Supplemental Materials for:

A Glial K/Cl Transporter Controls Neuronal Receptive-Ending Shape by Chloride Inhibition of an rGC

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

C. elegans methods

C. elegans were cultured as previously described (Brenner, 1974; Stiernagle, 2006). Bristol N2 strain was used as wild type. For all experiments, animals were raised at 20^{o}C (or alternate temperatures where noted) for at least two generations without starvation. Animals were picked as L4 larvae onto fresh plates and assayed 24 hours later, unless otherwise noted. Integration of extra-chromosomal arrays was performed using UV with or out without trioxalen (Sigma, T6137). Germ-line transformations by micro-injection to generate unstable extra-chromosomal array transgenes were carried out using standard protocols (Mello and Fire, 1995). For dietary supplementation with salts, sterile 3M solutions of KCl, NaCl or K-acetate were added to a final concentration of 150mM on plates. Plates were equilibrated for 48 hours before use.

Mutagenesis and mapping of genetic lesions

nsIs228 (*P$_{srtx-1}$:GFP*) animals were mutagenized with 75 mM ethylmethanesulfonate (EMS, Sigma M0880) for 4 hours at 20^{o}C. 10,800 F2 progeny were screened for AFD NRE morphology defects on an Axioplan 2 fluorescence microscope (Zeiss) with a 63x/1.4 NA objective (Zeiss) and dual-band filter set (Chroma, Set 51019). *gcy-8(ns335)* mapped to a 0.44 cM interval by Hawaiian SNIP-SNP mapping (Wicks et al., 2001). This interval was refined to 0.13 cM by deficiency mapping and the gene was identified by fosmid rescue and candidate gene analyses.

Strains and plasmids

Some strains listed below in Sections A and B were sourced from (a) the CGC, funded by NIH Office of Research Infrastructure Programs (P40 OD010440), (b) the International *C. elegans* Gene Knockout Consortium (*C. elegans* Gene Knockout Facility at the Oklahoma Medical Research Foundation, funded by the National Institutes of Health; and the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, funded by the Canadian Institute for Health Research, Genome Canada, Genome BC, the Michael Smith Foundation, and the National Institutes of Health) and (c) National BioResource Project (NBRP), Japan. Recombineered fosmids were obtained from The *C. elegans* TransgeneOme Project.

A. Mutants

LG1: *pde-1 (nj57), pde-5 (nj49), arl-13(gk513)*

LG2: *kcc-3(ok228), kcc-3(tm3649), pde-3 (nj59), pde-4(ok1290)*

LG3: *tax-4(p768), pde-2(tm3098)*

LG4: *gcy-8(tm949), gcy-8(ns335), gcy-18(nj38), gcy-23(ok797), cng-3(jh113), wsp-1(gm324), wsp-1(gk206830), egl-4(n478), egl-4(ad450), bbs-2(gk544)*

LG5: *osm-6(p811), ttx-1(p767),*

LGX: *dyf-11(mn392)*, *ttx-3(mg158)*

B. Integrated transgenes

nsIs228 was the control strain for all non-transgenic animals.

Strain	**Chromosome**	**Genotype**	**Reference**
nsIs228	I	$P_{srtx\text{-}1}$*:GFP*	(Colosimo et al., 2004). Extra-

			chromosomal array from Piali Sengupta's lab was integrated in this study.
nsIs143	X	*pF16F9.3:DsRed*	(Procko et al., 2011)
nsIs373	X	P_{srtxB}*:GFP*	AFD specific. Generated in this study by promoter bashing $P_{srtx\text{-}1}$
kyIs136	X	$P_{str\text{-}2}$*:GFP*	(Troemel et al., 1999)
oyIs44	V	*odr-1:RFP*	(L'Etoile and Bargmann, 2000)
nsIs109 and nsIs113		pF16F9.3:DT-A(G53E)	(Bacaj et al., 2008)

C. Extra-chromosomal transgenes and plasmids generated in this study

All transgenic arrays were generated with $P_{elt\text{-}2}$*:mCherry* (Armenti et al., 2014) as co-injection marker with three exceptions. *pde-1* and *kcc-3* fosmids had $P_{mig\text{-}24}$*:Venus* ((Abraham et al., 2007) as co-injection marker; *nsIs228* and *nsIs373* had $P_{unc\text{-}122}$*:RFP* (Miyabayashi et al., 1999) as marker and $P_{ver\text{-}1}$*:rab-1* constructs had *pRF4* (Gu et al., 1998) as co-injection marker. All transgenes are injected at 50 ng/ul unless otherwise noted.

