

Graded control of Purkinje cell outputs by cAMP through opposing actions on axonal action potential and transmitter release

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Running Title

Axon length defines control of synaptic outputs by cAMP

Footnotes

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Keywords

action potential, transmitter release, cAMP, axon, Purkinje cell

Table of Contents category: Neuroscience

Key points summary

- The information processing in the nervous system has been classically thought to rely on the axonal faithful and high-speed conduction of action potentials (APs).
- We demonstrate that the strength and timing of axonal outputs are weakened and delayed, respectively, by cytoplasmic cAMP depending on the axonal length in cerebellar Purkinje cells (PCs).
- Direct axonal patch-clamp recordings uncovered axon-specific attenuation of APs by cAMP through reduction of axonal Na⁺ currents.
- cAMP directly augments transmitter release at PC terminals without changing presynaptic Ca²⁺ influx or readily releasable pool of vesicles, although the extent is weaker compared to other CNS synapses.
- Two opposite actions of cAMP on PC axons, AP attenuation and release augmentation, together give rise to graded control of synaptic outputs in a manner dependent on the axonal length.

Abstract

All-or-none signaling by action potentials (APs) in neuronal axons is pivotal for the precisely timed and identical size of outputs to multiple distant targets. However, technical limitation to measure the signaling in small intact axons has hindered the evaluation of high-fidelity signal propagation. Here, using direct recordings from axonal trunks and/or terminals of cerebellar Purkinje cells in slice and culture, we demonstrate that the timing and amplitude of axonal outputs are gradually modulated by cAMP depending on the length of axon. During the propagation in long axon, APs were attenuated and slowed in conduction by cAMP via specifically decreasing axonal Na⁺ currents. Consequently, the Ca²⁺ influx and transmitter release at distal boutons are reduced by cAMP, counteracting its direct facilitating effect on release machinery as observed at various CNS synapses. Together, our *tour-de-force* functional dissection unveiled the axonal distance-dependent graded control of output timing and strength by intracellular signaling.

52

53 **Introduction**

54 Neuronal computation relies on axonal signaling with regenerative action potentials (APs) which
55 enable rapid information transfer over long distance (Llinás, 1988). Classically APs are regarded
56 as all-or-none type of rigid and faithful signals in terms of strength and velocity, and hence
57 unsusceptible to modification. Such high-fidelity axonal signaling has remained elusive because
58 of the technical hurdle for direct electrophysiological recordings from a distal compartment of an
59 intact axon which is hundreds of μm away from the soma. However, recent studies are changing
60 the classical view of such rigorous axonal AP conduction, highlighting an analogue signaling
61 capability of axons, that is, AP sometimes changes its amplitude, time course, and/or even
62 conduction velocity, in a manner dependent on neuronal activity or intracellular signaling like
63 cAMP (Shu *et al.* 2006; Debanne *et al.* 2011; Zbili & Debanne, 2019; Byczkiewicz *et al.* 2019;
64 Goillard *et al.* 2020; Lezmy *et al.* 2021). Such changes of APs are ascribed to the altered
65 membrane excitability at axon, which could be accompanied by changes of presynaptic Ca^{2+} influx
66 and the resultant transmitter release. In addition, presynaptic transmitter release is by itself
67 modulated by cAMP (Chavez-Noriega & Stevens, 1994; Weisskopf *et al.* 1994; Capogna *et al.*
68 1995; Salin *et al.* 1996; Kaneko & Takahashi, 2004; Meadows *et al.* 2021), suggesting a possibility
69 that the cAMP-caused changes of AP and Ca^{2+} influx would interact with the direct modification
70 of presynaptic release machinery to determine synaptic outputs.

71 To study the axonal AP propagation at a high temporal resolution together with biophysical
72 analysis of presynaptic release machinery, direct *tour-de-force* patch-clamp recordings from intact
73 long axons/terminals are essential. The axons/synapses in the CNS which allow direct functional
74 analysis have increased in number, such as axon terminals of mossy and/or parallel fibers and of
75 GABAergic interneurons in the hippocampus and cerebellum (Geiger & Jonas, 2000; Hu & Jonas,
76 2014; Ritzau-Jost *et al.* 2014; Kawaguchi & Sakaba, 2017; Trigo & Kawaguchi, 2023).
77 Nevertheless, simultaneous patch-clamp recordings from the presynaptic bouton and the

postsynaptic neuron are technically demanding, and in many cases the axon is short in nature or cut during the slicing procedure. Thus, cerebellar Purkinje cells (PCs) forming a long axon could be an ideal model, which allows to apply such a technique either in slice or primary culture to intact axonal trunks (~ 1 μm) and/or terminals (~ 2-3 μm ; Kawaguchi & Sakaba, 2015). Taking advantage of the technical merit of direct PC axonal recordings, we quantitatively measured how the AP propagation and the resultant transmitter release are modulated by cAMP, and found that the AP conduction velocity is lowered through shaping the waveform, which is accompanied by decreased membrane excitability via reduction of axonal Na^+ current. We also examined how the attenuated AP and cAMP pathway cooperatively affect the Ca^{2+} -triggered transmitter release from a PC bouton.

Methods

Animals

Wistar rats (Slc:Wistar, Japan SLC Inc.) of either sex were used in this study. All experimental procedures were performed in accordance with regulations on animal experimentation in Kyoto University and approved by the local committee in Graduate School of Science, Kyoto University (#202313). Rats were housed with a mother rat before weaning in filter-top cages with bedding at 20-24 °C with a 12/12 h light/dark cycle. Food and water were available *ad libitum*. Rats at P0-21 were euthanized by decapitation, and older ones by anaesthetization with isoflurane in air.

Cerebellar slices

Acute sagittal slices (200 μm thickness) of cerebellum were prepared from rats of either sex aged 12-16 or 24-32 days old for experiments on synaptic transmission, and 18-21 days old (to have enough EGFP signal in PC axons) for those on AP conduction. P24-32 rats were anaesthetized and transcardially perfused with an ice-cold extracellular solution containing the following (in mM):

125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 25 D-glucose, 0.4 Na-ascorbate, 3 myo-inositol, 2 Na-pyruvate, 2 CaCl₂, 1 MgCl₂, pH 7.3-7.4, osmolarity 300-320 mOsm/KgH₂O with continuous bubbling with mixed gas (95% O₂ and 5% CO₂). After decapitation, the cerebellum was quickly removed and cut with a Leica vibroslicer (VT1200S) in an ice-cold sucrose-based slicing solution containing the following (in mM): 60 NaCl, 120 Sucrose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 25 D-glucose, 0.4 Na-ascorbate, 3 myo-inositol, 2 Na-pyruvate, 0.1 CaCl₂, 3 MgCl₂, pH 7.3-7.4, osmolarity 300-370 mOsm/KgH₂O or that containing the following (in mM): 240 Sucrose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 25 D-glucose, 0.4 Na-ascorbate, 3 myo-inositol, 2 Na-pyruvate, 0.1 CaCl₂, 10 MgCl₂, pH 7.3-7.4, osmolarity 350-370 mOsm/KgH₂O with continuous bubbling with mixed gas. Slices were then incubated at 37 °C for 30 min to 1 h in an extracellular solution mentioned above. For experiments on AP conduction, PCs were EGFP-labeled by injection of an AAV vector serotype 2 under the control of CA promoter (AAV2-CA-EGFP) into the cerebellum of rats at postnatal days 2-6.

Cerebellar primary cultures

The method for preparing primary dissociated cultures of cerebellar neurons was similar to that in previous studies (Kawaguchi & Sakaba, 2015). Briefly, cerebella were dissected out from newborn rats and their meninges were removed. The cerebella were incubated in Ca²⁺ and Mg²⁺-free Hank's balanced salt solution containing 0.1% trypsin and 0.05% DNase for 15 min at 37°C. Cells were dissociated by trituration and seeded in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12)-based medium containing 2% fetal bovine serum (FBS). On the next day, ~ 80% of medium was replaced by basal medium eagle (BME)-based medium. After that, half of the medium was changed every 3-4 days. Cytosine arabinoside (AraC, 4 μM) was added to the medium to inhibit proliferation of glial cells, and PCs were transfected with EGFP at 4-5 days after seeding with AAV2-CA-EGFP. PCs could be visually identified by their large cell bodies and thick dendrites. An axon was clearly distinct from dendrites which have high density

of spines in a PC. Experiments were performed > 25 days after preparation of the culture.

Electrophysiology

Patch-clamp recordings were performed with an amplifier (EPC10, HEKA) mounted on an inverted Olympus IX71 microscope equipped with a $\times 40$, 0.95 numerical aperture (NA) objective or an upright Olympus BX51WI equipped with a $\times 60$, 1.0 NA objective at room temperature (20-24 °C), in an extracellular solution mentioned above for slices, or that containing the following for culture (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 2 CaCl₂, 1 MgCl₂, pH 7.3-7.4 adjusted by KOH and osmolarity 300-310 mOsm/KgH₂O. Images were obtained with a Zyla4.2 sCMOS camera (Andor). In some cases, a confocal microscope (FV1000, Olympus) was used to capture an image of EGFP-labelled PC axons in slice. In some experiments, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX, 10 μ M), picrotoxin (50 μ M), and tetrodotoxin (TTX, 1 μ M) were applied to the bath to inhibit glutamatergic EPSCs, GABAergic IPSCs, and APs, respectively. For cell-attached recordings in either culture or slices, patch pipettes were filled with the extracellular solution. For current-clamp recordings, K⁺-based internal solution with the following composition (in mM) was used: 155 K-gluconate, 7 KCl, 10 HEPES, 0.5 ethylene glycol bis (β -aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), 2 Mg-ATP, 0.2 Na-GTP, pH 7.3-7.4 adjusted by KOH and osmolarity 320-330 mOsm/KgH₂O. For voltage-clamp recording from an axon terminal of a PC, a patch pipette was filled with CsCl-based internal solution (pH 7.3-7.4 adjusted by CsOH and osmolarity 320-330 mOsm/KgH₂O) containing (in mM): 170 CsCl, 10 HEPES, 0.5 EGTA, 2 Mg-ATP, 0.2 Na-GTP, and recordings were performed in the presence of external TTX and tetraethylammonium (TEA, 2 mM). For postsynaptic recordings from PCs' target neurons, the internal solution was mixture of CsCl and Cs-gluconate-based solutions (pH 7.3-7.4 adjusted by CsOH and osmolarity 320-330 mOsm/KgH₂O) containing (in mM): 137 or 23 CsCl, 33 or 147 Cs-gluconate, 10 HEPES, 5 EGTA, 2 Mg-ATP, 0.2 Na-GTP. PCs' target glutamatergic projection neurons in the deep cerebellar nuclei (DCN) region in slice

