JCB

Ecm29-mediated Proteasomal Distribution Modulates Excitatory GABA Responses in the Developing Brain

Min Lee, Yen-Chen Liu, Chen Chen, Chi-Huan Lu, Shao-Tzu Lu, Tzyy-Nan Huang, Meng-Tsung Hsu, Yi-Ping Hsueh, and Pei-Lin Cheng

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April 8, 2019

Re: JCB manuscript #201903033

Dr. Pei-Lin Cheng
Institute of Molecular Biology
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Taipei 11529
Taiwan

Dear Dr. Cheng,

Thank you for submitting your manuscript entitled "Ecm29-mediated Proteasomal Distribution Modulates Excitatory GABA Responses in the Developing Brain". Thank you for your patience with the peer review process. The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that the reviewers are overall critical about the strength of the conclusions. They are concerned that the data linking Ecm29 to proteasome distribution and tethering at the AIS are not sufficiently clear and strong, and they're concerned that the binding studies with AnkG, Ecm29 and proteasome proteins are not clear enough. We agree with the reviewers, and with Rev#1's criticisms in particular, that there are significant concerns with the core premise that Ecm29 proteasomes co-localize with AnkG at the AIS. We are additionally concerned that several of the approaches lack specificity, including AnkG depletion as Rev#1 explains, and thus some of the observed effects may be indirect, secondary to other changes in the cell such as alterations in microtubule organization. These concerns, in addition to the many specific points raised, require revisions that in our view would be substantial and exceed a standard revision period. A lot more work would be needed to strengthen the current data and clarify the interplay between proteasome, Ecm29 and the AIS through AnkG, with additional perturbations of proteasome function as per Rev#2 and a clearer idea of why proteasome function is so important to be regulated at the AIS (e.g., providing evidence for NKCC1 degradation), as well as a resolution of the revs' questions around the Ecm29 KO data. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, we would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Erika Holzbaur, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This is an ambitious study that seeks to make a connection between increased seizure sensitivity in Ecm29 knockout mice, levels of the chloride importer NKCC1 at axon initial segments, and the developmental switch in response to GABAergic signaling. Overall, I had difficulty following the logic of this paper and in addition had multiple concerns related to experimental design and interpretation as well as a highly biased approach. Some examples are below.

1. Ecm29 is viewed by several groups as performing a quality control role for 26S proteasomes:
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The proteasome-interacting Ecm29 protein disassembles the 26S proteasome in response to oxidative stress.Wang X, Chemmama IE, Yu C, Huszagh A, Xu Y, Viner R, Block SA, Cimermancic P, Rychnovsky SD, Ye Y, Sali A, Huang L.J Biol Chem. 2017 Sep 29;292(39):16310-16320.
It is troubling that even though there are only 22 total papers in pubmed dealing with Ecm29, none of the above studies were cited and instead the authors focused on one paper linking Ecm29 to molecular motors.

2. A core premise of the paper is that Ecm29 and proteasomes co-localize with ankG at axon initial segments. However the data does not support such a co-localization. Actually Rpt5 (a surrogate for the proteasome) shows labeling of the cell body and proximal domains of both dendrites and axons. Comparison of intensity profile for ankG and Ecm29 reveals an inverse correlation: ankG rises while Ecm29 falls. The altered profile for ECM29 with ankG knockdown may reflect altered architecture of the proximal axon segment, or selection of a subset of neurons (which differ in the location of the AIS with respect to the cell body).

3. The use of oxygen and glucose depletion as a way to perturb the AIS suffers from lack of specificity. These neurons may be dying and undergoing apoptosis or other generalized consequences of cell death.

4. The effects of ankG depletion on axonal transport of proteasomes may be secondary to loss of microtubule bundling which accompanies loss of ankG (see Sobotznik et al. PNAS, 2009), and do not necessarily reflect direct interaction of ankG with proteasomes.
5. The Ecm29 knockout likely affects proteasomal function in all cells including multiple cell types in the nervous system, as well as all subcellular domains of these cells. It therefore is difficult to interpret the relatively subtle effects of Ecm29 knockout on reversal of Chloride from efflux to influx at day 7 instead of day 9. Similarly, seizure activity in Ecm29 ko mice is statistically significant but requires substantial doses of pentylenetetrazole and flurothyl.

Minor points
1. It is puzzling in figure 2B that MAP2 and ankG apparently co-localize in the control for OGD treatment. Normally MAP2 and ankG are each exclusively in either dendrites or axons respectively.
2. The rationale for using forskolin to enhance ankG Ecm29 interaction is not clear.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript by Lee et al. describes a novel mechanism of proteasome distribution in neurons, namely a retention via interaction with ankyrin G (ankG) along the proximal axon. This accumulation leads to a reduced NKCC1 concentration which is implicated in the GABA reversal from excitatory to inhibitory that occurs during neuronal maturation. In particular, the authors demonstrate the role of the protein Ecm29 in proper distribution of proteasome at the AIS and the downstream consequences of its absence in the Ecm29 -/- mouse.

This is an interesting study at the conceptual cell biological level, as it shows how the spatial regulation of protein degradation can play a role in protein distribution and ultimately contribute to important physiological processes. It also sheds light on a yet unexplored aspect of the AIS biology, where proteasome-related processes have not been studied so far. Despite these significant advances, several concerns need to be adressed in order to recommend acceptance for publication in the Journal of Cell Biology.

Major points

1. Results from Figure 1 show that ankG (and by extension the presence of the AIS scaffold) is important for selective retention of proteasomes at the AIS. However, there are a couple of technical questions that need to be clarified.
- How are intensity profiles traced, measured and averaged in order to obtain the average profiles shown in Fig. 1A-B and others? I could not find this in the Methods section.
- The quantitative impact of shRNA against ankG and OGD procedure is not specified beyond a "lower (by 10- 30% relative to wild-type)" mention in the Results. Does this mean that at least 70% of ankG remains after transfection with an shRNA against ankG? This would seem like an inefficient knockdown. Is it possible to present a graph of the absolute ankG intensities at the AIS in the different treatments (shRNA, OGD)? Similarly, the absolute levels for Ecm29 and Rpt1 would be useful. This is different from the graphs of Fig.1E that measure the relative intensity (i.e. concentration) against the distal axon value.
- If the shRNA against ankG has a significant knockdown efficacy, like in the example presented in Fig. 1A, it is not possible to use the ankG labeling to determine which neuronal process is the axon. How did the authors do it in this case?
- The kymographs from live-cell imaging acquisitions showing MV151 trajectories are very dense at

the AIS level, and the traced trajectories appear quite arbitrary given the complexity of the kymographs. It would be better to perform a pre-bleaching step of MV151 at the AIS in order to better isolate moving MV151-positive particles (which are the ones primarily quantified in Fig. 1D).

2. Results from Figure 2 relate to the molecular interaction between ankyrin G and proteasome components (Ecm29, Rpt1).However, the results are a bit confusing.
- The experiments involving heterologous AnkG expression (FRET, coIP) are done using 190-kDa ankG. However, the blots show the ankG band above 250 kDa (Fig. 2F), similar to the GST pull-down of endogenous brain ankyrin G in Sup. Fig. 1A, which should primarily show 270-kDa ankG. Can the authors comment on this discrepancy?
- Sup. Fig. 1B shows the interaction of the ankG spectrin-binding domain (SBD) with Kif5, contrasting with previous results from Barry et al. Dev Cell 2015, who showed interaction of Kif5 with the ankyrin-repeats in the membrane-binding domain. Citing this earlier work and commenting on this difference are advised.
- The overall mechanism of the ankG/Ecm29/Rpt1 interaction is really difficult to grasp. This is not helped by the beginning of the corresponding Results paragraph which states "We confirmed that proteasome mobility requires the adaptor protein Ecm29 [...] Therefore, Ecm29 may mediate proteasome tethering to the AIS through interacting with AnkG". Is Ecm29 favoring mobility or immobilization of proteasomes? From the binding data and competition between Ecm29 and Rpt1, the model I can come up with is that it is the AnkG-Rpt1 interaction that immobilizes proteasomes (hence immobility in Ecm29 -/- neurons) and the AnkG-Ecm29 interaction allows to free proteasomes from this immobilization (weakening the Ecm29/Rpt1 and ankG/Rpt1 interaction). But there is no clear presentation of an interaction model in the present manuscript that would integrate and make sense of this complex binding data.

