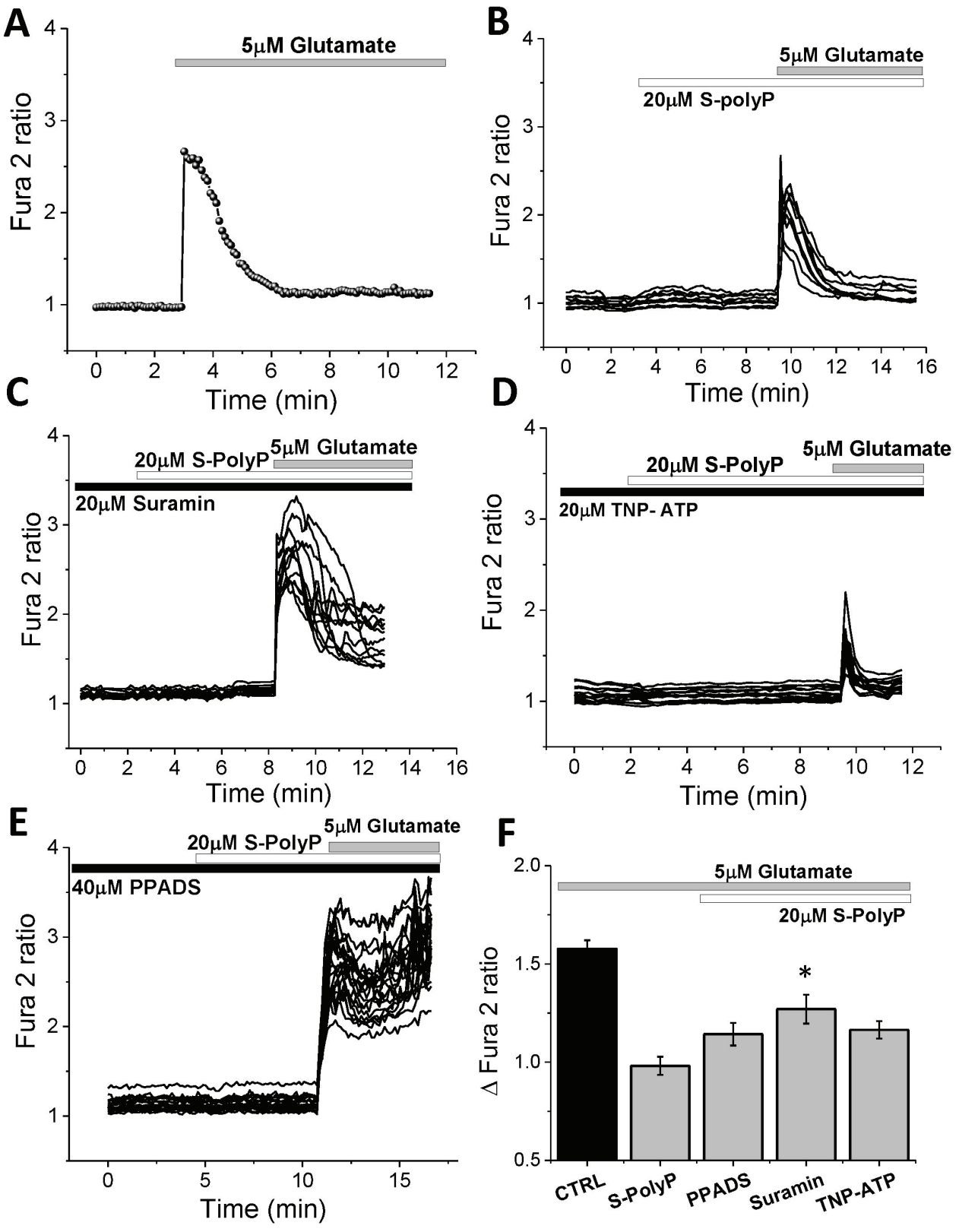

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