were identified by the relatively large cell bodies. Area size of cell bodies recorded in this study was $250 \pm 110 \mu\text{m}^2$ ($n = 18$ cells), in consistent with previous studies (Unsisaari *et al.* 2007). In measurements of voltage-gated currents through Na^+ , K^+ , and HCN channels, K^+ -based internal solution was used. When gluconate-based internal solution was used, the liquid junction potential (~ 15 mV) was corrected. KT5720 ($10 \mu\text{M}$) or ESI-09 ($100 \mu\text{M}$) was added to internal solution filled in the patch pipette to inhibit PKA or Epac, respectively. To increase internal cAMP, forskolin ($50 \mu\text{M}$) or cAMPS-Sp (1 mM) was added extracellularly to the bath or intracellularly through the patch pipette, respectively. In some experiments, dimethyl sulfoxide (DMSO, 0.05%) was applied to the bath as control.

Membrane potential of a PC was held at -70 mV unless otherwise stated. PCs' target postsynaptic neurons in either culture or slice were voltage-clamped at -70 to -120 mV for IPSC recordings to avoid unclamped Na^+ current. Series resistances at the PC soma, axon, and terminal were, $13 \pm 5 \text{ M}\Omega$, $64 \pm 17 \text{ M}\Omega$, and $137 \pm 56 \text{ M}\Omega$ (mean $\pm$ SD; $n = 27, 48$, and 45 cells), and were online-compensated by 40-60%. On-line series resistance compensation (20-60%) was applied for IPSC recordings from PCs' target cells. The remaining resistances were further corrected off-line for recordings from both presynaptic terminals and postsynaptic cells.

APs were elicited by current injection of 500-900 pA for 10 ms into PC soma in culture, or by extracellular electrical stimulation at ~ 50 V or ~ 15 mA for 10-200 μs with a glass pipette on the white matter in slices. In the recordings of AP conduction in slices, a glass pipette for stimulation was placed at $> 200 \mu\text{m}$ away from the recorded axonal bleb. To avoid the possible AP modulation at high frequency firing (Kawaguchi & Sakaba, 2015), axonal APs with intervals > 50 ms were used for analysis. eIPSCs in culture were accepted for analysis unless aberrant change in failure rate during recordings was observed.

Measurement of membrane capacitance (C_m) was done using sine +DC technique (Neher & Marty, 1982) implemented on the Patchmaster software (HEKA). Presynaptic terminals were held at -80 mV and the sine wave (1 kHz and the peak amplitude of 30 mV) was applied on the holding

potential. Because membrane conductance fluctuates during the depolarizing pulse due to large presynaptic conductance changes, C_m was usually measured ~ 50 ms after the depolarization.

Analysis of electrophysiological data

All obtained data were analyzed using Patchmaster and Igor Pro (WaveMetrics). APs, Ca^{2+} currents ($I_{Ca^{2+}}$), and postsynaptic currents (PSCs) were detected using Taro Tools extensions (<https://sites.google.com/site/tarotoolsregister/>) implemented in Igor Pro. To measure the distance between two sites for recording and stimulation, ImageJ (NIH) was used.

The onset of eIPSC was defined as the first time point at which the recorded current value showed change larger than 2SD of that at the basal condition. The AP conduction velocity was calculated by dividing the temporal difference between the somatic and axonal AP peaks by the distance between two sites. For mIPSC analysis, individual cumulative probability of inter-event intervals and amplitudes for each cell were calculated from ≥ 200 events which were ≥ 7 pA with appropriate time courses.

The current (I_{Na^+})-voltage (V_m) relation for axonal voltage-gated Na^+ currents was fitted by the following equation based on the Boltzmann function:

$$I_{Na^+} = G_{Na^+max} \cdot \frac{1}{1 + e^{\frac{V_{1/2} - V_m}{k}}} \cdot (V_m - E_{Na^+}) + G_{leak} \quad (1)$$

, where E_{Na^+} is the equilibrium potential of Na^+ . Fitting of I-V relationship for each axon yielded three key parameters for the Na^+ channel activation: relative peak conductance of Na^+ (G_{Na^+max}), voltage for half-maximal activation ($V_{1/2}$), and the slope factor (k).

To estimate the clamped area in a direct bouton recording, capacitive transients in response to hyperpolarizing pulses (10 or 20 mV) were used. Capacitive transients at a terminal followed a single exponential with time constants of 0.38 ± 0.12 ms ($n = 44$ cells), so that the clamped

terminal (and neighboring axon) size was estimated to be 3.2 ± 1.7 pF. Considering the high correlation between the clamped area size and the presynaptic $I_{Ca^{2+}}$ or C_m increase (Pearson's correlation coefficient, 0.78 or 0.49, respectively), $I_{Ca^{2+}}$ and C_m increase were normalized by the clamped membrane capacitance of individual boutons. Postsynaptic responses were evaluated by calculating inhibitory postsynaptic conductance (IPSG) obtained by dividing the IPSC amplitude by the driving force for Cl^- . For all fittings of data, the least square method was used. The sensitivity of C_m increase or synaptic responses to Ca^{2+} were obtained by fitting data assuming identical power dependency on Ca^{2+} which was apparently not affected by cAMPS-Sp.

Statistics

Data in all figures and table are presented as mean $\pm$ SD. The difference between groups was evaluated by paired t-test for paired groups or by unpaired Student's t-test for unpaired ones, unless otherwise stated. Two-way analysis of variance (ANOVA) was also used to analyze data shown in Fig. 5B and H. For multiple comparison, Dunnett's test was used in Fig. 5F. Spearman's correlation coefficients were used and tested based on Student's t-distribution. The difference or the correlation was considered significant when $p < 0.05$.

Results

Distance-dependent reduction of synaptic outputs from a PC axon by cAMP

We first examined the effect of cAMP on synaptic transmission at the synapse from a PC to a neuron in deep cerebellar nuclei (DCN). Cerebellar PCs project to both glutamatergic and GABAergic neurons in the DCN region (De Zeeuw & Berrebi, 1995). Whole-cell recordings were performed from a neuron with a relatively large cell body ($\sim 250 \mu\text{m}^2$) that corresponds to a glutamatergic projection neuron in DCN (Unsisaari *et al.* 2007) of acute slices prepared from cerebellum at P12-16 rats (Fig. 1A). Evoked IPSCs (eIPSCs) from PC axons were triggered by electrical stimulation of the white matter in the presence of AMPA receptor antagonist NBQX ($10 \mu\text{M}$) to avoid activation of GABAergic interneurons in local circuits of DCN, and were identified by almost all-or-none responses with large amplitude upon elevating stimulation intensity together with the tendency of frequency-dependent depression upon repetitive stimulation (Fig. 1A and B; Telgkamp & Raman, 2002). To increase cytoplasmic cAMP, we added the cell-permeable adenylyl cyclase activator forskolin ($50 \mu\text{M}$) to the external bath. In clear contrast to previous studies showing cAMP-induced facilitation of transmission at various CNS synapses (Chavez-Noriega & Stevens, 1994; Weisskopf *et al.* 1994; Capogna *et al.* 1995; Salin *et al.* 1996; Kaneko & Takahashi, 2004), unexpectedly, the eIPSC amplitude was not clearly facilitated, but rather depressed as the distance from the stimulation electrode to the recorded cell increased ($< 400 \mu\text{m}$, $100 \pm 26\%$; $> 400 \mu\text{m}$, $59 \pm 21\%$, $p = 0.0196$; Fig. 1B and C). In addition, forskolin significantly increased synaptic delay, the sum of two temporal parameters for the AP conduction and the subsequent exocytosis of synaptic vesicles, in a manner dependent on the distance ($r = 0.618$, $p = 0.0426$; Fig. 1B and D). Forskolin changed coefficient of variation (CV) of eIPSCs amplitude, but not time courses (Fig. 1E and F). Thus, cAMP tends to negatively regulate transmission at PC synapses.

Myelination of PC axons proceeds during early postnatal developmental stages and get almost completed around 2 weeks old, although its maturation is still in the process (Gianola *et al.* 2003). To test whether the cAMP-mediated modulation of PC outputs shown above is evident

even after the maturation of axon myelination, we also recorded eIPSCs at DCN in slices prepared from P24-32 rats. Similarly to PC synapses in young rats, eIPSC amplitude tended to be depressed by forskolin in a manner dependent on the axonal distance ($r = -0.857$, $p = 0.0137$; Fig. 1C), whereas synaptic delay became insensitive to forskolin ($p = 0.918$; Fig. 1D). Thus, matured PC axon with elaborate myelination during the development might somehow make the timing of synaptic outputs resistant to the cAMP-caused modulation while preserving the sensitivity of synaptic output strength to cAMP.

