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1 **Lateral diffusion of NKCC1 contributes to chloride homeostasis in**
2 **neurons and is rapidly regulated by the WNK signaling pathway**

3
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8
9 **Abstract:**

10 An upregulation of the Na⁺-K⁺-2Cl⁻ co-transporter NKCC1, the main chloride importer in
11 mature neurons, can lead to depolarizing/excitatory responses mediated by GABA_A
12 receptors and thus to hyperactivity. Understanding the regulatory mechanisms of NKCC1
13 would help prevent intra-neuronal chloride accumulation that occurs in pathologies with
14 defective inhibition. The cellular and molecular regulatory mechanisms of NKCC1 are poorly
15 understood. Here, we report in mature hippocampal neurons that GABAergic activity
16 controls the membrane diffusion and clustering of NKCC1 via the chloride-sensitive WNK1
17 kinase and the downstream SPAK kinase that directly phosphorylates NKCC1 on key
18 threonine residues. At rest, this signaling pathway has little effect on intracellular Cl⁻
19 concentration but it participates to the elevation of intraneuronal Cl⁻ concentration in
20 hyperactivity condition associated with an up-regulation of NKCC1. The fact that the chloride
21 exporter KCC2 is also regulated in mature neurons by the WNK1 pathway indicates that this
22 pathway will be a target of choice in the pathology.

1 **Keywords:**

2 Hippocampal neurons, chloride homeostasis, GABAergic transmission, lateral diffusion,
3 clustering, protein trafficking, signaling, single molecule localization microscopy.

4 **Introduction:**

5 Upon activation by GABA, GABA type A receptors (GABA_ARs) open a selective
6 chloride/bicarbonate conductance. The direction of chloride (Cl⁻) flux through the channel
7 depends on transmembrane Cl⁻ gradients. Therefore, Cl⁻ homeostasis critically determines
8 the polarity and efficacy of GABAergic transmission in the brain. Pharmaco-resistant
9 epilepsies are often associated with altered Cl⁻ homeostasis [1]. It is therefore crucial to
10 discover novel mechanisms regulating neuronal Cl⁻ homeostasis that may help develop new
11 and efficient treatment for these forms of epilepsy and other diseases associated with
12 impaired inhibition, such as neuropathies and neuropsychiatric disorders [2].

13 The increase in [Cl⁻]_i and subsequent depolarized shift in the reversal potential of GABA
14 (E_{GABA}) observed in adult epilepsy models are often attributed to reduced
15 expression/function of the neuronal K⁺-Cl⁻ cotransporter KCC2, responsible for Cl⁻ export [3].
16 Furthermore, up-regulation of the Na⁺-K⁺-Cl⁻ cotransporter NKCC1, which imports chloride
17 into neurons, also contributes to the increase in [Cl⁻]_i and depolarization of E_{GABA}. This has
18 been observed in the subiculum of patients with temporal lobe epilepsy (TLE) [4-5] as well
19 as in several *in vivo* and *in vitro* rodent models of epilepsy such as the glioma-induced [6],
20 the traumatic brain injury-induced [7] models of epilepsy, and the kainic acid and pilocarpine
21 epilepsy models in tissue slices [8-10]. Targeting NKCC1 in epilepsy with the inhibitor
22 bumetanide is protective: it abolished glioma-induced seizures in rats [6], it reduced the
23 frequency of interictal-like activities [4], the duration of ictal activities [10], and the sprouting
24 of mossy fibers [9].

1 Key cellular and molecular mechanisms regulate the membrane turnover of KCC2. They
2 involve activity-dependent regulation of the transporter membrane turnover and endocytosis
3 [11-12]. KCC2 is rapidly down-regulated by enhanced neuronal activity and glutamatergic
4 neurotransmission in mature neurons [13-15]. NMDA receptor-induced Ca^{2+} -influx leads to
5 protein phosphatase 1 (PP1)-dependent KCC2 Serine S940 (S940) dephosphorylation and
6 C-terminal domain cleavage by Ca^{2+} -activated protease calpain [14, 16-17]. This in turn
7 leads to increased lateral diffusion, endocytosis and degradation of KCC2 [13-14].
8 GABAergic signaling also tunes KCC2 at the neuronal membrane through $GABA_A$ R activity
9 and Cl^- -dependent phosphorylation of KCC2 Threonine 906 and 1007 (T906/1007) residues
10 [18]. Cl^- acts as a second messenger in this regulation by tuning the activity of the Cl^- sensing
11 With No lysine (K) serine-threonine kinase WNK1 and its downstream effectors Ste20
12 Proline Asparagine Rich Kinase (SPAK) and Oxidative Stress Response kinase 1 (OSR1)
13 [19].

14 Interestingly, this signaling not only promote KCC2 T906/T1007 but also NKCC1
15 T203/T207/T212 phosphorylation [20]. This results in a powerful modulation of Cl^- transport
16 by inhibiting KCC2 and activating NKCC1; both regulations leading to elevation in
17 intracellular Cl^- level in non-neuronal cells [21]. Therefore, inhibiting KCC2 and NKCC1
18 phosphorylation mediated by the WNK signaling might normalize the membrane
19 expression/function of transporters, thereby preventing the intracellular Cl^- buildup in
20 epilepsy and the intensity or emergence of epileptic seizures.

21 Our team has shown the contribution of lateral diffusion in the rapid control of KCC2
22 membrane stability and Cl^- neuronal homeostasis in response to changes in neuronal
23 activity [13,18]. The transporter alternates between periods of confinement within clusters
24 near synapses and periods of free movement outside the clusters. The free transporter can
25 then be targeted to the endocytic wells where it is internalized and then degraded or recycled

1 to the plasma membrane. These two pools of transporters are in dynamic equilibrium,
2 allowing the fine-tuning of synapses in response to local fluctuations in synaptic activity [11-
3 12]. Since changes in KCC2 mobility occur within tens of seconds [18], lateral diffusion is
4 probably the primary cellular mechanism modulating KCC2 membrane stability.

5 Recently, we showed with confocal microscopy that NKCC1 is targeted to the axonal and
6 somato-dendritic plasma membrane and that it forms clusters at the periphery of synapses
7 of hippocampal neurons [12]. Quantum Dot-based Single Particle Tracking (QD-SPT) in
8 living cultured hippocampal neurons indicated that NKCC1 explore large areas of the
9 somato-dendritic extrasynaptic membrane while others are confined near excitatory and
10 inhibitory synapses [12]. Transitions between NKCC1 confinement at/near synapses and
11 less constrained diffusion in extrasynaptic areas is reminiscent of KCC2 diffusion [13,18].
12 Therefore, similar to KCC2, NKCC1 responds to the “diffusion-trap” mechanism [12]. A rapid
13 activity-dependent regulation of NKCC1 diffusion/clustering, may thus locally affect its
14 function, and in turn GABA signaling.

15 Here we show that neuronal GABAergic activity rapidly regulates lateral diffusion and
16 membrane clustering of NKCC1 in mature hippocampal neurons in culture. Blocking or
17 activating GABA_A receptors with muscimol or gabazine confines the transporters to the
18 dendritic membrane. GABAergic activity would control diffusion and clustering of NKCC1 in
19 the dendrite via the chloride-sensitive kinase WNK1 and the downstream kinases SPAK and
20 OSR1 that would directly phosphorylate NKCC1 at key threonine phosphorylation sites
21 T203/T207/T212. Our results indicate that this regulation would be particularly effective in
22 regulating NKCC1 and chloride homeostasis in the somato-dendritic compartment under
23 hyperactive conditions, highlighting the value of targeting the WNK1/SPAK/OSR1 pathway
24 in inhibition-defective pathologies.

25

1 **Materials and Methods**

2 For all experiments performed on primary cultures of hippocampal neurons, animal
3 procedures were carried out according to the European Community Council directive of 24
4 November 1986 (86/609/EEC), the guidelines of the French Ministry of Agriculture and the
5 Direction Départementale de la Protection des Populations de Paris (Institut du Fer à Moulin,
6 Animalerie des Rongeurs, license C 72-05-22). All efforts were made to minimize animal
7 suffering and to reduce the number of animals used. Timed pregnant Sprague-Dawley rats
8 were supplied by Janvier Lab and embryos were used at embryonic day 18 or 19 as
9 described below.

10 **Neuronal culture.** Primary cultures of hippocampal neurons were prepared as previously
11 described [13] with some modifications in the protocol. Briefly, hippocampi were dissected
12 from embryonic day 18 or 19 Sprague-Dawley rats of either sex. Tissue was then trypsinized
13 (0.25% v/v), and mechanically dissociated in 1× HBSS (Invitrogen, Cergy Pontoise, France)
14 containing 10mM HEPES (Invitrogen). Neurons were plated at a density of 120×10^3
15 cells/ml onto 18-mm diameter glass coverslips (Assistent, Winigor, Germany) pre-coated
16 with 50 µg/ml poly-D,Lornithine (Sigma-Aldrich, Lyon, France) in plating medium composed
17 of Minimum Essential Medium (MEM, Sigma) supplemented with horse serum (10% v/v,
18 Invitrogen), L-glutamine (2 mM) and Na⁺ pyruvate (1 mM) (Invitrogen). After attachment for
19 3–4 h, cells were incubated in culture medium that consists of Neurobasal medium
20 supplemented with B27 (1X), L-glutamine (2 mM), and antibiotics (penicillin 200 units/ml,
21 streptomycin, 200 µg/ml) (Invitrogen) for up to 4 weeks at 37 °C in a 5% CO₂ humidified
22 incubator. Each week, one fifth of the culture medium volume was renewed.

23 **DNA constructs.** The pcDNA3.1 Flag YFP hNKCC1 HA-ECL2 (NT931) was a gift from Biff
24 Forbush (Addgene plasmid # 49063 ; <http://n2t.net/addgene:49063> ; RRID:Addgene_49063;
25 [13]. From this NKCC1-HA-Flag-mVenus plasmid, the following constructs were raised:

1 NKCC1-HA- Δ flag- Δ mVenus by truncation of the tags located on NKCC1 NTD, NKCC1-TA3-
2 Flag-mVenus and NKCC1-TA3- Δ Flag- Δ mVenus with mutation of T203/207/212 to Alanines,
3 and NKCC1-TA5-Flag-mVenus and NKCC1-TA5- Δ Flag- Δ mVenus with mutation of
4 T203/207/212/217/230 to alanines. Threonine nucleotide sequence was changed to GCA
5 for alanine substitution. The following constructs were also used: pCAG_rat KCC2-3Flag-
6 ECL2 [13], pCAG_KCC2-3Flag-ECL2 T906/1007E [18], eGFP (Clontech),
7 pCAG_GPHN.FingR-eGFP-CCR5TC [23] (gift from Don Arnold, Addgene plasmid # 46296 ;
8 <http://n2t.net/addgene:46296> ; RRID:Addgene_46296), homer1c-DsRed (kindly provided by
9 D. Choquet, IIN, Bordeaux, France), WNK1 with “kinase-dead, dominant-negative domain”
10 (WNK1-KD, D368A), “constitutively active” WNK1 (WNK1-CA, S382E) [24] (kindly provided
11 by I. Medina, INMED, Marseille), and SuperClomeleon [25] (kindly provided by G.J.
12 Augustine, NTU, Singapore). All constructs were sequenced by Beckman Coulter Genomics
13 (Hope End, Takeley, U.K).

14 **Neuronal transfection.** Neuronal transfections were carried out at DIV 13–14 using
15 Transfectin (BioRad, Hercules, USA), according to the manufacturers’ instructions
16 (DNA:transfectin ratio 1 μ g:3 μ l), with 1–2 μ g of plasmid DNA per 20 mm well. Simple
17 transfections of NKCC1-HA-Flag-mVenus plasmid concentration: 1 μ g. The following ratios
18 of plasmid DNA were used in co-transfection experiments : 1:0.4:0.4 μ g for NKCC1
19 constructs together with GPHN.FingR-eGFP and homer1c-DsRed ; 1:0.2 μ g for NKCC1
20 constructs with eGFP ; 0.7:0.7 μ g for NKCC1 constructs with WNK1-KD or WNK1-CA,
21 NKCC1 constructs with SuperClomeleon ; 0.7:0.7 μ g KCC2 constructs with
22 SuperClomeleon ; 0.5:0.5:0.5 μ g SCLM + KCC2 + NKCC1. Experiments were performed 7–
23 10 days post-transfection. SPT, STORM and chloride imaging experiments were performed
24 with Δ flag- Δ mVenus NKCC1 constructs. Standard epifluorescence microscopy with Flag-
25 mVenus NKCC1 constructs.

