

Excitatory Effect of GABAergic Axo-Axonic Cells in Cortical Microcircuits

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Axons in the cerebral cortex receive synaptic input at the axon initial segment almost exclusively from γ -aminobutyric acid–releasing (GABAergic) axo-axonic cells (AACs). The axon has the lowest threshold for action potential generation in neurons; thus, AACs are considered to be strategically placed inhibitory neurons controlling neuronal output. However, we found that AACs can depolarize pyramidal cells and can initiate stereotyped series of synaptic events in rat and human cortical networks because of a depolarized reversal potential for axonal relative to perisomatic GABAergic inputs. Excitation and signal propagation initiated by AACs is supported by the absence of the potassium chloride cotransporter 2 in the axon.

The position of AACs is unique in cortical microcircuits for two reasons: AACs exclusively innervate pyramidal cells and do not form synapses on other cells types; and the input to the axon initial segment of pyramidal cells is provided completely by AACs, apart from a few synapses arriving occasionally from basket cells (1–4). The input from AACs is surrounded by a high concentration of sodium channels (5). These synapses are the closest to the suggested site of the action potential initiation (5, 6). The effect of AACs on the postsynaptic cells is mediated by γ -aminobutyric acid type A (GABA_A) receptors. Thus, the polarity of postsynaptic responses is predominantly determined by chloride extrusion mechanisms (7–9) dominated by the potassium chloride cotransporter 2 (KCC2) in the mature cerebral cortex (10). To characterize the function of AACs in cortical microcircuits, we investigated the distribution of KCC2 and the reversal potential of GABAergic inputs in pyramidal cells and revealed that AACs evoke excitatory instead of inhibitory responses. We then confirmed the excitatory action of AACs in cortical networks.

Differential KCC2 expression in distinct types of neurons influences reversal potentials in various brain regions (10–13). However, quantification of this cotransporter in different regions of neurons is lacking. We used high-resolution immunolocalization to determine the subcellular distribution of KCC2 on layer 2/3 pyramidal cells of rat and human cortex (Fig. 1). In the rat, membranes of somata ($n = 38$) and membranes of axon initial segments ($n = 11$) contained higher densities of gold particles compared with background ($P < 0.0001$ for

soma, $P < 0.05$ for axon, Fig. 1B). Comparison of immunogold densities after the subtraction of background labeling (0.04 ± 0.01 gold/ μm^2) showed a decrease by a factor of ~ 44 from somatic to axon initial segment plasma membranes (from 1.34 ± 0.04 to 0.08 ± 0.03 gold/ μm^2 , $P < 0.0001$). Density levels dropped at the border between the hillock and the initial segment and remained stationary on individual axon initial segments measured up to $40 \mu\text{m}$ from the soma. We confirmed our results on the subcellular distribution of KCC2 in the

cortex of a human patient, where a ~ 52 -fold difference in labeling was detected between the membranes of somata ($n = 20$, 1.51 gold/ μm^2) and axon initial segments ($n = 4$, 0.04 gold/ μm^2) after the subtraction of background (0.01 gold/ μm^2).

Low KCC2 density and decreased chloride efflux support higher $[\text{Cl}^-]_{\text{in}}$, leading to depolarizing effects of GABA (7–9, 14, 15). Thus, the low concentration of KCC2 detected in the axon initial segment predicts that responses evoked by AACs are depolarizing at resting membrane potential. We directly compared the reversal potential of GABAergic inputs arriving at the perisomatic region of pyramidal cells elicited by AACs and basket cells in layers 2/3 of rat somatosensory cortex in vitro (Fig. 2A). We performed paired recordings of presynaptic interneurons and postsynaptic pyramidal cells using gramicidin perforated patches in order to keep the postsynaptic $[\text{Cl}^-]_{\text{in}}$ undisturbed. Basket cells ($n = 5$), targeting somata ($23 \pm 7\%$), dendritic shafts ($62 \pm 12\%$), and spines ($15 \pm 8\%$), as examined in the electron microscope, elicited unitary inhibitory postsynaptic potentials (IPSPs) with reversal potentials of -73.3 ± 3.0 mV (range, -67.1 to -76.6 mV). AACs were identified on the basis of light microscopic reconstructions showing axonal candles or cartridges specific to this cell type (3, 4) (Fig. 2B). The