We suspected that the negative modulation of PC outputs by cAMP might be ascribed to an issue of axonal viability in slice, and examined the effects of cAMP in culture, in which long axonal arborization (sometimes beyond 1 mm) is preserved from the soma to terminals. To specifically visualize PCs, we used an adeno-associated virus (AAV) vector, AAV2-CA-EGFP that preferentially infects PCs among neurons in the cerebellum (Kawaguchi & Sakaba, 2015). Presynaptic PC was current-clamped so that the membrane potential was around -70 mV, and APs were elicited by current injection (500 - 900 pA for 10 ms) to the soma, which caused eIPSCs in the voltage-clamped (at -80 to -100 mV) postsynaptic cell (Fig. 2A-D). eIPSCs showing large fluctuation in amplitude (in average 759 ± 905 pA) were negatively modulated by forskolin as the axonal length between two cells became longer ($< 400 \mu\text{m}$, $104 \pm 26\%$; $> 400 \mu\text{m}$, $73 \pm 15\%$, $p = 0.0436$; Fig. 2C-E), accompanied by an increase in the synaptic delay ($r = 0.818$, $p = 0.00208$; Fig. 2C, D and F). Forskolin little affected the 20-80% rise time or CV in amplitude of eIPSCs (Fig. 2G), although the somatic AP waveform and membrane resistance were a little, but significantly affected (Fig. 2H). Thus, it was concluded that cAMP delays and attenuates synaptic outputs in a PC depending on the axonal length.

cAMP attenuates and slows APs in a PC axon

Based on the slight change of somatic AP and membrane resistance, we hypothesized that the AP conduction might underlie the distance-dependent modulation of PC outputs by cAMP,

and simultaneously recorded spontaneous APs from both the soma and axon (Fig. 3A and B). EGFP fluorescence enabled us to precisely target a thin patch pipette to the axonal trunk. By measuring temporal difference between the peaks of spontaneous APs at the soma and axon, we evaluated the AP conduction velocity (Fig. 3B-D). Forskolin increased the time needed for the AP to reach the axonal recording site (at $452 \pm 145 \mu\text{m}$ away, $147 \pm 28 \%$; $0.97 \pm 0.51 \text{ ms}$ to $1.37 \pm 0.76 \text{ ms}$, $p = 0.00566$; Fig. 3C), indicating that cAMP slows down the AP conduction velocity in a PC axon ($569 \pm 235 \mu\text{m/ms}$ to $405 \pm 164 \mu\text{m/ms}$; Fig. 3D). In addition, the waveform of axonal cell-attached AP, but not that at the soma, tended to be slower in time course and smaller in amplitude after the forskolin application (soma, $97 \pm 16\%$, $p = 0.384$; axon, $59 \pm 10\%$, $p = 1.60 \times 10^{-4}$; Fig. 3B and E), implying that cAMP negatively regulates the AP conduction in axon, but not that around the soma.

The above observation in culture was again tested in slice. Injection of AAV2-CA-EGFP into the cerebellum at P2-6 fluorescently labeled PC axons a few weeks later, and a direct patch-clamp recording was performed from an axonal bleb, an enlarged structure formed at the cut end of the axon during slice preparation (Fig. 3F). APs were elicited by electrical stimulation with a glass pipette placed at $> 200 \mu\text{m}$ away from the recording site, and delay of the AP arrival from the stimulation site was measured (Fig. 3G). Supporting the data from culture preparation, forskolin increased the delay significantly ($109 \pm 8\%$, $p = 0.0402$; Fig. 3G and H). Together, our results indicate that cAMP decreases the AP conduction velocity in a PC, in a similar manner to that in a cortical pyramidal cell (Lezmy *et al.* 2021), but quite contrasts to the previous findings of increased AP conduction velocity by cAMP in the cerebellar mossy and parallel fibers (Byczkiewicz *et al.* 2019).

We next focused on the somatic and axonal AP waveforms during propagation in terms of their susceptibility to cAMP modulation. The soma and axon were whole-cell current-clamped so that the membrane potentials were around -70 mV except for the time triggering AP firings (Fig. 4A). An axon exhibited remarkable reduction in amplitude and slowed time course of AP

waveforms after the forskolin application (Fig. 4A and B, amplitude: $90 \pm 4\%$, $p = 1.57e-4$; 20-80% rise time: $129 \pm 15\%$, $p = 0.0198$; 80-20% decay time: $113 \pm 9\%$, $p = 0.0139$). Forskolin also affected AP waveforms recorded at the soma, although marginally (amplitude: $99 \pm 4\%$, $p = 0.0539$; 20-80% rise time: $109 \pm 13\%$, $p = 0.138$; 80-20% decay time: $104 \pm 4\%$, $p = 0.00264$; Fig. 4A and B). Trajectory of APs plotting the velocity of voltage change against the membrane potential indicated weakened drive for AP rising phase after the forskolin application markedly at axon (Fig. 4C and D). In addition, the AP conduction velocity showed almost linear relation to the axonal AP amplitude both before and after the forskolin application (Fig. 4E), suggesting that cAMP simply attenuates AP waveforms, accompanied by slowed conduction.

Reduction of axonal Na⁺ currents by cAMP

To explore the molecular mechanism for the axonal AP modulation by cAMP, we recorded voltage-activated currents from a PC axon, particularly focusing on voltage-gated Na⁺ (Nav) and K⁺ channels (Kv) which predominantly determine AP waveforms. As shown in Fig. 5A, step depolarization (from -70 mV) of axonal membrane caused a large transient inward current corresponding to Na⁺ influx, and the following sustained outward currents through K⁺ channels. Forskolin significantly reduced Na⁺ currents (I_{Na^+} , -1.42 ± 0.86 nA to -1.04 ± 0.71 nA at -20 mV, $p = 0.00433$), but not K⁺ currents (I_{K^+}), without changing the voltage sensitivity (Fig. 5A-D). In contrast, forskolin did not affect the somatically recorded I_{Na^+} (-4.51 ± 1.87 nA to -4.32 ± 1.81 nA at -20 mV, $p = 0.290$). Similarly, cytoplasmic dialysis of cAMPS-Sp, a cAMP analogue, through a patch pipette also decreased axonal I_{Na^+} , but not I_{K^+} (Table 1). Thus, the cAMP-caused AP attenuation at axon (shown in Fig. 4) would be ascribed mainly to the axon-specific reduction of Na⁺ influx, which would subsequently reduce the extent and/or timing of activation of K⁺ channels leading to slightly slower AP decays. Importantly, KT5720 (PKA inhibitor, 10 μ M) but not ESI-09 (Epac inhibitor, 100 μ M) halved the cAMP-caused reduction of axonal Na⁺ influx upon the forskolin application (Fig. 5E and F), suggesting that the negative regulation of Na⁺ channels by cAMP

elevation is dependent on phosphorylation by PKA, but not on Epac. These results indicate that the membrane excitability, mainly determined by the relative strength of Na^+ influx against K^+ efflux, is clearly decreased by the cAMP increase in a PC axon. In addition, we also recorded the hyperpolarization-activated currents (I_h), cation influx typically through HCN channels, by applying hyperpolarization pulses for 300 ms. In contrast to the cAMP-mediated changes of I_h in the cerebellar parallel and mossy fibers (Byczkowicz *et al.* 2019), forskolin little affected the I_h amplitude in a PC axon (Fig. 5G and H), probably due to the selective expression of cAMP-insensitive HCN1 in PCs (Wang *et al.* 2001; Notomi & Shigemoto, 2004). Thus, among voltage-dependent channels tested here, Nav channels were specifically and negatively regulated by the cytoplasmic cAMP increase in a PC axon.

Augmentation of release by cAMP in a PC terminal

The negative control of synaptic transmission by cAMP in a PC (shown in Fig. 1 and 2) contrasts to the well-known augmentation of transmitter release by forskolin reported in many previous studies on types of neurons, particularly at synapses characterized by loose coupling between voltage-gated Ca^{2+} channels (Cav) and release machinery, such as hippocampal mossy fiber boutons (Midorikawa & Sakaba, 2017). The tight Cav -release coupling in PC boutons (Díaz-Rojas *et al.* 2015) might preclude the cAMP-mediated facilitation. However, miniature IPSCs (mIPSCs) recorded from neurons targeted by PCs in culture showed forskolin-caused increase in the frequency (13.0 ± 6.7 Hz to 19.0 ± 9.6 Hz, $p = 0.0134$), without changes in the amplitude (45.3 ± 30.8 pA to 39.9 ± 16.6 pA, $p = 0.431$; Fig. 6). Thus, cAMP seems to augment transmitter release also in PC axon terminals.

Taking advantages of direct patch-clamp recording from a presynaptic bouton, we attempted to analyze the release augmentation by cAMP in PCs. Presynaptic Ca^{2+} currents ($I_{\text{Ca}^{2+}}$) in response to square pulse depolarization (to 0 mV for 1-50 ms) and the resultant presynaptic exocytosis, reflected by the changes of membrane capacitance (C_m), were voltage-clamp

recorded from an EGFP-labeled PC terminal (Fig. 7A). $I_{Ca^{2+}}$ was not significantly altered in the amplitude and kinetics (such as activation, inactivation, and deactivation) by cAMPS-Sp (Fig. 7B-D), similarly to other neurons (Kaneko & Takahashi, 2004; Midorikawa & Sakaba, 2017). In contrast, C_m tended to increase more efficiently (~ 2 fold) upon shorter depolarization pulses in the presence of cAMPS-Sp (Fig. 7B and E), although reaching the maximum upon longer pulses similar to the control condition (32.0 ± 19.3 and 34.0 ± 18.5 fF/pF upon 50 ms pulse, without and with cAMPS-Sp, $p = 0.843$; Fig. 7F). Thus, cAMP seems not to change the total size of readily releasable pool of synaptic vesicles, but to augment the efficiency of Ca^{2+} to trigger the exocytosis of vesicles even at 'tight coupling' presynaptic terminals of a PC.

Finally, to evaluate the interplay between the apparently counteracting actions of cAMP on presynaptic function, increased release probability and attenuated AP amplitude, we performed paired recordings from a presynaptic PC terminal and the target postsynaptic neuron (Fig. 8A). Two distinct AP waveforms, obtained from a PC axon terminal either in naïve condition or after the forskolin application (Fig. 8B), were applied to the patched terminal as voltage commands. The smaller AP waveform (90% in amplitude) decreased presynaptic $I_{Ca^{2+}}$ and postsynaptic current compared to those upon the naïve AP, irrespective of the presence of cAMPS-Sp ($I_{Ca^{2+}}$: $77 \pm 3\%$ and $79 \pm 7\%$; postsynaptic response: $70 \pm 10\%$ and $64 \pm 23\%$ without and with cAMPS-Sp, respectively; Fig. 8C and D). In contrast, the postsynaptic responses exhibited a tendency to be increased by cAMPS-Sp, showing ~ 3 fold larger postsynaptic response relative to the presynaptic $I_{Ca^{2+}}$ (Fig. 8E). Thus, cAMP augments vesicular exocytosis without affecting presynaptic Ca_v , counteracting the lowered release possibility due to the smaller Ca^{2+} influx upon the attenuated AP.