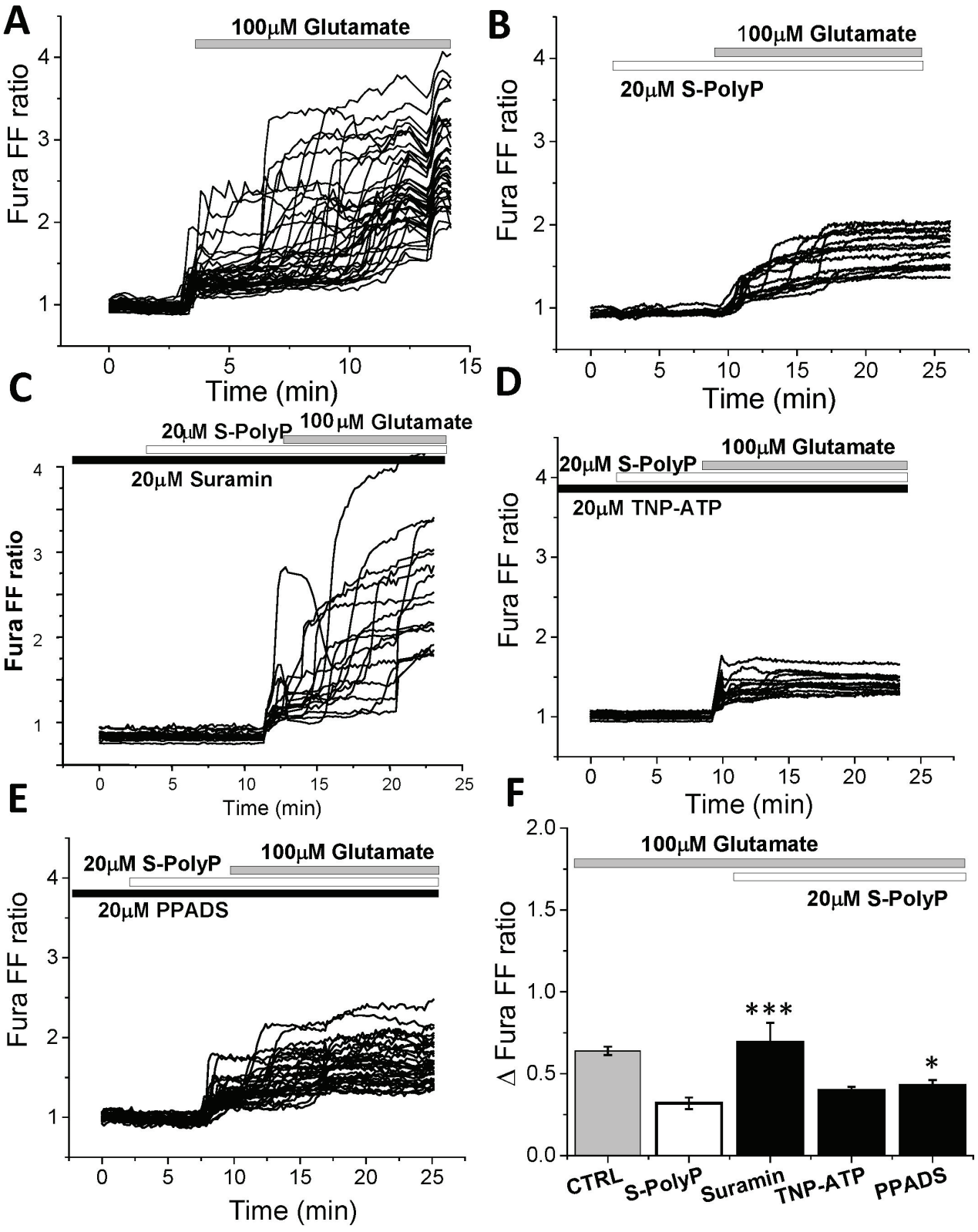

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