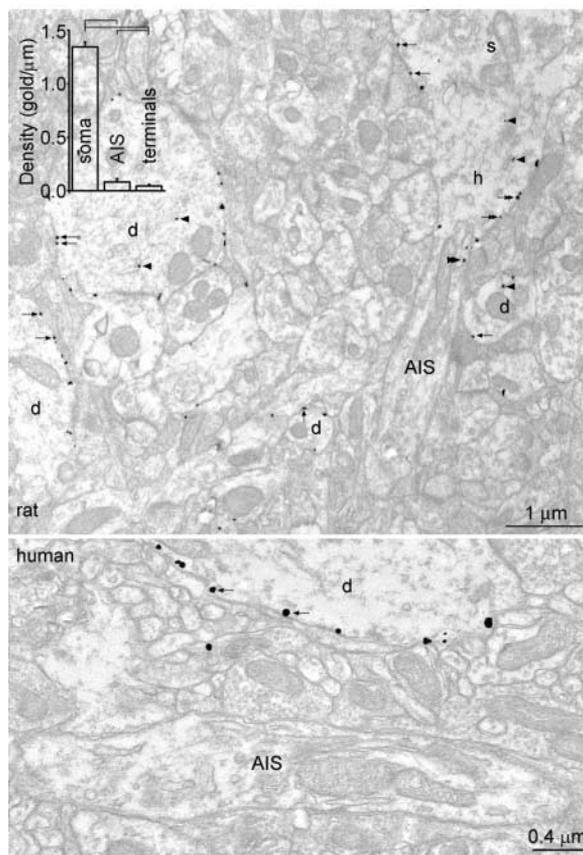


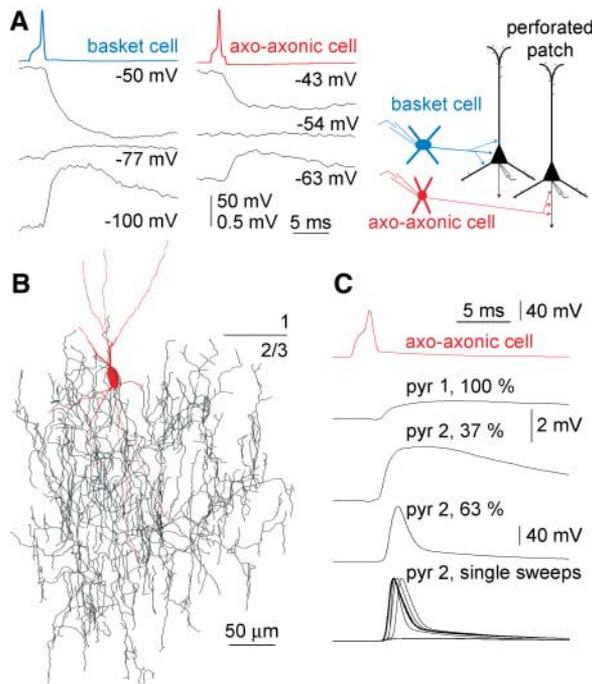
Fig. 1. Localization of KCC2 immunoreactivity in layer 2/3 of rat (top) and human (bottom) cerebral cortex. Gold particles labeling KCC2 (arrows) are predominantly found along somatic (s) and dendritic (d) membranes and cytoplasm (arrowheads). Gold particles (double arrows) are also attached to the membrane of the axon hillock (h), but the density of gold particles (double arrowheads) drops on the axon initial segment (AIS). (Inset) Quantitative evaluation of immunogold distribution of KCC2 on different subcellular compartments of cortical pyramidal cells. Bars indicate significant differences.