3. The results from the Ecm29 -/- KO are scattered between Fig. 1C (but not described in the corresponding Results section), Fig. 2G and Sup. Fig. 3. Moreover, the quantification of Rpt1 labeling in Sup. Fig. 3 (number of puncta) is not consistent with the quantification of Rpt1 labeling in other cases such as shRNA ankG or OGD in Fig. 1A (intensity profiles, no absolute quantification). It would be better to consolidate the Ecm29-/- results together and to unify the protein distribution quantifications (see also remark above on Fig.1 quantifications).

4. Is it possible to perturb the proteasome downstream of Ecm29 to clarify the role of the proteasome in AIS formation, plasticity and maintenance? Would it be possible to use shRNA of Rpt1 or Rpt1 -/- neurons?

5. The last part of the Results (Figure 7) show the perturbed AIS morphological developmental plasticity in Ecm29 -/- neurons. The results from preceding parts show that the GABAergic switch is delayed in Ecm29-/- neurons, which points to a delay in neuronal maturation. What the authors found for AIS position is that the distance between the AIS beginning and cell body shortened during normal neuronal maturation, but that this proximal shift occurred earlier in Ecm29-/- neurons, as the distance is the same in mature neurons (this only reported as a "larger proximal shift" in Ecm29 -/- neurons at div 7). This accelerated maturation is at odds with the delayed maturation seen with GABA properties. One could think of this accelerated AIS distance change being compensatory, as hinted by the first phrase of the corresponding Results text: "Finally, given AIS plasticity (Berger et al., 2018; Grubb and Burrone, 2010), we asked whether elevated NKCC1 levels and hyperexcitability observed in immature Ecm29 KO neurons promoted *compensatory* changes in AIS position or extent at early time-points (5-DIV to 14-DIV)". However, an AIS that shifts closer to the cell body earlier would make the neuron more excitable (if we accept the Grubb & Burrone point of view), which would be adding up to the early hyperexcitability attributed to GABA

properties in the preceding results, not compensate from it. Then, what is the conclusion from this AIS plasticity perturbation? I don't see the logic in the current conclusion for this part: "Overall, the precocious shift of AIS position in Ecm29 KO cortical neurons at 7-DIV supports the idea that increased neuronal excitability is primarily due to Ecm29 loss and NKCC1 accumulation (Figure 7D)".

Minor points
6. Results, p.7: "Given that AIS formation and the excitatory-to-inhibitory GABA polarity switch occur between 5-DIV and 14-DIV in cortical cultures". Is there a reference for the timing of the GABA polarity switch in cultures? Or is it the following experiments in the manuscript?

7. Related to this, premising the time of the GABA switch in vivo in the model used would clarify the relevance of the protein levels experiments in brain homogenates (Fig. 4). What is the equivalent of DIV7 (time of switch in culture) in vivo for these experiments? Is it P4?

8. The curves shown in Fig. 3A2 suggest that in the proximal axon of Ecm29 -/- neurons (third column), chloride efflux occurs at DIV 5 and DIV 7, and no exchange happens in DIV 9 and DIV 14 neurons (flat curve). This is at odds with the averaged results that show influx at DIV 9 and DIV 14 (Fig. 3B and 3C). Can the authors find a more representative example for Fig. 3A2?

9. Could the authors discuss the results form Wefelmeyer et al. PNAS 2015 and Muir & Kittler Front Cell Neuro 2014 that show mismatch between GABA innervation and AIS position after activity-induced plasticity? Could the perturbations in AIS developmental positioning (Fig. 7), conserved GABAergic innervation (Sup. Fig 6) and excitability phenotypes (Fig. 6) be considered under this angle?

Reviewer #3 (Comments to the Authors (Required)):

In this paper the authors have examined the role of the proteasome in the AIS. They propose that proteasome distribution in neurons indirectly controls chloride gradients by modulating the amount of NKCC1 in the axon initial segment, and that by controlling these chloride gradients the proteasome controls neuronal excitability. This is a very interesting and intriguing hypothesis. I think in general the quality of the results is quite good, and this paper is appropriate for JCB. There is a remarkable amount of work here including both in vitro and in vivo work. Nevertheless, I list several questions that should be addressed before the paper is complete.

1. On page 5, first paragraph, last sentence. I don't think the authors can immediately propose that ECM29/proteasome complexes are tethered to the AIS through AnkG. They should simply suggest that it is tethered at the AIS through some unknown mechanism. If you disrupt AnkG, you'll affect everything.
2. The results demonstrating Ecm29 binding to AnkG are very nice, but the localization of Ecm29 at the AIS could be improved. A) the SIM imaging is not compelling (Fig. S2) since we don't also see what the localization looks like in non-AIS domains. B) One property of AIS-restricted and ankG-interacting proteins is that they are retained at the AIS after detergent extraction (see Garrido et al., Science 2003 and Huang et al., 2017). I suggest the authors do the detergent extraction experiment and show that Ecm29 is retained at the AIS together with ankG.
3. Figure 1. The figure legend doesn't describe what the white or blue labels are. Why immunostain for Rpt5 and Ecm29 in both blue and green? (Fig1A, B). I don't understand the significance.
4. On page 7, end of first paragraph. I don't understand the conclusion about the findings and how

the authors think their results imply competition. Ecm29 and AnkG bind to each other (Fig. 2F) and form a complex. How do the authors think that when they are in a complex they compete for binding to the proteasome? If anything, it looks like the proteasome and Ecm29 compete for AnkG. You lose Ecm29 and you get more AnkG PLA signal at the AIS. I thought the model was that Ecm29 interacts with AnkG, thereby promoting recruitment of the proteasome to the AIS. But surprisingly, loss of ECM29 permits MORE proteasome in the AIS (Fig. 2G). Can the authors please clarify.

5. The results in Fig. 3 are very, very impressive and neat.

6. In Fig. 4, the authors attempt to draw a link between NKCC1 expression and Ecm29. They claim that there is increased NKCC1 in the ECM29 KO due to decreased turnover. While the immunostaining looks like there is more immunoreactivity at the AIS of Ecm29 KO mice, no experiments directly tests turnover rates. There are other potential explanations for their results: in the Ecm29 KO maybe trafficking to the AIS is more efficient? Maybe there is increased stability of membrane proteins? In short, no experiment actually demonstrates altered turnover rates. Indeed, doesn't Figure 4C2 argue for no change in surface protein levels? Why are there no immunoblots of NKCC1 for the surface fraction? In the Ecm29 KO you would expect to see increased levels.

7. As a preface to Fig. 6, the authors state they wanted to look at the relationship between neuronal excitability and Ecm29-mediated proteasomal distribution. The only data in the manuscript on proteasome distribution is found in Fig. 1C. I'm not convinced that proteasome localization depends on Ecm29. There is no analysis of proteasome localization in the Ecm29 KO. Trafficking is altered, so is the PLA in Fig. 2G. But why not also show labeling with MV151 in Ecm29 KO mice?

8. The physiology results (Fig. 7 and 8) are quite intriguing and consistent with a proximal shift of the AIS toward the cell body, leading to a more excitable neuron.

9. I think the most important question that remains unanswered is why is proximal axon NKCC1 preferentially targeted by the proteasome? Why aren't other AIS ion channels also targeted for degradation like NKCC1?

