Dendritic compartmentalization of chloride cotransporters underlies directional responses of starburst amacrine cells in retina

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Neurons that respond selectively to the direction of stimulus motion are a common feature of the nervous system. In the vertebrate retina, directionally selective (DS) ganglion cells respond well to stimulus motion in one (preferred) direction, but respond little or not at all to motion in the opposite (null) direction (1). Starburst amacrine cells (SACs), interneurons presynaptic to the DS ganglion cells, are an essential component of the mechanism that generates direction selectivity in the retina because selective elimination of this interneuron eliminates the directional light responses of DS ganglion cells (2, 3). Moreover, SAC dendrites generate DS light responses (4, 5), providing DS input to DS ganglion cells (5, 6). They hyperpolarize to stimuli that move centripetally from the periphery to their proximal dendrites but depolarize to stimuli that move centrifugally from their proximal dendrites to the periphery (5). However, although the DS light responses of SAC dendrites may function as the core directional process in the retina, the mechanisms that underlie them are not known.

In the retina, pharmacological studies have indicated that activation of GABA_A receptors, which open Cl⁻ channels, mediates direction selectivity (7). The cation-chloride cotransporters Na-K-2Cl (NKCC) and K-Cl (KCC) mediate the depolarizing and hyperpolarizing effects of GABA, respectively (8–11). NKCC transports Cl⁻ into cells so that the Cl⁻ equilibrium potential (ECr) is more positive than the resting membrane potential, resulting in a GABA-evoked depolarization. In contrast, KCC extrudes Cl⁻ so that the ECr is more negative than the resting membrane potential, resulting in a GABA-evoked hyperpolarization. Because selective blockade of NKCC and KCC by the loop diuretics bumetanide (BMN) and furosemide (FUR), respectively, and reduction of the transmembrane Cl⁻ gradient eliminates the directional responses of DS ganglion cells and SACs (5), we investigated how NKCC and KCC activity and localization underlie the directional responses of SACs.

Results

Light Responses of SACs to Moving Stimuli. SACs express GABA and glutamate receptors along their dendrites (12–14), and their light responses exhibit a glutamate-mediated center and a GABA-mediated surround receptive field (RF) organization (15–17). Fig. 1 shows that the light responses of SACs to moving stimuli consist of two components, a slow, GABA-mediated component that is DS and primarily responsive to surround illumination, and a faster, glutamate-mediated component that is responsive to central illumination, but not DS. Fig. 14 illustrates that when a slit moved through the RF center and surround, both the slow DS response component and the faster, non-DS component were produced. Fig. 1B shows that the SAC did not generate the fast depolarization (or the fast OFF hyperpolarization) if the slit did not stimulate the RF center. That is, the SAC produced a slow hyperpolarization to a slit stimulus that moved centripetally from the periphery through the surround but did not reach the RF center, and produced a slow depolarization when the slit stimulus reversed direction and moved centrifugally. The slow hyperpolarization and slow depolarization to stimulus motion in the surround in the centripetal and centrifugal directions, respectively, represent the DS light response of the SAC dendrite.

Dark-adapted SACs exhibited similar resting membrane potentials and responses to moving slit stimuli when recorded with whole-cell patch-clamp electrodes (Fig. 1C) and with intracellular sharp microelectrodes (Fig. 1D), a means of monitoring neurons that does not significantly alter their intracellular milieu. Specifically, the average dark resting potential (see Materials and Methods) and light response amplitudes and dynamics (see Fig. 1 C and D) of SACs were similar when recordings were

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Abbreviations: BMN, bumetanide; ChAT, choline acetyltransferase; DS, directionally selective; FUR, furosemide; RF, receptive field; SAC, starburst amacrine cell.

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obtained with sharp intracellular and whole-cell patch-clamp electrodes, a finding that strongly suggests that our whole-cell patch-clamp recording data reveal the electrical characteristics and light response properties of in vivo SACs.