1 **Peptide treatment and pharmacology.** The following peptides and drugs were used:
2 myristoylated dynamin inhibitory peptide (50 μ M; Tocris Bioscience), TTX (1 μ M; Latoxan,
3 Valence, France), R,S-MCPG (500 μ M; Abcam, Cambridge, UK), S-MCPG (250 μ M;
4 HelloBio), Kynurenic acid (1 mM; Abcam), gabazine (10 μ M; Abcam), muscimol (10 μ M;
5 Abcam), WNK463 (10 μ M, MedChemTronica), closantel (10 μ M; Sigma), bumetanide (5 μ M,
6 Abcam). R,S-MCPG and S-MCPG were prepared in equimolar concentrations of NaOH;
7 TTX in 2% citric acid (v/v); closantel in DMSO (Sigma). Equimolar DMSO concentrations
8 were used for control experiments in these conditions. For SPT experiments, neurons were
9 transferred to a recording chamber, pre-incubated in presence of drugs and/or peptide at
10 31 °C for 10 min in imaging medium (see below for composition) and used within 45 min in
11 presence of the appropriate drug for imaging. For immunofluorescence experiments, drugs
12 were added directly to the culture medium for 30 min in a CO₂ incubator set at 37 °C. The
13 imaging medium consisted of phenol red-free minimal essential medium supplemented with
14 glucose (33 mM; Sigma) and HEPES (20 mM), glutamine (2 mM), Na⁺-pyruvate (1 mM),
15 and B27 (1X) from Invitrogen. The 138mM [Cl⁻] extracellular solution was composed of 2
16 mM CaCl₂, 2 mM KCl, 3 mM MgCl₂, 10 mM HEPES, 20 mM glucose, 126 mM NaCl, 15 mM
17 Na⁺ methane sulfonate; the 0 mM [Cl⁻] extracellular solution was made of 1 mM CaSO₄, 2
18 mM K⁺ - methane sulfonate, 2 mM MgSO₄, 10 mM HEPES, 20 mM glucose, 144 mM Na⁺ -
19 methane sulfonate.

20 **Live cell staining for single-particle imaging.** Neurons were labelled as described
21 previously. Briefly, cells were incubated for 8 min at 37 °C with primary antibodies against
22 HA (rabbit, 1:250, Cell signaling Technology, cat #C29F4). After washes, cells were
23 incubated for 1 min with F(ab')₂-Goat anti-Rabbit IgG (H+L) Secondary Antibody QDot
24 emitting at 655 nm (1 nM; Invitrogen) in PBS (1 M; Invitrogen) supplemented with 10%
25 Casein (v/v) (Sigma).

1 **Single-particle tracking and analysis.** Cells were imaged as previously described using
2 an Olympus IX71 inverted microscope equipped with a 60X objective (NA 1.42; Olympus)
3 and a 120W Mercury lamp (X-Cite 120Q, Lumen Dynamics). Individual images of gephyrin-
4 YFP and homer1c-DsRed, and QD real time recordings (integration time of 30 ms over 1200
5 consecutive frames) were acquired with an ImagEM EMCCD camera and MetaView
6 software (Meta Imaging 7.7). Cells were imaged within 45 min following appropriate drugs
7 pre-incubation. QD tracking and trajectory reconstruction were performed with homemade
8 software (Matlab; The Mathworks, Natick, MA) as described in [26]. One to two sub-regions
9 of dendrites were quantified per cell. In cases of QD crossing, the trajectories were
10 discarded from analysis. Trajectories were considered synaptic when overlapping with the
11 synaptic mask of gephyrin-mRFP or homer1c-GFP clusters, or extrasynaptic for spots four
12 pixels (760 nm) away. The inclusion area was increased relatively to previous studies on
13 KCC2 [13,18] due to the low numbers of NKCC1 trajectories recorded with a 380 nm
14 distance. Expanding the radius did actually not change results for perisynaptic NKCC1
15 lateral diffusion, suggesting its clusters are located further away from synapses than KCC2
16 ones. Values of the mean square displacement (MSD) plot vs. time were calculated for each
17 trajectory by applying the relation :

$$18 \quad MSD(n\tau) = \frac{1}{N-n} \sum_{i=1}^{N-n} [(x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2]$$

19 where τ is the acquisition time, N is the total number of frames, n and i are positive integers
20 with n determining the time increment. Diffusion coefficients (D) were calculated by fitting
21 the first four points without origin of the MSD vs. time curves with the equation: $MSD(n\tau) =$
22 $4Dn\tau + \sigma$; where σ is the spot localization accuracy. Depending on the type of lamp used
23 for imaging, the QD pointing accuracy is ~20–30 nm, a value well below the measured
24 explored areas (at least 1 log difference). The explored area of each trajectory was defined

1 as the MSD value of the trajectory at two different time intervals of 0.42 and 0.45 s [27].
2 Synaptic dwell time was defined as the duration of detection of QDs at synapses on a
3 recording divided by the number of exits as detailed previously [26]. Dwell times ≤ 5 frames
4 were not retained. The number of QD vary from one cell to another and from one condition
5 to another in a given experiment.

6 **Chloride imaging.** Neurons were imaged at 33 °C in a temperature-controlled open
7 chamber (BadController V; Luigs & Neumann) mounted onto an Olympus IX71 inverted
8 microscope equipped with a 60# objective (1.42 numerical aperture (NA); Olympus). CFP
9 and YFP were detected using Lambda DG-4 monochromator (Sutter Instruments) coupled
10 to the microscope through an optic fiber with appropriate filters (excitation, D436/10X and
11 HQ485/15X; dichroic, 505DCXR; emission, HQ510lp; CFP and YFP filters from Chroma
12 Technology). Images were acquired with an ImagEM EMCCD camera (Hamamatsu
13 Photonics) and MetaFluor software (Roper Scientific). Mean background fluorescence
14 (measured from a nonfluorescent area) was subtracted and the ratio F480/F440 was
15 determined. Z-stack images (16-bit; 512 × 512) were typically acquired every 30 s for 5
16 minutes, with an integration time of 30 ms. Regions of interest (ROIs) were selected for
17 measurement if they could only be measured over the whole experiment.

18 **Immunocytochemistry.** NKCC1-HA-Flag-mVenus membrane expression and clustering
19 was assessed with staining performed after a short fixation at room temperature (RT) in
20 paraformaldehyde (PFA; 4% w/v; Sigma) and sucrose (20% w/v; Sigma) solution in 1× PBS.
21 The cells were then washed in PBS and incubated for 30 min at RT in goat serum (GS; 3%
22 v/v; Invitrogen) in PBS to block non-specific staining. Neurons were then incubated for 60-
23 180 min at RT with HA antibody (rabbit, 1:250, Cell signaling Technology, cat #C29F4) in
24 PBS–GS blocking solution. After washing, neurons were incubated with Cy™3 AffiniPure
25 Donkey Anti-Rabbit IgG (H+L) (1.9 µg/ml; Jackson ImmunoResearch, cat #111-165-003) for

1 standard epifluorescence assays, or Alexa Fluor® 647 AffiniPure Donkey Anti-Rabbit IgG
2 (H+L) (2 µg/ml, Jackson ImmunoResearch, cat #711-605-152) for super-resolution
3 experiments, in PBS-GS solution. The coverslips were then washed, and mounted on slides.
4 Coverslips were mounted on slides with mowiol 844 (48 mg/ml; Sigma). Sets of neurons
5 compared for quantification were labeled and imaged simultaneously.

6 **Fluorescence image acquisition and analysis.** Image acquisition was performed using a
7 ×100 objective (NA 1.40) on a Leica (Nussloch, Germany) DM6000 upright epifluorescence
8 microscope with a 12-bit cooled CCD camera (Micromax, Roper Scientific) run by
9 MetaMorph software (Roper Scientific, Evry, France). Quantification was performed using
10 MetaMorph software (Roper Scientific). To assess NKCC1-HA clusters, exposure time was
11 fixed at a non-saturating level and kept unchanged between cells and conditions. For the
12 dendritic intensity and clustering analysis, the ROI was precisely traced around focused
13 dendrites, and global ROI pixel intensity was measured. For the clustering images were
14 flatten background filtered (kernel size, 3 × 3 × 2) to enhance cluster outlines, and a user
15 defined intensity threshold was applied to select clusters and avoid their coalescence.
16 Clusters were outlined and the corresponding regions were transferred onto raw images to
17 determine the mean NKCC1-HA cluster number, area and fluorescence intensity. For whole-
18 dendrite intensity measurements to estimate the membrane pool fraction of NKCC1, mean
19 pixel intensity of Venus emission and mean pixel intensity of Cy3-tagged membrane NKCC1
20 were computed, and then the ratio was calculated before the background flattening. The
21 dendritic surface area of the region of interest was measured to determine the number of
22 clusters per pixel. For each culture, we analyzed ~10 cells per experimental condition.

23 **STORM microscopy.** Stochastic Optical Reconstruction Microscopy (STORM) imaging on
24 fixed samples was conducted on an inverted N-STORM Nikon Eclipse Ti microscope with a
25 100x oil immersion objective (NA 1.49) and an Andor iXon Ultra EMCCD camera (image

1 pixel size, 160 nm), using specific lasers for STORM imaging of Alexa 647 (640 nm). Videos
2 of 30,000 frames were acquired at frame rates of 50 ms. The z position was maintained
3 during acquisition by a Nikon perfect focus system. Single-molecule localization and 2D
4 image reconstruction was conducted as described in [28] by fitting the PSF of spatially
5 separated fluorophores to a 2D Gaussian distribution. The position of fluorophore were
6 corrected by the relative movement of the synaptic cluster by calculating the center of mass
7 of the cluster throughout the acquisition using a partial reconstruction of 2000 frames with a
8 sliding window [28]. STORM images were rendered by superimposing the coordinates of
9 single molecule detections, which were represented with 2D Gaussian curves of unitary
10 intensity. To correct multiple detections coming from the same Alexa 647 molecule, we
11 identified detections occurring in the vicinity of space (2σ) and time (15 s) as belonging to
12 the same molecule. The surface of NKCC1 clusters and the densities of NKCC1 molecules
13 per square nanometer were measured in reconstructed 2D images through cluster
14 segmentation based on detection densities. The minimal thresholds to determine clusters
15 were 1% intensity, 0.1 per nm^2 minimum detection density and 10 detections. The resulting
16 binary image was analyzed with the function “regionprops” of Matlab to extract the surface
17 area of each cluster identified by this function. Density was calculated as the total number
18 of detections in the pixels (STORM pixel size = 20 nm) belonging to a given cluster, divided
19 by the area of the cluster.

20 **Statistics.** Sampling corresponds to the number of quantum dots for SPT, number of
21 cultures or animals for biochemistry, cells for ICC and chloride imaging. Sample size
22 selection for experiments was based on published experiments, pilot studies, as well as in-
23 house expertise. All results were used for analysis except in few cases. For imaging
24 experiments (chloride and calcium imaging, SPT, immunofluorescence), cells with signs of
25 suffering (apparition of blobs, fragmented neurites) were discarded from the analysis. Data
26 representation was usually done with boxplots or cumulative frequency plots. The statistical

1 test to compare two groups was either Welch t-test when normality assumption was met (Q-
2 Q plots and cumulative frequency fit), otherwise Mann-Whitney test was performed to
3 assess the presence of a dominance or no between the two distributions. For variables
4 following a log-normal distribution, such as variables obtained from SPT and STORM assays,
5 we applied the $\log(\cdot)$ function after division by the control group's median. For super-
6 resolution experiments, as an important variability could be observed between different cells
7 in a same coverslip, a balanced random selection of clusters across neurons, conditions
8 and cultures was performed, then variables from each culture were divided by the median
9 of the control group. Results from different cultures were pooled and $\log(\cdot)$ was applied, then
10 the Mann-Whitney U value was computed. The process was repeated 1000 times and the
11 p-value was determined from U distribution using the basic definition of the p-value. For SPT
12 analysis, note that each QD is associated with 3 EA values, thus the sample size is 3 times
13 greater. Statistical analysis were performed with R version 3.6.1 (R Core Team (2019). R: A
14 language and environment for statistical computing. R Foundation for Statistical Computing,
15 Vienna, Austria. Package used: ggplot2, matrixStats). Statistical tests were performed
16 between a condition and its control only using cultures where both conditions were tested.
17 Differences were considered significant for p-values less than 5% (*p < 0.05; **p < 0.01; ***p
18 < 0.001; NS, not significant).