Dr. Erika Holzbaur, PhD
Monitoring Editor, *Journal of Cell Biology*
Dr. Melina Casadio
Senior Scientific Editor, *Journal of Cell Biology*
The Rockefeller University Press
950 Third Ave., 2nd Floor
New York, NY 10022

October 15, 2019

Dear Drs. Holzbaur and Casadio:

Enclosed please find our resubmitted manuscript entitled, "Ecm29-mediated Proteasomal Distribution Modulates Excitatory GABA Responses in the Developing Brain" for your consideration for publication in *Journal of Cell Biology*.

Over the past five months, we performed additional experiments and controls to address reviewers' comments, and now provide more evidence in support of Ecm29-dependent proteasome localization to the axon initial segment (AIS). Specifically, we performed additional sets of co-localization assays using a detergent extraction method to confirm direct association of Ecm29 and proteasomes with detergent-resistant AIS structures in hippocampal neurons. These results, together with new experiments using the NavII-III loop to target the protein degradation reporter GFPu to the AIS membrane, strongly support Ecm29 and proteasome function-dependent control of local protein homeostasis at the AIS. Moreover, new pulse-chase experiments verified that Ecm29 modulates stability of NKCC1 protein, providing a mechanistic basis for the timely developmental switch of intrinsic neuronal properties governed by $[Cl^-]$ balance and AIS structural plasticity.

Our detailed responses to reviewers' comments are provided on separate pages. We believe that these new results address reviewers' questions and hope you will now re-consider our manuscript as suitable for publication.

Pei-Lin Cheng, Ph.D.
Associate Research Fellow
Institute of Molecular Biology
Academia Sinica, Taiwan

(reviewer's comments in italics)
Reviewer #1 (Comments to the Authors (Required)):

This is an ambitious study that seeks to make a connection between increased seizure sensitivity in Ecm29 knockout mice, levels of the chloride importer NKCC1 at axon initial segments, and the developmental switch in response to GABAergic signaling. Overall, I had difficulty following the logic of this paper and in addition had multiple concerns related to experimental design and interpretation as well as a highly biased approach. Some examples are below.

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We thank the referee for suggesting additional references. All of these papers are now cited in the Introduction section of the revised manuscript (please see Page 3, second paragraph).

2. A core premise of the paper is that Ecm29 and proteasomes co-localize with ankG at axon initial segments. However the data does not support such a co-localization. Actually Rpt5 (a surrogate for the proteasome) shows labeling of the cell body and proximal domains of both dendrites and axons. Comparison of intensity profile for ankG and Ecm29 reveals an inverse correlation: ankG rises while Ecm29 falls. The altered profile for ECM29 with ankG knockdown may reflect altered architecture of the proximal axon segment, or selection of a subset of neurons (which differ in the location of the AIS with respect to the cell body).

We have now performed additional experiments to confirm Ecm29/AnkG co-localization at the AIS membrane. To do so we undertook non-ionic detergent (Triton X-100) extraction experiments using DIV7 hippocampal cultures and showed that both proteasomes and Ecm29 were retained in the AnkG-positive AIS region after detergent treatment (0.02% Triton, 37°C, 2 min). Proteasome retention in the detergent-resistant AIS required Ecm29 expression, as staining of the proteasome subunits Rpt1 and Rpt5 was abolished in Ecm29 KD neurons after extraction (see

new data in revised Figure 2D). Also, a GST pull-down assay supported the idea that Ecm29 expressed in either P21 rat cortical lysates or in Neuro2a cell lysates associated with the C-terminus, but not the N-terminus, of GST-AnkG (please see Figure S2B and S2C). Furthermore, super-resolution images obtained using structured illumination microscopy (SIM) in DIV7 neurons revealed a similar spatial organization with a periodicity of ~190 nm for proteasome subunits (such as Rpt1, Rpt5, and 20S CP) and the AnkG N-terminal domain, but not for signals outside the AIS region (see new Figure S3C). These findings support the idea that these proteins co-localize at the AIS.

3. The use of oxygen and glucose depletion as a way to perturb the AIS suffers from lack of specificity. These neurons may be dying and undergoing apoptosis or other generalized consequences of cell death.

We agree and are aware that prolonged OGD conditions may be harmful to cells. We note that OGD conditioning used our cultures was as short as 30 min, followed by a 30 min of normoxia and recovery in regular neuronal culture medium overnight (see Materials and Methods). The optimal OGD procedures were determined from a serial OGD time-response assessment (the original confocal images and the graphs showing traces of AnkG intensity are now shown in new Figure S1 and attached below for the referee's perusal). Finally, we did not observe signs of axonal beading (a feature of unhealthy neurons) prior to immunofluorescent staining in any assessments of AIS integrity or proteasome distribution after OGD conditioning.

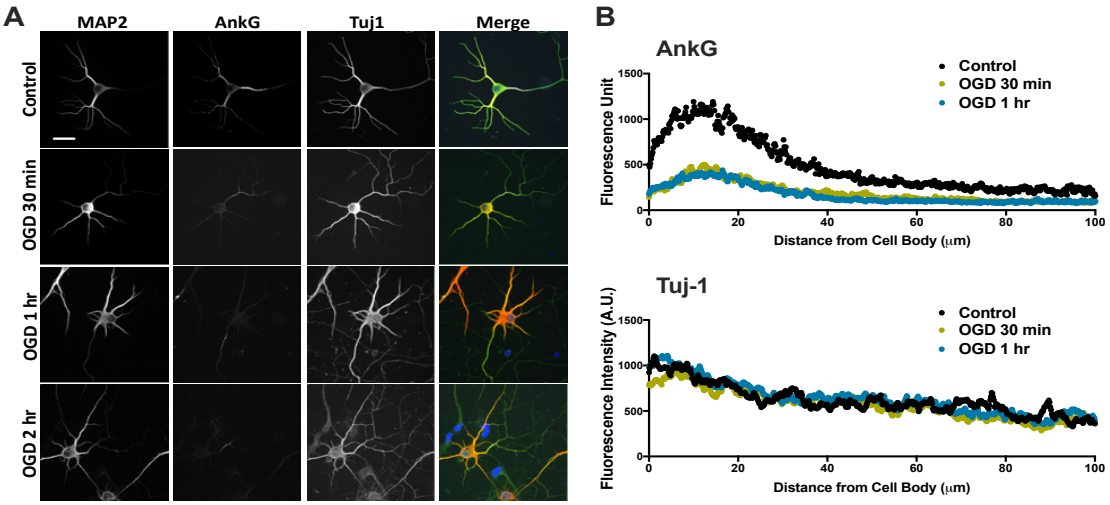

Figure Q3. OGD time-response assessment for AIS disruption and axonal beading. (A) Representative images of DIV8 neurons post to exposure to OGD condition at different time points, immunostained with dendritic maker MAP2, Neuron-specific class III β-tubulin ("Tuj-1"), AIS marker AnkG, as indicated. Note that neurons under 30 min OGD condition showed no sign of tubulin beading. Scale, 20 μm. (B) Traces showing AIS disruption and no axonal beading with or without OGD conditioning for 30 min or 1 hr. Data represents mean fluorescence intensity (n>30 cells for each group) of AnkG and Tuj-1 along axons.

4. The effects of ankG depletion on axonal transport of proteasomes may be secondary to loss of microtubule bundling which accompanies loss of ankG (see Sobotznik et al. PNAS, 2009), and do not necessarily reflect direct interaction of ankG with proteasomes.