Starburst Cells Exhibit Both NKCC and KCC Activity and Expression. Although previous results demonstrated that NKCC and KCC contribute to the DS responses of SACs (5), they did not show whether the cotransporters are expressed by the SACs themselves, or alternatively, whether they mediate the directional responses of SACs through the retinal network. We thus investigated whether BMN and FUR altered the DS responses of individual SAC dendrites in the intact rabbit retina when the drugs were introduced into the cells via the patch pipettes during whole-cell recording so that only the cotransporters on the cell under study were selectively blocked. As shown in Fig. 2, pipette application of either drug eliminated or greatly reduced the DS light responses of SAC dendrites (A–D) and the initial transient hyperpolarization (C and D), but it did not eliminate the non-DS, RF center light response that is likely glutamate-mediated (C and D). In addition, FUR (25 μM) depolarized (average depolarization = 4.2 ± 1.5 mV, n = 28) and BMN (10 μM) hyperpolarized (average hyperpolarization = −17.3 ± 2.6 mV, n = 13) every cell studied. These results indicate that SAC dendrites exhibit both NKCC and KCC activity and that NKCC and KCC are tonically and highly active and work together to contribute to the DS responses of SACs (5), they did not show whether the cotransporters are expressed by the SACs themselves, or alternatively, whether they mediate the directional responses of SACs through the retinal network. We thus investigated whether BMN and FUR altered the DS responses of individual SAC dendrites in the intact rabbit retina when the drugs were introduced into the cells via the patch pipettes during whole-cell recording so that only the cotransporters on the cell under study were selectively blocked. As shown in Fig. 2, pipette application of either drug eliminated or greatly reduced the DS light responses of SAC dendrites (A–D) and the initial transient hyperpolarization (C and D), but it did not eliminate the non-DS, RF center light response that is likely glutamate-mediated (C and D). In addition, FUR (25 μM) depolarized (average depolarization = 4.2 ± 1.5 mV, n = 28) and BMN (10 μM) hyperpolarized (average hyperpolarization = −17.3 ± 2.6 mV, n = 13) every cell studied. These results indicate that SAC dendrites exhibit both NKCC and KCC activity and that NKCC and KCC are tonically and highly active and work together to contribute to the DS responses of SACs (5), they did not show whether the cotransporters are expressed by the SACs themselves, or alternatively, whether they mediate the directional responses of SACs through the retinal network. We thus investigated whether BMN and FUR altered the DS responses of individual SAC dendrites in the intact rabbit retina when the drugs were introduced into the cells via the patch pipettes during whole-cell recording so that only the cotransporters on the cell under study were selectively blocked. As shown in Fig. 2, pipette application of either drug eliminated or greatly reduced the DS light responses of SAC dendrites (A–D) and the initial transient hyperpolarization (C and D), but it did not eliminate the non-DS, RF center light response that is likely glutamate-mediated (C and D). In addition, FUR (25 μM) depolarized (average depolarization = 4.2 ± 1.5 mV, n = 28) and BMN (10 μM) hyperpolarized (average hyperpolarization = −17.3 ± 2.6 mV, n = 13) every cell studied. These results indicate that SAC dendrites exhibit both NKCC and KCC activity and that NKCC and KCC are tonically and highly active and work together to contribute to the DS responses of SACs (5), they did not show whether the cotransporters are expressed by the SACs themselves, or alternatively, whether they mediate the directional responses of SACs through the retinal network. We thus investigated whether BMN and FUR altered the DS responses of individual SAC dendrites in the intact rabbit retina when the drugs were introduced into the cells via the patch pipettes during whole-cell recording so that only the cotransporters on the cell under study were selectively blocked. As shown in Fig. 2, pipette application of either drug eliminated or greatly reduced the DS light responses of SAC dendrites (A–D) and the initial transient hyperpolarization (C and D), but it did not eliminate the non-DS, RF center light response that is likely glutamate-mediated (C and D). In addition, FUR (25 μM) depolarized (average depolarization = 4.2 ± 1.5 mV, n = 28) and BMN (10 μM) hyperpolarized (average hyperpolarization = −17.3 ± 2.6 mV, n = 13) every cell studied. These results indicate that SAC dendrites exhibit both NKCC and KCC activity and that NKCC and KCC are tonically and highly active and work together to contribute to the DS responses of SACs (5), they did not show whether the cotransporters are expressed by the SACs themselves, or alternatively, whether they mediate the directional
investigated whether their DS light responses are mediated sections clearly showed NKCC2 labeling on the cell body (varicosities (Fig. 4 highest density of KCC2 label was on the distal dendrites and Gavrikov et al. inuition of the label more distally (Fig. 4 proximal dendrites of Alexa-filled SACs with progressive dim- and the retinas were then stained with antibodies against NKCC2 (types 1–4). ChAT-labeled displaced SACs showed specific NKCC2 (C) and KCC2 (F) labeling. Moreover, transverse vibratome sections clearly showed NKCC2 labeling on the cell body (+) and proximal dendrites (arrows) of SACs (A–C) and faint KCC2 labeling on SAC cell bodies (+) (F). Images represent single 1-μm-thick optical slices. (Scale bars: 10 μm.)

generate the DS responses of SAC dendrites by driving $E_C$ in the positive and negative directions, respectively.