Discussion

In this study, taking advantages of direct patch-clamp recordings from an intact long axon of PC

in culture and from an axonal cut end in acute slice, we found that AP is reduced in size by cytoplasmic cAMP specifically in axon through reduction of I_{Na^+} , which results in slower AP conduction along the axon and hence longer delay in synaptic outputs. In addition, presynaptic Ca^{2+} influx is also reduced by cAMP via attenuation of AP, counteracting the facilitatory effect of cAMP on transmitter release machinery. As a result of interplay of the intricate mechanisms, in contrast to the classical view of axon as an all-or-none high-fidelity signal conductor, the temporal precision and intensity of output from PC axons are coordinately modulated by cAMP in a graded manner dependent on the axonal length.

Analogue modulation of axonal signaling

Presynaptic AP waveform is a critical determinant of synaptic strength because it sets the maximal probability and time course of Ca_v opening (Sabatini & Regehr, 1997; Borst & Sakmann, 1998, 1999; Geiger & Jonas, 2000; Bischofberger *et al.* 2002; Kawaguchi & Sakaba, 2015; Zbili *et al.* 2020). Axonal AP waveform changes upon high frequency firing in axons of a variety of neurons, such as cerebellar PCs (Kawaguchi & Sakaba, 2015), hippocampal mossy fibers (Geiger & Jonas, 2000; Engel & Jonas, 2005; Alle & Geiger, 2006), and Calyx of Held in the auditory pathway (Taschenberger & von Gersdorff, 2000). It would be interesting to look at the constancy of output timing during repetitive firing in those neurons. On the other hand, other neurons, such as fast spiking GABAergic interneurons in the hippocampus, the layer 5 of cerebral cortex, or the cerebellar molecular layer, exhibit stable AP waveforms in an axon even at very high frequency firing (Hu & Jonas, 2014; Ritzau-Jost *et al.* 2021; Trigo & Kawaguchi, 2023), although modulation of AP width by inactivation of K_v channels was also reported (Rowan & Christie, 2017). Furthermore, axonal AP waveforms or firings have been shown to be affected by local receptors for various transmitters such as GABA and purine (Trigo *et al.* 2010; Zorrilla de San Martin *et al.* 2017; Gomez *et al.* 2020).

Our paired recordings from the soma and axonal trunk yield the AP conduction velocity in a PC axon as $\sim 570 \mu\text{m/ms}$ at room temperature (see Fig. 3), which compromises the velocity around $770 \mu\text{m/ms}$ studied in slice at physiological temperature (Clark *et al.* 2005), when considering the temperature sensitivity of AP conduction as 1.5-2 fold per 10°C changes (Q_{10}) (Chapman, 1967). The AP conduction velocity was here slowed down as a result of altered AP waveforms by cAMP through lowered membrane excitability, leading to delayed synaptic outputs from terminals. On the other hand, increased membrane excitability by cAMP through activating HCN channels was also shown in previous studies to change the axonal AP conduction velocity in cerebellar and cortical neurons, although the direction of speed change was diverse: speeding in cerebellar parallel and mossy fibers (Byczkiewicz *et al.* 2019), whereas slowing in cortical pyramidal cells (Lezmy *et al.* 2021). In addition, the decay phase of presynaptic AP by itself also affects the synaptic latency by changing the timing of Ca^{2+} influx, as shown in cortical synapses between layer 5 neurons (Boudkkazi *et al.* 2011). Thus, the timing of neuronal outputs is fine-tuned by dynamic interplay of multiple mechanisms to determine the APs, which would be central for neuronal signal integration.

As shown in Fig. 1, axonal length-dependent graded modulation of synaptic output strength and timing in a coordinated manner by cAMP was evident at PCs in the early developmental stage in rats (postnatal day 12-16), but tended to become specific to the amplitude of synaptic transmission at the older age around ~ 4 weeks after birth. The maturation of axonal myelination in process during that developmental period (Gianola *et al.* 2003) might secure the saltatory conduction of APs in PC axons, minimizing the effect of cAMP-caused slight reduction of AP amplitude on the activation of Na^+ channels accumulated at the node of Ranvier. Nevertheless, the axonal length-dependent graded control of synaptic output intensity by cAMP is preserved even after the maturation of cerebellar cortical circuit.

Mechanism of AP modulation in axon

We demonstrated negative regulation of Na⁺ channels by cAMP through PKA, but not through Epac, specifically at axon (see Fig. 5), leading to the attenuated AP, in line with previous reports (Scheuer, 2011). PCs are reported to express various types of Nav, such as Nav1.1, 1.4, 1.6, 1.7, 1.8 and 1.9 by *in situ* hybridization data from Allen Mouse Brain Atlas (Lein *et al.* 2007; but see Schaller & Caldwell, 2003), and it remains unclear what type of Nav is responsible for axonal component susceptible to the down-regulation by cAMP, which should be clarified in future studies. The axon-specific reduction of I_{Na}⁺ by cAMP implies different subtypes of Nav located at the soma and axon in PCs, which makes it feasible to selectively control the axonal AP waveform without affecting input-output conversion by AP initiations around the soma. On the other hand, in contrast to previous studies showing cAMP-caused modulation of axonal HCN currents in other neurons (Byczkiewicz *et al.* 2019; Lezmy *et al.* 2021), PC axons showed no clear change of I_h upon cAMP elevation (see Fig. 5G and H), probably because of selective expression of cAMP-insensitive HCN1 in PCs (Wang *et al.* 2001; Notomi & Shigemoto, 2004). Thus, different neuronal axons equipped with distinct types of channels have diverse mechanisms to change the AP waveform and conduction. Other than regulation of Nav, numbers of previous studies have provided evidences for changes of AP waveforms through modulation of K⁺ channels in various types of neuronal axons (Hoppa *et al.* 2014; Begum *et al.* 2016; Rowan & Christie, 2017; Cho *et al.* 2020).

Release machinery facilitated by cAMP

cAMP is involved in the presynaptic long-term potentiation expressed as the augmented transmitter release at low release probability synapses, characterized by relatively loose coupling between Ca_v and release machinery, such as hippocampal mossy fibers (Midorikawa & Sakaba, 2017) and cerebellar parallel fibers (Kawaguchi & Sakaba, 2017). Thus, the mechanism how cAMP augments transmitter release has attracted attention (Cheung *et al.* 2006; Kaeser-Woo *et al.* 2013). A recent study applying direct patch-clamp recordings combined with fluorescent

imaging of vesicle fusion to the plasma membrane and/or super-resolution fluorescent microscopy on hippocampal mossy fibers demonstrated that cAMP shortens the coupling distance between Ca_v and release machinery (Fukaya *et al.* 2021). This shift of Ca_v -release coupling from loose to tight is thought to be reflected as the increased release probability, as experimentally characterized by the higher resistance of transmission to slow Ca^{2+} chelator EGTA in the cytoplasm. Although the shortening of the distance of Ca_v to release machinery could be an efficient way to positively modulate transmission, it is unlikely that such a mechanism strongly operates at tight-coupling synapses because of the limited margin for further shortening of the coupling distance. As shown in this study, PC boutons which show EGTA-resistant tight Ca_v -release coupling (Díaz-Rojas *et al.* 2015), still undergo cAMP-mediated increase in release probability. Relatively weak facilitation of release by cAMP in PC boutons (~ 2-3 fold) compared to mossy fiber boutons (~ 4 fold; Weisskopf *et al.* 1994) might be ascribed to the distinct synaptic design in terms of Ca_v -release coupling. Previous studies using paired pre- and postsynaptic recordings together with Ca^{2+} uncaging methods at Calyx of Held synapses clarified that cAMP potentiates transmitter release by increasing Ca^{2+} sensitivity of release machinery rather than by changing either the coupling distance or the size of readily releasable pool (Yao & Sakaba, 2010), in a similar manner to PCs as shown here. Recently, it was shown that PKA and/or Epac-mediated modulation of RIM1 and Munc-13 at active zones underlies the cAMP-mediated enhanced release (Wang *et al.* 2023). Similar molecular mechanisms might operate also at the tight-coupling PC boutons, which is an important issue to be addressed in future studies.

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Additional information

Data availability statement

All the data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare no competing interests.

Author contributions

K.F. and S.K. designed the study; K.F. and T.I. performed the experiments; K.F. analyzed and K.F. and S.K. interpreted the data; all authors wrote and edited the manuscript and approved the final version of it, and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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	control (n = 6)	cAMPS-Sp (n = 6)	p value
I_{Na^+} (nA)	-1.44 ± 0.45	-0.78 ± 0.31	0.0185
I_{K^+} (nA)	2.58 ± 0.62	2.13 ± 1.10	0.409
leak current (pA)	-31.2 ± 15.9	-95.2 ± 35.1	0.00226
apparent R_m ($M\Omega$)	362.0 ± 136.5	149.1 ± 68.7	0.00663
R_s ($M\Omega$)	58.6 ± 5.6	69.2 ± 11.4	0.0668
soma-axon distance (μm)	358.8 ± 192.8	377.5 ± 133.6	0.849

Table 1. Summary of cAMPS-Sp effects on axonal voltage-gated currents in PCs

Mean $\pm$ SD of I_{Na^+} (at -20 mV) and I_{K^+} (at 0 mV) amplitude, leak current, apparent membrane resistance (R_m), series resistance (R_s), and distance from soma in PC axonal recordings without (control) and with cAMPS-Sp. n = 6 for each. P values for student's t-test between control and cAMPS-Sp are also shown.

