Contribution of extrasynaptic \( \text{N}-\text{methyl-D-aspartate} \) and adenosine A1 receptors in the generation of dendritic glutamate-mediated plateau potentials

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Thin basal dendrites can strongly influence neuronal output via generation of dendritic spikes. It was recently postulated that glial processes actively support dendritic spikes by either ceasing glutamate uptake or by actively releasing glutamate and adenosine triphosphate (ATP). We used calcium imaging to study the role of NR2C/D-containing \( \text{N}-\text{methyl-D-aspartate} \) (NMDA) receptors and adenosine A1 receptors in the generation of dendritic NMDA spikes and plateau potentials in basal dendrites of layer 5 pyramidal neurons in the mouse prefrontal cortex. We found that NR2C/D receptor subunits contribute to the amplitude of synaptically evoked NMDA spikes. Dendritic calcium signals associated with glutamate-evoked dendritic plateau potentials were significantly shortened upon application of the NR2C/D receptor antagonist PPDA, suggesting that NR2C/D receptors prolong the duration of calcium influx during dendritic spiking. In contrast to NR2C/D receptors, adenosine A1 receptors act to abbreviate dendritic and somatic signals via the activation of dendritic \( \text{K}^+ \) current. This current is characterized as a slow-activating outward-rectifying voltage- and adenosine-gated current, insensitive to 4-aminopyridine but sensitive to TEA. Our data support the hypothesis that the release of glutamate and ATP from neurons or glia contribute to initiation, maintenance and termination of local dendritic glutamate-mediated regenerative potentials.

1. Introduction

Identifying the mechanisms of dendritic integration is necessary for understanding the function of the brain in health and disease. When adequately clustered in time and space, afferent glutamatergic inputs have the ability to give rise to long-lasting and high-amplitude regenerative dendritic plateau potentials [1]. Regenerative dendritic spikes may amplify synaptic inputs and influence action potential (AP) initiation [2]. More importantly, the existence of multiple locations within thin dendrites, where these regenerative events can take place, enhances the computational power of an individual neuron, because each dendrite or specialized dendritic segment can serve as an autonomous decision-making computational unit [3,4]. Two classes of dendritic regenerative potentials, \( \text{N}-\text{methyl-D-aspartate} \) (NMDA) spikes and plateau potentials, exhibit distinct electrical (somatic) and calcium (dendritic) waveforms [5]. NMDA spikes are predominantly subthreshold events at the cell body, while dendritic plateau potentials reach much larger depolarization amplitudes and drastically longer durations. Electrical recordings from the cell body reveal that NMDA spikes are pointy and brief transients (half-width approx. 50 ms) [6], while plateau potentials can last for several hundred milliseconds [7]. Dendritic calcium imaging revealed that NMDA spikes are coupled with fleeting calcium transients highly localized at the synaptic input site [8], while dendritic plateau potentials are accompanied by strong calcium influx that spreads along the entire dendritic branch [9].
Studies of the ionic basis of dendritic spikes suggest that several receptor channels in the postsynaptic membrane contribute to the depolarizing charge [8,10,11]. Receptor channels located outside the synaptic cleft may also contribute charge to dendritic spikes [5,12–14]. Besides the afferent glutamatergic axons, glial processes may also release glutamate that contributes to the gluta-
mate build up (glutamate threshold) required to bring a dendrite into an UP state, which in turn brings the cell body into an UP state [1,15]. The current knowledge about specific neuronal and glial membrane conductances involved in the generation and maintenance of long-lasting dendritic plateau potentials is incomplete. The biophysical mechanisms recruited by the activation of extrasynaptic glutamate receptors are poorly understood due to the technical limitations that preclude physi-
ological measurements in thin dendrites and the lack of drugs specific for extrasynaptic versus synaptic receptor channels. We performed calcium-sensitive dye imaging, synaptic stimulation, glutamate iontophoresis, and used selective receptor subunit antagonists to investigate the membrane conductances underlying glutamate-mediated dendritic regenerative events.

This study is based on the hypothesis that both dendritic events, NMDA spikes and glutamate-mediated plateau poten-
tials, are driven by five critical processes: (i) synchronous activation of spatially segregated presynaptic glutamatergic terminals [16], (ii) failure of nearby glial processes to clear the bulk of extracellular glutamate [5,12,13], (iii) stimulation of extrasynaptic NR2C/D receptors on spine necks and dendritic shafts by spilled-over glutamate [12,13,17,18], (iv) glutamate-
dependent release of adenosine from either neurons or glia, or both [19,20], and (v) activation of adenosine-sensitive dendritic K⁺ current, which imposes negative feedback on the amplitude and duration of dendritic depolarizations. The aforementioned five processes (i–v) synergistically contribute to the generation of local dendritic regenerative events.