Because rabbit SACs express GABA$_A$ receptors (12, 14), we investigated whether their DS light responses are mediated by endogenous GABA$_A$ receptor activation. Application of gabazine (SR95331, 50–100 μM), a specific GABA$_A$ receptor antagonist, consistently hyperpolarized the cells by an average of 6.5 ± 2.4 mV ($n = 10$), eliminated the hyperpolarizing response elicited by annular stimulation of the RF surround (Fig. 2F) and eliminated their DS light responses, but did not eliminate the non-DS, RF center light response (Fig. 2E) that is likely glutamate-mediated. In contrast, application of TPMPA (50 μM), a specific GABA$_C$ receptor antagonist, had little effect (data not shown).

To identify the specific subtypes of NKCC and KCC expressed by SACs, rabbit retinas were double-labeled with antibodies against choline acetyltransferase (ChAT), which specifically stains SACs (18, 19), and against NKCC (types 1 and 2) or KCC (types 1–4). ChAT-labeled displaced SACs showed specific NKCC2 (Fig. 3C) and KCC2 (Fig. 3F) labeling. Similar NKCC2 and KCC2 labeling was also observed on ChAT-labeled SACs in the amacrine cell layer (data not shown). In contrast, NKCC1, KCC1, KCC3, and KCC4 labeling was not observed on SACs (data not shown).

**Differential Dendritic Compartmentalization of NKCC2 and KCC2.**

Because SACs express both NKCC2 and KCC2 (Fig. 3) and because selective blockade of NKCC2 and KCC2 activity eliminates the DS light responses of SAC dendrites (Fig. 2), we examined whether NKCC2 and KCC2 are differentially located on the proximal and distal portions of SAC dendrites, respectively. Individual displaced SACs were injected with Alexa 488, and the retinas were then stained with antibodies against NKCC2 or KCC2. The highest density of NKCC2 label was on the proximal dendrites of Alexa-filled SACs with progressive diminution of the label more distally (Fig. 4B). In contrast, the highest density of KCC2 label was on the distal dendrites and varicosities (Fig. 4D). Quantification of the NKCC2 and KCC2 staining patterns on SAC dendrites indicates that different dendritic compartments express NKCC2 and KCC2, with NKCC2 predominating proximally and KCC2 predominating distally (Fig. 4E).

Because NKCC2 and KCC2 are preferentially located on the proximal and distal portions of SAC dendrites, respectively, the [Cl$^-$], should be lower in the distal compared with the proximal dendrites; and as a result, $E_{GABA}$ should be more negative distally than proximally. We thus measured $E_{GABA}$ along both the proximal and more distal portions of individual SAC dendrites after synaptic block. GABA application onto the proximal portion of the SAC dendritic tree evoked responses that consistently reversed at a holding potential ~10 mV more positive than what occurred when GABA was puffed onto a more distal portion of the dendritic tree of the same cell (Fig. 5A–C).

Because KCC2 is preferentially located on SAC distal dendrites (Fig. 4), selective blockade of KCC2 with FUR should shift $E_{GABA}$ at the distal dendrite to a more positive value. Average $E_{GABA}$ at the more distal dendrites was ~10 mV more positive after application of 25 μM FUR (Fig. 5D). BMN (10 μM) application did not alter $E_{GABA}$ at the distal dendrites (data not shown). The finding that FUR (25 μM) eliminated the proximal-distal difference in $E_{GABA}$ not only indicates that the more negative $E_{GABA}$ at SAC distal dendrites is mediated by KCC2 but also that the proximal-distal difference in $E_{GABA}$ does not result from a space-clamp or other measurement artifact. In addition, the finding that FUR (25 μM) did not block the effects of GABA (Fig. 5D), that is, GABA evoked voltage responses of similar amplitude when puffed in the presence or absence of FUR, indicates that FUR did not alter the GABA$_A$ receptor-mediated conductance. This is consistent with the finding that SACs express GABA$_A$ receptors that do not contain α6 or α4 subunits (12), because FUR only antagonizes GABA$_A$ receptors that contain α6 and α4 subunits (20). The selective inhibitory effect of FUR (25 μM) for starburst KCC2 compared with NKCC2 is shown by the consistently opposite effects of FUR (25 μM) and BMN (10 μM), a selective inhibitor of NKCC2 at 10 μM (8–11), and can be attributed to the finding that the low concentration (25 μM) of FUR used here is ineffective against many NKCC1 and NKCC2 subtypes (8, 9, 11, 21), suggesting that SACs express a FUR-insensitive NKCC2 subtype.