Extra-chromosomal array (nsEx) number	**Plasmid**	**Genotype**
3936, 3937, 3938, 3939	pAS300	*Psrtx-1B*:GFP
4236, 4260, 4381	pAS406	*Psrtx-1B*:mCherry

-	pAS178	*Psrtx-1*:SL2:GFP
3944, 3945, 3946, 3947	Recombineered fosmid	*Pgcy-8*:*gcy-8*:GFP (20 ng/ul)
3565, 3566	WRM0629cG03	*Pgcy-8*:*gcy-8* (10 ng/ul)
3976, 4015, 3977	pAS185	*Psrtx-1*:GCY-8:SL2:GFP
3973, 3966, 3974	pAS306	*Psrtx-1*:GCY-8(E974A):SL2:GFP
4189, 4190, 4194, 4162, 4311	pAS400	*Psrtx-1:*GCY-8(D976A):SL2:GFP
5634, 5635, 5636, 5637	pAS257	*Psrtx1:*GCY-8(S110A)*:SL2:GFP*
5638, 5639, 5640, 5641	pAS260	*Psrtx1:*GCY-8(S110E)*:SL2:GFP*
4080, 4063, 4064,4076	pAS330	*Psrtx-1:*GCY-8 (HCAT):SL2:GFP
4164, 4165, 4166	pAS351	*Psrtx-1:*GCY-8(KHD):SL2:GFP
4163, 4496, 4497, 4506, 4498	pAS353	*Psrtx-1:*GCY-8(KHD+HCAT):SL2:GFP
4442, 4443, 4444, 4574	pAS430	*Psrtx-1B:*GCY-8(TM+KHD+HCAT):SL2:GFP
4040, 4041, 4042, 4043	pAS316	*Psrtx-1:*hNPR1:SL2:GFP
4037, 4038, 4039	pAS314	*Psrtx-1:*hNPR3:SL2:GFP
4195, 4188, 4191, 4192	pAS404	*Psrtx-1:*hGUCY2D:SL2:GFP
4605, 4617, 4580, 4606	pAS457	*Psrtx-1*:hNPR1(E1006A):SL2:GFP
4621, 4622, 4623	pAS467	*Psrtx-1:*hGUCY2D(E1010A):SL2:GFP
5062, 5063, 5613, 5614	pAS506	*Psrtx1:*GUCY2D(G686E):SL2:GFP
4607, 4646, 4624	pAS468	*Psrtx-1:*PDE1B:SL2:GFP
4730, 4804, 4767	Recombineered	*Ppde-1*:PDE-1:GFP

	fosmid	
3282, 3283, 3297	WRM0636cF12	*Pwsp-1:wsp-1*
4842, 4843 4853, 4854	pAS245	*Psrtx-1:*WSP-1A:SL2:GFP
2680, 2655, 2769, 2770	pAS125	*Pver-1:*RAB-1(S25N)
4809, 4838, 4839, 4810	pMK179.1	*Pkcc-3:*KCC-3 + pF53F4.13:mCherry
LX1161	pJT68A	*Pkcc-3:gfp*
4995, 4996, 4997	Recombineered fosmid	*Pkcc-3-1:*KCC-3:GFP
5615, 5616, 5617, 5618	pAS250	*pF53F4.13*:KCC-3 cDNA:SL2:mCherry (1 ng/ul)
5650, 5651, 5652	pAS265	*pF53F4.13:*KCC-3 cDNA:mCherry (1 ng/ul)
n.a.	pAS214	*p*CMV:FLAG:GCY-8
n.a.	pAS221	*p*CMV:FLAG:GCY-8(G707E)
n.a.	pAS227	*p*CMV:FLAG:GCY-8(E1057A)
n.a.	pAS222	*p*CMV:FLAG:hNPR-1
n.a.	pAS501	*p*CMV:FLAG:hNPR-1(G680E)
n.a	pAS265	*p*CMV:FLAG:GCY-8(S110E)
n.a	pAS270	*p*CMV:FLAG:GCY-8(C141S)