2. Material and methods

(a) Brain slice and electrophysiology

Swiss Webster mice (P21–P36) were anaesthetized with iso-
flurane inhalation, decapitated and brains were extracted with the head immersed in ice-cold, artificial cerebrospinal fluid (ACSF), according to an animal protocol approved by the Center for Laboratory Animal Care, University of Connecticut. ACSF contained (in mM) 125 NaCl, 26 NaHCO₃, 10 glucose, 2.3 KCl, 1.26 KH₂PO₄, 2 CaCl₂ and 1 MgSO₄, pH 7.4. Coronal slices (300 μm) were cut from frontal lobes. All experimental measurements were performed at 32–34°C. Whole-cell recordings were made from visually identified layer 5 (L5) pyramidal neurons within the ventral medial prefrontal cortex (PFC), including prelimbic and infralimbic cortex. Intracellular solution contained (in mM) 135 K-glucosе, 2 MgCl₂, 3 Na₂-ATP, 10 Na₂-
phosphocreatine, 0.3 Na₂-GTP and 10 Hepes (pH 7.3). Electrical signals were amplified with Multiclamp 700B and digitized with two input boards: (i) Digidata Series 1322A ( Molecular Devices, Union City, CA, USA) and (ii) Neuroplex (RedShirtImaging, Decatur, GA, USA). Only cells with a membrane potential more negative than −50 mV (not corrected for junction potential), and AP amplitudes exceeding 70 mV (measured from the baseline) were included in this study. Calcium-sensitive dye Oregon Green BAPTA-1 (OGB1) and fluorescent dye Alexa Fluor 594 (AF594) were purchased from Invitrogen, Carlsbad, CA, USA, and dissolved in intracellular solution, at concentration of 150 and 50 μM, respectively. Neurons were filled through whole-cell patching pipettes 30–40 min before optical recordings.

(b) Dendritic voltage and calcium imaging

Multi-site dendritic imaging was performed on an Olympus BX51WI microscope, equipped with 40× objective, two camera ports and a metal halide lamp (Lumen 200, Prior Scientific) for epi-illumination. Functional dendritic imaging was performed with a NeuroCCD camera (80 × 80 pixels, RedShirtImaging, LLC, Decatur, GA, USA). Calcium-dye signals were sampled at 40–125 Hz full frame rate. Neutral density filters were used to reduce the intensity of epi-illumination light during positioning and focusing. Optical filters were purchased from Chroma Tech-
nology (Rockingham, VT, USA) and Omega Optical (Brattleboro, VT, USA). Filters for AF594 were a Chroma exciter HQ380/20× (570–590 nm bandpass), dichroic Q595LP and emitter HQ600/60 m (600–660 nm bandpass). The filter cube for OGB1 con-
tained an Omega exciter 500AF25, dichroic 525DRLP and emitter 530ALP. Optical signal amplitudes are expressed as ΔF/F, where F represents the resting light intensity at the begin-
ing of the optical trace, and ΔF represents the change in peak fluorescence during the biological signal.

(c) Synaptic stimulation and glutamate microiontophoresis

Stimulation electrodes were pulled from borosilicate glass with filament (1.5 mm outer diameter; 0.8 inner diameter). Synaptic stimulation pipettes (7 MΩ) were filled with regular ACSF and glutamate stimulation pipettes (40–50 MΩ) were filled with 200 mM Na-glutamate (pH = 9). A programmable stimulator, Master-8, and a stimulus isolation unit, IsoFlex (A.M.P.L., Jerusa-
lem, Israel), were used to generate current pulses for both synaptic stimulation and glutamate iontophoresis. Following the spread of fluorescent dyes into the dendrites, the stimulation pipettes were positioned in mid-regions of basal branches, between 65 and 130 μm from the cell body. Navigation of the flexible glass pipettes through the slice tissue was achieved with the aid of a ‘fourth axis’ (concomitant engagement of both X- and Z-axes), available on Sutter Instruments M-285 motORIZED micromanipulator. The intensities of current pulses for synaptic stimulation (two pulses, inter-stimulus interval 20 ms, pulse duration 100 μs, current intensity 50–200 nA), or of single glutamate microiontophoresis puffs (duration 5 ms, intensity 10–25 nA), were adjusted to produce a long-lasting somatic depolarization (half-width 200–500 ms) with or without the addition of sodium APs atop the plateau depolarization. The reported stimulation current intensities are nominal values obtained from the IsoFlex settings.