19 **Results**

20 Opposite effects of GABAergic activity on NKCC1 mobility in the axon vs. the dendrite

21 We questioned whether inhibitory GABAergic transmission influences NKCC1 lateral
22 diffusion in mature (21-23 days in vitro, DIV) hippocampal cultured neurons using quantum-
23 dot based single particle tracking (QD-SPT). We explored the impact of a pharmacological
24 activation or blockade of GABA_ARs in the presence of TTX + KYN + MCPG to block
25 glutamatergic activity and compared to the TTX + KYN + MCPG "control" condition. First,

1 we report that in control conditions, NKCC1 diffuses along the axon (Figure S1A) and in the
2 somato-dendritic compartment (Fig. 1A). Neurons were then acutely exposed to the
3 GABA_AR agonist muscimol (10 μM) or competitive antagonist gabazine (10 μM), two drugs
4 that were shown using electrophysiological recordings to increase or block GABA_AR-
5 mediated inhibition in mature hippocampal cultured neurons [18]. We observed that, when
6 exposed to gabazine or muscimol, exploration of individual QDs in the axon was increased
7 to bigger areas, compared to QDs in control conditions (Figure S1A). The slope of the mean
8 square displacement (MSD) as a function of time was increased for trajectories recorded in
9 the presence of gabazine and muscimol compared to controls (Figure S1B), indicating
10 reduced confinement of NKCC1 in the axon. This was accompanied by an increase in the
11 diffusion coefficient (Figure S1C) and the explored area (Figure S1D). Therefore, **NKCC1**
12 **confinement is reduced in the axon under conditions of GABAergic activity blockade.**

13 Moreover, we found that GABAergic signaling oppositely regulated the mobility of NKCC1
14 in the axon vs. the dendrite. In contrast to what we found in the axon, we showed that QDs
15 explored smaller areas of the dendritic membrane following exposure to gabazine or
16 muscimol, compared to the control condition (Fig. 1A). Analysis performed on the bulk
17 population (extrasynaptic + synaptic) of dendritic trajectories revealed that the MSD function
18 displayed a less steep slope for trajectories recorded in the presence of muscimol or
19 gabazine as compared with control (Fig. 1B), indicative of increased confinement upon
20 GABA_AR activation or blockade. Consistent with this observation, the median diffusion
21 coefficient and explored area values of dendritic NKCC1 were also significantly decreased
22 upon muscimol and gabazine application (Fig. 1 C, D respectively). Thus, **the lateral**
23 **diffusion of NKCC1 on the dendrite is regulated by inhibitory GABAergic**
24 **transmission: the transporters being slowed down and confined in response to an**
25 **increase or decrease in GABAergic activity.**

1 We then analyzed the effects of gabazine and muscimol on the diffusive behavior of NKCC1
2 in extrasynaptic and synaptic domains. Muscimol and gabazine reduced the transporter
3 mobility and surface exploration of individual QDs (Fig. 1E). Quantitative analysis on
4 populations of QDs revealed an impact of the treatments on diffusion coefficient and
5 explored area both in the extrasynaptic membrane and at excitatory and inhibitory synapses
6 (Fig. 1 F-G). The effect was greater on the diffusion coefficient than on the explored area for
7 NKCC1 trajectories in the vicinity of excitatory synapses and vice versa for trajectories near
8 inhibitory synapses (Fig. 1 F-G). **Thus, NKCC1 exhibits increased diffusion constraints**
9 **at extrasynaptic sites and at the periphery of synapses upon GABA_AR activation or**
10 **blockade.**

11 NKCC1 is targeted to endocytic zones where they are stored upon GABAergic activity
12 changes.

13 The increased confinement of NKCC1 induced by changes in GABA_AR activity would result
14 in its recruitment to membrane clusters or targeting to endocytic zones where the transporter
15 would be stored there until later use or internalized and recycled back to the membrane or
16 sent for degradation. We observed that acute exposure to muscimol or gabazine increased
17 the confinement of NKCC1 in endocytic zones as observed in neurons transfected with
18 clathrin-YFP for individual trajectories (Fig. 1H) or for hundreds of molecules (Fig. 1 I-J).
19 Moreover, blocking clathrin-mediated endocytosis with an inhibitory peptide prevented the
20 slow down and increased confinement of the whole NKCC1 population upon gabazine or
21 muscimol treatment (Fig. 1 K-L). **We therefore concluded that the treatment of neurons**
22 **with muscimol or gabazine increased the confinement of NKCC1 transporters in**
23 **endocytic zones in the dendrites.**

24 To investigate whether the increased confinement of the transporter to endocytic zones
25 induced by GABA_AR agonists and antagonists is accompanied by an increase in its

1 internalization, we analyzed the surface pool of NKCC1 (by calculating the ratio of the mean
2 fluorescence intensity of the surface / surface + intracellular pool of NKCC1 (Fig. 2 A-B).
3 The ratio remained unchanged (Fig. 2B) after exposure to muscimol or gabazine for 30
4 minutes, indicating that **changes in GABA_AR activity do not affect the membrane**
5 **stability of NKCC1**. This is consistent with the regulation of KCC2 lateral diffusion by
6 GABAergic inhibition, which allows for rapid regulation of clustering and thus transporter
7 membrane function without requiring transporter internalization [18]. We therefore examined
8 whether changes in GABA_AR-dependent inhibition resulted in alteration in NKCC1 clustering
9 in hippocampal neurons. Using conventional epifluorescence, we reported that muscimol
10 significantly reduced by 1.28-fold the density of NKCC1 clusters at the surface of transfected
11 neurons (Fig. 2C). Furthermore, a 30 min exposure to muscimol reduced by 1.1-fold the
12 mean size of NKCC1 clusters (Fig. 2D), as compared with untreated cells. In contrast,
13 muscimol did not affect the mean fluorescence intensity of the clusters (Fig. 2E). These
14 results indicate that the **increased confinement of NKCC1 in endocytic zones induced**
15 **upon muscimol treatment is accompanied by a rapid reduction in its membrane**
16 **clustering**. Unlike muscimol, gabazine did not noticeably alter the density of NKCC1
17 clusters (Fig. 2C). However, it significantly reduced the size (Fig. 2D) and intensity (Fig. 2E)
18 of these clusters, suggesting transporter loss within clusters.

19 Since NKCC1 cluster size is at the limit of the resolution of a standard epifluorescence
20 microscope, we further analyzed the effect of the treatments on NKCC1 clustering using
21 super-resolution STORM. NKCC1 form round-shaped clusters along the dendrites (Fig. 2F).
22 We report that neuronal exposure to gabazine or muscimol altered the nanoscopic
23 organization of NKCC1 (Fig. 2F). The muscimol treatment significantly decreased by 1.16
24 fold the average size of NKCC1 nanoclusters (Fig. 2G). This effect was not accompanied by
25 a decrease in the number of molecules detected per cluster (Fig. 2H). In fact, the density of
26 particles detected per cluster was significantly increased by 1.43 fold after muscimol

1 exposure (Fig. 2I), indicating molecular compaction. This effect coupled with the loss of
2 NKCC1 clusters observed with standard epifluorescence and the increased confinement of
3 the transporter in endocytic zones **is consistent with a muscimol-induced escape of**
4 **NKCC1 transporters from membrane clusters followed by their recruitment and**
5 **storage in endocytic zones.**

6 STORM microscopy revealed that gabazine treatment induced a significant decrease in the
7 size of NKCC1 clusters (by 0.62-fold, Fig. 2G), accompanied by a 1.5-fold reduction in the
8 number of molecules per cluster (Fig. 2H), in agreement with the notion that transporters
9 escaped clusters. This effect was not associated with a change in the density of molecules
10 per cluster (Fig. 2I), reporting no change in the compaction of molecules within the cluster.
11 **Although the effects of muscimol and gabazine differ on the nanoscale organization**
12 **of NKCC1, both treatments lead to the escape of transporters from clusters, which**
13 **are then rapidly captured in the endocytic zones where they are stored.**

14 Intracellular chloride levels tune NKCC1 surface diffusion and clustering

15 We then investigated whether changes in $[Cl^-]_i$ could explain the effects of manipulations of
16 GABA_AR activity on the diffusion of NKCC1, as shown for KCC2 [18]. We lowered $[Cl^-]_i$ by
17 substituting extracellular Cl^- with methane sulfonate in the imaging medium and tested its
18 effect on NKCC1 diffusion. The decrease in $[Cl^-]_i$ increased NKCC1 surface exploration for
19 individual trajectories located at the periphery of synapses and at distance (Fig. 3A).
20 Quantification revealed that this treatment had no significant effect on the diffusion
21 coefficient of NKCC1 on dendrites either for extrasynaptic or synaptic trajectories (Fig. 3B).
22 On the other hand, lowering $[Cl^-]_i$ decreased the confinement of extrasynaptic transporters
23 (Fig. 3C), while the confinement of synaptic transporters was unchanged (Fig. 3C). We then
24 asked if the reduced confinement observed at extrasynaptic sites concerned transporters
25 stored in endocytic zones. We found that the diffusion coefficient and surface exploration of

1 NKCC1 were significantly increased for transporters located at distance of clathrin-coated
2 pits (Fig. 3 D-F) while the mobility of transporters in endocytic zones was not modified upon
3 lowering $[Cl^-]_i$ (Fig. 3 E-F). Therefore, reducing $[Cl^-]_i$ does not increase the confinement of
4 NKCC1 in endocytic zones. **Altogether, our results provide evidence that lowering**
5 **intracellular chloride levels removes diffusion constraints onto NKCC1, which move**
6 **faster in the membrane, probably by being relieved from endocytic zones.**

7 We then determined whether this relief in diffusion constraints of the transporter was
8 associated with its membrane redistribution. Quantification of the ratio of the surface pool of
9 NKCC1 over the total pool of NKCC1 revealed that lowering $[Cl^-]_i$ by extracellular Cl^-
10 substitution increased NKCC1 immunoreactivity on the dendrites (Fig. 3G). This was
11 accompanied by a 1.25 fold increase in the amount of NKCC1 detected at the cell surface
12 (Fig. 3H). This increase in the membrane stability of NKCC1 was accompanied by an
13 increase in its clustering. The treatment did not alter the number of clusters detected at the
14 cell surface (Fig. 3I). However, lowering $[Cl^-]_i$ level led to a 1.12 fold increase in the median
15 size of NKCC1 clusters (Fig. 3J) and a 1.4 fold increase in their median fluorescence
16 intensity (Fig. 3K). These results indicate a **chloride-dependent regulation of NKCC1**
17 **diffusion-capture, consistent with a homeostatic regulation of the transporter.**

18 The WNK signaling pathway regulates NKCC1

19 The chloride-sensitive WNK signaling pathway regulates NKCC1 activity [21]. We assessed
20 the role of this pathway in NKCC1 clustering using overexpression of constitutively active
21 (WNK-CA) or kinase-dead (WNK-KD) WNK1 [24] and using WNK1 (WNK463) inhibitor. The
22 overexpression of WNK-CA had no effect on NKCC1 surface exploration and mobility for
23 individual trajectories (Fig. 4A) and population of QDs (Fig. 4 B-C). Similarly, overexpressing
24 WNK-CA did not alter the surface expression level of NKCC1 (Fig. 4 D-E) nor the number
25 of NKCC1 clusters (Fig. 4F) nor the size and intensity of these clusters (Fig. 4 G-H). Based

1 on these results, we concluded that, under basal activity conditions, **activation of the WNK1**
2 **pathway does not affect the diffusion, expression and distribution of NKCC1 at the**
3 **dendritic surface in mature hippocampal neurons.**

4 Conversely, we studied the effects of an inhibition of the WNK1 signaling pathway on NKCC1
5 surface expression and clustering following overexpression of WNK-KD or after acute
6 exposure to a pan-WNK antagonist (WNK-463). An acute blockade for 30 min of WNK1 with
7 WNK463 had no effect on the membrane stability of NKCC1 (Fig. 4 I-J) while blocking WNK1
8 activity for 7 DIV by overexpressing WNK-KD significantly reduced the membrane stability
9 of NKCC1 (Fig. 4 I-J). On the other hand, WNK inhibition using genetic or pharmacological
10 approaches significantly altered the clustering of NKCC1 by decreasing respectively to 2
11 fold and 1.6 fold the number of NKCC1 clusters (Fig. 4K). This was not accompanied by a
12 reduction in the size of the clusters upon WNK463 treatment or WNK-KD overexpression
13 (Fig. 4L). However, WNK-KD overexpression decreased to 2-fold NKCC1 cluster intensity
14 (Fig. 4M). Therefore, **inhibiting the WNK1 signaling pathway in basal activity**
15 **conditions reduces NKCC1 membrane stability and clustering.**

16 We then studied the contribution of the WNK1 effectors SPAK and OSR1 in the regulation
17 of NKCC1 membrane diffusion, stability and clustering using the SPAK/OSR1 inhibitor
18 closantel [29]. An acute exposure of neurons to closantel rapidly reduced the surface
19 explored by individual QDs (Fig. 5A). This was accompanied by a 1.14-fold reduction in
20 NKCC1 diffusion coefficients (Fig. 5B) and by a 1.28-fold decrease in its explored area (Fig.
21 5C), revealing **increased NKCC1 diffusion constraints as compared with control.** This
22 effect on diffusion was however not accompanied by a change in the surface expression of
23 NKCC1 (Fig. 5 D-E), nor by a change in its clustering as determined by standard
24 epifluorescence on the number of NKCC1 clusters (Fig. 5F), as well as on the size and
25 intensity of these clusters (Fig. 5 G-H). However, the analysis of NKCC1 clusters using

1 super-resolution imaging (Fig. 5I) revealed that a 30 min exposure to closantel reduced by
2 1.3-fold the cluster size (Fig. 5J). This effect was not accompanied by a significant change
3 in the number of particles detected per cluster (Fig. 5K). However, closantel increased by
4 1.75-fold the density of molecules per cluster (Fig. 5L) as compared with untreated cells,
5 indicative of molecular compaction. Therefore, the closantel-induced confinement of NKCC1
6 is accompanied by a rapid alteration in the nanoscale organization of the transporter. **Taken**
7 **together these results show that the WNK1/SPAK/OSR1 pathway regulates the**
8 **membrane dynamics, stability and clustering of NKCC1 in mature neurons. The fact**
9 **that an activation of the WNK1 pathway (by overexpressing WNK-CA) has no effect**
10 **on NKCC1 membrane dynamics, expression and clustering suggests that this**
11 **pathway is active in mature neurons and regulates NKCC1 diffusion-capture.**

12 The WNK signaling pathway targets key threonine residues on NKCC1

13 WNK kinases promote NKCC1 T203/T207/T212 phosphorylation [24], which in turn results
14 in NKCC1 activation [21]. In order to test the involvement of NKCC1-T203/207/212/217/230
15 phosphorylation in the regulation of NKCC1 diffusion, we expressed NKCC1 constructs
16 harboring mutations of T203/T207/T212 or T203/T207/T212/T217/T230 to alanine (TA3 and
17 TA5, respectively) that mimic dephosphorylated states. The mobility and exploration of
18 individual NKCC1 T203/207/212/217/230A was decreased relative to WT especially for
19 extrasynaptic QDs (Fig. 6A). This was reflected by a 1.2 fold lower speed (Fig. 6B) and a
20 1.48 fold increased confinement (Fig. 6C) of QDs in the extrasynaptic membrane without
21 changing the diffusion coefficient or the surface area explored at the inhibitory and excitatory
22 synapses (Fig. 6 B-C). **Therefore, the dephosphorylation of NKCC1 on key threonine**
23 **residues confines the transporter in the extrasynaptic membrane. In agreement with**
24 **a regulation of NKCC1 by the WNK1 signaling pathway, these results indicate that a**
25 **proportion of NKCC1 is phosphorylated on T203/207/212 in mature neurons. This**