It is important to note that GABA application to proximal dendrites (Fig. 5A) and onto distal dendrites ~100 μm from the soma (Fig. 5B) produced large amplitude responses, indicating that proximal dendrites and the portion of the dendrites 100 μm from the soma were adequately current clamped and that the $E_{GABA}$ measurements were accurate, although they were likely an underestimate of the actual proximal-distal difference in $E_{GABA}$ (see Materials and Methods). However, GABA application onto dendrites 150–200 μm from the soma produced very small amplitude responses (data not shown), suggesting that this more distal portion was weakly electrically linked with the soma and not adequately clamped. The lack of control of the membrane potential of the more distal dendrites probably resulted from a significant intracellular resistance that accumulates along the dendrites so that the membrane potential of the more distal dendrites is different from that in the soma.

**Discussion**

We have demonstrated in this article that (i) SACs express both NKCC2 and KCC2; (ii) the selective blockade of NKCC2 and KCC2 located on the SAC dendrites eliminates the DS responses of SACs and shows that the cotransporters work in opposition and are tonically and highly active; (iii) NKCC2 and KCC2 are preferentially located on the proximal and distal dendrites, respectively; and (iv) GABA receptor activation at the proximal dendrite produces a depolarization that is mediated by NKCC2, whereas GABA receptor activation at the more distal dendrite...
produces a hyperpolarization that is mediated by KCC2. These findings indicate that the differential distribution of NKCC2 on the proximal dendrites and KCC2 on the distal dendrites of SACs results in a GABA-evoked depolarization and hyperpolarization at the NKCC2 and KCC2 compartments, respectively, and underlies the DS responses of the dendrites. Moreover, the effects of gabazine on SACs strongly support the view that endogenous GABA depolarizes the proximal dendrites and hyperpolarizes the distal dendrites. Specifically, the finding that GABA_A receptor blockade hyperpolarized SACs (Fig. 2 E and F) indicates that the primary effect of endogenous GABA_A receptor activation that is detectable at starburst somata is a sustained depolarization that depends on NKCC2 activity in the central portion of SACs. In contrast, GABA_A receptor blockade eliminated the hyperpolarizing response elicited by annular stimulation of the RF surround (Fig. 2F), indicating that surround activation of GABA_A receptors hyperpolarizes SACs.

Because synaptic vesicles are located at the distal (but not the proximal) portions of SAC dendrites (22), GABA release (23–25) from the SAC distal dendrite will likely occur when the dendrite is depolarized by light stimuli that move in the centrifugal direction. In contrast, GABA release from the distal SAC dendrite will be minimal when the dendrite is hyperpolarized by light stimuli that move in the centripetal direction. The directional release of GABA from SAC dendrites that point in the null direction of DS ganglion cells and that are located on the null side of the ganglion cells will thus confer null direction inhibition on DS ganglion cells (5, 6).

Although our results indicate that NKCC2 and KCC2 on SAC dendrites work in opposition and are tonically and highly active (Fig. 2), the degree to which they drive local $E_{Cl}$ along the dendrites is uncertain. It is theoretically possible for NKCC2, which utilizes the average transmembrane cation ($Na^+$ and $K^+$) gradient, to drive $E_{Cl}$ at the proximal dendrite to $(E_{Na} + E_{K})/2$ ($= −31.9$ mV in our experimental conditions), and for KCC2, which utilizes the transmembrane K$^+$ gradient, to drive $E_{Cl}$ at the distal dendritic tip to $E_{K}$ ($= −94.7$ mV in our experimental conditions). The finding that blockade of KCC2 by FUR depolarized SACs by $≈4$ mV and that blockade of NKCC2 by BMN hyperpolarized SACs by $≈17$ mV (Fig. 2) suggests that the difference in local $E_{Cl}$ between the proximal and distal portions of the dendrites is at least 21 mV. However, a proximal-distal difference of 21 mV would occur if SACs only had a $Cl^-$ conductance. Because SACs, like other neurons, have significant $Na^+$ (26) and $K^+$ conductances (16, 27) in addition to a $Cl^-$ conductance, the $Na^+$ and $K^+$ conductances will reduce the size of the changes in membrane potential that result when BMN and FUR block the $Cl^-$ cotransporters and alter $E_{Cl}$. As a result, the actual proximal-distal dendritic difference in local $E_{Cl}$ should be greater than 21 mV. For example, if the $Cl^-$ conductance were half the total SAC conductance, the proximal-distal difference in local $E_{Cl}$ would double to 42 mV.