(d) Drugs

Stock solutions of ifenprodil (Tocris Bioscience), (2S*,3R*)-1-
(phenanthrene-2-carboxylic acid (PPDA; Tocris) and 8-cyclopentyl-1,3-dimethylxanthine (CPT: Sigma-Aldrich) were dissolved in dimethylsulfoxide. Adenosine (Sigma), 4-aminopyridine (4-AP; Tocris) and tetraethylammonium chloride (TEA; Tocris) and theophylline (Sigma) were dissolved in water. Final concentrations in the bath: 4-AP = 2 mM, TEA = 2 mM and theophylline = 1 mM. PPDA was used at two concentra-
tions: 1 μM and 2 μM. Adenosine (40 mM) was loaded into sharp micropipettes (50–60 MΩ). Brief pulses of adenosine (duration 8 ms or 500 ms) were iontophoretically ejected onto the cell body from a distance 10–20 μm away from the cell body.

(e) Data analysis

Analysis of optical data, including spatial averaging, high-pass and low-pass filtering, was conducted with Neuroplex v. 8.0.0 (RedShirtImaging). For numerical analysis, calcium signals were selected from dendritic segments closest to the glutamate stimulation site. To process off-line calcium-imaging data, we
applied a Butterworth high-pass filter at 0.1 Hz cut-off and a Gaussian low-pass filter at 30 Hz cut-off. Electrical recordings were analysed in Clampfit v. 10.1 (Molecular Devices, Sunnyvale, CA, USA). Plateau duration was measured at half amplitude of the slow component of somatic depolarization. In some experiments, we used glutamate microiontophoresis pulses to evoke three successive plateau depolarizations in the same recording sweep. Although the amplitude and durations of three successive plateau events are stable, in the numerical analysis we normalize the first, second and third plateau potential in drug condition using control values for the first, second or third plateau potential in the control condition, respectively. This procedure eliminates the possibility that, for example, the third plateau in drug is compared with the first plateau in control. Statistical tests were performed using Excel (Microsoft). All statistics were done on data points after normalization unless otherwise specified. Paired Student’s t-tests were used for comparing data obtained from the same neuron (in two different conditions). Unpaired Student’s t-tests were used for data obtained from different neurons. Significance was set as p-value < 0.05 (one asterisk), and high significance as p < 0.01 (two asterisks). Values are presented as mean ± s.e.m.

3. Results

(a) N-methyl-D-aspartate spikes: NR2C/D receptors

To test if NR2C and NR2D (NMDA receptor subunits) receptors contribute to voltage and calcium waveforms of dendritic NMDA spikes, L5 pyramidal neurons were filled with Ca2+-sensitive dye, OGB1 (figure 1a), and two synaptic shocks were delivered in the vicinity of a basal dendrite [6,13]. The mean peak amplitude of the NMDA spike in somatic recordings (figure 1b, patch, NMDA spike) was 11.5 ± 0.6 mV (n = 51 measurements in eight cells). The mean peak amplitude of the dendritic Ca2+ signal at the synaptic stimulation site (figure 1b, patch, Ca2+ region of interest—ROI) was 11.2 ± 0.7% (∆F/F), in the same sample of eight cells. Dendritic Ca2+ signals were tightly restricted to the synaptic stimulation site as previously shown [5,8]. The synthetically evoked dendritic NMDA spikes were challenged with a potent antagonist of NR2C and D subunits, PPDA (2 μM). The drug perfusion phase lasted 15–20 min (figure 1b, black horizontal bar). As the drug entered the recording chamber, dual electrical and optical recordings were made every 1–3 min. This allowed us to divide the drug perfusion phase into three time periods (a, b and c). In the first time period, marked as period ‘a’, 2–5 min following the start of the drug perfusion, the peak amplitude of the somatic voltage transient was reduced to 78 ± 4% compared with the control amplitudes established before drug application of 100 ± 1% (eight cells). In the second and third time periods, ‘b’ and ‘c’, the voltage transients were reduced to 69 ± 4% and 60 ± 3%, respectively (figure 1d, Soma-Vm, n = 8 cells). Similar amplitude reductions affected the accompanying Ca2+ transients in the basal dendrite, 80 ± 11%, 78 ± 9% and 45 ± 6%, in time periods ‘a’, ‘b’ and ‘c’, respectively (figure 1d, Dend.-Ca2+). In the period of time when PPDA first enters the recording chamber (period ‘a’), the amplitude of the dendritic NMDA spike was significantly reduced (figure 1c) in all neurons tested (n = 8). Given the sixfold greater sensitivity of NR2CD over NR2AB subunits to PPDA [21], these data suggest that NR2CD subunits are activated and contribute to the voltage and calcium waveforms of dendritic NMDA spikes.