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Fig. 2. Depolarizing effect of AACs. **(A)** Unitary interactions from basket and AACs to pyramidal cells reveal different reversal potentials for perisomatic and axonal GABAergic inputs (-72 and -54 mV, respectively). **(B)** Reconstruction of an AAC in the layer 2/3 of somatosensory cortex of the rat (soma and dendrites, red; axons, black). **(C)** Simultaneous triple recording of an AAC (red) and two pyramidal cells (black, pyr 1 and 2) recorded with axonal $[Cl^-]_i$ in whole-cell mode. Single action potentials in the AAC elicited subthreshold depolarizing GABAergic postsynaptic potentials (dIPSPs) on pyramid 1 and triggered postsynaptic action potentials in the majority of trials on pyramid 2 from resting membrane potential.



reversal potential of GABAergic responses evoked by AACs was more depolarized ($n = 7$; -51.1 ± 5.4 mV; range: -42.5 to -58.3 mV, $P < 0.001$). Experiments in which the postsynaptic cells were recorded in whole-cell configuration with an intracellular solution producing a reversal potential of -48.2 ± 4.4 mV ($n = 9$) for GABAergic responses (Fig. 2B). Applying these conditions, AACs evoked exclusively subthreshold responses from the resting membrane potential (-72 ± 2 mV) in 15 pyramidal cells with amplitudes of 0.75 ± 0.4 mV. However, in two pyramidal cells in which the amplitude of the subthreshold responses was 3.1 and 1.2 mV, single spikes in two presynaptic AACs elicited postsynaptic action potentials with a probability of 62 and 57%, peak latencies of 2.48 and 2.61 ms, and a temporal variance of 0.35 and 0.58 ms, respectively. Inputs from basket cells could not evoke action potentials at similar circumstances in spite of having a larger amplitude 2.53 ± 1.81 (range, 0.33 to 5.43 mV, $n = 33$).

The experiments described above show that AACs elicit depolarizing, excitatory responses at axon initial segments of postsynaptic pyramidal cells. Although our whole-cell recordings suggest that spike-to-spike coupling might occur between AACs and pyramidal neurons, a clear demonstration of spike transmission requires experiments in which the pyramidal cells are undisturbed. Thus, we looked for the synaptic output of pyramidal neurons left unrecorded in the network to detect their firing (16, 17). In supragranular cortical lay-

ers, pyramidal neurons are the exclusive locally triggered sources of glutamatergic excitatory postsynaptic potentials (EPSPs) and the only targets of AACs (4). EPSPs triggered by AACs are therefore sufficient for detecting pyramidal cell firing. We applied simultaneous recordings from up to four neighboring AAC and various cortical neurons using an intracellular solution with low $[Cl^-]_i$ at membrane potentials of -56 ± 3 mV in order to easily discriminate EPSPs and IPSPs in the rat and human cortex (Fig. 3). Single spikes in rat AACs triggered temporally fixed series of multiple synaptic events with reliabilities characteristic to unitary, single cell-to-single cell transmission (Fig. 3, A to E). 22 out of 48 AACs (46%) elicited short latency (0.81 ± 0.31 ms) unitary IPSPs in pyramidal cells ($n = 9$), which were followed by longer latency (3.71 ± 0.90 ms, $P < 0.001$), presumably disynaptic EPSPs on the same ($n = 3$) or different ($n = 5$) pyramidal cells and on various interneurons ($n = 19$). Application of the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) ($10 \mu\text{M}$) reversibly blocked these EPSPs ($n = 3$, Fig. 3, E and F). Disynaptic EPSPs were highly reliable (failure rate: $25.8 \pm 20.7\%$), and their kinetic properties were similar to monosynaptic EPSPs evoked by pyramidal cells on the same types of postsynaptic neurons. Disynaptic, recurrent EPSPs were detected while we recorded the firing pattern of several AACs ($n = 18$, Fig. 3B) and were similar to depolarizing afterpotentials found in hippocampal AACs (18). In these cells, as in the hippocampal AACs, spike doublets

were also identified during spontaneous firing. Abandoning and repatching of the recorded AACs ($n = 4$) with a new pipette did not change the identified synaptic signal sequences, which excluded the possibility that multiple events could be activated by interactions between the recording pipette and parts of different cells (Fig. 3C). Pharmacological experiments showed that AACs are capable of recruiting local glutamatergic cells through GABAergic synapses ($n = 7$, Fig. 3, E and F). The specific GABA_A receptor antagonist gabazine ($20 \mu\text{M}$) reversibly blocked monosynaptic IPSPs, as well as disynaptic EPSPs. The dual sensitivity of disynaptic EPSPs to gabazine and NBQX rules out the involvement of gap junctions in the underlying circuit. We also recorded from human layer 2/3 axo-axonic cells ($n = 4$) and confirmed GABAergic recruitment of local pyramidal cells (Fig. 3D). Moreover, a human example also showed that second-order spikes in pyramidal cells initiated by axo-axonic GABAergic synapses project powerfully to neighboring interneurons and trigger third-order action potentials. Third-order feedback EPSPs similar to depolarizing afterpotentials were also detected in human AACs.