In response to the reviewer's comment, we have now conducted immunostaining with III beta-tubulin (Tuj1) to determine microtubule bundle integrity in AnkG KD neurons. We found comparable Tuj-1 staining intensity and patterns between scramble-siRNA control and AnkG-siRNA KD neurons (see newly revised Figure 1A and 1B). In a proteasome transport assay (see Figure 1C and 1D), we found that the percentage of MV-151-labeled proteasomes traversing the proximal axon increased in AnkG KD neurons, a phenotype opposite to that seen following treatment with the microtubule-disrupting agent nocodazole (Figure 1C and 1D). Also, new in vitro and in vivo binding assays support the idea that AnkG, motors, Ecm29, and proteasomes form complexes (see Figure S2 and new Figure 2A and 2C). Although we cannot exclude the possibility of perturbed axo-dendritic polarity following AnkG KD, these results support, at least in part, the idea that AnkG protein modulates proteasome transport behavior via direct physical association with Ecm29/proteasome complexes.

5. The Ecm29 knockout likely affects proteasomal function in all cells including multiple cell types in the nervous system, as well as all subcellular domains of these cells. It therefore is difficult to interpret the relatively subtle effects of Ecm29 knockout on reversal of Chloride from efflux to influx at day 7 instead of day 9. Similarly, seizure activity in Ecm29 ko mice is statistically significant but requires substantial doses of pentylenetetrazole and flurothyl.

The relationship between PalmPalm-ClopHensor intensity and [Cl^-] is inverse, such that the time of the switch in directionality of GABA-induced chloride flux in Ecm29 KO neurons was delayed, not accelerated. Based on the reviewer's comment, we now provide three sets of new experiments that support the idea that Ecm29 loss affects chloride dynamics by altering NKCC1 degradation. First, levels of AIS-localized and membrane surface NKCC1 protein increased in young Ecm29 KO neurons, as assessed by immunofluorescent staining and use of a surface protein biotinylation assay (see new Figure 3B and 3C). Second, the relative half-life of NKCC1 protein increased in Ecm29 KO neurons as assessed by a new pulse-chase assay (see new Figure 3D), a phenotype rescued by ectopic expression of the Ecm29 N-terminus ("Ecm29ΔC-mCherry" in new Figure 3D). Third, blocking NKCC1 activity by treating young Ecm29 KO neurons with the potent NKCC1 inhibitor bumetanide or by ectopic expression of Ecm29 protein rescued the prolonged excitatory GABAergic response seen in these cells (in Figure 4A3).

Thus, we feel that two days of a prolonged intrinsic GABAergic excitatory response in Ecm29 KO neurons (as reflected by the GABA-induced chloride efflux) caused by elevated NKCC1 levels was sufficient to render these neurons hyper-excitable, due to their high plasticity. This idea is supported by electrophysiological findings of NKCC1-dependent hyperexcitability in layer 5 pyramidal neurons of the medial prefrontal cortex in brain slices obtained from perinatal (P7-P9) Ecm29 KO mice (shown in Figure 6). We also provide new evidence that perinatal blockade of NKCC1 activity at P7 by i.p. bumetanide administration is sufficient to rescue hyper-susceptibility to chemically-induced seizures in Ecm29 KO mice (see new Figure 5A6 and 5C).

Due to the lack of reagents useful to modulate proteasome activity in subcellular compartments, however, we could not assess an effect of local proteasome dysregulation on chloride ion homeostasis. Thus, as an alternative, we performed

experiments using the sodium channel loop Na_vII-III to target the protein degradation reporter GFPu to the AIS membrane (see new Figure 2D) and also conducted a biotinylated membrane protein pull down assay (see new Figure 3B). Both assays indicated increased NKCC1 abundance and protein stability in the AIS membrane of Ecm29 KO neurons. Although we cannot exclude potential effects of non-cell-autonomous signals on neurons, these results support, at least in part, the idea that Ecm29/proteasome complexes confer spatial and temporal control of NKCC1 abundance, in turn governing chloride dynamics in maturing neurons (see new Figure 2D, and 3B).

Finally, we used standard guidelines to administer pentylenetrazole (PTZ) and flurothyl and doses were carefully calculated based on body weight (B.W.=~ 21g-25g for young adult (P90) mice). Also, optimal PTZ doses were determined from dose-response assessment to prevent overdose (see new Figure 5B3, and Materials and Methods section).

Minor points

1. It is puzzling in figure 2B that MAP2 and ankG apparently co-localize in the control for OGD treatment. Normally MAP2 and ankG are each exclusively in either dendrites or axons respectively.

We agree and now show new more representative images in Figure 1B.

2. The rationale for using forskolin to enhance ankG Ecm29 interaction is not clear.
Forskolin was used to determine whether AnkG-Ecm29 interaction on an AIS-like membrane domain in HEK293T cells was modulated by a reagent that promotes neuronal maturation.

Reviewer #2 (Comments to the Authors (Required)):

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1. Results from Figure 1 show that ankG (and by extension the presence of the AIS scaffold) is important for selective retention of proteasomes at the AIS. However, there are a couple of technical questions that need to be clarified.

- How are intensity profiles traced, measured and averaged in order to obtain the average profiles shown in Fig. 1A-B and others? I could not find this in the Methods section.

- The quantitative impact of shRNA against ankG and OGD procedure is not specified beyond a "lower (by 10- 30% relative to wild-type)" mention in the Results. Does this mean that at least 70% of ankG remains after transfection with an shRNA against ankG? This would seem like an inefficient knockdown. Is it possible to present a graph of the absolute ankG intensities at the AIS in the different treatments (shRNA, OGD)? Similarly, the absolute levels for Ecm29 and Rpt1 would be useful. This is different from the graphs of Fig.1E that measure the relative intensity (i.e. concentration) against the distal axon value.

- If the shRNA against ankG has a significant knockdown efficacy, like in the example presented in Fig. 1A, it is not possible to use the ankG labeling to determine which neuronal process is the axon. How did the authors do it in this case?

- The kymographs from live-cell imaging acquisitions showing MV151 trajectories are very dense at the AIS level, and the traced trajectories appear quite arbitrary given the complexity of the kymographs. It would be better to perform a pre-bleaching step of MV151 at the AIS in order to better isolate moving MV151-positive particles (which are the ones primarily quantified in Fig. 1D).

We thank the referee for carefully reading the manuscript and for helpful suggestions. We have now added detailed information relevant to quantification to the revised Material and Methods section. Briefly, for fluorescence intensity profiles shown in Figure 1A and 1B, intensity values were subtracted by the value at the distal axon (i.e., a data point taken 100 um away from cell body), and then converted to Arbitrary Units. To calculate intensity relative to the distal axon value shown in Figure1E, the average intensity of AIS region (AIS start and end positions were defined as positions of 50% of peak AnkG intensity values at either tail of the curve) was

normalized to that of the distal axon (a 20 um segment between data points at 60 and 80 um).

Relevant to knockdown effects, we now state “led to significantly lower levels (10-30% relative to wild-type) of AnkG signals” in the revised Results section (“led to significantly lower levels (10-30% relative to wild-type) of AnkG signals”; Page 5, second line from the bottom). Based on quantitative analysis, only 10-30%, not 70%, of AnkG remained after shRNA KD or the OGD procedure. The graph showing the original fluorescence intensity and traces is attached below for the referee’s perusal.

The control scramble siRNA and AnkG siRNA used in the experiments were labelled with fluorescein, which allowed us to track neuronal processes of the siRNA transfected neurons and to identify which process was the axon based on morphological features (i.e., a process at least 3x longer than other neurites) in AnkG knockdown neurons after DIV5.

Based on the referee’s suggestion, we tested several sub-bleaching protocols on live-cell imaging acquisitions. However, due to significant phototoxicity effects and dim MV151 signals after the sub-bleaching procedure was applied at the AIS region, we could not obtain a sufficient number of moving particles to assess MV151 trajectories.