(b) Plateau potentials: NR2C/D receptors

To test if NR2C and NR2D receptors contribute to voltage and calcium waveforms of dendritic plateau potentials, L5 pyramidal neurons were filled with OGB1 (figure 1e) and brief (duration 5 ms) iontophoretic pulses of glutamate were delivered in the vicinity of a basal dendrite. Each experimental sweep consisted of three glutamate pulses separated by a 2.5 s interval. To prevent AP firing during glutamatergic stimulations, neurons were briefly hyperpolarized by negative current injection (duration 11 s) to bring the resting membrane potential into the range between −70 and −80 mV. Glutamate-evoked plateau potentials were recorded electrically at the cell body (figure 1f) and optically in the dendrite (figure 1g). The mean peak amplitude of the glutamate-evoked plateau in somatic recordings (figure 1f, patch soma) was 14.4 ± 1.3 mV (n = 15 control measurements for four cells) for the first plateau in the sequence. The mean amplitudes of the second and third plateau in the train were 13.2 ± 1.3 mV and 12.8 ± 1.3 mV, respectively. The mean durations (half-width) of the first, second and third plateau were 413 ± 26 ms, 415 ± 21 ms, and 429 ± 20 ms, respectively (n = 15 control measurements obtained in four cells). Unlike dendritic NMDA spikes, dendritic plateau potentials propagate from the site of initiation (figure 1e, glut.) in two directions, towards the cell body and towards the distal dendritic tip (ROI 6), causing influx of Ca2+ throughout the entire dendritic branch [9]. At the site of glutamate input (ROIs 2 and 3), the calcium influx is dominated by NMDA receptors, while distally (ROI 6) and proximally from the input site (not shown) the Ca2+ signals are mediated by dendritic voltage-gated Ca2+ channels [9]. Glutamate-evoked dendritic plateau potentials were challenged by 1 μM PPDA. As the drug entered the recording chamber, dual electrical and optical recordings were made at 1–3 min intervals. This allowed us to divide the drug perfusion phase into three time periods (a, b and c). In the first time period, marked as period ‘a’, 2–5 min following the start of the drug perfusion, the peak amplitude of the somatic voltage transient was reduced to 92 ± 1% (n = 48 plateaus in four cells) compared with the control amplitudes established before drug application of 100.0 ± 1% (n = 39 plateaus in four cells). In the second and third time periods, ‘b’ and ‘c’, the voltage transients were reduced to 82 ± 2% (n = 45 plateaus in four cells) and 84 ± 2% (n = 30 plateaus in four cells), respectively (figure 1h). In the same sample as above (30–48 recordings in four cells), the mean relative amplitudes of the accompanying Ca2+ transients (measured at the glutamate stimulation site) in time periods ‘a’, ‘b’ and ‘c’ were 110 ± 3%, 94 ± 3% and 80 ± 8%, respectively (figure 1h, Dend.-Ca2+). Bath application of 1 μM PPDA reduced the durations of dendritic Ca2+ transients in time periods ‘a’, ‘b’ and ‘c’ to 88 ± 2%, 70 ± 2%, and 75 ± 3%, respectively.

Bath application of 2 μM PPDA for 10–15 min reduced the amplitude of dendritic Ca2+ transients to 71 ± 3% (n = 29 measurements in six cells) compared with control recordings of 100% ± 1% obtained before application of drug (n = 39 measurements in six cells). The duration of the dendritic Ca2+ signal was reduced to 54 ± 3%. During bath application of 2 μM PPDA for 10–15 min, the somatic voltage transients were reduced in amplitude down to 75 ± 3% and duration down to 62 ± 4% (figure 1h, Soma-Vm). Bath application of a mixture of non-selective NMDAR
antagonists, 50 μM APV and 10 μM MK801, was performed on a sample of six cells using whole-cell recordings only. During the drug application phase, the amplitude of the somatic plateau depolarization was reduced to 54 ± 3% and duration to 89 ± 4% compared with control recordings made in the same neurons before drug application (n = 18 recordings obtained in six cells). The reductions in voltage and Ca\(^{2+}\) signals obtained with 1 μM PPDA at 10–15 min (period ‘c’) were less than with 2 μM PPDA (10–15 min) (figure 1h) or after using the mixture of 50 μM APV and 10 μM MK801 (figure 1h). In the period of time when 1 μM PPDA first enters the recording chamber (period ‘a’), and the concentration of PPDA is less than 1 μM, the duration of the dendritic calcium plateau was reduced (figure 1h). Given the sixfold greater sensitivity of NR2C/D over NR2AB subunits to PPDA [21], these data suggest that NR2CD subunits are activated and contribute to the calcium waveforms of dendritic plateau potentials.
and 108 recordings in the drug condition, obtained in nine cells. (1) TTX, tetrodotoxin. (2) Application of CPT, were normalized, averaged and displayed in the bar diagram. Basal dendrites. Neurons were briefly hyperpolarized to prevent AP firing. The amplitudes and durations of voltage waveforms obtained before (control) and after 28 recordings in drug. Electrical signals (Vm):

\[ V_m = \frac{A_{\text{dendritic plateau}}}{V_m} \]

The mean amplitude (figure 2b, Ca^2+ Amp) and duration (Ca^2+ Dur) of the dendritic calcium signal, and the mean duration of the somatic plateau depolarization (figure 2b, Vm Dur) were significantly greater in the presence of CPT than in the controls (\( p < 0.01, n = 81 \) control and 108 drug-treatment traces obtained from nine cells). Given that the duration of calcium signal depends on the duration of the dendritic plateau potential [28] and that the duration of dendritic plateau potential is faithfully represented in the somatic depolarization envelope [9], we tested if CPT had changed the relationship between electrical and calcium signal durations. The duration ratio between dendritic calcium and somatic voltage signals was significantly greater in CPT compared with control recordings (figure 2b, Ca/Vm).