1 **differs from the KCC2 transporter for which regulation by the WNK1 signaling was**
2 **only observed when GABA_AR activity was challenged [18].**

3 We have previously shown in similar experimental preparations that an acute application of
4 muscimol induces an increase in $[Cl^-]_i$ [18]. This is corroborated by the inhibition of WNK1
5 and dephosphorylation of NKCC1 [18]. Knowing that the phosphorylation of NKCC1 by
6 WNK1 regulates its activity in non-neuronal cells [30], we wanted to know if the membrane
7 stability and clustering of the mutated transporter was altered compared to that of the WT.
8 Our results show that the surface pool of NKCC1 T203/T207/T212A is decreased (by 1.1
9 fold) compared to that of the WT transporter (Fig. 6 D-E). This decrease in the membrane
10 stability of the transporter was accompanied by a 1.6 fold and a 1.7 fold decrease in the
11 cluster density of NKCC1 T203/T207/T212A and NKCC1 T203/T207/T212/T217/T230A (Fig.
12 6F), compared to the WT. The remaining NKCC1 T203/T207/T212A and NKCC1
13 T203/T207/T212/T217/T230A clusters were not changed in size or fluorescence intensity
14 compared to WT (Fig. 6 G-H). This effect is reminiscent of that observed upon muscimol
15 treatment (Fig. 2) or WNK1 inhibition (Fig. 4). Importantly, NKCC1
16 T203/T207/T212/T217/T230A prevented the muscimol-induced decrease in NKCC1
17 clustering (Fig. 6 I-K). **We conclude that GABA_AR-dependent regulation of NKCC1**
18 **membrane stability, diffusion and clustering involves phosphorylation of its**
19 **T203/T207/T212/T217/T230 residues.**

20 Functional impact of NKCC1 regulation by the WNK signaling pathway in mature
21 hippocampal neurons

22 Our work describes a regulation of NKCC1 membrane stability and clustering through
23 GABAergic activity. This regulation involves the phosphorylation of the transporter by the
24 WNK/SPAK/OSR1 signaling pathway. To assess the functional relevance of this regulation,
25 we looked at the impact of NKCC1 phosphorylation on $[Cl^-]_i$. We used SuperClomeleon [25]

1 to quantify potential changes in intracellular chloride concentration. Changes of
2 concentration were inferred from changes in YFP/CFP ratios (Fig. 7A). As a control condition,
3 we compared the YFP/CFP ratio of cells transfected with KCC2-WT vs KCC2-T906/T1007E
4 i.e. a construction mimicking the phosphorylated state of the transporter with a reduced
5 capacity to extrude chloride ions, notably by modifying its membrane stability and clustering
6 [18, 24]. An important decrease in the ratio was observed in cells transfected with KCC2-TE
7 as compared to KCC2-WT, reflecting a higher $[Cl^-]_i$ (Fig. 7B). Thus, this approach allows the
8 measurement of $[Cl^-]_i$ elevation resulting from manipulations of chloride co-transporter
9 membrane expression and thereby function. We then tested whether the expression of
10 endogenous or recombinant NKCC1 significantly impacted $[Cl^-]_i$ in our neuronal preparation.
11 First, we determined the impact of an acute blockade of NKCC1 activity with the NKCC1
12 inhibitor bumetanide (5 μ M) on the YFP/CFP ratio in neurons transfected with
13 SuperClomeleon alone. The YFP/CFP ratio was comparable in both bumetanide-exposed
14 and non-bumetanide-exposed neurons (Fig. 7C). These results are in agreement with data
15 suggesting that **at rest, endogenous NKCC1 do not significantly influence $[Cl^-]_i$ in**
16 **mature hippocampal neurons** [31-32]. Similarly, expression of the recombinant NKCC1-
17 WT in mature neurons did not increase $[Cl^-]_i$ compared to neurons expressing the chloride
18 probe alone, nor did it increase their sensitivity to bumetanide (Fig. 7C). This suggests that
19 **the neuron tightly regulates the level of recombinant NKCC1 present at the cell**
20 **membrane.**

21 If the expression of NKCC1-WT does not influence $[Cl^-]_i$, then it is not surprising that a loss
22 of function of the transporter cannot be detected. Indeed, we found that NKCC1-
23 T203/T207/T212/T217/T230A, which shows a defect in membrane clustering compared to
24 WT, has no effect on either the YFP/CFP ratio or the response to bumetanide (Fig. 7C).
25 However, since NKCC1 plays an important role on $[Cl^-]_i$ in mature neurons under conditions
26 where KCC2 is down-regulated [4, 9, 18, 32], we performed additional analyses on neurons

1 co-transfected with the mutant transporter KCC2-T906/1007E, which has a reduced chloride
2 extrusion capacity [24] and Fig. 7A). Over-expression of KCC2-T906/1007E was preferred
3 to the expression of a shRNA against KCC2 because this strategy led to neuronal death
4 when NKCC1 was expressed in concert. Interestingly, neuronal death was not observed
5 when the shRNA against KCC2 was expressed alone but only when its expression was
6 combined with that of NKCC1 (data not shown). This indicates that the recombinant NKCC1
7 transporter is functional in neurons and that the influx of chloride through NKCC1 in neurons
8 in the absence of chloride ion extrusion capacity is toxic. In agreement with previous works
9 [31-32], this result also means that in mature neurons KCC2 is the major regulator of $[Cl^-]_i$.
10 However, no effect of the endogenous NKCC1 or of the recombinant NKCC1-WT or NKCC1-
11 T203/T207/T212/T217/T230A was observed on the YFP/CFP ratio, nor on bumetanide
12 sensitivity in conditions of low KCC2 activity (Fig. 7D). We concluded **that at rest, in**
13 **conditions of normal or reduced KCC2 expression, endogenous or exogenous**
14 **NKCC1 transporters are not significantly contributing to $[Cl^-]_i$ in mature hippocampal**
15 **cultured neurons.**

16 Since NKCC1 is overexpressed in the adult epileptic brain and that this overexpression
17 contributes to increase seizure susceptibility [3, 7], we tested the contribution of the WNK1
18 signaling in the regulation of the membrane stability and function of NKCC1 in pathological
19 conditions. For this purpose, neurons were acutely exposed to the convulsing agent 4-
20 Amminopyridine (4-AP), a blocker of the voltage-dependent K^+ channels responsible for
21 membrane repolarization. This experiment was performed in absence of TTX + KYN +
22 MCPG. The “4-AP condition” was compared to the control condition in the absence of any
23 drug. We previously showed that an acute exposure of hippocampal neurons to 4-AP
24 induces KCC2 endocytosis [13]. Conversely, we show here that this treatment rapidly
25 increases the membrane expression of NKCC1 (Fig. 7E). Pre-treatment of neurons with the
26 inhibitor WNK-463 prevented the increase in surface expression of NKCC1 induced by 4-

1 AP (Fig. 7E), implicating WNK1 in the upregulation of NKCC1 at the neuronal surface.

2 Although we have shown that NKCC1 does not participate in the regulation of intracellular
3 chloride levels under basal activity conditions in mature hippocampal neurons (Fig. 7 C-D),
4 we show here the contribution of NKCC1 to neuronal chloride homeostasis under
5 pathological conditions. Indeed, chloride imaging revealed that an acute exposure to 4-AP
6 induced a significant increase in intracellular chloride levels in neurons (Fig. 7 F-G). This
7 effect was blocked by pre-incubating the neurons with the WNK antagonist or by blocking
8 NKCC1 activity with bumetanide (Fig. 7 F-G), thus directly implicating the WNK signaling
9 and NKCC1 in this regulation. **Thus, we propose that 4-AP-induced hyperactivity**
10 **activates the WNK pathway, which by phosphorylating NKCC1 on key threonine**
11 **residues, increases its membrane expression and clustering leading to intracellular**
12 **chloride influx and decreased efficacy of GABAergic transmission.**

13 Discussion

14 We have studied, in mature hippocampal neurons, the cellular and molecular mechanisms
15 regulating the co-transporter NKCC1, which transports chloride ions inside neurons. We
16 showed that the transporter displays a heterogeneous distribution at the plasma membrane:
17 it is either diffusely distributed and freely mobile in the membrane or it is organized in
18 membrane clusters where it is slowed down and confined. Here, we show that this
19 distribution and behavior can be rapidly tuned by GABAergic activity changes. In particular,
20 acute GABA_AR activation or inhibition with muscimol or gabazine respectively causes the
21 escape of transporters from membrane clusters and its translocation to endocytic zones
22 where it is confined. GABA_AR-mediated regulation of NKCC1 membrane distribution uses
23 chloride as a secondary messenger and the Cl⁻-sensitive WNK/SPAK pathway, which in turn
24 affects the phosphorylation of a series of threonine residues on NKCC1. At rest, these
25 modifications have little effect on [Cl⁻]_i but they could participate to the accumulation of Cl⁻

1 in neurons in pathological conditions associated with an up-regulation of NKCC1.

2 An increase in clustering generally correlates with a slowing down and confinement of the
3 molecule in a sub-cellular compartment e.g. synapses for neurotransmitter receptors.
4 Conversely, a dispersion of molecules from clusters implies a lifting of the diffusion brakes.
5 Usually, the molecule is confined thanks to its binding to scaffolding proteins that anchors it
6 to the cytoskeleton. However, the membrane molecule can escape from this confined region
7 by lateral diffusion. This is the case of excitatory glutamate receptors and inhibitory
8 GABA_ARs [33-34], as well as of ion transporters such as the chloride co-transporter KCC2
9 [11-12]. However, NKCC1 has a different diffusion behavior. The transporter was restricted
10 in its movement when its membrane clustering was decreased (gabazine and muscimol
11 conditions) while its diffusion was not significantly changed when its clustering increased
12 (low chloride condition). This could be explained by the fact that a low proportion of NKCC1
13 transporters is clustered in the membrane while a more significant proportion of them is
14 present in endocytic zones where they are confined and stored (in particular upon GABA_AR
15 activity changes).

16 Acute blockade of glutamatergic activity by the TTX+KYN+MCPG drug cocktail confines
17 NKCC1 to the axon [35]. This suggests that spontaneous glutamatergic activity in contrast
18 makes NKCC1 mobile along the axon. Here we investigated the role of GABAergic
19 transmission on NKCC1 diffusion in the axon. We show that blocking GABA_AR-mediated
20 inhibition by adding gabazine to the bath in the presence of TTX+KYN+MCPG to prevent
21 the indirect effects of gabazine on excitation, removes the constraints on NKCC1 diffusion
22 in the axon. Conversely, activation of GABA_AR by muscimol in the presence of
23 TTX+KYN+MCPG slows NKCC1 in the axon. Thus, GABAergic and glutamatergic activity
24 have opposite effects on NKCC1 diffusion in the axon. We propose that an increase in
25 spontaneous glutamatergic activity homeostatically regulates NKCC1 diffusion in the axon

1 to compensate for the treatment-induced increase in activity by decreasing the
2 depolarizing/excitatory effect of GABA_AR in the axon. Here, in contrast, muscimol-mediated
3 GABA_AR solicitation confines NKCC1 in the axon to enhance depolarizing GABA to
4 counteract the increased inhibition. The effects of inhibition can also be attributed to
5 homeostatic regulation of excitatory GABAergic transmission in the axon with potential
6 effects on neurotransmitter release [36] and action potential firing [31].

7 Of note, the effects of gabazine on NKCC1 diffusion in the dendrite and axon are opposite
8 highlighting distinct regulatory mechanisms. In the case of regulation by glutamatergic
9 transmission, different effects were also observed in the dendrite vs. the axon. Future
10 experiments will tell whether this difference is due to variations in intracellular chloride
11 concentration (with a higher concentration in the axon than in the dendrite) and activation of
12 the WNK pathway or to different molecular mechanisms.

13 In the dendrites of mature neurons, we observed a similar effect of GABA_AR activation or
14 inhibition on the diffusion and clustering of dendritic NKCC1. In both cases, the transporter
15 was sent to endocytic zones and confined there. The fact that there was no change in the
16 global pool (surface + intracellular) of the transporter indicates that it is stored in the
17 endocytic zones without being internalized and degraded. These endocytic zones have been
18 shown to constitute reserve pools of neurotransmitter receptors, which can, depending on
19 the synaptic demand, be released and reintegrated into the diffusing pool of receptors [37].

20 In the case of NKCC1, this reserve pool would allow a rapid increase in the transporter
21 availability in the plasma membrane, for example in pathological situations in which an up-
22 regulation of NKCC1 has been observed [9].

23 Muscimol by activating the GABA_AR raises $[Cl^-]_i$ [18]. An increase in $[Cl^-]_i$ inhibits the activity
24 of WNK1 and SPAK, OSR1 kinases [38-39], leading to the dephosphorylation of NKCC1-
25 T203/207/212/217/230 [40-42], and reduced transporter activity [43-44]. We have shown

1 that following exposure to muscimol, NKCC1 escaped from membrane clusters and was
2 confined in endocytic zones. Thus, transition of the transporter between membrane clusters
3 and endocytic zones by lateral diffusion would allow modulating rapidly its availability in the
4 membrane and its activity. The effects of muscimol are compatible with NKCC1-
5 T203/207/212/217/230 dephosphorylation. Pharmacological (WNK-463 or closantel) or
6 genetic (WNK-KD) blockade of the WNK/SPAK pathway or the expression of NKCC1 TA3
7 or NKCC1 TA5 mutants that mimic NKCC1 dephosphorylation have the same effects as
8 muscimol: they restrict NKCC1 in their movement and reduced the membrane clustering of
9 the transporter. The demonstration that the effect of muscimol directly involve
10 dephosphorylation of NKCC1-T203/207/212/217/230 was provided by the fact that the effect
11 of muscimol on NKCC1 clustering can be prevented when the mutant NKCC1-TA5 was
12 exposed to the drug, compared to WT.