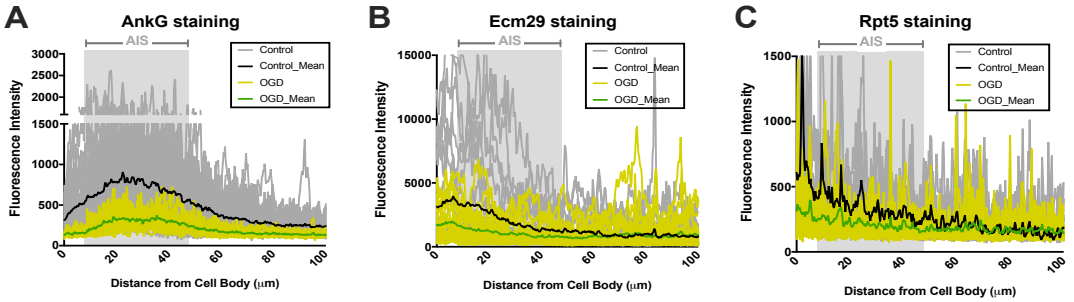

Figure Q1. Line plots, all from confocal images of 7-DIV neurons co-immunostained with antibodies against AIS marker AnkG (A), Ecm29 (B), and proteasome subunit Rpt5 (C), indicating protein distribution based on AnkG, Ecm29, Rpt5 staining alone the axon.

2. Results from Figure 2 relate to the molecular interaction between ankyrin G and proteasome components (Ecm29, Rpt1).However, the results are a bit confusing. - The experiments involving heterologous AnkG expression (FRET, coIP) are done using 190-kDa ankG. However, the blots show the ankG band above 250 kDa (Fig. 2F), similar to the GST pull-down of endogenous brain ankyrin G in Sup. Fig. 1A, which should primarily show 270-kDa ankG. Can the authors comment on this discrepancy?

Close proximity of ectopically expressed 190-kDa AnkG in the co-IP assay (now shown in Figure 2A) to the 250 kDa mark may be due to its fusion with 27kDa GFP. We observed a similar size shift when we used a GFP-270-kDa AnkG expression construct, in which the band appeared above the 300 kDa mark and was slightly larger than that predicted by molecular weight. Despite these shifts, both 190 kDa and 270 kDa AnkG forms interact with Flag-tagged full-length Ecm29 when expressed in HEK293T cells. The data are shown below for the referee’s perusal.

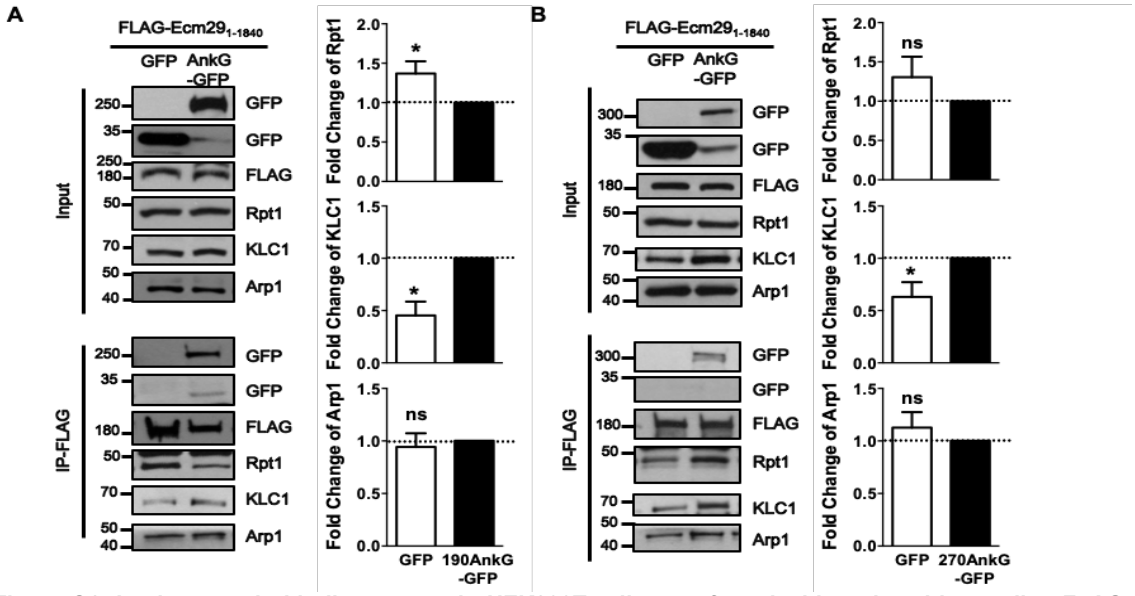

Figure Q2. In vivo protein binding assays in HEK293T cells transfected with a plasmid encoding FLAG-tagged full-length Ecm29 (FLAG-Ecm291-1840) and plasmids encoding 190AnkyrinG-GFP (A), 270AnkyrinG-GFP (B), or control GFP vectors, as indicated.

- Sup. Fig. 1B shows the interaction of the ankG spectrin -binding domain (SBD) with Kif5, contrasting with previous results from Barry et al. Dev Cell 2015, who showed interaction of Kif5 with the ankyrin-repeats in the membrane-binding domain. Citing this earlier work and commenting on this difference are advised.

Based on this suggestion we now cite Barry's work (see Page 7, line 15-16). We feel that our domain mapping results obtained from Neuro2a cell lysates (original Figure S1C, now shown in Figure S2C) agree with, rather than contradict, Barry's finding of binding of the Tail-domain of KIF5B to the N-terminal membrane-binding domains of AnkG seen in HEK293T cell lysates (Barry et al. 2014). However, binding affinity of Kif5 domains to AnkG depends on context, as in brain lysates we found that AnkG membrane-binding domains exhibited weaker, but detectable, interaction with Kif5 protein than the ankG spectrin-binding domain (in Figure S2B).

- The overall mechanism of the ankG/Ecm29/Rpt1 interaction is really difficult to grasp.
This is not helped by the beginning of the corresponding Results paragraph which states "We confirmed that proteasome mobility requires the adaptor protein Ecm29 [...] Therefore, Ecm29 may mediate proteasome tethering to the AIS through interacting with AnkG". Is Ecm29 favoring mobility or immobilization of proteasomes? From the binding data and competition between Ecm29 and Rpt1, the model I can come up with is that it is the AnkG-Rpt1 interaction that immobilizes proteasomes (hence immobility in Ecm29 -/- neurons) and the AnkG-Ecm29 interaction allows to free proteasomes from this immobilization (weakening the Ecm29/Rpt1 and ankG/Rpt1 interaction). But there is no clear presentation of an interaction model in the present manuscript that would integrate and make sense of this complex binding data.

We now present a clearer model for AnkG/Ecm29/Rpt1 interaction based on in vivo findings, in vitro protein interaction assays (new Figure 2A and Figure S2), and new results from super-resolution 3D structured illumination microscopy (3D-SIM-Microscopy) (in revised Figure S3B and S3C), and detergent extraction experiments (new Figure 2C), which replace PLA findings (original Figure 2D-2G). The extraction assay, in particular, revealed that tethering of proteasomes to the AIS requires Ecm29 protein, as we found that proteasome/AnkG co-localization in the AIS region was significantly suppressed following Ecm29 KD (see new Figure 2C).

In the revision we have now omitted PLA assays previously reported as we feel that the antibody (AnkG antibody clone H215 that targeted the AnkG C-terminus) used in those assays was not sensitive enough to assess proteasome-AnkG associations.

3. The results from the Ecm29 -/- KO are scattered between Fig. 1C (but not described in the corresponding Results section), Fig. 2G and Sup. Fig. 3. Moreover, the quantification of Rpt1 labeling in Sup. Fig. 3 (number of puncta) is not consistent with the quantification of Rpt1 labeling in other cases such as shRNA ankG or OGD in Fig. 1A (intensity profiles, no absolute quantification). It would be better to consolidate the Ecm29-/- results together and to unify the protein distribution quantifications (see also remark above on Fig.1 quantifications).