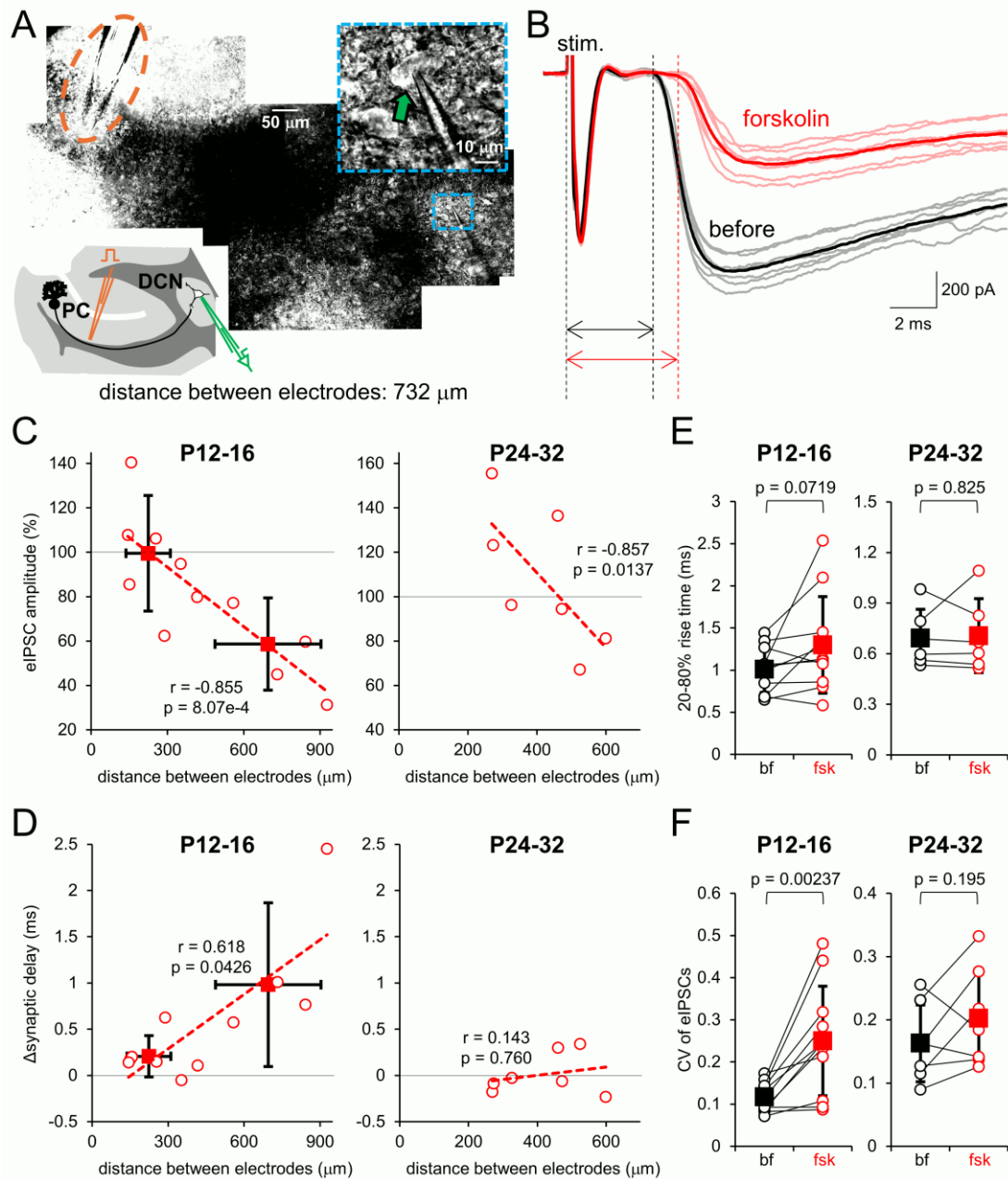


Figure 1. Distance-dependent reduction and delay of synaptic outputs from PCs by cAMP in acute slices

A, Scheme of slice recordings of eIPSC from DCN neurons (green), triggered by electrical stimulations at white matter (orange). Inset, magnified image of the recorded DCN neuron. **B**, Representative (gray, pink) and averaged (black, red) eIPSCs before and after the forskolin application. Synaptic delays are highlighted by double arrows. **C**, **D**, Normalized amplitudes (**C**) and changes in synaptic delay (**D**) of eIPSCs after the forskolin application relative to before, plotted against the distance between two electrodes. Data for P12-16 (left) and P24-32 (right). Linear fits to the data are shown by dotted lines. **E**, **F**, 20-80% rise time (**E**) and CV (**F**) of eIPSCs before (bf) and after the forskolin (fsk) application. Data for individual cells (open circles) and mean $\pm$ SD (closed squares) at P12-16 (left, $n = 11$) and P24-32 (right, $n = 7$) are shown.

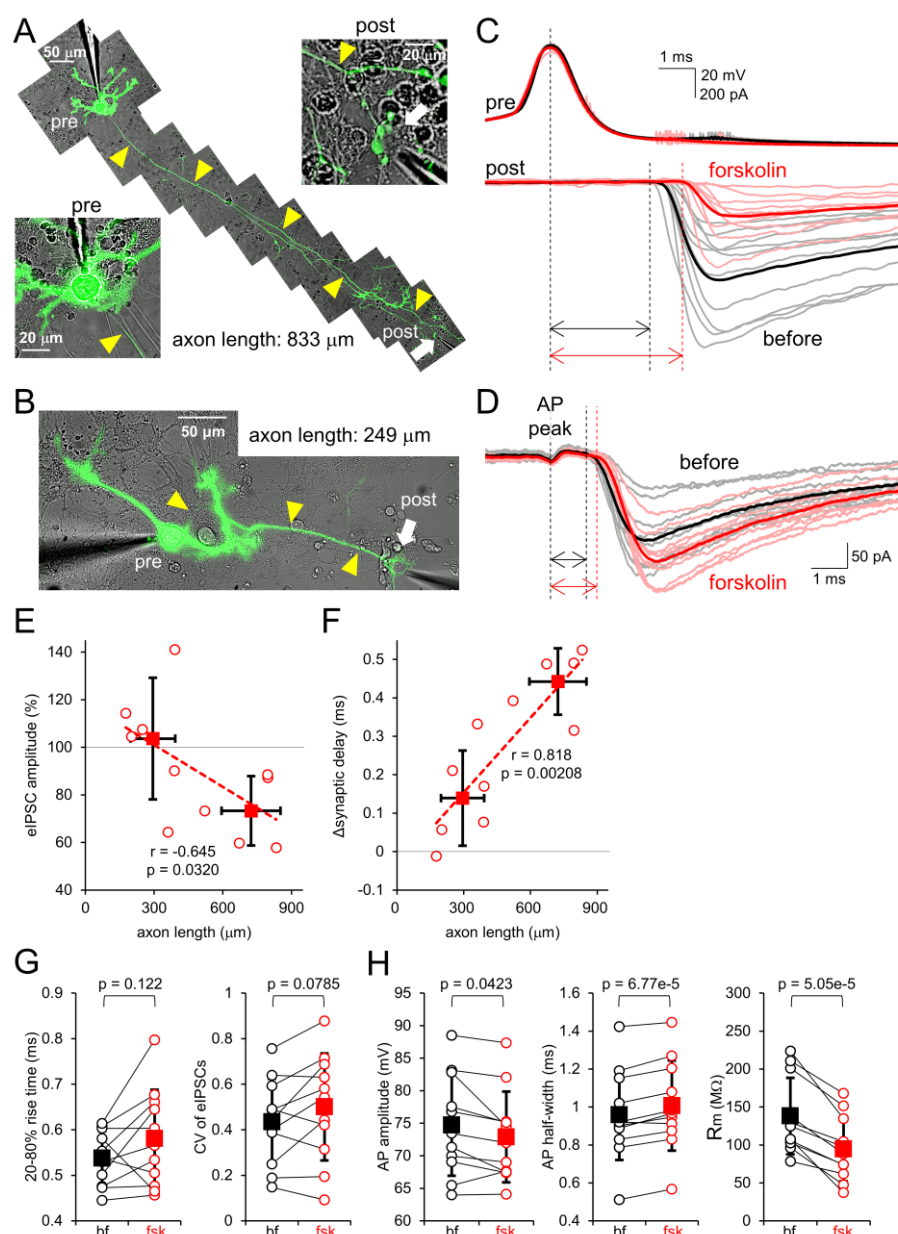


Figure 2. Distance-dependent reduction and delay of synaptic outputs from PCs by cAMP in culture

A, B, Paired whole-cell recordings from the presynaptic EGFP-labeled PC soma and the postsynaptic neuron with long (A) or short (B) distance between cells. Yellow arrowheads indicate the axon. **C, D**, Representative (gray, pink) and averaged (black, red) traces of presynaptic APs and eIPSCs recorded from cell pairs shown in A or B before and after the forskolin application. Traces are aligned by the peak time of APs. **E, F**, Normalized amplitudes (E) and changes in synaptic delay (F) of eIPSCs after the forskolin application plotted against the distance between two cells. Linear fits to the data are shown by dotted lines. **G, H**, 20-80% rise time (G, left) and CV (G, right) of eIPSCs, AP amplitudes (H, left) and half-widths (H, middle), and estimated membrane resistances (R_m , H, right) at PC soma before (bf) and after the forskolin (fsk) application. $n = 11$. Data for individual cells (open circles) and mean $\pm$ SD (closed squares) are shown.