13 In contrast, treatment of neurons with gabazine, by blocking the activity of GABA_ARs,
14 decreases dendritic [Cl⁻]_i. In non-neuronal cells, low chloride activates WNK1/SPAK [38-39]
15 by auto-phosphorylation of WNK1 S382 residue. Then, WNK phosphorylates in cascade
16 SPAK on S373 and OSR1 on S325 [45], that in turn phosphorylate NKCC1 on
17 T203/207/212/217/230 and increase the surface expression and activity of the transporter
18 [44, 46]. We have shown that this signaling cascade is operant in mature hippocampal
19 neurons: gabazine activates the WNK1/SPAK/OSR1 pathway by phosphorylation thus
20 inducing the phosphorylation of KCC2-T906/1007 as well as NKCC1-T203/207/212/217/230
21 [18]. If muscimol decreases NKCC1 clustering and confines it to endocytic zones, gabazine
22 treatment should conversely induce the escape of the transporter from endocytic zones
23 thereby increasing its clustering and function in the membrane. Although we observed that
24 a decrease in [Cl⁻]_i by substituting chloride with methane sulfonate decreases the
25 confinement of NKCC1 and increases its membrane stability and clustering, treatment of
26 neurons with gabazine did not reproduce this effect. On the contrary, gabazine confined the

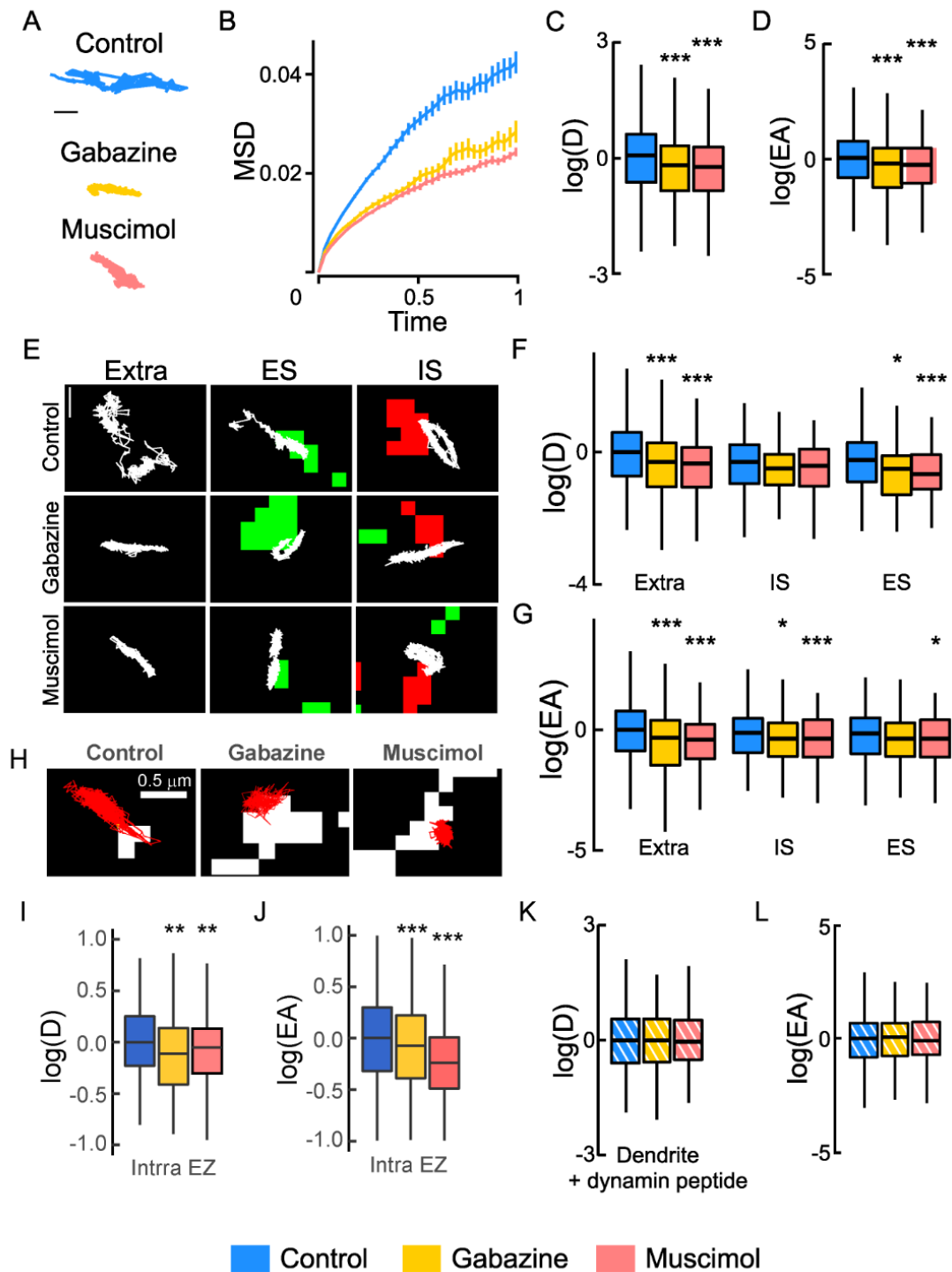
1 transporter and induced the loss of its clustering just as muscimol did. However, we have
2 shown that antagonizing inhibition with gabazine reduces surface expression of KCC2 by
3 increasing lateral diffusion and endocytosis of the transporter [18]. This results in reduced
4 intracellular chloride extrusion capacity of the neuron leading to a significant increase in $[Cl^-]_i$
5 as monitored by SuperClomeleon imaging [18]. KCC2 is more effective in regulating $[Cl^-]_i$
6 than NKCC1 in dendrites of mature neurons [47-48]. We therefore hypothesize that the
7 regulation of NKCC1 by gabazine is not due to a decrease in $[Cl^-]_i$ but instead to an increase
8 in $[Cl^-]_i$ following the regulation of KCC2 by the WNK1/SPAK/OSR1 pathway. Thus, changes
9 in the membrane expression of KCC2 (under control of the WNK1/SPAK/OSR1 pathway)
10 would condition that of NKCC1, thus allowing $[Cl^-]_i$ to be maintained at a low level in mature
11 neurons. This would explain why expressing recombinant NKCC1-WT in mature neurons
12 does not significantly increase its expression at the membrane nor does it increase $[Cl^-]_i$.
13 This suggests that the level of expression of NKCC1 at the plasma membrane is under the
14 control of KCC2 and its tuning of $[Cl^-]_i$.

15 Nevertheless, we show that the membrane stability and clustering of NKCC1 can be rapidly
16 regulated by lateral diffusion and that this mechanism is rapidly controlled by GABAergic
17 inhibition and the WNK1/SPAK/OSR1 pathway on the dendrites of mature neurons.
18 Although this pathway has little influence on the amount/function of NKCC1 at the neuronal
19 surface under basal activity conditions, we propose that it may play a role in pathological
20 situations associated with increased expression levels of NKCC1 based on our 4-AP data.
21 Interestingly, in the pathology, upregulation of NKCC1 is often accompanied by a down-
22 regulation of KCC2 at the neuronal surface [3, 16]. KCC2 is also regulated by diffusion-
23 capture. We have shown that a short exposure of neurons to the convulsive agent 4-AP
24 increases the lateral diffusion of KCC2, which escapes from the clusters, is internalized and
25 degraded [13]. Thus, lateral diffusion would be a general mechanism to control the
26 membrane stability of chloride co-transporters. Moreover, the fact that KCC2 is also

1 regulated in mature neurons by the WNK1/SPAK/OSR1 pathway [18] and that this regulation
2 has an inverse effect on membrane stability, clustering and function of KCC2 indicates that
3 this pathway is a target of interest in the pathology. Inhibition of the pathway would prevent
4 the loss of KCC2 and the increase of NKCC1 at the surface of the neuron, thus preventing
5 the abnormal rise of $[Cl^-]_i$ in the pathology and the resulting adverse effects.

1

2 Figure legends



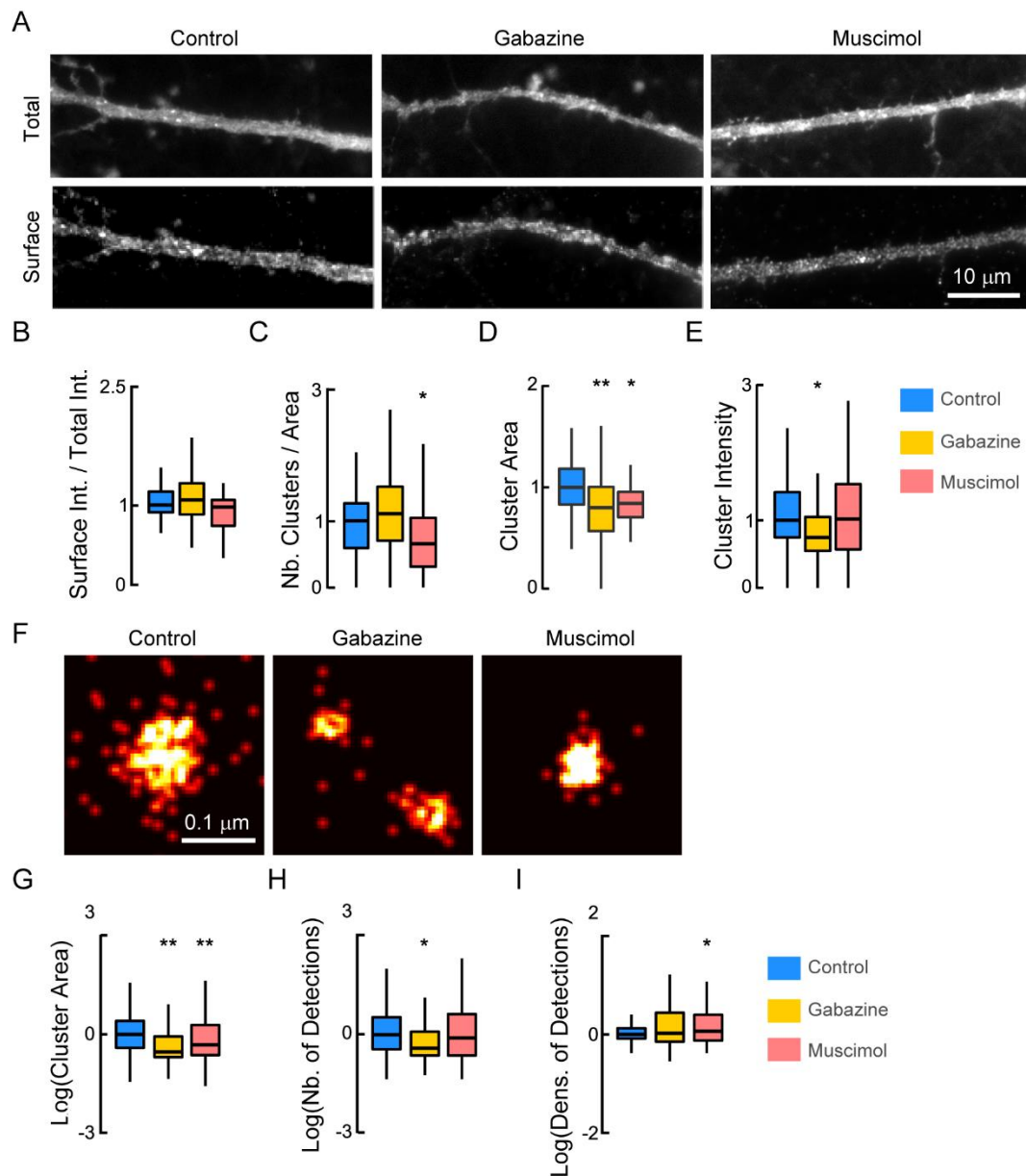
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4 **Fig. 1. GABA_AR activity regulates NKCC1 membrane dynamics.** A, Examples of NKCC1

5 trajectories showing reduced surface exploration in the presence of gabazine or muscimol.