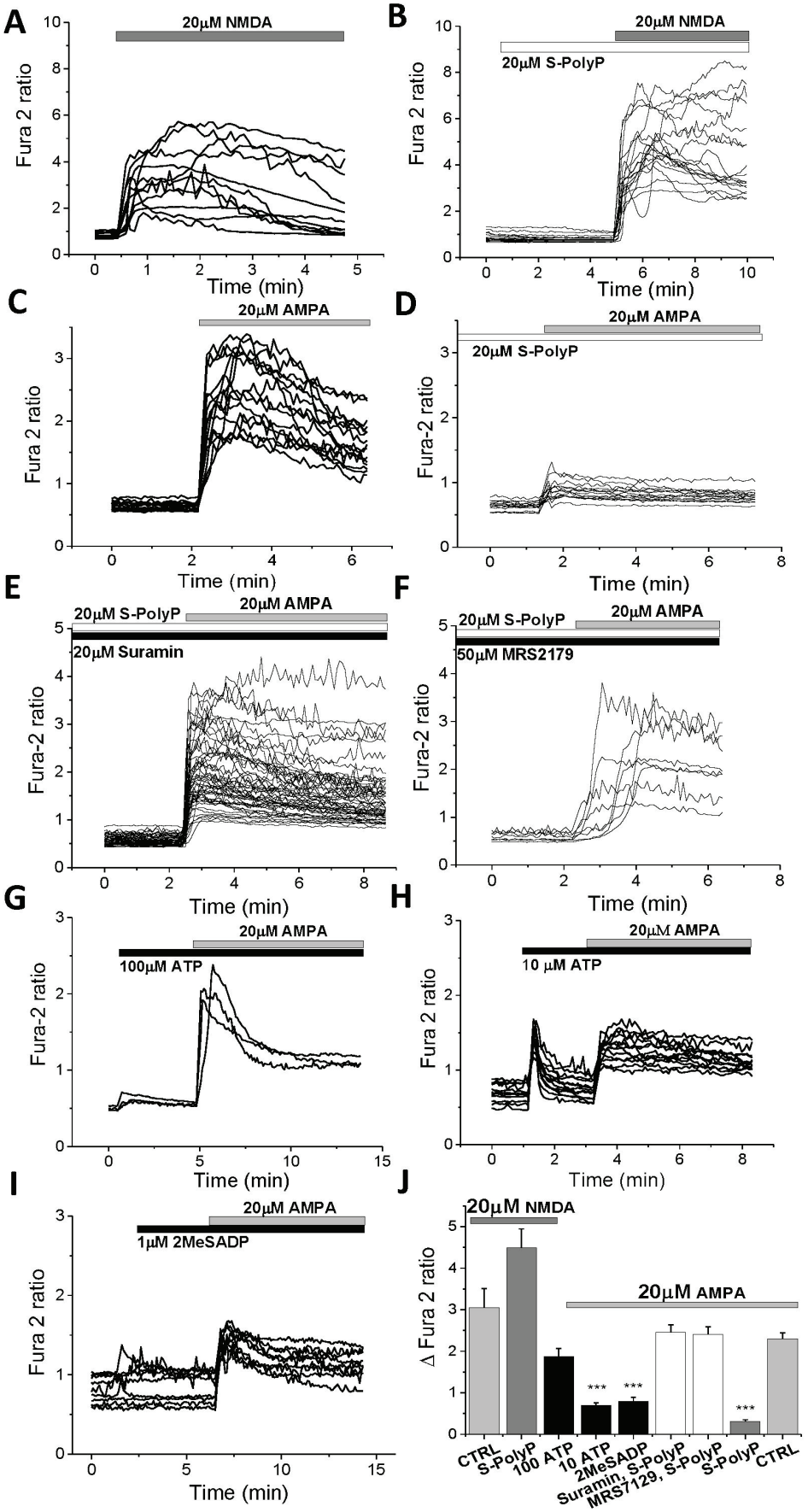