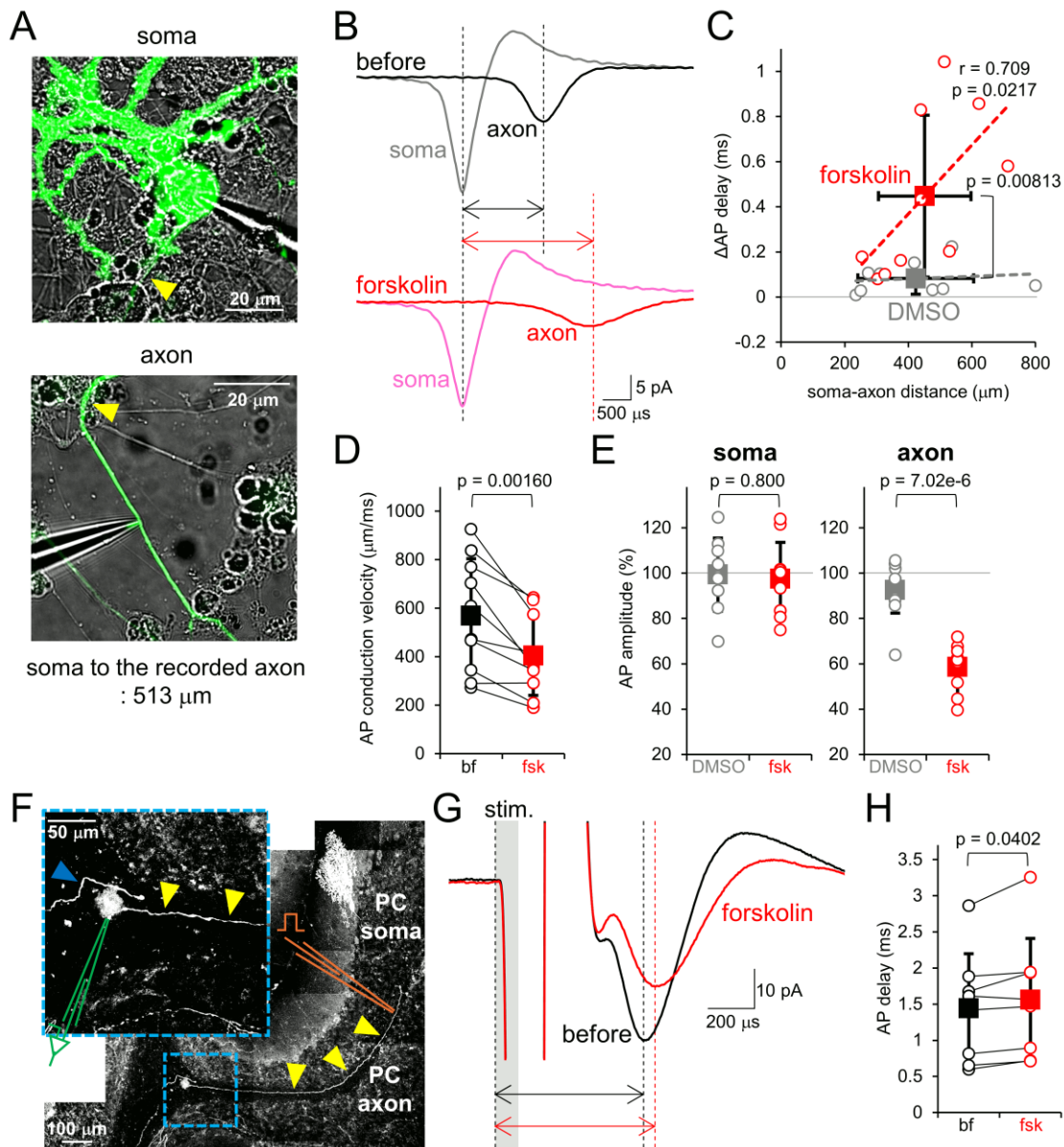


Figure 3. cAMP slows APs in a PC axon

A, Simultaneous patch-clamp recordings from the PC soma (top) and axon (bottom). Yellow arrowheads indicate the axon. **B**, Representative averaged APs obtained by cell-attach recordings at the soma (gray, pink) and axon (black, red), aligned by the peak of somatic APs before and after the forskolin application. **C**, Changes in delay in spontaneous AP arrival to the axon, plotted against the distance between the recorded somatic and axonal sites. $n = 9$ (DMSO) and 10 (forskolin). Linear fits to the data are shown by dotted line. **D**, Calculated AP conduction velocities before (bf) and after the forskolin (fsk) application. $n = 10$. **E**, Normalized amplitudes of somatic (left) and axonal (right) spontaneous APs after the DMSO or forskolin application. **F**, EGFP-labeled PC axons (yellow arrowheads; blue, another axon) in slice and the recorded axonal bleb (green in magnified image). A stimulation electrode (orange) was placed $\sim 950 \mu\text{m}$ away from the recorded bleb. **G**, **H**, Representative averaged traces (**G**) and delays (**H**) of cell-attached axonal APs before and after the forskolin application. $n = 8$. Data for individual cells (open circles) and mean $\pm$ SD (closed squares) are shown.

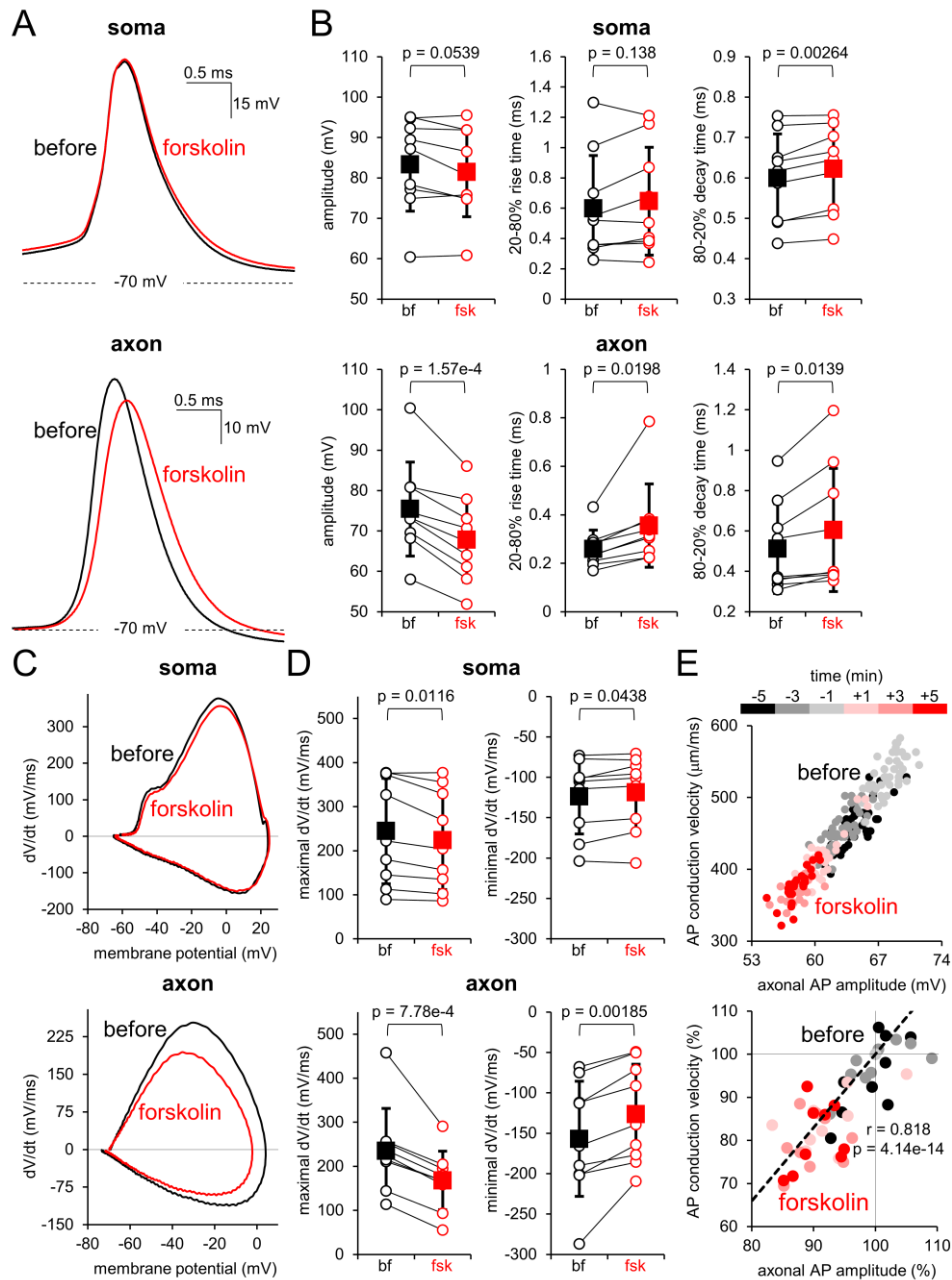


Figure 4. cAMP attenuates APs in a PC axon

A, B, Representative averaged traces (**A**), amplitudes (**B**, left), 20-80% rise time (**B**, middle), and 80-20% decay time (**B**, right) of APs recorded at the soma (top) and axon (bottom) elicited by somatic current injection before (bf) and after the forskolin (fsk) application. **C, D**, Representative phase plots (dV/dt vs V; **C**), maximal (**D**, left) and minimal (**D**, right) dV/dt of somatic (top) and axonal (bottom) APs before and after the forskolin application. Data for individual cells (open circles) and mean $\pm$ SD (closed squares) are shown. **E**, Relationship between the axonal AP conduction velocity and amplitude in a representative cell (top) and individual cells (bottom). Time of individual AP recording is represented by graded colors (from 5 min before (black) to 5 min after (red) the forskolin application). In the bottom graph, averaged AP conduction velocity and amplitude are normalized by the values at 1 min before the forskolin application for individual cells. $n = 9$. Linear fits to the data are shown by a dotted line.