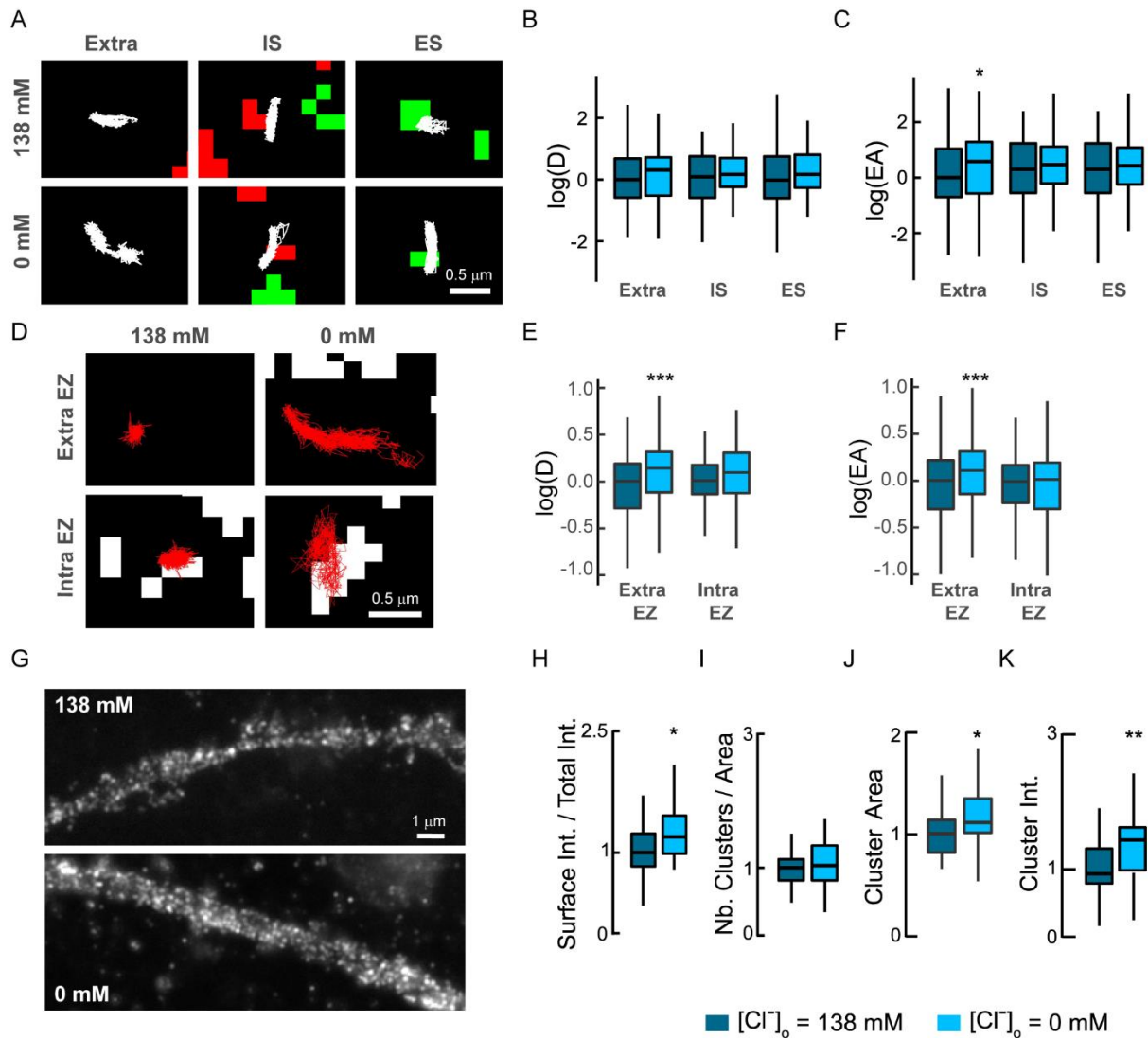
1 Scale bar, 0.5 μm . **B**, Time averaged MSD functions in control (blue) vs. gabazine (yellow)
2 or muscimol (orange) conditions show increased confinement upon gabazine or muscimol
3 application. **C-D**, Boxplots of $\log(D)$ of NKCC1 in control condition (blue) or upon application
4 of gabazine (yellow) or muscimol (orange) showing reduced diffusion upon gabazine or
5 muscimol application. $N = 1558$ QDs (control, 41 cells), $n = 387$ QDs (gabazine, 18 cells),
6 Welch t-test, $p = 1.3 \cdot 10^{-6}$, $n = 545$ QDs (muscimol, 27 cells), Welch t-test, $p = 2.1 \cdot 10^{-12}$, 5
7 cultures. **D**, Median explored area EA in control vs. gabazine or muscimol conditions show
8 increased confinement upon gabazine (Welch t-test, $p = 2 \cdot 10^{-8}$) or muscimol (Welch t-test,
9 $p = 4.2 \cdot 10^{-11}$) application. **E**, Trajectories (white) overlaid with fluorescent clusters of
10 recombinant homer1c-DsRed (green) or gephyrin-Finger-YFP (red) to identify extrasynaptic
11 trajectories (extra), trajectories at excitatory (ES) and inhibitory synapses (IS). Scale bar, 0.4
12 μm . **F-G**, $\log(D)$ (**F**) and EA (**G**) of NKCC1 are decreased upon gabazine or muscimol
13 application as compared with control condition. Note that the effect is more pronounced for
14 extrasynaptic trajectories than for ES or IS trajectories. Diffusion coefficient (D): Extra, $n =$
15 899 QDs (control), 227 QDs (gabazine), $p = 2.9 \cdot 10^{-5}$, $n = 268$ QDs (muscimol) $p = 2.2 \cdot 10^{-9}$;
16 IS, $n = 244$ QDs (control), 79 QDs (gabazine) $p = 0.16$; $n = 142$ QDs (muscimol) $p = 0.19$;
17 ES, $n = 415$ QDs (control), $n = 81$ QDs (gabazine) $p = 0.021$, $n = 135$ QDs (muscimol) $p =$
18 0.00046. Explored area (EA): Extra, gabazine $p = 2.9 \cdot 10^{-5}$, muscimol $p = 2.2 \cdot 10^{-9}$; IS,
19 gabazine $p = 0.16$, muscimol $p = 0.19$; ES, $n = 415$ QDs (control), $n = 81$ QDs (gabazine) p
20 $= 0.021$, $n = 135$ QDs (muscimol) $p = 0.00046$. **H**, NKCC1 trajectories in control vs. gabazine
21 or muscimol conditions in relation with endocytic zones identified by the presence of clathrin-
22 YFP clusters. Scale bar, 0.5 μm . **I-J**, Reduced diffusion coefficient (**I**) and explored area (**J**)
23 of NKCC1 within endocytic zones upon muscimol and gabazine treatment. Diffusion
24 coefficient (D): Ctrl $n = 117$ QDs, 48 cells, Gbz $n = 145$ QDs, 64 cells, $p = 0.0014$; Ctrl $n =$
25 273 QDs, 35 cells, Musc $n = 247$ QDs, 28 cells, $p = 0.0056$, 3 cultures. Explored area (EA):
26 Gbz, $p = 5.46 \cdot 10^{-5}$ and Musc, $p = 2.2 \cdot 10^{-16}$, 3 cultures. **K-L**, No effect of gabazine or muscimol

1 on $\log(D)$ (K) and EA (L) of NKCC1 in conditions of blockade of endocytosis. Diffusion
 2 coefficient (D): Bulk Ctrl n = 491 QDs, Gbz n = 238 QDs, p = 0.6 and Musc n = 243 QDs, p
 3 = 0.61, 5 cultures. Explored area (EA): Gbz, p = 0.84 and Musc, p = 0.81, 5 cultures. B, F, I,
 4 K: D in $\mu\text{m}^2.\text{s}^{-1}$; C: MSD in μm^2 vs time (s); D, G, J, L: EA in μm^2 .



5
 6 **Fig. 2. Regulation of NKCC1 membrane clustering by GABA_A-mediated inhibition.** A,
 7 Conventional microscopy of HA surface staining in hippocampal neurons (DIV 21)
 8 expressing recombinant NKCC1-HA in absence (Ctrl) or presence of gabazine (Gbz), or
 9 muscimol (Musc) for 30 min. Scale bar, 10 μm . B, Quantification of the ratio of the surface

1 pool of NKCC1 over the total pool of NKCC1 in control (blue), gabazine (yellow), and
2 muscimol (orange) conditions showing no significant changes of surface NKCC1 after
3 gabazine or muscimol treatment. Ctrl n = 30 cells, Gbz, n = 41 cells, MW test p = 0.87, Musc
4 n = 38 cells, p = 0.17, 8 cultures. **C-E**, Quantification of NKCC1-HA cluster number (**C**), area
5 (**D**), and intensity (**E**) shows reduced density and size of NKCC1 clusters upon muscimol
6 treatment while gabazine treatment reduced the size and intensity of NKCC1 clusters.
7 Values were normalized to the corresponding control values. The MW test was used for data
8 comparison. Gabazine: cluster number (nb) p = 0.49, area p = 0.003, intensity p = 0.017.
9 Muscimol: cluster nb p = 0.027, area p = 0.029, intensity p = 0.92. **F-I**, STORM showing that
10 gabazine and muscimol treatments alter NKCC1 nanoclusters at the surface of hippocampal
11 neurons. **F**, Representative STORM images of NKCC1 at the surface of neurons exposed
12 30 min to gabazine or muscimol. Scale bar, 0.1 μm . **G**, Quantification of NKCC1 cluster area
13 shows reduction in nanocluster size upon gabazine and muscimol treatment. Ctrl n = 550
14 nanoclusters, Gbz n = 192 nanoclusters, Monte-Carlo simulations of the MW test p = 0.002,
15 Musc n = 410 nanoclusters, p = 0.004, 4 cultures. **H**, Quantification of the number of particles
16 detected per nanocluster showing reduced number of detection upon gabazine (Monte-
17 Carlo simulations of MW test, p = 0.012) but not muscimol (p = 0.3) exposure. **I**,
18 Quantification of the density of NKCC1 molecules per square micrometer highlighting denser
19 NKCC1 packing upon neuronal exposure to muscimol (Monte-Carlo simulations of MW test,
20 p = 0.016) but not gabazine (p = 0.97). C: μm^{-1} , D: μm^2 , G: nm^2 , H: μm^{-1} , I: μm^{-2} .



1

2 **Fig. 3. Lowering intracellular chloride increases the membrane diffusion, clustering**

3 **and stability of NKCC1.** A, NKCC1 trajectories in high vs. low extracellular Cl⁻

4 concentration in the extrasynaptic area (extra), and at inhibitory (IS) or at excitatory (ES)

5 synapses. Scale bar, 0.5 μm. B, No major effect of a reduction of Cl⁻ concentration on log(D)

6 (B) of NKCC1. Note the increase in log(EA) for extrasynaptic trajectories (C) in conditions

7 of low chloride. Diffusion coefficient (D): extra, low Cl⁻ n = 128 QDs, high Cl⁻ n = 93 QDs,

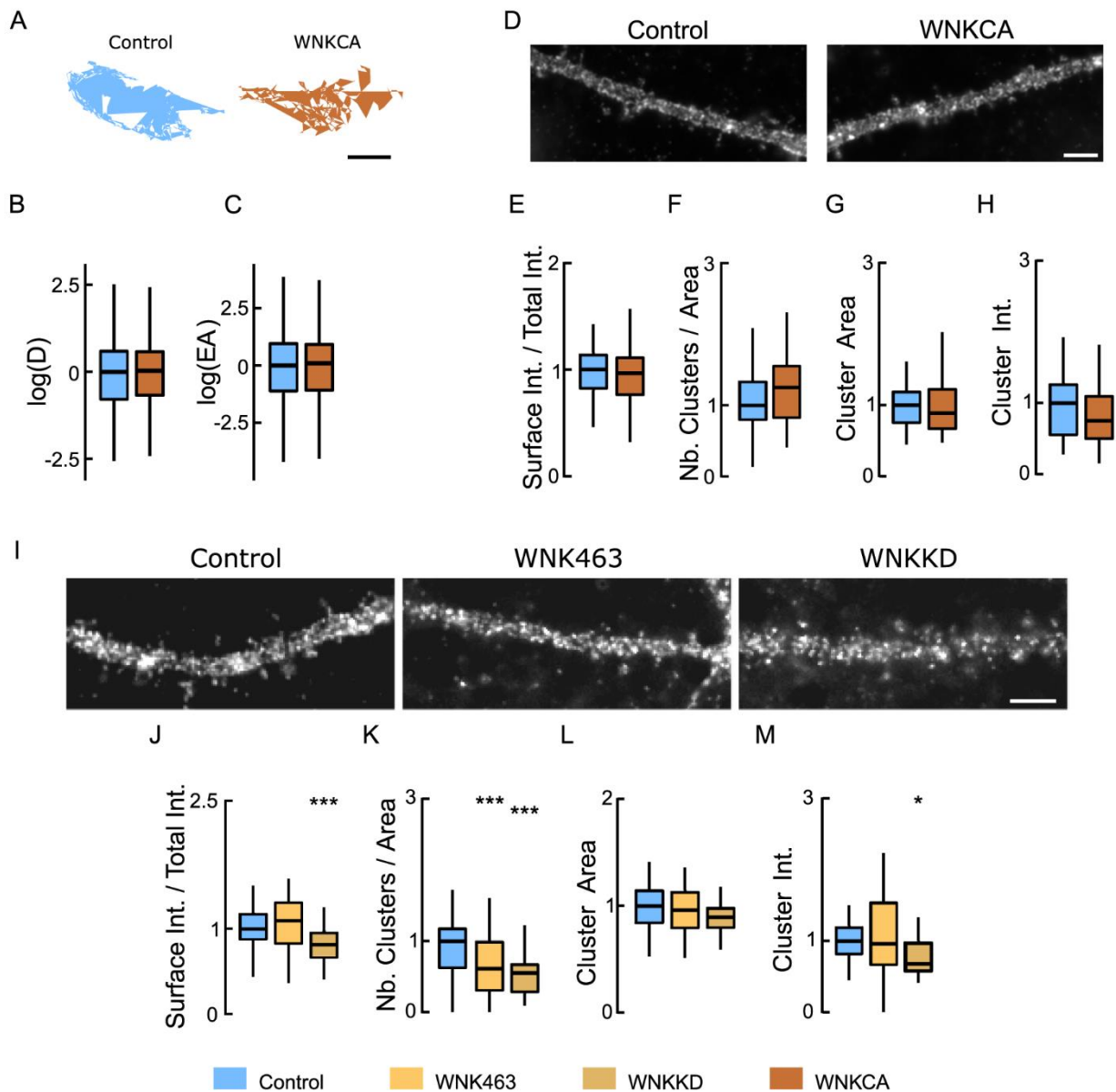
8 Welch t-test p = 0.89; IS, low Cl⁻ n = 64 QDs, high Cl⁻ n = 56 QDs, Welch t-test p = 0.74; ES,

9 low Cl⁻ n = 62 QDs, high Cl⁻ n = 68 QDs, Welch t-test p = 0.36, 2 cultures. Explored area

10 (EA): extra, low Cl⁻ Welch t-test p = 0.01; IS, Welch t-test p = 0.6; ES, Welch t-test p = 0.6.