(c) Adenosine A1 receptors

Adenosine is found in synaptic vesicles together with adenosine triphosphate (ATP) and neurotransmitters, and it can be co-released from neurons and glia during intense network activity [19, 20, 22]. The major effect of adenosine is an inhibition of neuronal activity mediated via A1 receptors [20, 23–27]. In the following experiments, we tested if adenosine A1 receptors are activated during glutamate-induced dendritic plateau depolarizations. Glutamate pulses (5 ms) were delivered on mid-segments of basal dendrites to produce dendritic plateau potentials [7] manifested by sustained somatic depolarizations and dendritic calcium plateaus (figure 2a, ROI 2). Bath application of adenosine A1 receptor antagonist CPT (10–20 \( \mu \)M) caused a significant broadening of dendritic calcium transients at the glutamate stimulation site (figure 2a, ROI 2, compare grey and black trace). The mean amplitude (figure 2b, Ca^2+ Amp) and duration (Ca^2+ Dur) of the dendritic calcium signal, and the mean duration of the somatic plateau depolarization (figure 2b, Vm Dur) were significantly greater in the presence of CPT than in the controls (\( p < 0.01, n = 81 \) control and 108 drug-treatment traces obtained from nine cells). Given that the duration of calcium signal depends on the duration of the dendritic plateau potential [28] and that the duration of dendritic plateau potential is faithfully represented in the somatic depolarization envelope [9], we tested if CPT had changed the relationship between electrical and calcium signal durations. The duration ratio between dendritic calcium and somatic voltage signals was significantly greater in CPT compared with control recordings (figure 2b, Ca/Vm).

In the next series of experiments, we studied somatic voltage waveforms only (no Ca^2+ imaging). The Ca^2+-sensitive dye, OGB1, was omitted from the intracellular solution and instead of TTX (figure 2a) we used hyperpolarizing pulses to block AP firing (figure 2c). Under these conditions, CPT caused a decrease in somatic voltage amplitude of 15.5 ± 2%, and an increase in somatic voltage duration of 28.7 ± 1% (\( n = 72 \) recordings in control, \( n = 96 \) recordings in drug, obtained in three cells, figure 2c, bar diagram).

Next, we replaced CPT with another adenosine receptor antagonist, theophylline, which is less potent and less selective for A1 receptors than CPT. Using the same experimental set-up as in figure 2a, bath application of theophylline (1 mM) caused an increase in dendritic Ca^2+ amplitude and duration of 30.4 ± 5% and 25.2 ± 3%, respectively (\( n = 24 \) recordings in control, \( n = 28 \) recordings in drug, obtained in four cells). Bath application of theophylline (1 mM) caused a decrease in somatic voltage amplitude of 6.7 ± 2% and increase in duration of 3.7 ± 1% (figure 2d). Taken together, these data suggest that activation of adenosine A1 receptors causes shortening of voltage and calcium waveforms that underlie glutamate-mediated dendritic plateau potentials.

(d) Adenosine-induced current

In the next series of experiments, we tested the effects of local application of adenosine in PFC L5 pyramidal neurons. Brief pulses of adenosine (duration 8 ms) were iontophoretically ejected from sharp micropipettes onto the cell body (figure 3a, (c) Adenosine-induced current)....
Figure 3. Adenosine-activated voltage-activated current. (a) Adenosine (Ado) microiontophoresis (pulse duration = 8 ms) delivered on the cell body caused a small and transient hyperpolarization in each neuron tested (n = 10). An average of nine sweeps obtained from the same neuron are shown below a single sweep from that set. Bottom trace: vertical tic marks the timing of the Ado pulse. (b) Neuron filled with QX314 and bathed in TTX. A series of voltage commands (−110 to +20 mV) produced outward current only when paired with the release of Ado (Ado iontophoresis). (c) TTX (1 μM) is in the patch pipette. Timing of the voltage commands (voltage, −110 to +20 mV) and timing of Ado iontophoresis are both shown by dashed lines. The corresponding current traces are displayed above. One current trace (trace Q, obtained at −90 mV) is copied and shown separately. At −90 mV voltage command, the Ado iontophoresis failed to generate an outward current (trace Q). A family of current traces obtained in the absence of Ado (not shown) was subtracted from the family of traces obtained with Ado (shown) and used to create an I−V plot (inset). The I−V plot is an average of 5 I−V curves obtained in five neurons. (d) TTX (1 μM) is in the pipette. Grey thick line represents a current trace obtained in response to a voltage step from −70 to +20 mV. During the steady state of the voltage-activated current (I_{VD}), the Ado iontophoresis (I_{Ado}) was used to generate an additional outward current, termed Ado-activated current (I_{Ado}). The amplitudes of I_{VD} and I_{Ado} are measured against the baseline-1 (bl-1) and baseline-2 (bl-2), respectively. The I_{Ado} double arrowhead does not accurately reflect the 400 ms time point used for the quantification of the I_{Ado}. Bath application of TEA induces a significant change in the amplitude of I_{Ado} (n = 4). A bath application of 4-AP (n = 6) induced a significant change in the amplitude of I_{Ado}. *p < 0.05, **p < 0.01.