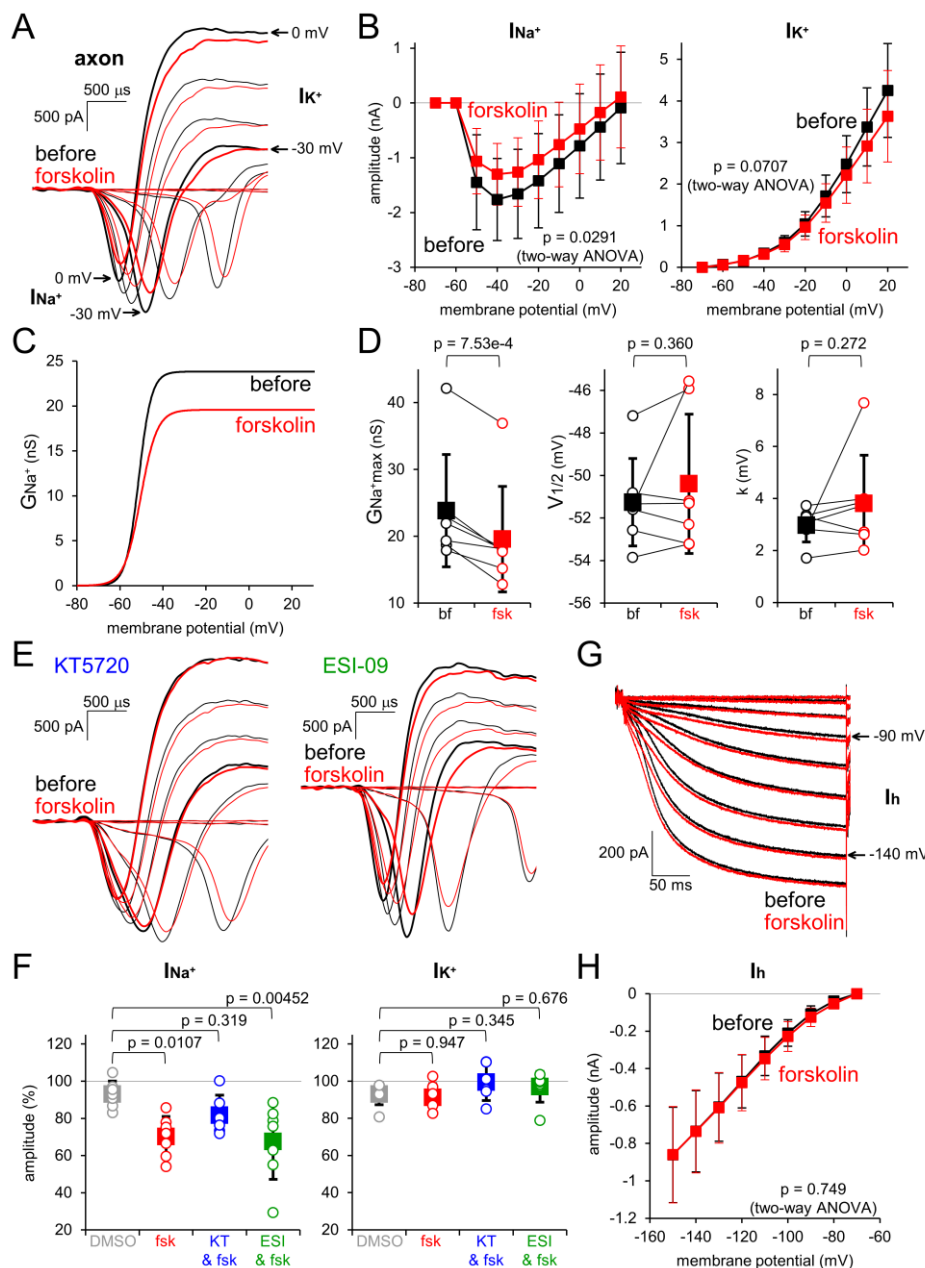


Figure 5. Reduction of PC axonal Na^+ currents by cAMP

A, B, Representative traces (A) and current-voltage (I-V) relations (B) of I_{Na^+} and I_{K^+} recorded from a PC axon upon depolarization steps before and after the forskolin application. $n = 7$. **C, D**, Voltage-dependent activation profile of axonal Na^+ conductance (C) and three parameters (D): peak conductance (G_{Na^+max} , left), half-maximal activation voltage ($V_{1/2}$, middle), and slope factor (k , right) before (bf) and after the forskolin (fsk) application. Three parameters were calculated from the recorded dataset shown in B. **E, F**, Representative traces (E) and normalized amplitudes (F) of axonal I_{Na^+} and I_{K^+} (at -20 mV and 0 mV, respectively) before and after the forskolin application in the absence or presence of KT5720 (KT) or ESI-09 (ESI). Dunnett's test was used for statistical comparison. $n = 7$ (DMSO), 7 (fsk), 6 (KT & fsk), and 7 (ESI & fsk). Data for individual cells (open circles) and mean $\pm$ SD (closed squares) are shown. **G, H**, Representative traces (G) and I-V relations (H) of axonal I_h upon hyperpolarization steps before and after the forskolin application. See also Table 1.

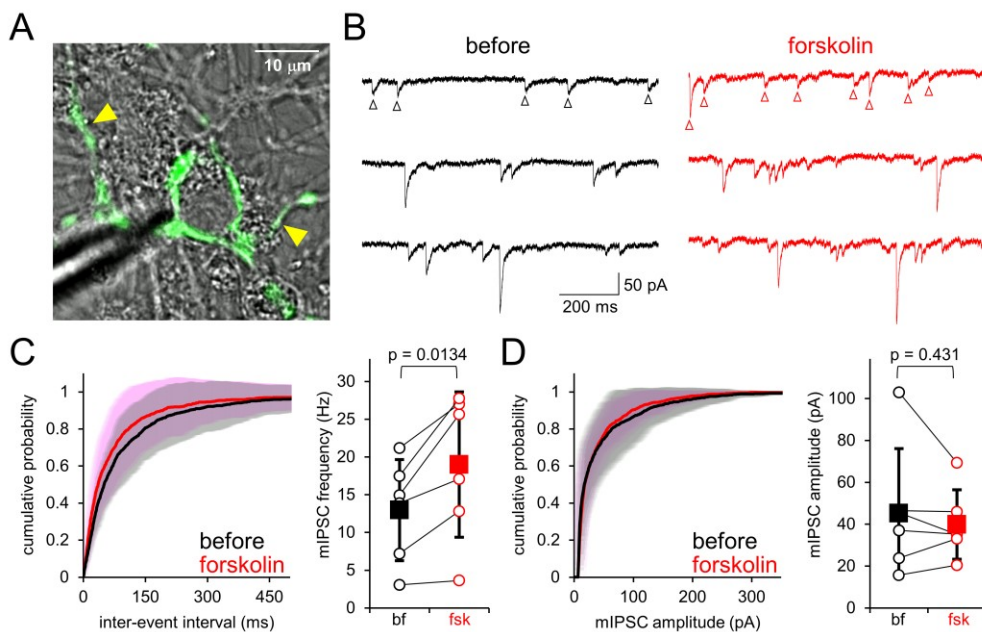


Figure 6. cAMP increases mIPSC frequency without changing amplitude in PCs' target neurons

A, Image of mIPSC recordings from a PCs' target neuron surrounded by EGFP-positive PC boutons. Yellow arrowheads show PC axons. **B**, Representative traces of mIPSCs (open arrowheads) before and after the forskolin application. **C**, **D**, Left, Cumulative probability distribution of inter-event intervals (**C**) and amplitudes (**D**) of mIPSCs before and after the forskolin application. SD is also shown by thin colors. Right, Frequency (**C**) and amplitude (**D**) of mIPSCs before (bf) and after the forskolin (fsk) application. $n = 6$. Data for individual cells (open circles) and mean $\pm$ SD (closed squares) are shown.

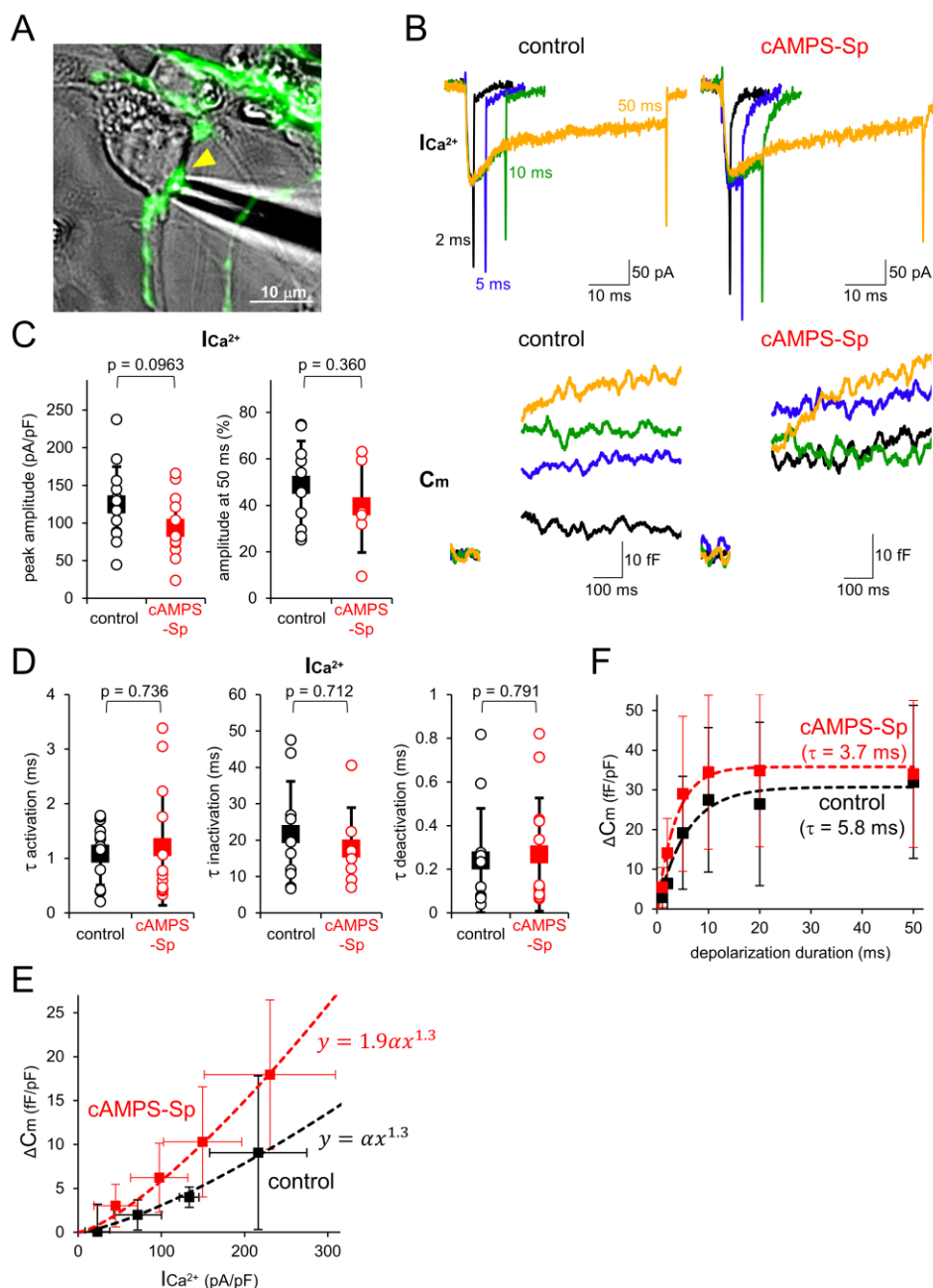


Figure 7. cAMP facilitates release without changing presynaptic Ca^{2+} influx and RRP