Plasmids

GCY-8 PLASMIDS: cDNA for *gcy-8* was PCR amplified from a mixed stage cDNA library in two segments using an internal Hind3 segment and cloned into pAS178 by triple ligation using Xma1/Nhe1 sites flanking either end. This generated the pAS185 plasmid, which was used as template for all subsequent domain manipulations. *gcy-8:gfp* fusion plasmids were made by triple ligation of appropriately modified pAS185 fragments to enable in frame translation with gfp. Mutations were introduced by site directed mutagenesis in the appropriate fragment and triple ligating with the appropriate fragment and vector.

HUMAN rGC PLASMIDS: Human NPR1 (BC063304.1), NPR-3 (BC131540.1) and GUCY2D (BC148421.1) were obtained from the mammalian gene collection through GE Dharmacon/Thermo Fisher. Each cDNA was cloned into pAS178 by simple or triple ligation and this was used as template for all subsequent cloning. Mutations were introduced by site directed mutagenesis in the appropriate fragment and triple ligating with the appropriate fragment and vector.

PDE-1B PLASMID: The *pde-1B* isoform was PCR amplified from a mixed stage cDNA library and ligated into pAS178 using Xma-1/Nhe-1 flanking sites.

WSP-1A PLASMID**:** The *wsp-1a* isoform was PCR amplified from pGO101 (gift from Grigorios Oikinomou, SS lab) and ligated into pAS465 or pAS178 using Xma-1/Sal-1 flanking sites.

KCC-3 PLASMIDS: The *kcc-3* expression construct including the 5' and 3' UTR sequences fused in frame to GFP were a gift from Jessica Tanis, Michael Koelle lab. P_{kcc-3}::*kcc-3* was constructed by amplifying the coding sequence of kcc-3 and inserting it in frame with

the UTR sequences of pJT68A. The first seven of the fifteen introns in this gene were included in this construct. pAS250, 255 and 265 were generated by amplifying the KCC-3 cDNA from a mixed stage *C. elegans* cDNA library and inserting into pAS465, pSGEM or pAS512 respectively using BamH1/Sal1 flanking sites. For pAS265, a single nucleotide deletion of the stop codon was done by site directed mutagenesis to put the cDNA in frame with mCherry. The sequence of the cDNA we isolate suggests that 87 nucleotides of the annotated intron 8 on WormBase are in fact coding sequence. This adds 29 amino acids of a stretch of conserved residues, including a Cys conserved in all KCC, but not NKCC genes across species (data not shown and Figure 1E). This finding is consistent with KCC-3 being a member of the SLC12A4/KCC family.

FLAG:rGC PLASMIDS: All constructs to generate N-terminal FLAG tagged protein were made by amplifying cDNA sequences in two fragments with appropriate flanking restriction enzyme sites and triple ligating into the pFLAG:CMV vector (Sigma E6783). Mutant proteins were generated by subsequent site-directed mutagenesis of wild type rGC cDNA inserts.