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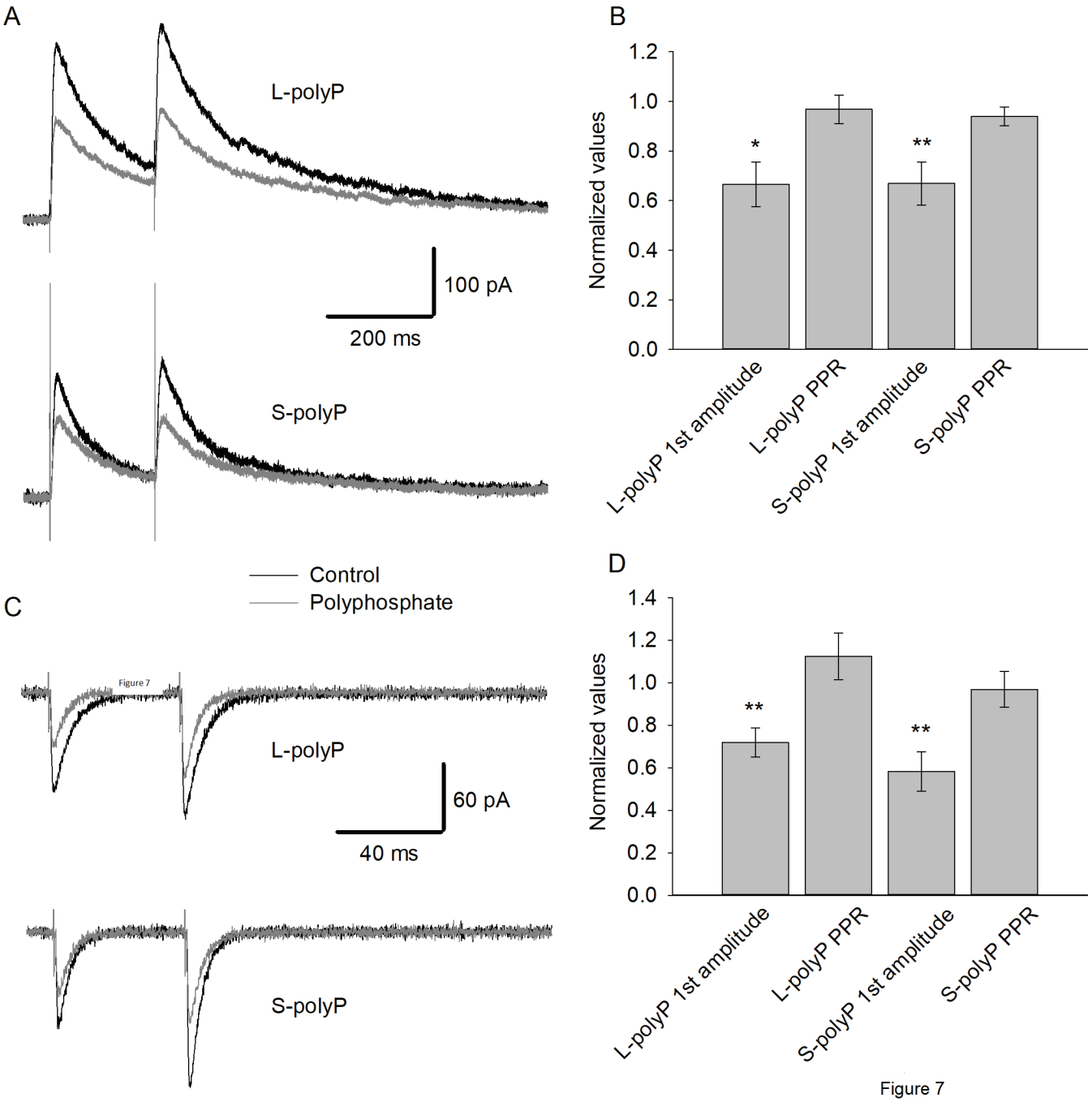

