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REST/NRSF-MEDIATED INTRINSIC HOMEOSTASIS PROTECTS NEURONAL NETWORKS FROM HYPEREXCITABILITY

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 03 April 2013

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, both referees find the analysis of interest. However they also raise a number of important concerns that would have to be addressed in order to consider publication here. Given the referees' comments, I would like to invite you to submit a revised version of the manuscript that addresses the concerns raised. I should add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

In this manuscript the authors explore the role of the transcriptional repressor REST in homeostatic plasticity of intrinsic excitability following activity elevation with 4AP. The results suggest that REST plays a role in down-regulating Na channel expression following activity-elevation, and contributes to network level homeostasis of firing. While the homeostatic regulation of Na channel density has been established previously, the pathways that control this regulation are largely unknown so the data are of interest. While the results are interesting and generally sound, they are in some cases overstated. Further, there are a number of issues surrounding data controls, presentation and analysis that need addressing.

Major points

1. A major issue is that firing rates (and other measures of activity) in these cultures is increasing dramatically over time (Fig. 6) and does not appear to plateau. The authors describe these cultures as "mature" at 16 DIV, but a glance at Fig. 6e makes it clear that this is not accurate since activity increases 3-4 fold between 16 and 26 DIV. Since most of the other firing rate data in the manuscript is normalized to control, these baseline effects are not apparent in (for example) Fig. 1f. Because everything is normalized it is very difficult to determine how big the effects of manipulating REST are, relative to these developmental changes in activity. Note that the normalization method is not described; I had assumed values were normalized to values just before 4AP treatment, but it appears they must be normalized to control values at each time point, otherwise the developmental rise in activity would be apparent in these figures. The authors never really address this baseline change in activity, but this phenomenon needs some explaining in the context of a manuscript on homeostasis of firing. What happens to REST levels during this period of time? Does knocking down REST accelerate this change, or is this completely independent of REST? What is the magnitude of the REST effects on activity relative to these baseline changes?

2. To accurately determine intrinsic excitability FI curves should be generated with synaptic transmission blocked; otherwise differences in synaptic conductance can contribute to differences in the FI curves. Were they? Also, why are all the parameters in fig 2c-f reported as normalized values, rather than the actual values? This makes it impossible to assess whether the parameters they measure are in the usual range. It would be much more informative to see the actual values.

3. The effects of OE and KD of REST do not seem to fully account for the 4AP-induced changes in excitability. For example, the magnitude of the effect of OE on the FI curves is less extreme than the effects of 4AP, yet the effects on Na current are larger. This is not discussed.

4. In figures 7 and 8 all conditions reported are in 4AP, and there is no comparison to matched control data. This makes it impossible to assess how fully KD of REST prevents the effects of 4AP. A comparison of the FI curves in Fig 7b with those in Fig. 2b or 5c suggests that there is only a partial block of the change in FI curve, consistent with the effects of OE. It is not surprising that 4AP might initiate changes that are not mediated by REST, but the authors side-step this issue and discuss the data as though changes in REST can fully account for the changes in excitability. Results should be presented relative to matched control data and this issue discussed in the manuscript.

5. A similar comment holds for the network activity data in Fig 8; in Fig. e-f no control (non-4AP treated) data are shown, so it is not apparent how KD+ 4AP compares to matched control condition.

6. My guess is the authors can make the case that REST mediates some of the effects of elevated activity on intrinsic excitability, but need to acknowledge clearly that changes in REST cannot account for all the changes in intrinsic excitability, nor all the network adaptation to 4AP treatment. Whether the effects represent a "central role" or a more minor role is not entirely clear at the moment given the lack of appropriate control comparisons.

Minor points

7. This is not really a "new form of intrinsic homeostasis" as claimed in the abstract; the phenomenon is well-described, but the role of REST is novel.

8. Is the change in Na inactivation significant?

9. Why does REST and Na channel expression return to baseline at 96 hr, when excitability is still depressed? The authors need to discuss this.