In addition to providing a physiological–morphological basis of the DS light responses of SACs, our article demonstrates that different compartments of individual CNS dendrites express two distinct transporter types so that a single neurotransmitter depolarizes and hyperpolarizes the different dendritic compartments. The processes of interneurons in the retina and elsewhere in the CNS may express $Cl^-$ cotransporters as well as other transporter, channel (27), and synaptic proteins in different functional compartments. In fact, it has been reported that single interneurons in the midbrain and hippocampus contain com-
GABA was applied onto the proximal dendrite (A) with a highly radial and symmetric appearance, possesses an subcellular level. This means by which the CNS encodes complex information at the subcellular level. In summary, our findings indicate that the SAC, an interneuronal process could provide a mechanism for the directional action flow/neural activity in one direction will depolarize the neuronal process to distinguish between and compare different GABAergic inputs. Second, spatial segregation of GABA-evoked depolarizing and hyperpolarizing responses along a neuronal process could enable that spatial segregation of GABA-evoked depolarizing and hyperpolarizing responses of individual interneuronal processes in the CNS? First, because GABA puffed on the distal dendrite did not reach the proximal dendrite. Dye included in the puff pipette confirmed this (n = 4). However, the measured proximal-distal difference in $E_{\text{GABA}}$ (see Fig. 5) is likely an underestimate of the actual difference for two reasons. First, because GABA puff application at the proximal dendrite necessarily reached the more distal dendrite, the effective distance between the proximal and distal GABA applications was somewhat less than 100 $\mu$m. Second, although SAC dendrites 100 $\mu$m from the soma were adequately clamped (see Fig. 5), they may not have been as well clamped as the soma, so that the driving force for the $\text{Cl}^-$ current ($V_m - E_{\text{GABA}}$) elicited by GABA application onto this more distal portion of the dendrites may have been slightly attenuated compared with the $\text{Cl}^-$ current elicited by GABA application onto the proximal dendrite (30). In addition, due to the cable properties of the dendrites, the response to GABA, which was measured at the soma, may have been slightly smaller in amplitude when GABA was applied more distally compared with when it was applied proximally. It is thus likely that the difference in $E_{\text{GABA}}$ between the proximal dendrites and the distal dendrites 150–200 $\mu$m from the soma is >10 mV and more closely approximates the difference obtained after application of BMN and FUR (see Discussion).

**Immunohistochemistry.** NKCC2 immunoreactivity was localized with a rabbit polyclonal antiserum to a 15-aa C-terminal sequence of rat NKCC2 (Chemicon AB3562P) (1:200). This amino acid sequence is common to each of the three known rabbit KCC in different dendritic compartments, can mediate neural computations in the brain.

**Materials and Methods**

**Preparation.** Retinal eyecups were obtained after deep general (urethane, 1.5 g/kg, i.p.) and local intraorbital (2% Xylocaine) anesthesia of New Zealand white rabbits (2.5 kg) (5). Animal care and use followed all federal and institutional guidelines. Superfusate (pH 7.4, 34–35°C) contained 117.0 mM NaCl, 3.1 mM KCl, 10.0 mM glucose, 2.0 mM CaCl$_2$, 1.2 mM MgSO$_4$, 30.0 mM NaHCO$_3$, 0.5 mM Na$_2$PO$_4$, and 0.1 mM L-glutamine. SACs were selectively labeled by intraocular injection of 0.3 $\mu$g of DAPI 1 day before experiments (5, 19). DAPI-labeled, displaced SACs were identified with brief UV illumination, and infrared illumination was used for microelectrode manipulation. Biocytin injection confirmed SAC identity.