We have now reorganized our report of results in Ecm29$^{-/-}$ KO neurons. We added quantitative results of MV-151 transport behavior and of “Rpt5” distribution for Ecm29-/- KO neurons to revised Figure1 (see Figure 1D and 1E, and new FigureS7A). Summaries of patterns of proteasome subunits (Rpt1, Rpt5, and 20S core particle) in wild-type and Ecm29$^{-/-}$ KO neurons are now shown in new Figure S7A. Although an AIS retention pattern was evident for proteasome subunits tested (Rpt1, Rpt5, and 20S CP) in DIV7 wild-type neurons, we noted differences in fluorescence signals between Rpt1 and Rpt5 staining: Rpt1 displays a dense punctate pattern, while the Rpt5 pattern is more diffuse, probably due to antibodies used. Thus we quantified Rpt1 distribution profiles by calculating the density of Rpt1 punctae (as in original Figure S3), while we show the Rpt5 distribution profile as relative Rpt5 intensity in the AIS region versus that in the distal axon (see Figure 1 and new Figure S7A). Representative pictures and quantitative results for Rpt1, Rpt5, and 20sCP are now shown in new FigureS7A for the referee’s perusal.

4. Is it possible to perturb the proteasome downstream of Ecm29 to clarify the role of the proteasome in AIS formation, plasticity and maintenance? Would it be possible to use shRNA of Rpt1 or Rpt1 -/- neurons?

Yes. In response to this question we knocked down the Rpt1 expression by siRNA approach to assess the role of proteasome on AIS dynamics. After Rpt1 KD in neurons we observed similar AIS structural dynamics as that seen in Ecm29-deficient neurons or neurons treated with MG132 or lactacystin. All showed normal AIS structure on DIV5 but exhibited accelerated AIS position shifts prior to DIV7. These findings are shown in new Figure 2D (showing increased protein stability in the AIS region of Rpt1 KD neurons) and Figure 7B (showing accelerated AIS re-positioning in DIV7 Rpt1 KD neurons).

*5. The last part of the Results (Figure 7) show the perturbed AIS morphological developmental plasticity in Ecm29 -/- neurons. The results from preceding parts show that the GABAergic switch is delayed in Ecm29-/- neurons, which points to a delay in neuronal maturation. What the authors found for AIS position is that the distance between the AIS beginning and cell body shortened during normal neuronal maturation, but that this proximal shift occurred earlier in Ecm29-/- neurons, as the distance is the same in mature neurons (this only reported as a "larger proximal shift" in Ecm29 -/- neurons at div 7). This accelerated maturation is at odds with the delayed maturation seen with GABA properties. One could think of this accelerated AIS distance change being compensatory, as hinted by the first phrase of the corresponding Results text: "Finally, given AIS plasticity (Berger et al., 2018; Grubb and Burrone, 2010), we asked whether elevated NKCC1 levels and hyperexcitability observed in immature Ecm29 KO neurons promoted *compensatory* changes in AIS position or extent at early time-points (5-DIV to 14-DIV)". However, an AIS that shifts closer to the cell body earlier would make the neuron more excitable (if we accept the Grubb & Burrone point of view), which would be adding up to the early hyperexcitability attributed to GABA properties in the preceding results, not compensate from it. Then, what is the conclusion from this AIS plasticity perturbation? I don't see the logic in the current conclusion for this part: "Overall, the precocious shift of AIS position in Ecm29 KO cortical neurons at 7-DIV supports the idea that increased neuronal excitability is primarily due to Ecm29 loss and NKCC1 accumulation (Figure 7D)".*

We appreciate these comments and now provide a clearer explanation at the end of the Results. Specifically, we replaced “compensatory changes” with “a perturbed AIS plastic morphological response” in the Abstract (Page 2, line 14) and with “structural changes” in the Results section (Page 17, line 4) and changed the last sentence of the Results to read, "Overall, our findings suggest that altered neuronal excitability caused by Ecm29 loss, proteasome dysfunction, and/or NKCC1 accumulation in immature neurons perturbs developmental AIS positioning (Figure D)” (Page 17, last two lines and Page 18, first two lines). Also, we have changed language used in our previous submission relevant to a “precocious” proximal shift in the AIS start position. We now state that accelerated changes in the AIS position in neurons seen after Ecm29 loss or proteasome inhibition morphologically reflect altered intrinsic electrical properties and abnormally elevated excitability, and that changes in AIS positioning do not necessarily indicate precocious maturation. These statements appear in the Results (please see Page 17, 8th and 13th lines from the bottom) and Discussion (Page 20, last 6 lines of second paragraph) sections.

Minor points

6. Results, p.7: "Given that AIS formation and the excitatory-to-inhibitory GABA polarity switch occur between 5-DIV and 14-DIV in cortical cultures". Is there a reference for the timing of the GABA polarity switch in cultures? Or is it the following experiments in the manuscript?

Yes, the occurrence of the GABA switch was recently demonstrated in hippocampal cultures by monitoring GABA-induced Ca2+ transients and reversal potentials (Leonzino et al., 2016). Those authors found that the switch occurred prior to DIV8, a

time point similar to what we observed by Ca2+ imaging and PalmPalm-ClopHensor experiments for the switch (original Figure 3 and 4A, now shown in new Figure 4).

7. Related to this, premising the time of the GABA switch in vivo in the model used would clarify the relevance of the protein levels experiments in brain homogenates (Fig. 4). What is the equivalent of DIV7 (time of switch in culture) in vivo for these experiments? Is it P4?

We have now added sentences defining the time of the GABA switch as reported in studies of rodent hippocampus and cortex (Page 10, second paragraph, 9th-12th lines from the bottom). Based on experiments performed in CA3 pyramidal cells in rat hippocampus (Ben-Ari et al, 1989; Swann et al., 1989), the switch from depolarizing to hyperpolarizing GABAergic signaling occurs at approximately P5. The time of DIV7-9 in rat hippocampal culture would be equivalent to P5-P7 in rat hippocampus or cortex.

8. The curves shown in Fig. 3A2 suggest that in the proximal axon of Ecm29 -/- neurons (third column), chloride efflux occurs at DIV 5 and DIV 7, and no exchange happens in DIV 9 and DIV 14 neurons (flat curve). This is at odds with the averaged results that show influx at DIV 9 and DIV 14 (Fig. 3B and 3C). Can the authors find a more representative example for Fig. 3A2?

We have replaced this data with more representative traces, now shown in Figure 4A2 (replacing previous Figure 3A2).

9. Could the authors discuss the results form Wefelmeyer et al. PNAS 2015 and Muir & Kittler Front Cell Neuro 2014 that show mismatch between GABA innervation and AIS position after activity-induced plasticity? Could the perturbations in AIS developmental positioning (Fig. 7), conserved GABAergic innervation (Sup. Fig 6) and excitability phenotypes (Fig. 6) be considered under this angle?

We now discuss the findings from Wefelmeyer et al. PNAS 2015 and Muir & Kittler Front Cell Neuro 2014 in the Discussion section (Page 20, second paragraph). Specifically, both studies found that chorionic depolarization of mature neurons led to distal shifts in the AIS position, while positions of inhibitory GABAergic synapses, that is, axo-axonic connections in proximal axons, were not changed. Wefelmeyer et al propose that spatial mismatch between GABAergic synapses and the AIS causes a higher shunting inhibition of GABAergic synapses that increases firing threshold and renders neurons less excitable. Our study, which was performed at an earlier developmental time point before GABA polarity switch, reports a different type of plastic AIS positioning in response to local protein homeostasis stresses and [Cl-] dynamics. We observed a proximal shift of the AIS toward the cell body when young pyramidal neurons were still excitable by GABA, a developmental AIS positioning that was NKCC1 activity-dependent. Conversely, altered NKCC1 accumulation in proximal axons caused by proteasome dysregulation would not only accelerate proximal AIS shifts but perturb the homeostatic regulation of GABAergic inputs and the AIS, leading to a hyper-excitable neuron.