10. It is not meaningful to refer to these changes in excitability etc as "downscaling". That term was coined to refer specifically to proportional scaling of postsynaptic current amplitudes across synapses.

Referee #2

The paper by Pozzi et al. identifies a novel and interesting link between transcriptional regulation mediated by REST/NRSF and homeostatic regulation of intrinsic excitability. This new finding contributes mechanistic insight into homeostatic regulation of neuronal firing properties and intrinsic excitability. Using Ca2+ imaging and electrophysiology, the authors show that the K+ channel blocker 4AP enhances overall network activity in cortical neuron cultures, but this effect slowly reverses over the next 48-96 hrs. These results support the idea that homeostatic adaptations renormalize network activity during sustained 4AP exposure, and are consistent with the previous literature using other pharmacological tools to enhance network activity (e.g., GABA-A receptor antagonists). The authors then show that a decrease in intrinsic neuron excitability likely contributes to this renormalization, an effect that is accompanied by decreases in voltage-gated Na+ currents and voltage-gated Na+ channel expression. Most significantly, the authors demonstrate that REST/NRSF plays an important role in these effects, as REST/NRSF overexpression reduces Na+ currents and excitability, whereas RNAi-mediated knockdown of REST/NRSF prevents the renormalization of firing activity during prolonged 4AP exposure. Overall, the data and manuscript are thorough, well-presented, and support these conclusions. However, I have several concerns that need to be addressed by the authors.

Major Concerns
1) The authors correlate the changes they observe in Na+ currents with the decrease in excitability measured during prolonged current pulses, but they never actually demonstrate that the changes in Na+ currents are responsible for the excitability changes. Chronic 4AP treatment appears to augment spike-frequency adaptation, a parameter that has been traditionally associated more with recruitment of Ca2+-activated K+ currents than voltage-gated Na+ currents. The authors provide no data on a potential role for altered Ca2+-activated K currents nor do they demonstrate that a decrease in voltage-gated Na+ currents can reproduce the changes in spike-frequency adaptation that are observed. Can low doses of TTX reproduce the excitability changes they observe, i.e., a decrease in instantaneous firing frequency without a change in action potential threshold? Alternatively, it may be informative to examine post-burst after hyperpolarizations to gain some insight into whether Ca2+-activated K+ currents are regulated. At the very least, some discussion of alternative mechanisms is necessary, which would include some rationale for how changes in Na channel expression can account for the changes in firing patterns observed.
2) It is surprising to me that the prominent decrease in NaV expression (> 50%) observed following 96 hrs of 4AP (Figure 3) is not accompanied by changes in AP threshold or rheobase (Figure 2). By contrast, overexpression of REST leads to a significant increase in rheobase, while shRNA-mediated REST knockdown significantly reduces rheobase. This discrepancy between 4AP treatment and REST expression is puzzling and not discussed by the authors. Assuming that this is not a measurement issue (see below), does this discrepancy simply reflect a more prominent reduction in Na+ currents after manipulation of REST expression? Or, does this potentially reflect regulation of other ion channels by chronic 4AP that act to offset the higher threshold one would expect from a decrease in Na+ channel density? A final possibility is that Na+ current measurements at the cell soma do not reflect Na+ channel density at the axon initial segment (AIS), with the AIS more intimately associated with threshold.
3) Related to the point above, I have some concerns regarding the threshold calculations, which were done with Minianalysis software. The threshold for firing is important information because the