Our results show that instead of exclusively inhibiting the axon initial segment, AACs can act as unique excitatory neurons in the cortex. High sodium channel density and/or sodium channels with a shifted voltage dependence at relatively proximal parts of the axon (5, 19) and the high-density synaptic input in an area with a very high surface-to-volume ratio favor the output-generating effectiveness of depolarizing inputs from AACs. The unique proximity of input from AACs to the first axonal branch point suggested as the site of spike initiation in neurons might also contribute to the generation of AAC-triggered EPSPs in the network (6). Although ephaptic or gap-junctional coupling was proposed for functional interaction between axons as a mechanism for signal propagation in neural circuits (17), the dual sensitivity to GABA_A and AMPA receptor antagonists of network events triggered by AACs shows the requirement of chemical synapses. The ionic equilibrium for GABAergic inputs is dynamically regulated by cellular and network mechanisms (7–9, 14). Intraneuronal chloride gradients are important in neural computation (12, 13) and depolarized GABA reversal potentials in interneurons could lead to GABAergic excitation within networks of GABAergic cells (10, 13, 20). The membrane potential of pyramidal cells and thus the effectiveness of axo-axonic spike transmission is regulated by local circuits through dynamic

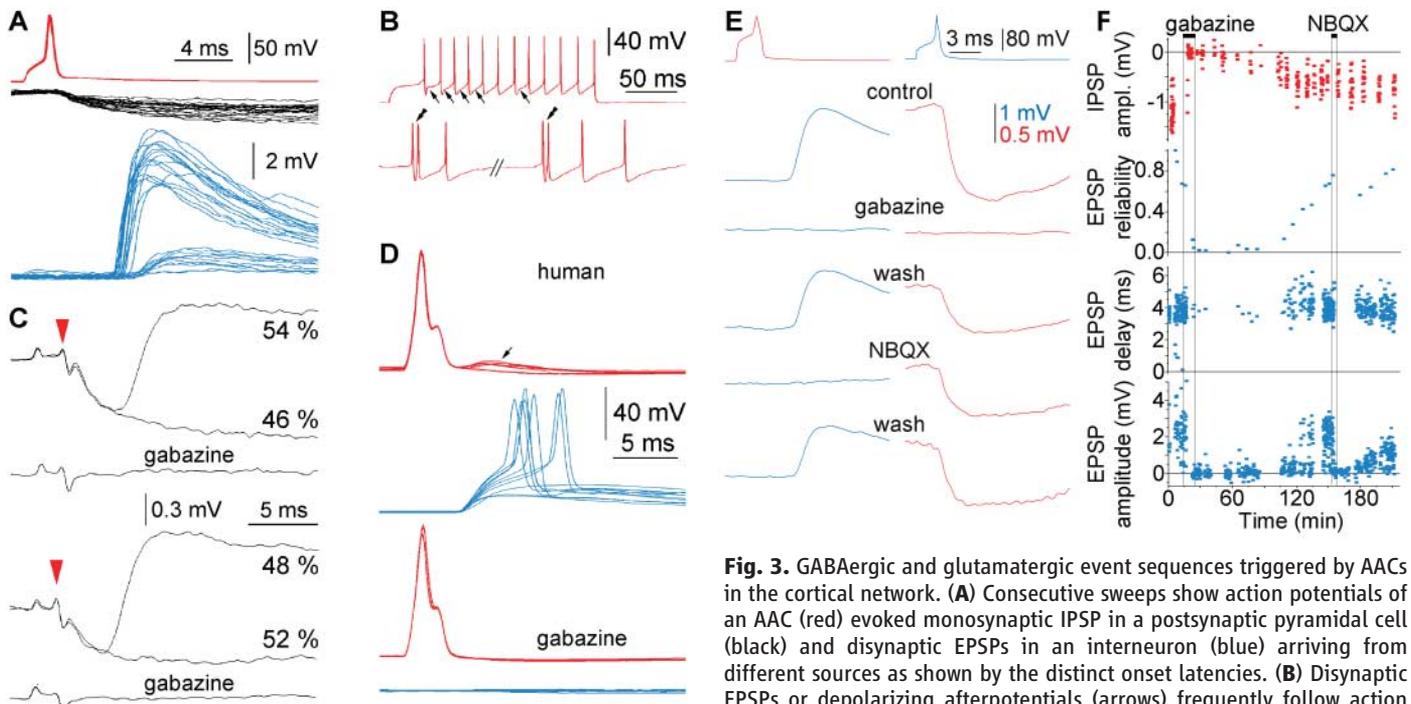


Fig. 3. GABAergic and glutamatergic event sequences triggered by AACs in the cortical network. **(A)** Consecutive sweeps show action potentials of an AAC (red) evoked monosynaptic IPSP in a postsynaptic pyramidal cell (black) and disynaptic EPSPs in an interneuron (blue) arriving from different sources as shown by the distinct onset latencies. **(B)** Disynaptic EPSPs or depolarizing afterpotentials (arrows) frequently follow action potentials elicited by current injections in AACs (top) and occasional spike doublets occur (double arrowheads) during spontaneous firing (bottom). **(C)** Spikes in an AAC (red triangles) elicited monosynaptic IPSPs, which were followed by convergent disynaptic EPSPs in 54% of trials in a pyramidal cell (black). Application of gabazine (20 μ M) blocked IPSPs, as well as the EPSP. Repatching the presynaptic cell during washout with a different recording pipette did not alter the IPSP-EPSP sequence and its sensitivity to gabazine. **(D)** A human axo-axonic