Reviewer #3 (Comments to the Authors (Required)):

In this paper the authors have examined the role of the proteasome in the AIS. They propose that proteasome distribution in neurons indirectly controls chloride gradients by modulating the amount of NKCC1 in the axon initial segment, and that by controlling these chloride gradients the proteasome controls neuronal excitability. This is a very interesting and intriguing hypothesis. I think in general the quality of the results is quite good, and this paper is appropriate for JCB. There is a remarkable amount of work here including both in vitro and in vivo work. Nevertheless, I list several questions that should be addressed before the paper is complete.

1. On page 5, first paragraph, last sentence. I don't think the authors can immediately propose that ECM29/proteasome complexes are tethered to the AIS through AnkG. They should simply suggest that it is tethered at the AIS through some unknown mechanism. If you disrupt AnkG, you'll affect everything.

We thank the referee for the suggestion. We have changed the last sentence to read, "these findings suggest that Ecm29/proteasome complexes are tethered to the AIS via unknown mechanisms, rather than by actin filaments." (Page 6, last line).

2. The results demonstrating Ecm29 binding to AnkG are very nice, but the localization of Ecm29 at the AIS could be improved. A) the SIM imaging is not compelling (Fig. S2) since we don't also see what the localization looks like in non-AIS domains. B) One property of AIS-restricted and ankG-interacting proteins is that they are retained at the AIS after detergent extraction (see Garrido et al., Science 2003 and Huang et al., 2017). I suggest the authors do the detergent extraction experiment and show that Ecm29 is retained at the AIS together with ankG.

We have performed additional experiments to determine whether Ecm29/proteasome complexes are tethered to the AIS via Ecm29-dependent AnkG association. We performed non-ionic detergent extraction of DIV7 hippocampal cultures as suggested and showed that both proteasomes and Ecm29 were retained in the AnkG-positive AIS region after detergent treatment (0.02% Triton X-100, 37°C, 2 min). Such proteasome retention requires Ecm29 expression, as staining of proteasome subunits Rpt1 and Rpt5 was abolished in extracted neurons after Ecm29 KD (new Figure 2C).

We now include the full original SIM images of a neuron co-stained with antibodies against AIS proteins (N- or C-AnkG), proteasome subunits (20SCP), and/or Ecm29 in the new Figure S3B and 3C. Quantification of results indicates that both AnkG intensity and the extent of Ecm29-AnkG co-localization in distal axons were lower than that seen in the AIS domain.

3. Figure 1. The figure legend doesn't describe what the white or blue labels are. Why immunostain for Rpt5 and Ecm29 in both blue and green? (Fig1A, B). I don't understand the significance.

We apologize for confusion and have now added a sentence to the figure legend to describe the rightmost (blue) panels, which show ROI (dashed boxes) of the AIS

represented at higher magnification, with Rpt5 or Ecm29 staining intensity indicated by a linear pseudocolor scale (see Figure 1A and 1B).

4. On page 7, end of first paragraph. I don't understand the conclusion about the findings and how the authors think their results imply competition. Ecm29 and AnkG bind to each other (Fig. 2F) and form a complex. How do the authors think that when they are in a complex they compete for binding to the proteasome? If anything, it looks like the proteasome and Ecm29 compete for AnkG. You lose Ecm29 and you get more AnkG PLA signal at the AIS. I thought the model was that Ecm29 interacts with AnkG, thereby promoting recruitment of the proteasome to the AIS. But surprisingly, loss of ECM29 permits MORE proteasome in the AIS (Fig. 2G). Can the authors please clarify.

Again, we are sorry for confusion and now clarify our model based on in vivo findings, in vitro protein interaction assays (new Figure 2A and Figure S2), and new experimental results from detergent extraction experiments (new Figure 2C). The extraction assay, in particular, confirmed that AIS tethering of proteasomes requires Ecm29 protein, as proteasome/AnkG co-localization in the AIS was significantly suppressed in Ecm29 KD cells (new Figure 2C).

We note that our previous interpretation of PLA assays might be incorrect due to use of the AnkG antibody clone H215, which targets the AnkG C-terminus. Thus we replaced the original PLA analysis (original Figure 2D and 2G) with our new detergent extraction experiment (new Figure 2C) and with data derived from super-resolution 3D structured illumination microscopy (3D-SIM-Microscopy) (see revised Figure S3).

5. The results in Fig. 3 are very, very impressive and neat.

We thank the referee for the positive comment.

6. In Fig. 4, the authors attempt to draw a link between NKCC1 expression and Ecm29. They claim that there is increased NKCC1 in the ECM29 KO due to decreased turnover. While the immunostaining looks like there is more immunoreactivity at the AIS of Ecm29 KO mice, no experiments directly tests turnover rates. There are other potential explanations for their results: in the Ecm29 KO maybe trafficking to the AIS is more efficient? Maybe there is increased stability of membrane proteins? In short, no experiment actually demonstrates altered turnover rates. Indeed, doesn't Figure 4C2 argue for no change in surface protein levels? Why are there no immunoblots of NKCC1 for the surface fraction? In the Ecm29 KO you would expect to see increased levels.

Based on the referee's suggestion, we tested several commercially available NKCC1 antibodies for use in immunoblotting of the membrane fraction from P0 and P14 mouse cortical lysates (revised Figure 3B). Among those tested, only clone T4 NKCC1 antibody (from DSHB) was sensitive enough to detect surface NKCC1 protein (145-205 kDa, larger than the calculated M.W. based on the data sheet provided by DSHB). Our new quantitative analysis of NKCC1 expression as assessed by biotin labeling and affinity purification of plasma membrane proteins with an NKCC1 antibody (clone T4) supports the conclusion that Ecm29 loss blocks

down-regulation of surface NKCC1 protein in perinatal brain lysates. This data is now shown in new Figure 3B.

Based on the referee's inquiry, we provide three sets of new data to support the conclusion that NKCC1 up-regulation in Ecm 29 KO neurons is due to altered protein degradation. First, we used an AIS-located protein turnover reporter composed of an AnkG binding loop of Na_vII-III fused short half-life form of GFP (GFPu) to show more durable GFPu accumulation after MG132 pre-treatment (2.5 μM, 30 min) or shRNA-mediated knock-down of Rpt-1 or Ecm29 expression at the AIS (in new Figure 2D). Second, our new pulse-chase analysis of NKCC1 stability showed increased half-life of NKCC1 in Ecm29 KO hippocampal neurons (to >9 hours compared to ~6 hours in WT neurons). Also, ectopic expression of the proteasome/AnkG binding form of Ecm29 (Ecm29ΔC) rescued enhanced NKCC1 stability (see new Figure 3D). Third, real-time RT-PCR analysis excluded the possibility that NKCC1 accumulation in Ecm20 KO neurons was due to transcriptional changes (see new Figure S6). Overall, these findings support the idea that Ecm29 modulates the rate of NKCC1 turnover in immature hippocampal neurons.

7. As a preface to Fig. 6, the authors state they wanted to look at the relationship between neuronal excitability and Ecm29-mediated proteasomal distribution. The only data in the manuscript on proteasome distribution is found in Fig. 1C. I'm not convinced that proteasome localization depends on Ecm29. There is no analysis of proteasome localization in the Ecm29 KO. Trafficking is altered, so is the PLA in Fig. 2G. But why not also show labeling with MV151 in Ecm29 KO mice?

Based on these comments we revised the manuscript to include immunofluorescence staining of proteasome subunits (Rpt1 and Rpt5, in new Figure S7A) and quantitative results indicating the trajectory of MV151-labeled proteasomes (in revised Figure 1D and 1E; also see Video 1) in WT and Ecm29 KO neurons. Based on these findings, we are confident that proteasome distribution in maturing neurons is regulated by Ecm29 expression.