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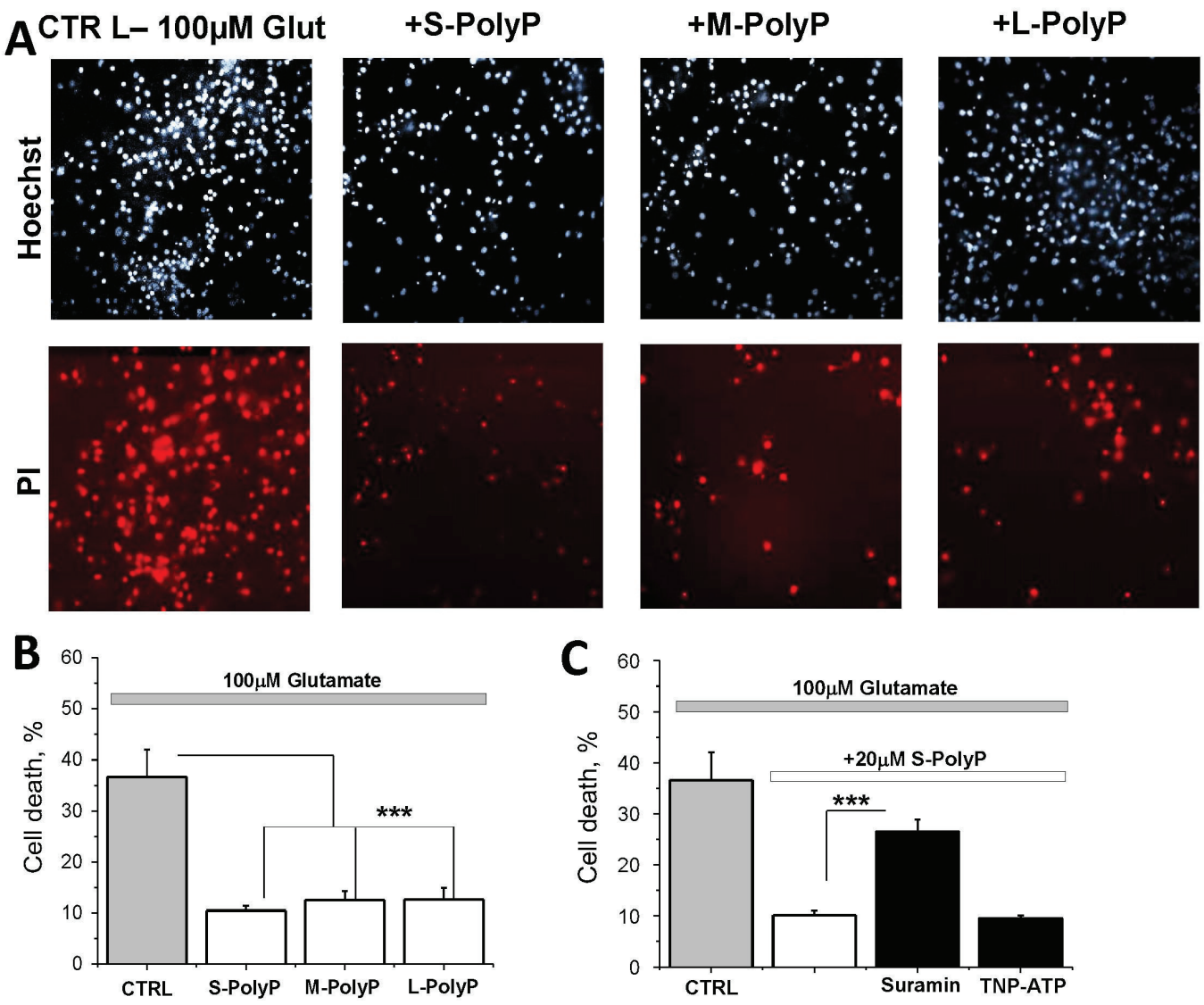

Figure 8