down regulation of sodium channels found following 4AP treatment might be expected to increase in threshold, but this is not observed by the authors. I wonder if the 5 kHz sampling rate is not high enough to detect the fast fluctuations in membrane potential that would potentially be revealed would with higher sampling, such as 20 kHz. Secondly, the threshold is reported as normalized, but it is unclear how this normalization was done. What does a number above or below 1 mean? Is greater than 1 an increase or decrease in threshold? It would be clearer to report the raw values in mV. Additionally, the action potential(s) chosen for analysis of threshold is not stated. Measuring threshold on top of a current injection is not ideal, but has been done using the rheobase action potential - the first action potential elicited by the current injection.
4) The results from the PI assay used to measure cell vitality is certainly consistent with the notion that the cells are not dying in the presence of chronic 4AP. However, it is not necessarily a sensitive measure of cell health, either. It would be useful to report the passive membrane properties of the neurons, as this is likely a better indicator of overall health than is the PI assay.
5) In figure 8, the authors use broad lentiviral expression of the shRNAs to measure the impact of REST/NRSF knockdown on renormalization of network activity. This data is very nice and convincing, but does not address the impact of these changes at the level of single cells in the network. Ultimately, the changes in intrinsic excitability should act to normalize firing patterns within individual cells. If the shRNA is expressed sparsely, do transfected neurons exhibit cell-autonomous changes in the firing rates/bursts of spontaneous activity, or do all neurons need to have shRNA expression to see the results in figure 8?

Specific Comments
1) The authors report that the maximal rising slope of the AP is diminished following chronic 4AP. Some traces that exemplify this effect would be nice to include.
2) In figure 2, plotting the Normalized Threshold in Fig.2e next to example traces in Fig.2a implies that the analysis was done on multiple action potentials. This should be clarified.
3) Figure 2a should include a voltage scale bar.
4) In the Western Blot shown in Figure 3e, the Na/K pump band is overexposed. Is this the exposure used for the quantification? The figure legend indicates the methods are described in "MM" however this information seems to be omitted from the manuscript. A lower exposure of this band should be provided and the authors should clarify how densiometric measurements were made.
5) In the "Protein extraction and western blotting" section on page 27 the authors indicate that the proteins were quantified by DC Protein Assay and "80 mg were separated by 10% SDS-PAGE." I am assuming that this is formatting issue with greek letters, and the loading material was 80 micrograms - is this correct?

1st Revision - authors' response 25 July 2013

ANSWERS TO REVIEWERS' COMMENTS
The EMBO Journal
Re: **EMBOJ-2013-84901R**.

Referee #1

In this manuscript the authors explore the role of the transcriptional repressor REST in homeostatic plasticity of intrinsic excitability following activity elevation with 4AP. The results suggest that REST plays a role in down-regulating Na channel expression following activity-elevation, and contributes to network level homeostasis of firing. While the homeostatic regulation of Na channel density has been established previously, the pathways that control this regulation are largely unknown so the data are of interest. While the results are interesting and generally sound, they are in some cases overstated. Further, there are a number of issues surrounding data controls, presentation and analysis that need addressing.

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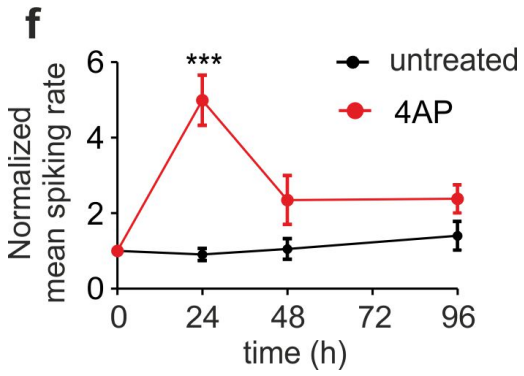
1. A major issue is that firing rates (and other measures of activity) in these cultures is increasing dramatically over time (Fig. 6) and does not appear to plateau. The authors describe these cultures as "mature" at 16 DIV, but a glance at Fig. 6e makes it clear that this is not accurate since activity increases 3-4 fold between 16 and 26 DIV. Since most of the other firing rate data in the manuscript is normalized to control, these baseline effects are not apparent in (for example) Fig. 1f. Because everything is normalized it is very difficult to determine how big the effects of manipulating REST are, relative to these developmental changes in activity. Note that the normalization method is not described; I had assumed values were normalized to values just before 4AP treatment, but it appears they must be normalized to control values at each time point, otherwise the developmental rise in activity would be apparent in these figures. The authors never really address this baseline change in activity, but this phenomenon needs some explaining in the context of a manuscript on homeostasis of firing. What happens to REST levels during this period of time? Does knocking down REST accelerate this change, or is this completely independent of REST? What is the magnitude of the REST effects on activity relative to these baseline changes?