8. The physiology results (Fig. 7 and 8) are quite intriguing and consistent with a proximal shift of the AIS toward the cell body, leading to a more excitable neuron.

We thank the referee for the positive comments.

9. I think the most important question that remains unanswered is why is proximal axon NKCC1 preferentially targeted by the proteasome? Why aren't other AIS ion channels also targeted for degradation like NKCC1?

We agree and have added discussion relevant to substrate preference of the Ecm29/proteasome complexes to the Results (see Page 12, second paragraph, lines 13-18) and Discussion (Page 19, 7th-15th lines from the bottom) sections. In answer to this question, it possible that proximal axon NKCC1 abundance in maturing neurons is greater than that of other AIS ion channels or receptors. We conclude this based on expression profiles of factors analyzed during the GABAergic switch, which is concomitant with the decline of NKCC1 RNA transcripts and constitutes a time window when NKCC1 protein abundance and function predominate over KCC2, Na_v channels, Kainate, AMPA, and NMDA receptors. In this scenario, NKCC1 in the proximal axon may be a relatively accessible substrate (given its abundance) at the

AIS for the proteasome relative to other ion channels; hence a shift in the NKCC1 decline curve caused by proteasome dysregulation would become evident. We examined this possibility using two stable isotopically-labeled amino acids in cell culture (SILAC)-based mass spectrometry (MS)/MS approaches (spike-in SILAC). Indeed, we found that a group of proteins whose abundance gradually declines during the GABAergic switch (from DIV5 to DIV10) shows an altered pattern in Ecm29 KO neurons with a delayed decay (from DIV7 to DIV10) (see supplemental Figure below, Cluster 1). We do not include these preliminary results in the revised manuscript but provide them here for the referee's perusal.

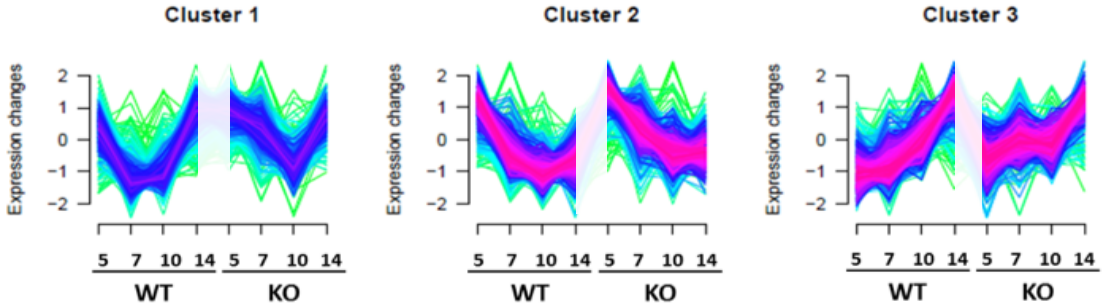

Figure Q9. Differentially expressed protein profile of wild-type ("WT") and Ecm29$^{-/-}$ (KO) neurons identified by spike-in SILAC approach. Proteins in cluster 1 (n= 462) exhibit a strong downregulation from DIV5 to DIV10 in WT neurons, but not in KO neurons. Proteins in cluster 2 (n=586) exhibit a gradual downregulation from DIV5 to DIV 14 in both WT and KO neurons. Proteins in cluster 3 (n=500) exhibit a gradual increase from DIV 5 to DIV14 in both WT and KO neurons. NKCC1 (encoding by SLC12A1) belongs to cluster 1.

November 5, 2019

RE: JCB Manuscript #201903033R-A

Dr. Pei-Lin Cheng
Institute of Molecular Biology
No. 127 Academia Road, Section2, Nankang,
Taipei 11529
Taiwan

Dear Dr. Cheng,

Thank you for submitting your revised manuscript entitled "Ecm29-mediated Proteasomal Distribution Modulates Excitatory GABA Responses in the Developing Brain". You will see that our returning reviewers -- who assessed the full revision in depth -- are now supportive of publication. We appreciated the extensive revisions made to the work and agree with their evaluations. You will see that Rev#1 however disagrees with the focus on the AIS and asks that you broaden the interpretation and revise the discussion to extend the model to somatodendritic GABAergic synapses. No new experimentation is needed. We would be happy to publish your paper in JCB pending final revisions to the text to address these final points and revisions necessary to meet our formatting guidelines (see details below).

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3) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. :
- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style. Suggested revised statement to meet these requirements:
Lee et al show that the local abundance of the chloride importer NKCC1 and timely emergence of GABAergic inhibition are modulated by proteasome distribution, including through interactions of proteasomes with the proteasome adaptor Ecm29 and the axon initial segment scaffold protein ankyrin-G.

4) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 1AB (including side panels), 2D1, 3C1C2 (side panels), 4A1,

7A1C1, S3B1, S5ABCD (magnifications), S7A (magnifications), S7C
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: 2A1 (unit labels), 3A1, B1 (unit labels), 3D2, S2A (unit labels), S2BC

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Please indicate n/sample size/how many experiments the data are representative of: 7C3, S3C2

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Erika Holzbaur, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This is an interesting and ambitious revised manuscript presenting data supporting a role for localized proteosomal degradation of NKCC1 in the developmental switch from excitatory to inhibitory GABAergic signaling. The paper has been improved with revision, but some substantive concerns remain. These could be addressed without new experiments, but will require revision of the text.
A major issue is the narrow focus on the axon initial segment. GABAergic synapses primarily are targeted to somatodendritic sites, which should be explicitly acknowledged. This would allow the authors to more accurately present their data. A few examples: NKCC1 and proteosomal subunits are present in both somatodendritic and axonal compartments; GABA-induced fluxes reverse in both axons and dendrites in Ecm29 k/o neurons (Fig 4 A2); Ecm29 k/o increased NKCC1 in both axons and dendrites (Fig 3C1); overall levels of NKCC1 were elevated in Ecmko brains even though only a small fraction of NKCC1 is localized at the AIS (arguing for a global and not AIS-specific effect). In addition, the 190 kDa isoform of ankG in this study localizes to postsynaptic sites (see Penzes 2014 Neuron paper), while the 480 kDa ankG isoform is localized in both the AIS and

somatodendritic compartments (see Tseng WC et al PNAS, 2015; Jenkins PM et al., PNAS, 2015; Freal et al., J Neuroscience, 2016). The super-resolution data in this study supports the localization of 480 kd ankG at the AIS since Ig against N-terminal and C-terminal domains give different patterns, likely due to spatial separation by the large exon-encoded sequence of 480 kda ankG (similar to findings of Letterier et al., 2015). The authors could consider adding clarifying statements to the introduction and discussion to the effect that although they have focused on the AIS, their findings also extend to somatodendritic GABAergic synapses.

Other issues;
-NKCC1 bands in western blots in Fig 3 B1 are difficult to see
-start sites for the AIS in cultured neurons exhibit large variation, and the functional significance of the modest statistical differences between WT and Ecm29 k/o is not clear. The authors could consider removing this figure.

Reviewer #3 (Comments to the Authors (Required)):

This is an extensively revised version that has addressed all of my concerns. I think the detergent extraction experiments are very nice and compelling. This paper is a tremendous amount of work and is the first to show how protein turnover may work through the proteasome and Ecm29. It also provides an explanation for the phenotypes of the mice lacking. Overall, it is both conceptually and technically superb.

I only have a few minor editing comments:

1. in Fig. B2, the P0 and P14 labels for NKCC1 are missing
2. IN Fig. S2, I think the title is misleading. There are no data for 'in the AIS'. The statement is only true without the 'in the AIS'. It should be removed.