A, Direct patch-clamp recording from a presynaptic PC bouton (yellow arrowhead). **B**, Representative presynaptic $I_{Ca^{2+}}$ (top) and C_m increase (bottom) upon 2, 5, 10 and 50 ms depolarization (to 0 mV) without (control) or with cAMPS-Sp. **C**, $I_{Ca^{2+}}$ amplitudes at the peak (left) or at 50 ms relative to the peak (right) during the depolarization pulse without or with cAMPS-Sp. **D**, Time constants for activation (D, left), inactivation (D, middle), and deactivation (D, right) of $I_{Ca^{2+}}$ without or with cAMPS-Sp. Data for individual cells (open circles) and mean $\pm$ SD (closed squares) are shown. **E**, Relationship between the maximal $I_{Ca^{2+}}$ (tail current) and the C_m increase upon 1 or 2 ms depolarization pulses without or with cAMPS-Sp. C_m increase showed the 1.3th power dependency on $I_{Ca^{2+}}$ (dotted lines). **F**, C_m increase upon 1-50 ms depolarization pulses without or with cAMPS-Sp. $n = 14$ (control) and 13 (cAMPS-Sp) boutons. $I_{Ca^{2+}}$ and C_m increase are normalized by the size of clamped area.

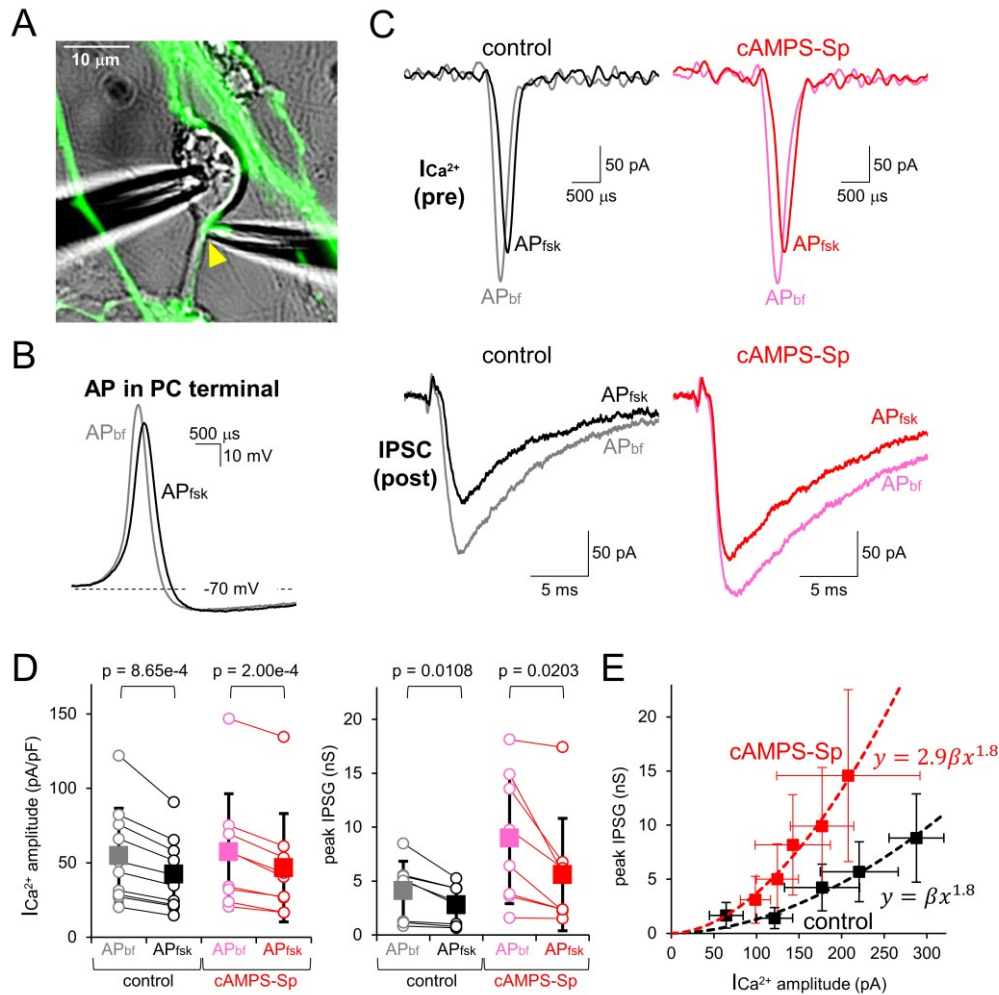
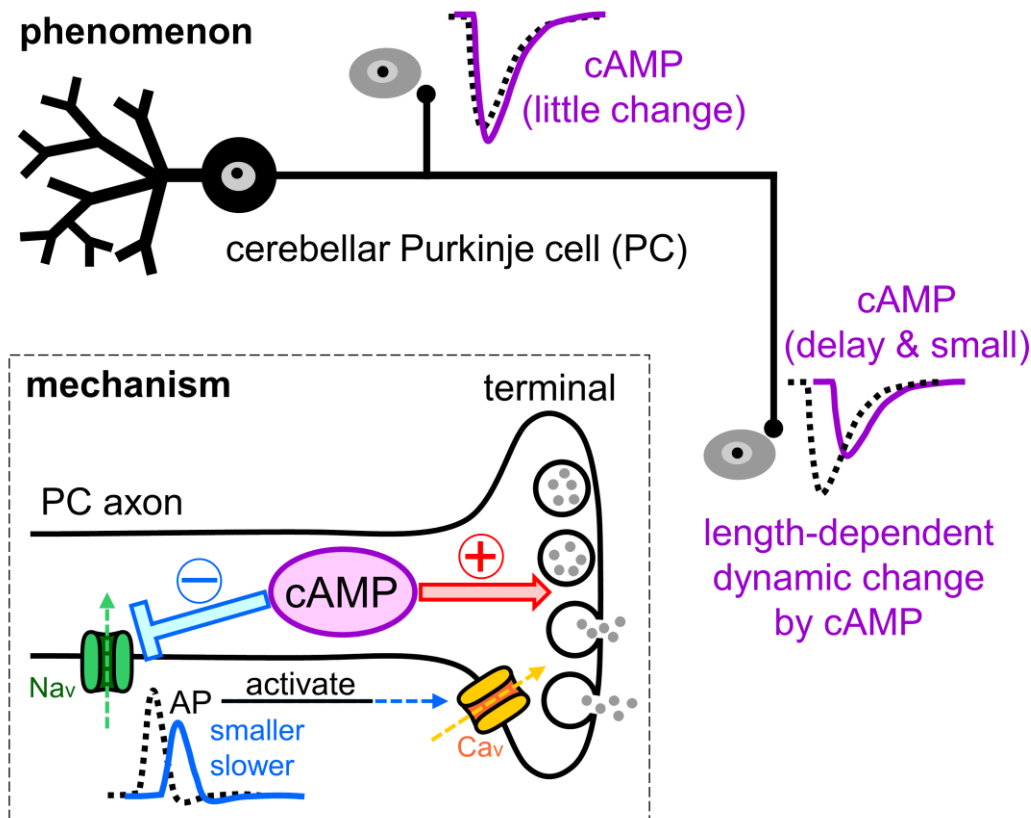


Figure 8. Release augmentation by cAMP counteracts the weakened Ca^{2+} influx and release by AP attenuation

A, Paired recordings from a presynaptic PC bouton (yellow arrowhead) and its postsynaptic neuron. **B**, AP waveforms recorded from a PC terminal before (gray, AP_{bf}) and after (black, AP_{fsk}) the forskolin application. **C-E**, Representative traces (**C**), amplitudes (**D**), and relationship (**E**) of presynaptic $I_{Ca^{2+}}$ and postsynaptic responses upon presynaptic AP command (AP_{bf} or AP_{fsk}) without (control) or with cAMPS-Sp. In **D**, $I_{Ca^{2+}}$ amplitudes are normalized by the size of clamped area. Data for individual cells (open circles) and mean $\pm$ SD (closed squares) are shown. $I_{Ca^{2+}}$: $n = 10$ (control) and 9 (cAMPS-Sp); IPSC: $n = 8$ for each. In **E**, the postsynaptic conductance change (peak IPSC) showed the 1.8th power dependency on $I_{Ca^{2+}}$ (dotted lines).



Abstract figure legend

In this study, we show that amplitude and timing of synaptic outputs are gradually modulated by cAMP in a manner dependent on axonal length in cerebellar Purkinje cells (PCs). Direct patch-clamp recordings from axonal trunks and/or terminals revealed two opposite actions of cAMP on transmitter release at PC axon terminals, giving rise to the axonal length-dependent graded regulation of outputs: (1) attenuation and delay of action potentials (APs) during propagation in a long axon through suppression of axonal voltage-gated Na⁺ channels (Nav), resulting in reduction of presynaptic Ca²⁺ influx via voltage-gated Ca²⁺ channels (Cav); (2) direct facilitation of transmitter release without affecting Cav and readily releasable vesicles.