cartoon). At resting membrane potential (range −59 to −66 mV), the focal application of adenosine caused a small hyperpolarization of the cell body (figure 3a) with a mean amplitude of 3.4 ± 0.3 mV and half-width of 137 ± 29 ms (n = 10). This corroborates the previous report that L3–L6 prefrontal cortical pyramidal neurons hyperpolarize between 1.2 and 10.8 mV following adenosine application [29]. In voltage clamp experiments, the adenosine-activated current exhibited very slow activation dynamics and strong dependence on voltage (figure 3b, with Ado). At −90 mV voltage command, the adenosine application failed to trigger any detectable current in all neurons tested (figure 3c, trace Q). The adenosine-mediated current was only observed at depolarized voltages (figure 3b,c). In order to study the voltage dependence of the adenosine-activated neuronal current, the current traces were generated with and without adenosine application and then subtracted from each other. The I−V plots generated using such processed traces (the results of subtraction) revealed that the adenosine-activated outward current is outward-rectifying (figure 3c, inset, I−V plot). Our experimental protocol consisting of voltage commands and adenosine iontophoresis in the middle of the voltage step (figure 3c) was used to separately measure the voltage-dependent (I_{VD}) and adenosine-dependent neuronal currents (I_{Ado}). The amplitude of I_{VD} was determined by calculating the difference between the baseline-1 (figure 3d, bl-1) and the steady-state phase of the voltage-only-activated current (figure 3d, double arrowhead-I_{Ado}). Likewise, the amplitude of I_{Ado} was determined by calculating the difference between the baseline-2 (figure 3d, bl-2) and the adenosine-activated current at time point 400 ms after its onset (figure 3d, double arrowhead-I_{Ado}). In the next series of experiments, we explored the pharmacological properties of I_{VD} and I_{Ado}.

Bath application of a non-selective K+ channel antagonist, TEA (4 mM), had a little effect on the amplitude of I_{VD} (figure 3d, compare control and TEA trace in the first period of time, bl-1). While exerting only a modest effect on I_{VD}, TEA caused a relatively large reduction in I_{Ado} (figure 3d, compare control and TEA trace in the second...
period of time, bl-2). More specifically, in the presence of TEA, the amplitudes of \( I_{\text{VD}} \) and \( I_{\text{Ado}} \) were reduced to 80 ± 36% and 44 ± 12%, respectively \((n = 4)\). The TEA-induced change in \( I_{\text{Ado}} \) amplitude was statistically significant compared with control values obtained in the same neurons before application of TEA (paired \( t \)-test, \( p < 0.05 \), \( n = 4 \)). A 15 min wash of TEA with drug-free ACSF produced a partial recovery of both \( I_{\text{VD}} \) and \( I_{\text{Ado}} \) (figure 3d, TEA, wash). Experiments with another K⁺ channel antagonist, 4-AP, had an opposite effect on the two currents. Bath application of 4-AP (2 mM) caused a large reduction in the amplitude of \( I_{\text{VD}} \) and a modest reduction in the amplitude of \( I_{\text{Ado}} \). More specifically, in the presence of 4-AP, the amplitudes of \( I_{\text{VD}} \) and \( I_{\text{Ado}} \) were reduced to 30 ± 5% and 75 ± 11% \((n = 4)\) compared with control values before application of 4-AP. The change in \( I_{\text{VD}} \) amplitude was statistically significant (paired \( t \)-test, \( p < 0.01 \), \( n = 6 \)). A 15 min wash of 4-AP with drug-free ACSF produced a partial recovery of both \( I_{\text{VD}} \) and \( I_{\text{Ado}} \) (figure 3d, 4-AP, wash). Together, these experiments indicate that activation of adenosine receptors on L5 pyramidal cells in the mouse PFC facilitates the activation of a voltage-sensitive K⁺ current. This adenosine-activated voltage-activated current has slow dynamics of activation (figure 3c, With Ado) and exhibits outward rectification (figure 3c, I–V plot); it is sensitive to TEA, and insensitive to 4-AP (figure 3d).