Electron microscopy

For transmission EM, animals were prepared and sectioned using standard methods (Lundquist et al., 2001). Imaging was performed with an FEI Tecnai G2 Spirit BioTwin transmission electron microscope equipped with a Gatan 4K · 4K digital camera. Focused ion beam scanning EM (FIB-SEM) was performed at the NY Structural Biology Center. A modified high-contrast en bloc staining OTO method (Seligman et al., 1966) was applied

to the FIB-SEM specimen preparation. Sodium thiocarbohydrazide (TCH) was used to bind the primary osmium stain. Then the en-block stain was enhanced by a second round of osmium fixation. Glutaraldehyde (1%) was added to provide effective protein crosslinking in tissues. The serial sections prior to the region-of-interest were searched and confirmed by TEM imaging before the critical portion of the specimen had been reached. The samples for SEM were mounted on a stub of metal with silver adhesive, coated with 40-60 nm of Palladium using a Denton vacuum sputter coater. The image collection was performed by a Dual beam FEI Helios 650 Focused Ion Beam Scanning Electron Microscope (FIB-SEM) with a CCD camera with field of view of 4096 x 3536 pixels. Serial backscattered scanning electron micrographs were collected from the specimen surface after each 30 nm layer had been milled away. Beam voltage was 2keV and beam current was 0.4nA. Accurate image registration was achieved by applying a scale-invariant features algorithm (Lowe, 1999) to the raw image stacks.

Cell culture

HEK293T cells were grown using standard procedures at 37^{o}C and 5%$C0_2$ in DMEM-C [DMEM media (Gibco 11995-065) supplemented with 10% heat inactivated fetal bovine serum, L-Glutamine, MEM-NEAA and Penicillin/Streptomycin]. Transfections were performed using Lipofectamine 2000 (Invitrogen, 11668-027) as per manufacturer's protocol. pCMV:GFP:Pac (Campeau et al., 2009) was used as a co-transfection reagent at 1:10 dilution for each test (i.e. pAS) plasmid. Puromycin efficacy was determined by serial dilution of antibiotic concentration and assaying viability of un-transfected

HEK293T cells at 24 and 48 hours. GFP positive transfected cells were selected for stable integrations and maintained in DMEM-C media supplemented with 4ug/ml puromycin (Sigma P9620). Expression of FLAG tagged constructs was verified by lysing cells in RIPA buffer (Sigma, R0278) and Western blotting equal amounts of total protein extract as quantified by Bradford assay (Bio Rad 500-0202). Detection was performed using anti-FLAG antibody (Sigma, F7425) and Clean-Blot IP detection kit (HRP) reagents (Thermo Scientific, 21232). Protein expression level of each construct was normalized to expression of hNPR-1 with alpha-tubulin as a loading control. Westerns were done in triplicate and averaged for normalization. Experiments were done at normal physiological temperature for each protein.

Thermotaxis behavior

Thermotaxis assays were performed on a 18°-26°C linear temperature gradient (Hedgecock and Russell, 1975; Mori and Ohshima, 1995). Animals were synchronized and the staged progeny were tested on the first day of adulthood. Briefly, animals were washed twice with S-Basal and spotted onto the center of a 10-cm plate warmed to room temperature and containing 12 mL of NGM agar. The plate was placed onto the temperature gradient (17-26^{o}C) with the addition of 5 mL glycerol to its bottom to improve thermal conductivity. At the end of 45 mins, the plate was inverted over chloroform to kill the animals and allowing easy counting of animals in each bin. The plates have an imprinted 6x6 square pattern which formed the basis of the 6 temperature bins. Each data point is the average of 2-5 assays with ~150 worms/assay.

RNAi

Plasmids expressing double-stranded RNA (dsRNA) were obtained from the Ahringer library (Kamath et al., 2003). An empty vector was used as the negative control. RNAi was performed by plating L4 *nsIs228; rrf-3*(*pk1426*) animals onto RNAi bacteria and allowing them to feed (Timmons and Fire, 1998). 24 hours later, adults were moved to a fresh plate with RNAi bacteria and their progeny were assayed 3-5 days later for defects in AFD receptive ending shape.

Statistics

Statistical significances (*p-values*) were computed using the two-sample proportion Z test with normal approximation to the binomial at StatCrunch (www.statcrunch.com), except for Figure 5B where the unpaired two sample *t*-test was used.

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