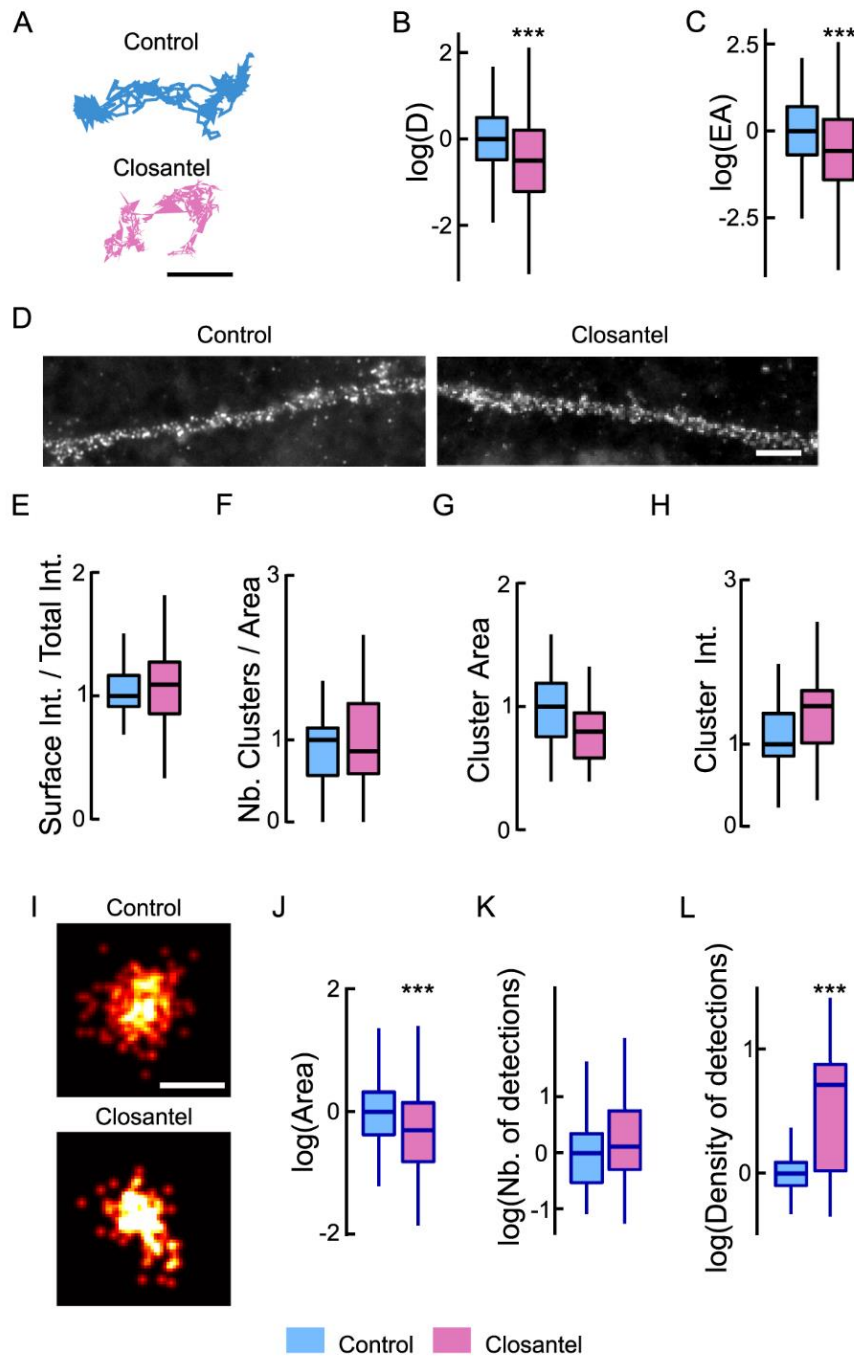
11 D, Examples of NKCC1 trajectories (red) inside and outside clathrin-YFP fluorescent (white)

1 endocytic zones in conditions of low vs. high chloride extracellular Cl⁻ concentration. Scale
2 bar, 0.5 μm. **E-F**, lowering intracellular chloride levels increases log(D) and log(EA) of
3 NKCC1 located outside endocytic zones while the diffusion of NKCC1 inside endocytic
4 zones is unchanged. Diffusion coefficient (D): extra EZ, low Cl⁻ n = 209 QDs, high Cl⁻ n =
5 238 QDs, Welch t-test p = 0.0006; intra EZ, low Cl⁻ n = 91 QDs, high Cl⁻ n = 105 QDs, Welch
6 t-test p = 0.23, 2 cultures. Explored area (EA): extra EZ, p = 7.12 10⁻⁸; intra EZ, p = 0.41. **G-**
7 **K**, Lowering intracellular chloride increases surface detection and clustering of NKCC1. **G**,
8 HA surface staining in neurons expressing recombinant NKCC1-HA in in high vs. low
9 extracellular Cl⁻ concentration for 30 min. Scale bar, 1 μm. **H**, Quantification of the ratio of
10 the surface pool of NKCC1 over the total pool of NKCC1 in high (dark blue), and low (light
11 blue) Cl⁻ concentration showing increase of NKCC1 surface staining upon reduction of Cl⁻
12 concentration. Low Cl⁻ n = 35 cells, high Cl⁻ n = 40 cells, Welch t-test p = 0.01, 3 cultures. **I-**
13 **K**, Quantification of NKCC1-HA cluster number (**I**), area (**J**), and intensity (**K**) shows
14 increased size and intensity of NKCC1 clusters upon reduction of Cl⁻ concentration. Values
15 were normalized to the corresponding control values. Cluster Number (Nb) MW test p = 0.2,
16 area MW test p = 0.018, intensity MW test p = 0.0056. B, E: D in μm².s⁻¹; C, F: EA in μm²;
17 I : μm⁻¹; J : μm².



1 **Fig. 4. Inhibiting WNK1 reduces the clustering of NKCC1 at the neuronal surface.** A,
 2 Representative trajectories of NKCC1 in neurons expressing constitutively-active WNK1
 3 (WNK-CA, blue) vs. control (gray). Bar : 0.4 μ m. B-C, WNK-CA over-expression does not
 4 change NKCC1 diffusion at the plasma membrane, with no effect on D (B) and EA (C).
 5 Diffusion coefficient (D): Bulk, Ctrl n = 305 QDs, WNK-CA n = 358 QDs, Welch t-test p =
 6 0.81, 3 cultures; Explored area (EA): Welch t-test p = 0.49. D, HA surface staining in
 7 hippocampal neurons expressing recombinant NKCC1-HA together with WNK1-CA or a
 8 control plasmid. Scale bar, 4 μ m. E, Overexpressing WNK-CA does not modify the level of
 9 NKCC1 expressed at the cell surface. Ctrl n = 45 cells, WNK-CA: n = 43 cells, Welch t-test
 10

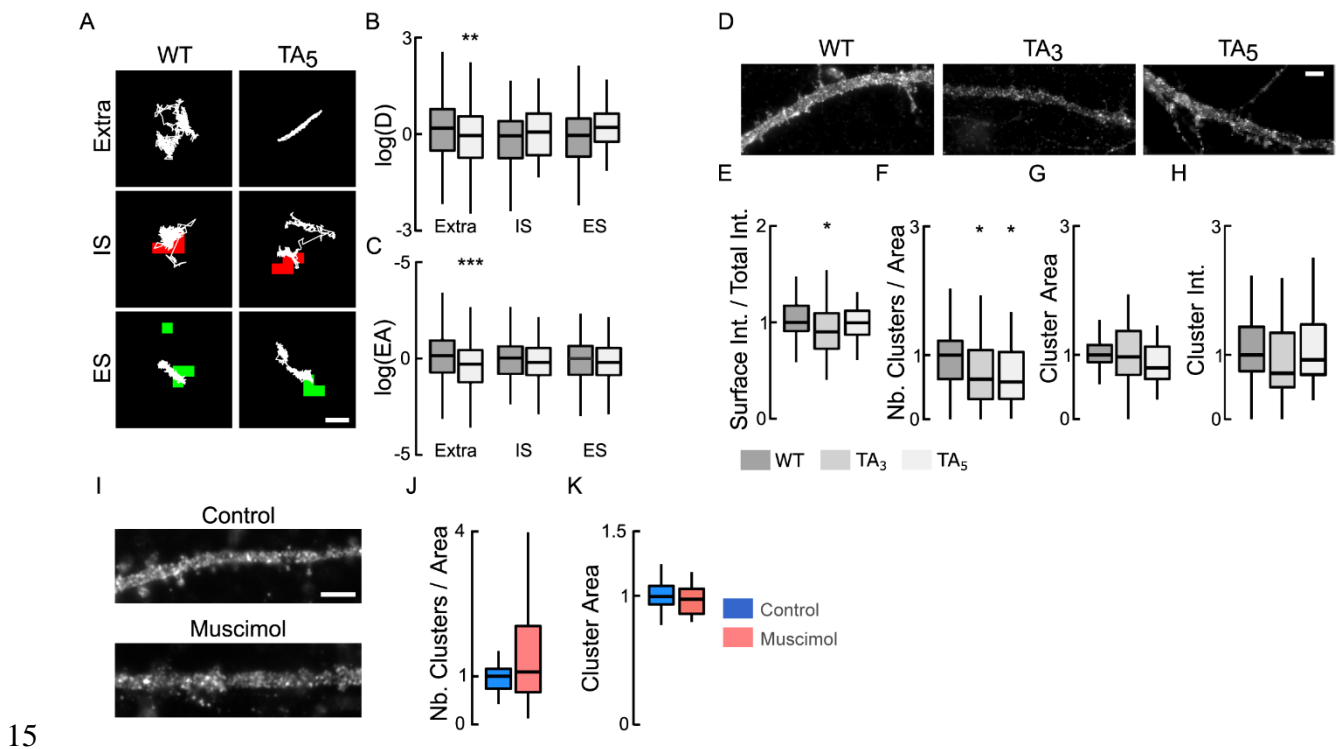
1 P = 0.26, 3 cultures. **F-H**, No effect of WNK-CA expression on NKCC1 cluster number (**F**),
2 area (**G**), and intensity (**H**). Values were normalized to the corresponding control values.
3 Cluster Number (Nb) MW test p = 0.14, area MW test p = 0.32, intensity MW test p = 0.25.
4 **I**, Impact of an inhibition of WNK1 on HA surface staining of neurons transfected with
5 NKCC1-HA. WNK1 was inhibited either by incubating the neurons for 30 min with a specific
6 inhibitor WNK-463 or by co-expressing kinase-dead WNK1 (WNK1-KD). Scale bar, 4 μ m. **J**,
7 Incubating the neurons with WNK-463 (orange) does not modify the surface level of NKCC1
8 compared to controls (blue), however over-expression of WNK-KD (brown) sharply reduced
9 NKCC1 surface levels. WNK-463 experiment: Ctrl n = 24 cells, WNK-463 n = 25 cells, Welch
10 t-test p = 0.58. WNK-KD experiment: Ctrl n = 34 cells, WNK-KD n = 24 cells, Welch t-test p
11 = 0.0096, 4 cultures. **K-M**, Loss of NKCC1 clusters (**K**) and reduced cluster size (**L**) but not
12 cluster intensity (**M**) upon WNK1 activity suppression with either WNK-KD or WNK-463, as
13 compared to control conditions. Values were normalized to the corresponding control values.
14 The MW test was used for data comparison. WNK-KD experiment: Cluster Number (Nb) p
15 = 0.45 10^{-6} , area p = 0.012, intensity p = 0.0031. WNK-463 experiment: Cluster Number (Nb)
16 p = 0.0008, area p = 0.86, intensity p = 0.83.