4. Discussion

(a) NR2C/D receptors

NMDA receptors are of interest in several fields of neurobiology including development, synaptic plasticity, neurological disorders and brain injury. Here, we used an NR2C/D antagonist to probe the contribution of these receptor channels to glutamate-mediated dendritic plateau potentials. We were able to trigger synaptically evoked NMDA spikes [6] and glutamate-evoked plateau potentials [7] in basal dendrites of L5 pyramidal neurons in the mouse mPFC. The generation of both types of dendritic spikes involved the activation of NR2C/D receptors. NR2C/D-containing NMDA receptors have been shown to be preferably positioned at extrasynaptic and perisynaptic sites, outside the postsynaptic density, along the sides of dendritic spines and dendritic shafts, as well as in presynaptic terminals [30]. Extrasynaptic NMDA receptors are highly mobile and constantly in transit to and from synapses [31,32]. Glutamate can escape the restricted volume of the synaptic cleft and activate extrasynaptic NMDA receptors [17,33,34]. This scenario is more likely to occur during intense synaptic activity [35]. Two-photon calcium-imaging experiments revealed that during synaptically evoked NMDA spikes, glutamate diffuses from nearby axon terminals to activate extrasynaptic NMDA receptors on both spines and dendritic shafts [13]. Dendritic depolarization, the accompanying local calcium entry and neurotransmitter spillover during dendritic spikes, can activate a variety of receptors and channels located outside the narrow synaptic cleft [13,14,36].

To block NR2C/D receptors, we used PPDA (figure 1). Based on the glutamate binding assays at recombinant receptors expressed in cultured cells, the PPDA Ki values are 0.096, 0.125, 0.31 and 0.55 μM for NR2C, NR2D, NR2B and NR2A subunits, respectively [21]. It is likely that the PPDA concentrations needed to block NMDARs in neurons positioned 50 μm below the surface of a brain slice will be higher than those obtained in cultured cells. Using neurons in brain slices and 15–20 min bath application of 1 μM PPDA, we obtained approximately 15% reduction in plateau amplitude. With 2 μM PPDA, we obtained approximately 25% reduction in plateau amplitude. Non-selective antagonists (mixture of APV and MK801) produced a 43% reduction in signal amplitude (figure 1h, APV). In this study, the PPDA application phase was divided into three successive periods (a–c). In the first 2–5 min of 1 μM PPDA application, when the drug first entered the volume of the recording chamber (period ‘a’), the PPDA concentration is notably lower than 1 μM, because the drug-rich solution exiting the perfusion line is mixing with drug-free solution in the recording chamber. At low concentration, the PPDA is expected to block the most sensitive NR2C and NR2D receptors [21]. Interestingly, blocking the NR2C/D receptors in period ‘a’ exerted no change in Ca²⁺ plateau amplitude, but rather a change in Ca²⁺ plateau duration (figure 1h, Dend.-Ca²⁺, period ‘a’). The NR2C/D subunits (putative extrasynaptic receptors) have a much slower deactivation time than the NR2A subunits (putative synaptic NMDA receptors) [37]. In other words, the extrasynaptic NMDA receptors remain open for a longer period of time. This may explain the prolonged influx of Ca²⁺ observed during glutamate-mediated dendritic plateau potentials [9]. Our finding that blockage of NR2C/D receptors affects the duration but not the amplitude of dendritic Ca²⁺ signals (figure 1h, period ‘a’) is consistent with very long-lasting current but small peak amplitude of NR2C/D-mediated currents [37].

(b) Adenosine A1 receptor

(i) Source of adenosine

During dendritic UP states [1,15], glutamate spillover leads to the activation of metabotropic glutamate receptors on glial cells [38]. The ensuing intracellular accumulation of Ca²⁺ in glial processes causes a vesicular release of ATP in the extracellular milieu [39–41]. Extracellular ATP rapidly breaks down to adenosine via the help of membrane-bound ectonucleotidases [42]. Although ATP hydrolysis is considered to be the major source of adenosine build-up, adenosine can also be directly released from glial processes [43–45]. The release of ATP from neurons is more controversial [46].

(ii) Adenosine effects

ATP or adenosine is released in response to strong synaptic activity. Adenosine binds to A1 receptors, located in both presynaptic and postsynaptic neuronal membranes [47,48] and also in astrocytic membranes [49]. Activation of adenosine A1 receptors predominantly leads to inhibition of neuronal activity. The mechanism of the adenosine inhibitory action is either presynaptic, through blockade of voltage-gated Ca²⁺ channels (reduction of glutamate release), or postsynaptic, through activation of K⁺ channels [25,27,50–55]. The coupling of A1 adenosine receptors to Gi/Go proteins influences numerous pathways through which adenosine can modulate and control membrane excitability and synaptic transmission [20,24].