**Electrophysiology.** Patch-clamp recording of dark-adapted, displaced SACs was used. Whole-cell electrodes, which had resistances of 5–6 M$\Omega$, contained 14.0 mM KCl, 100.0 mM K-glucuronate, 5.0 mM EGTA, 5.0 mM Heps, 3.0 mM MgATP, 0.5 mM Na$_3$GTP, 0.5 mM CaCl$_2$, 20.0 mM Na$_2$-phosphocreatine, and 4.1 mM NaHCO$_3$. The average resting potential of SACs was $-49.8 \pm 1.8$ mV (n = 45). In control experiments (n = 8) in which FUR and BMN were not added to the patch pipettes, the average resting potential and the light responses of the cells remained unchanged for at least 30 min. In control experiments in which sharp microelectrodes were used for intracellular recording, the average resting potential of displaced SACs was $-52.8 \pm 3.4$ mV (n = 6). Sharp microelectrodes contained 1 M K-acetate and had resistances of $\approx 150$ M$\Omega$.

$E_{\text{GABA}}$ was measured by pressure ejecting (6 psi, 100 msec) GABA (0.5 mM) from 5- to 6-$\mu$m-diameter tip pipettes. GABA was applied onto SAC proximal and distal dendrites with synaptic transmission blocked with cobalt (2 mM), while the membrane potential of the cells was shifted between $-90$ and $-40$ mV by using constant current pulses. The potential at which GABA did not evoke a response corresponded to $E_{\text{GABA}}$. In these experiments, the superfusate flowed in the same direction as the centrifugal direction of the dendrite under study so that GABA puffed on the distal dendrite did not reach the proximal dendrite. Dye included in the puff pipette confirmed this (n = 4). Hence, the measured proximal-distal difference in $E_{\text{GABA}}$ (see Fig. 5) is likely an underestimate of the actual difference for two reasons. First, because GABA puff application at the proximal dendrite necessarily reached the more distal dendrite, the effective distance between the proximal and distal GABA applications was somewhat less than 100 $\mu$m. Second, although SAC dendrites 100 $\mu$m from the soma were adequately clamped (see Fig. 5), they may not have been as well clamped as the soma, so that the driving force for the $\text{Cl}^-$ current ($V_m - E_{\text{GABA}}$) elicited by GABA application onto this more distal portion of the dendrites may have been slightly attenuated compared with the $\text{Cl}^-$ current elicited by GABA application onto the proximal dendrite (30). In addition, due to the cable properties of the dendrites, the response to GABA, which was measured at the soma, may have been slightly smaller in amplitude when GABA was applied more distally compared with when it was applied proximally. It is thus likely that the difference in $E_{\text{GABA}}$ between the proximal dendrites and the distal dendrites 150–200 $\mu$m from the soma is $>10$ mV and more closely approximates the difference obtained after application of BMN and FUR (see Discussion).
NKCC2 splice variants and is located on the cytoplasmic face of the membrane (11). All staining in rabbit retina cryostat and vibratome sections was abolished by preincubation of the antisera with the NKCC2-specific peptide (Chemicon AG205), a procedure analogous to Western blot analysis with respect to determining antibody specificity. The finding that BMN, a selective NKCC inhibitor at 10 μM (8–11), hyperpolarized SACs and eliminated their DS light responses when dialyzed into individual cells (see Fig. 2 A) also demonstrates that SACs contain functional NKCC. KCC2 was localized with a rabbit polyclonal antiserum AB 144P (Chemicon) both preabsorption and Western blot analysis (31). ChAT was determined for each slice and recorded as a separate (blue) channel by using the ImageJ RGB colocalization plug-in. z-projections were then generated by using the ImageJ RGB projection plug-in, and the images were converted to Tiff format. Photoshop was used to assess the percent ratio of colocalized pixels within proximal, intermediate, and distal compartments, based on unique, previously described SAC morphological characteristics (27, 32).

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Determination of NKCC2 and KCC2 Staining Patterns on Starburst Dendrites. Iontophoretically filled (Alexa Fluor 488; Molecular Probes A-10440) DAPI-labeled displaced SACs were double-labeled for NKCC2 or KCC2 as above, except that the antisera were visualized with Alexa Fluor 647 goat anti-rabbit Fab2 (Molecular Probes A11070). A Zeiss laser confocal microscope was used for image and data acquisition. Images were scanned at a 0.7-μm optical thickness. Image stacks were processed with ImageJ (http://rsb.info.nih.gov/ij) and Photoshop 7.0 (Adobe). Colocalized pixels (coincident red and green channels) were determined for each slice and recorded as a separate (blue) channel by using the ImageJ RGB colocalization plug-in. Z-projections were then generated by using the ImageJ RGB projection plug-in, and the images were converted to Tiff format. Photoshop was used to assess the percent ratio of colocalized pixels within proximal, intermediate, and distal compartments, based on unique, previously described SAC morphological characteristics (27, 32).