Cultured neurons are generally considered mature at 14-16 days in vitro (DIV) because at this stage of development both the inhibitory and excitatory synapses are completely formed and appear structurally and functionally mature, each neuron expresses all the voltage-gated conductances driving action potentials, and spontaneous neuronal activity is well established at the network level. However, it is also true that during in vitro development, the overall network activity of an healthy culture changes over time due to several factors such as an increase in the synaptic contact number, a higher expression level of voltage channels, etc. In other words the neuronal network reconstituted in vitro represents a system that never reaches stability in terms of activity.
Thus, the Referee is perfectly right in asking to evaluate the homeostatic responses, involving prolonged treatments, with respect to the control developmental profile of parallel, untreated cultures. Whenever possible, e.g. in patch clamp recordings, we have shown the data in absolute values for treated and untreated groups. For Ca^{2+} imaging studies (e.g. Fig. 1c) and biochemical data (PCR and immunoblots; e.g. Fig. 4) that are performed on independent sample cultures, we have normalized each data point to the activity level of untreated neurons recorded at the same

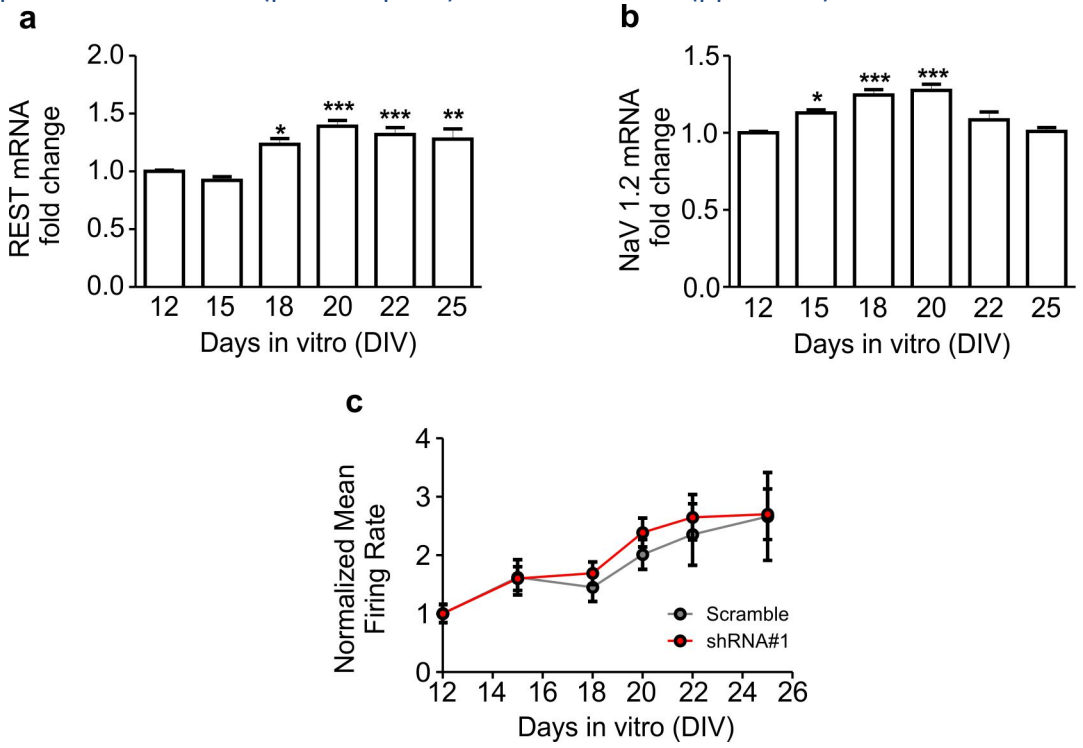
stage of development. In case of MEA recordings, when the very same culture can be repeatedly measured during development thanks to the non invasive character of the technique, we represented network activity in treated and untreated samples by normalizing the data to the values of the very same culture before 4AP treatment. Such normalization is necessary, given the intrinsic variability in the basal spiking rates due to differences in cell culture density between different preparations.
We made a new version of Fig. 1f (reported below) by following the latter procedure, now clearly described in the respective legends and the "Methods" section.