1

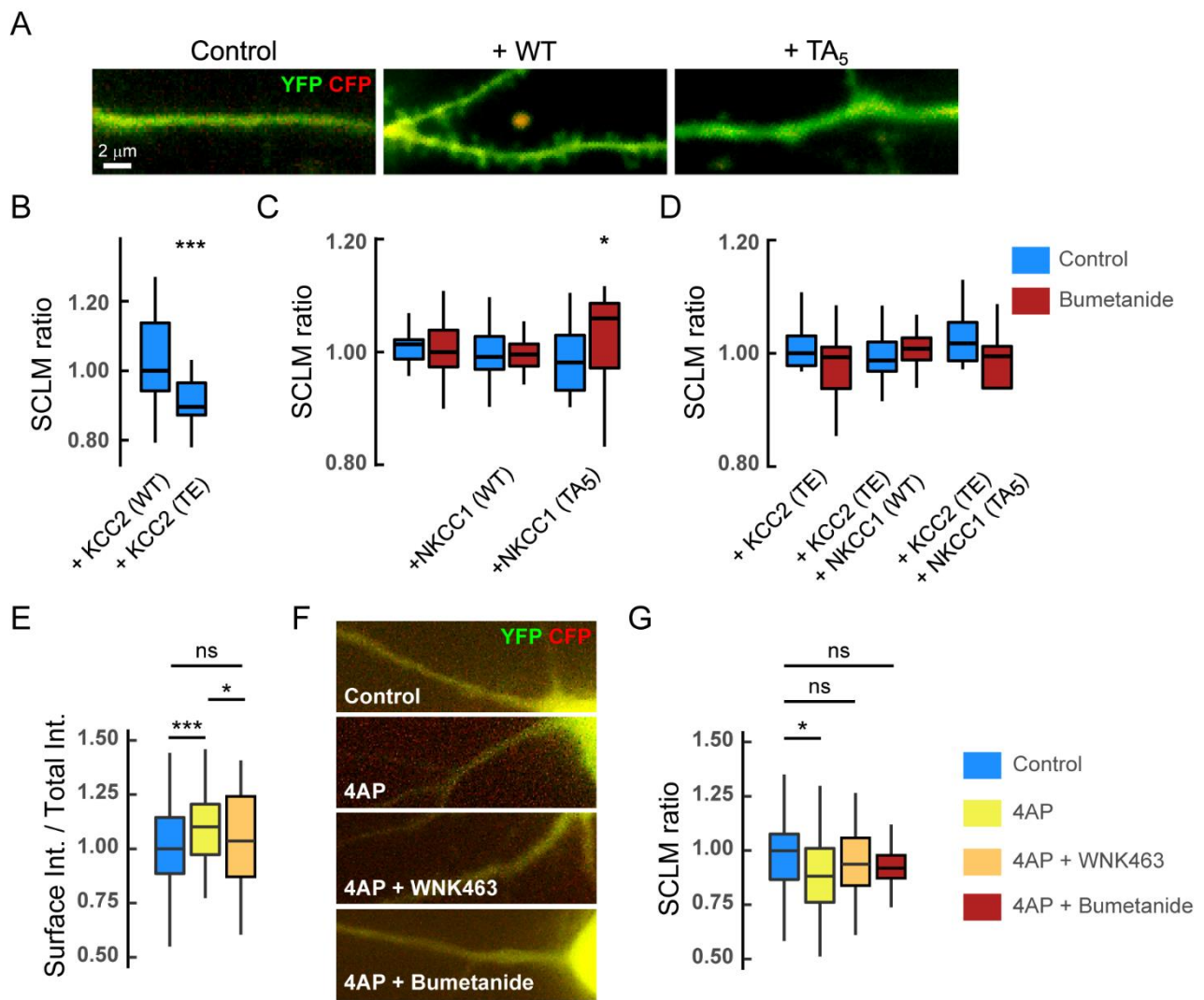
2 **Fig. 5. Inhibiting SPAK-OSR1 activity tunes NKCC1 membrane diffusion and**
 3 **clustering.** A, NKCC1 trajectories showing reduced surface exploration in the presence of
 4 closantel. Scale bar, 0.5 μm . B-C, Log (D) (B) and EA (C) of NKCC1 are decreased upon
 5 closantel application (pink) as compared with control condition (blue), indicating reduced
 6 mobility and increased confinement. Diffusion coefficient (D): Bulk, Ctrl n = 433 QDs,
 7 closantel n = 371 QDs, Welch t-test $p = 5.5 \cdot 10^{-5}$, 2 cultures. Explored area (EA): Welch t-

1 test $p = 4.8 \cdot 10^{-11}$. **D-H**, Standard epifluorescence microscopy showing no significant effect
 2 of closantel on NKCC1 membrane immunoreactivity. **D**, HA surface staining in hippocampal
 3 neurons expressing recombinant NKCC1-HA treated or not with closantel. Scale bar, 4 μm .
 4 **E**, Closantel treatment does not alter the surface expression level of NKCC1. Ctrl $n = 51$
 5 cells, closantel $n = 61$ cells, Welch t-test $p = 0.49$, 7 cultures. **F-H**, Closantel has no impact
 6 on NKCC1 cluster number (**F**), area (**G**), and intensity (**H**). Cluster Number (Nb) MW test p
 7 = 0.6, area MW test $p = 0.09$, intensity MW test $p = 0.81$. **I-L**, STORM imaging showing that
 8 closantel affects NKCC1 organization at the nanoscale. **I**, Representative images of NKCC1
 9 at the surface of neurons exposed 30 min to closantel vs control condition. Scale bar, 0.1
 10 μm . Quantification of NKCC1 cluster area (**J**), number of particles per cluster (**K**) and density
 11 of detections in the clusters (**L**) reveal reduction in cluster size upon closantel treatment. Ctrl
 12 $n = 218$ clusters, closantel $n = 147$ clusters, 2 cultures. Cluster area: Monte-Carlo
 13 simulations of the MW test $p < 0.001$; Nb detection: MW test $p = 0.058$, density: MW test p
 14 < 0.001 .



16 **Fig. 6. NKCC1 membrane dynamics, stability and clustering is dependent on NKCC1**

1 **phosphorylation of T203/207/212/217/230. A,** Examples of NKCC1-
2 T203/207/212/217/230 (WT) and NKCC1-T203/207/212/217/230A (TA₅) trajectories in
3 resting condition at extrasynaptic site (extra), at inhibitory (IS) and excitatory glutamatergic
4 (ES) synapses. Scale bar, 0.5 μm. **B-C,** Log(D) (**B**) and EA (**C**) show that the dephospho-
5 mimetic NKCC1-TA₅ (light gray) was slower and more confined than NKCC1-WT (gray) at
6 extrasynaptic sites but not near synapses. Diffusion coefficient (D): WT: extra, n = 189 QDs,
7 IS, n = 42 QDs, ES n = 30 QDs; TA₅ : extra, n = 166 QDs, IS, n = 33 QDs, ES n = 34 QDs;
8 from 67 cells and 2 cultures. Welch t-test: extra p = 0.0088, IS p = 0.28, ES p = 0.73.
9 Explored area (EA): extra p = 4.5 10⁻⁴, IS p = 0.71, ES p = 0.45. **D,** HA surface staining in
10 hippocampal neurons expressing recombinant NKCC1-WT vs NKCC1-TA₃ or NKCC1-TA₅
11 in resting conditions. Scale bar, 4 μm. **E,** Expression of NKCC1-TA₃ is slightly reduced at
12 the plasma membrane as compared to NKCC1-WT. WT (dark gray) n = 68 cells, TA₃ (gray)
13 n = 43 cells, TA₅ (light gray) n = 36 cells (9 cultures) ; WT vs TA₃ Welch t-test p = 0.041, WT
14 vs TA₅ p = 0.87, 9 cultures. **F-H,** Quantification of cluster number (**F**), area (**G**) and intensity
15 (**H**) for NKCC1-TA₃, NKCC1-TA₅ vs. NKCC1-WT. Note the reduced density of cluster (**F**) for
16 NKCC1-TA₃ and NKCC1-TA₅ as compared to NKCC1-WT. Cluster Number (Nb) WT vs TA₃
17 MW test p = 0.042, WT vs TA₅ MW test p = 0.014; area: WT vs TA₃ MW test p = 0.46, WT
18 vs TA₅ MW test p = 0.22; intensity WT vs TA₃ MW test p = 0.3, WT vs TA₅ MW test p = 0.86.
19 **I,** HA surface staining in hippocampal neurons expressing recombinant NKCC1-TA₅
20 exposed or not to muscimol. Scale bar, 4 μm. **J-K,** Muscimol application has no effect on
21 NKCC1-TA₅ cluster number: MW test, P = 0.45 (**J**) and area: MW test, P = 0.38 (**K**). Control:
22 26 cells, muscimol : 22 cells, 3 cultures.



1

2 **Fig. 7. Functional impact of NKCC1 regulation by the WNK signaling on chloride**
3 **homeostasis.** **A-D,** In basal activity conditions, expression of recombinant WT or
4 dephospho-mimetic NKCC1 have no major impact on $[Cl^-]_i$ in mature hippocampal neurons.
5 **A,** composite images of CFP (red) and YFP (green) in neurons expressing SuperClomeleon
6 (SCLM) alone (Control) or in combination with NKCC1-T203/207/212/217/230 (WT) or
7 NKCC1-T203/207/212/217/230A (TA₅) in resting conditions. Scale bar, 2 μ m. **B,** CFP/YFP
8 fluorescence ratio in hippocampal neurons expressing SCLM together with KCC2-
9 T906/T1007 (WT) or phospho-mimetic KCC2-T906/T1007E (TE). KCC2-WT n = 36 cells,
10 KCC2-TE n = 39 cells, MW test $p = 7.1 \cdot 10^{-6}$, 3 cultures. **C,** CFP/YFP fluorescence ratio in
11 neurons expressing SCLM alone or in combination with NKCC1-WT or NKCC1-TA₅ before
12 (blue) and after (red) 10-30 min application of bumetanide. SCLM Ctrl n = 20 cells, Bumet n

1 = 17 cells; SCLM + NKCC1-WT Ctrl n = 18 cells, Bumet n = 20 cells; SCLM + NKCC1-TA₅
2 Ctrl n = 21 cells, Bumet n = 16 cells; 3 cultures. Ctrl vs Bumet: SCLM MW test p = 0.96,
3 SCLM + NKCC1-WT MW test p = 0.53, SCLM + NKCC1-TA₅ MW test p = 0.029. SCLM vs
4 SCLM + NKCC1-WT, MW test p = 0.71, SCLM vs SCLM + NKCC1-TA₅, MW test p = 0.69;
5 SCLM + NKCC1-WT vs SCLM + NKCC1-TA₅, MW test p = 0.89. **D**, CFP/YFP fluorescence
6 ratio in neurons expressing the same recombinant proteins than in B but in the presence of
7 KCC2-TE before (blue) and after (red) bumetanide treatment. SCLM + KCC2-TE Ctrl n = 15
8 cells, Bumet n = 15 cells, MW test p = 0.15; SCLM + KCC2-TE + NKCC1-WT Ctrl n = 13
9 cells, Bumet n = 16 cells, MW test p = 0.56 ; SCLM + KCC2-TE + NKCC1-TA₅ Ctrl n = 21
10 cells, Bumet n = 8 cells, MW test p = 0.092; SCLM + KCC2-TE vs SCLM + KCC2-TE +
11 NKCC1-WT, MW test p = 0.65, SCLM vs SCLM + KCC2-TE + NKCC1-TA₅, MW test p = 0.2,
12 SCLM + KCC2-TE + NKCC1-WT vs SuperClomeleon + KCC2-TE + NKCC1-TA₅, MW test
13 p = 0.12. 2 cultures. **E**, Quantification of the ratio of the surface pool of NKCC1 over the total
14 pool of NKCC1 in control (blue), 4-AP (yellow), and 4-AP + WNK463 (orange) conditions
15 showing a significant increase in surface NKCC1 upon 4-AP treatment, an effect prevented
16 by pre-incubation of neurons with WNK463 (orange). Ctrl n = 63 cells, 4-AP, n = 64 cells,
17 MW test p = 0.00046, 4-AP + WNK463 n = 57 cells, 4-AP vs. 4-AP + WNK463 p = 0.03, Ctrl
18 vs. 4-AP + WNK463 p = 0.19, 4 cultures. **F**, Overlay images of CFP (red) and YFP (green)
19 in neurons expressing SuperClomeleon (SCLM) and NKCC1 in control vs. 4-AP, 4-AP +
20 WNK463 or 4-AP + Bumetanide conditions. Scale bar, 2 μ m. **G**, CFP/YFP fluorescence ratio
21 in neurons expressing SCLM and NKCC1 in control (blue) vs. 4-AP (yellow), 4-AP +
22 WNK463 (orange) or 4-AP + Bumetanide (red) conditions. Ctrl n = 53 cells, 4-AP n = 48
23 cells, MW test p = 0.01; 4-AP + WNK463 n = 41 cells, MW test p = 0.20; 4-AP + Bumetanide
24 n = 29 cells, MW test p = 0.21, 3-6 cultures.

1 **Supplementary Materials:**

2 The following supporting information can be found: Figure S1: NKCC1 diffusion in the axon
3 is increased upon GABA_AR blockade.

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19 **Author contributions:**

20 SL conceptualized the project, designed the experiments and supervised the experimental
21 work. SL and EC prepared the figures and wrote the paper. EC and JG performed single
22 particle tracking experiments and analyzed the data. EC, JG and SB performed conventional

1 wide field microscopy and analyzed the data. EC performed STORM and analyzed the data.
2 EC and JG conducted chloride imaging and analyzed the data. MR prepared the
3 hippocampal cultures.

4 **Institutional Review Board Statement:**

5 For all experiments performed on primary cultures of hippocampal neurons, animal
6 procedures were carried out according to the European Community Council directive of 24
7 November 1986 (86/609/EEC), the guidelines of the French Ministry of Agriculture and the
8 Direction Départementale de la Protection des Populations de Paris (Institut du Fer à Moulin,
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10 **Informed Consent Statement:**

11 Not applicable

12 **Data Availability Statement:**

13 The data that support the findings of this study are available from the corresponding author
14 upon reasonable request. The transfer of plasmids generated for this study will be made
15 available upon request. A Materials Transfer Agreement may be required.

16 **Conflicts of Interest:**

17 The authors declare no competing interests in relation to the submitted work.

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