cell (red) shows recurrent, disynaptic EPSPs (arrow) on single consecutive sweeps following the action potential, which elicited disynaptic EPSPs capable of initiating third-order spikes in a simultaneously recorded interneuron (blue). Disynaptic EPSPs and downstream spikes were blocked by gabazine (20 μ M). **(E and F)** Both GABAergic and glutamatergic mechanisms are necessary for reliable triggering of disynaptic EPSPs. **(E)** An interneuron (blue) elicited a unitary IPSP in an AAC (red), and the AAC triggered a disynaptic EPSP in the interneuron. Application of gabazine (20 μ M) blocked both responses (averages of 20 traces) with a recovery on washout. NBQX (10 μ M) reversibly eliminated the disynaptic EPSP and had no effect on the IPSP. **(F)** The reliability (averages of ten consecutive sweeps) and single amplitudes of the disynaptic EPSPs showed recovery in parallel with the amplitude of the IPSP, but the onset delay of disynaptic EPSPs remained relatively stationary.

spike doublets occur (double arrowheads) during spontaneous firing (bottom). **(C)** Spikes in an AAC (red triangles) elicited monosynaptic IPSPs, which were followed by convergent disynaptic EPSPs in 54% of trials in a pyramidal cell (black). Application of gabazine (20 μ M) blocked IPSPs, as well as the EPSP. Repatching the presynaptic cell during washout with a different recording pipette did not alter the IPSP-EPSP sequence and its sensitivity to gabazine. **(D)** A human axo-axonic cell (red) shows recurrent, disynaptic EPSPs (arrow) on single consecutive sweeps following the action potential, which elicited disynaptic EPSPs capable of initiating third-order spikes in a simultaneously recorded interneuron (blue). Disynaptic EPSPs and downstream spikes were blocked by gabazine (20 μ M). **(E and F)** Both GABAergic and glutamatergic mechanisms are necessary for reliable triggering of disynaptic EPSPs. **(E)** An interneuron (blue) elicited a unitary IPSP in an AAC (red), and the AAC triggered a disynaptic EPSP in the interneuron. Application of gabazine (20 μ M) blocked both responses (averages of 20 traces) with a recovery on washout. NBQX (10 μ M) reversibly eliminated the disynaptic EPSP and had no effect on the IPSP. **(F)** The reliability (averages of ten consecutive sweeps) and single amplitudes of the disynaptic EPSPs showed recovery in parallel with the amplitude of the IPSP, but the onset delay of disynaptic EPSPs remained relatively stationary.