(iii) A1 antagonist, CPT

The inhibition caused by activation of adenosine A1 receptors can be blocked by application of a competitive antagonist, CPT,
which belongs to a family of xanthine chemicals, together with other stimulant drugs such as caffeine, theophylline and theobromine [56]. Application of CPT has previously been reported to antagonize the effects of adenosine and induce an increase in neuronal activity by potentiating glutamatergic evoked excitatory postsynaptic currents after train stimulation [57,58]. In this study, the effects of CPT on the presynaptic regulation of glutamate release were circumvented by microiontophoresis of glutamate. Our data revealed that durations (half-widths) of both voltage and calcium transients were significantly enlarged during bath perfusion with CPT or theophylline (figure 2). This means that during intense excitatory glutamatergic barrages, a release of adenosine from neurons and/or glia would assist in shortening the duration of glutamate-mediated plateau potentials via the opening of K⁺ channels [27,59–61]. The release of adenosine from neurons or glial cells can act as a protective mechanism against glutamate overstimulation in two ways: (i) by inhibiting glutamate release from presynaptic terminals and (ii) by downregulating the synaptic activity level during ischaemic-like conditions [62–65] or during an UP state in slow wave sleep [22,66,67]. It was shown that extracellular concentration of adenosine tends to escalate during seizures and after stroke, possibly as a homeostatic feedback mechanism for preventing tissue damage and by acting as an endogenous anticonvulsant [68–70]. Another important link between glutamate-mediated sustained depolarizations and adenosine is the sleep–wake cycle. In the cerebral cortex, levels of adenosine increase during wakefulness and during sleep deprivation [22,71]. Blocking the A1 receptor or blockade of the astrocytic release of ATP prevents cognitive deficits resulting from sleep deprivation [67].

(iv) Adenosine current
The adenosine-activated voltage-sensitive dendritic K⁺ channel involved in the shortening of dendritic plateau potentials (figure 2) fits a profile of an outward rectifier K⁺ channel (figure 3), which is normally sensitive to the quaternary ammonium pore blocker TEA on the extracellular side, but peculiarly insensitive to 4-AP, a drug that blocks transient (inactivating) dendritic K⁺ channels [72]. Previous reports have identified a K⁺ current, with the same aforesaid pharmacological profile, that resembles that of the slow-activating (delayed) rectifier channel, which was sensitive to TEA and insensitive to 4-AP. The K⁺ channels with such pharmacological and biophysical profile are likely to contain Kv2 subunits [73]. We found that the more the cell becomes depolarized, the larger the K⁺ current becomes in the presence of adenosine (figure 3b,c). During the plateau phase of dendritic plateau potential, the dendritic membrane is depolarized by approximately 40 mV, while the cell body is experiencing only a 20 mV depolarization [7]. This voltage difference between dendrite and soma will cause a differential activation of I_{Ado} in the active (glutamate-receiving) dendrite versus inactive dendrite or soma. The amplitude of I_{Ado} in the active dendritic segment will be significantly greater than in any other part of the same neuron, which creates the basis for spatially restricted purinergic modulation of dendritic excitability, similar to the spatially restricted dopaminergic modulation of dendritic excitability [74]. In other words, a deluge of glutamate engulfing only one dendritic segment [1,5,16] would not generate inhibitory adenosine transients competent to alter the physiology of other dendrites belonging to the same cell.

(v) Purinergic modulation of dendritic excitability: spatial and temporal aspects
The local activation of dendritic K⁺ current (I_{Ado}) can assure that strongly activated dendrites will enter a period of reduced excitability (refractory state), while at the same time, dendrites not experiencing strong glutamatergic input will have a preserved excitability and preserved ability to respond to small synaptic inputs. The proposed A1 receptor-mediated purinergic modulation of dendritic excitability depends on the coincidence of adenosine release and membrane depolarization (figure 3b). As strong glutamatergic inputs trigger (i) dendritic UP states [1,15] and (ii) ATP/adenosine release [41,75,76], the purinergic modulation of dendritic excitability will be spatially and temporally restricted to dendrites experiencing dendritic UP states. The results of this study show that blockade of adenosine A1 receptors removes the suppressing effects of dendritic K⁺ current thus extending the dendritic depolarization, as well as the associated calcium influx in the specific input-receiving dendrite. Thus, we can postulate that adenosine receptor signalling and astrocytic activity exert modulatory influences on glutamate-evoked plateau potentials in cortical networks.

Data accessibility. The NEUROPLEX software for data acquisition and data analysis is available from: http://www.redshirtimaging.com/redshirt_neuro/software_home.html.

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