changes in the proportional balance of in excitation and inhibition during diverse cortical tasks (21, 22). Our results paradoxically suggest that postsynaptic hyperpolarization or “down” state and sodium channel deactivation helps axo-axonic spike triggering, but “up” states, depolarization, and sodium channel inactivation could limit axo-axonic inputs to shunting or hyperpolarizing. AACs, therefore, might have a bistable role in neural circuits. Indeed, firing of AACs stereotypically precedes or follows activation of pyramidal cells depending on the operational state of the network in vivo (23, 24). Simultaneous activation of a fraction of the several hundred postsynaptic pyramids innervated by a single AAC (3, 4) could lead to synchronous recruitment of network activity as observed during the onset of cortical ripples (24). AACs do not target GABAergic interneurons (3, 4); therefore, they are well suited for initiating repeatable event sequences in the cortical microcircuit (25, 26) through selective spike triggering in pyramidal cells followed by downstream recruitment of inhibition that

enforces spatiotemporal fidelity in signal propagation (27, 28).

References and Notes

- J. Szentagothai, M. A. Arbib, *Neurosci. Res. Program Bull.* **12**, 305 (1974).
- P. Somogyi, *Brain Res.* **136**, 345 (1977).
- T. F. Freund, G. Buzsáki, *Hippocampus* **6**, 347 (1996).
- P. Somogyi, G. Tamas, R. Lujan, E. H. Buhl, *Brain Res. Brain Res. Rev.* **26**, 113 (1998).
- C. M. Colbert, D. Johnston, *J. Neurosci.* **16**, 6676 (1996).
- B. A. Clark, P. Monsivais, T. Branco, M. London, M. Hausser, *Nat. Neurosci.* **8**, 137 (2005).
- U. Misgeld, R. A. Deisz, H. U. Dodt, H. D. Lux, *Science* **232**, 1413 (1986).
- K. J. Staley, B. L. Soldo, W. R. Proctor, *Science* **269**, 977 (1995).
- J. A. Payne, C. Rivera, J. Voipio, K. Kaila, *Trends Neurosci.* **26**, 199 (2003).
- C. Rivera *et al.*, *Nature* **397**, 251 (1999).
- M. Martina, S. Royer, D. Pare, *J. Neurophysiol.* **86**, 2887 (2001).
- A. Gulacsi *et al.*, *J. Neurosci.* **23**, 8237 (2003).
- K. E. Gavrikov, A. V. Dmitriev, K. T. Keyser, S. C. Mangel, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 16047 (2003).
- B. E. Alger, R. A. Nicoll, *Nature* **281**, 315 (1979).
- A. T. Gullledge, G. J. Stuart, *Neuron* **37**, 299 (2003).
- B. Katz, R. Mileti, *J. Physiol.* **192**, 407 (1967).
- D. Debanne, *Nat. Rev. Neurosci.* **5**, 304 (2004).
- E. H. Buhl *et al.*, *J. Neurophysiol.* **71**, 1289 (1994).
- G. Stuart, N. Spruston, B. Sakmann, M. Hausser, *Trends Neurosci.* **20**, 125 (1997).
- J. Chavas, A. Marty, *J. Neurosci.* **23**, 2019 (2003).
- M. Steriade, A. Nunez, F. Amzica, *J. Neurosci.* **13**, 3252 (1993).
- Y. Shu, A. Hasenstaub, D. A. McCormick, *Nature* **423**, 288 (2003).
- Y. Zhu, R. L. Stornetta, J. J. Zhu, *J. Neurosci.* **24**, 5101 (2004).
- T. Klausberger *et al.*, *Nature* **421**, 844 (2003).
- M. Abeles, *Corticonics* (Cambridge Univ. Press, Cambridge, 1991).
- Y. Ikegaya *et al.*, *Science* **304**, 559 (2004).
- F. Pouille, M. Scanziani, *Nature* **429**, 717 (2004).
- R. D. Traub, M. A. Whittington, I. M. Stanford, J. G. Jefferys, *Nature* **383**, 621 (1996).
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References

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