As suggested by the Reviewer, we have also studied whether REST/NRSF expression levels change during the physiological development of neuronal cultures, particularly during the time window in which the neuronal activity significantly increases.
To this aim, we investigated by rtPCR analysis whether the in vitro development of the neuronal network from 12 to 25 div was associated with endogenous changes in REST/NRSF and Nav1.2 mRNA levels. We found that REST/NRSF mRNA levels, constant during the early stages of in vitro maturation, increased between 15 and 20 div to reach a steady-state level (see figure below panel a). Over a similar time window, the Nav1.2 mRNA was also up-regulated (see figure below panel b). These temporal profiles indicate that Nav 1.2 expression during this developmental phase is regulated by several factors and that the parallel changes in REST/NRSF and Nav 1.2 mRNAs are the expression of a general maturation of the network (increase in synapse number and in the expression of voltage-gated conductances, etc.), as reflected in the progressive increase in network activity (see figure below lower panel).
We have also studied the effect of REST/NRSF knocking-down on the increase in network activity observed during the in vitro development. Neuronal cultures have been infected with either REST/NRSF specific shRNA or the scrambled sequence, and the spiking rate was monitored between 12 and 25 DIV. We found that the down-regulation of the endogenous REST/NRSF levels did not affect the age-dependent increase of spontaneous network activity normally occurring during in vitro maturation (see figure below panel c). Note that in this new set of experiments the increase in the mean firing rate is lower than that one reported in Fig. 6. Such variability is quite expected in neuronal culture where both cell density and the maturation rate can vary significantly among different culture preparations.

Overall this new evidence indicates that REST/NRSF expression can increase both during the slow age-dependent enhancement of spontaneous neuronal activity and upon the fast transient 4AP-mediated augmentation of neuronal firing. However, only the latter process, which trigger an homeostatic response of the network, is tightly REST/NRSF-dependent and can be finely modulated over time by the expression level of this transcription factor. We think that these new results complete and extend the knowledge of REST/NRSF-mediated effects. Thus, we have

inserted new figures in supplementary material (Suppl. Fig. 4 and Suppl. Fig. 7d), and new paragraphs in the "Results" (p. 8 and p. 13) and "Discussion" (pp. 19-20) sections.



2. To accurately determine intrinsic excitability FI curves should be generated with synaptic transmission blocked; otherwise differences in synaptic conductance can contribute to differences in the FI curves. Were they? Also, why are all the parameters in fig 2c-f reported as normalized values, rather than the actual values? This makes it impossible to assess whether the parameters they measure are in the usual range. It would be much more informative to see the actual values.

The current clamp recordings for investigating intrinsic excitability (including the FI curves) were all performed in the presence of synaptic transmission blockers (APV 50 μM, Bicuculline 30 μM and CNQX 10 μM), as reported in the "Methods". However, for the sake of clarity, this information has now been added to the legend to Fig. 2 and in the description of the results. Moreover, as suggested by the Referee, all data shown in Figs. 2, 5 and 7 are now presented as absolute values. We have improved the analysis of the action potential properties reported in Fig. 2 by increasing the sampling frequency from 10 to 50 KHz. We have also added representative action potential traces (new Fig. 2d) and the plot of the time derivative of the voltage (dV/dt) versus voltage (the so-called phase-plane plot, new Fig. 2e) (Bean et al., 2007).

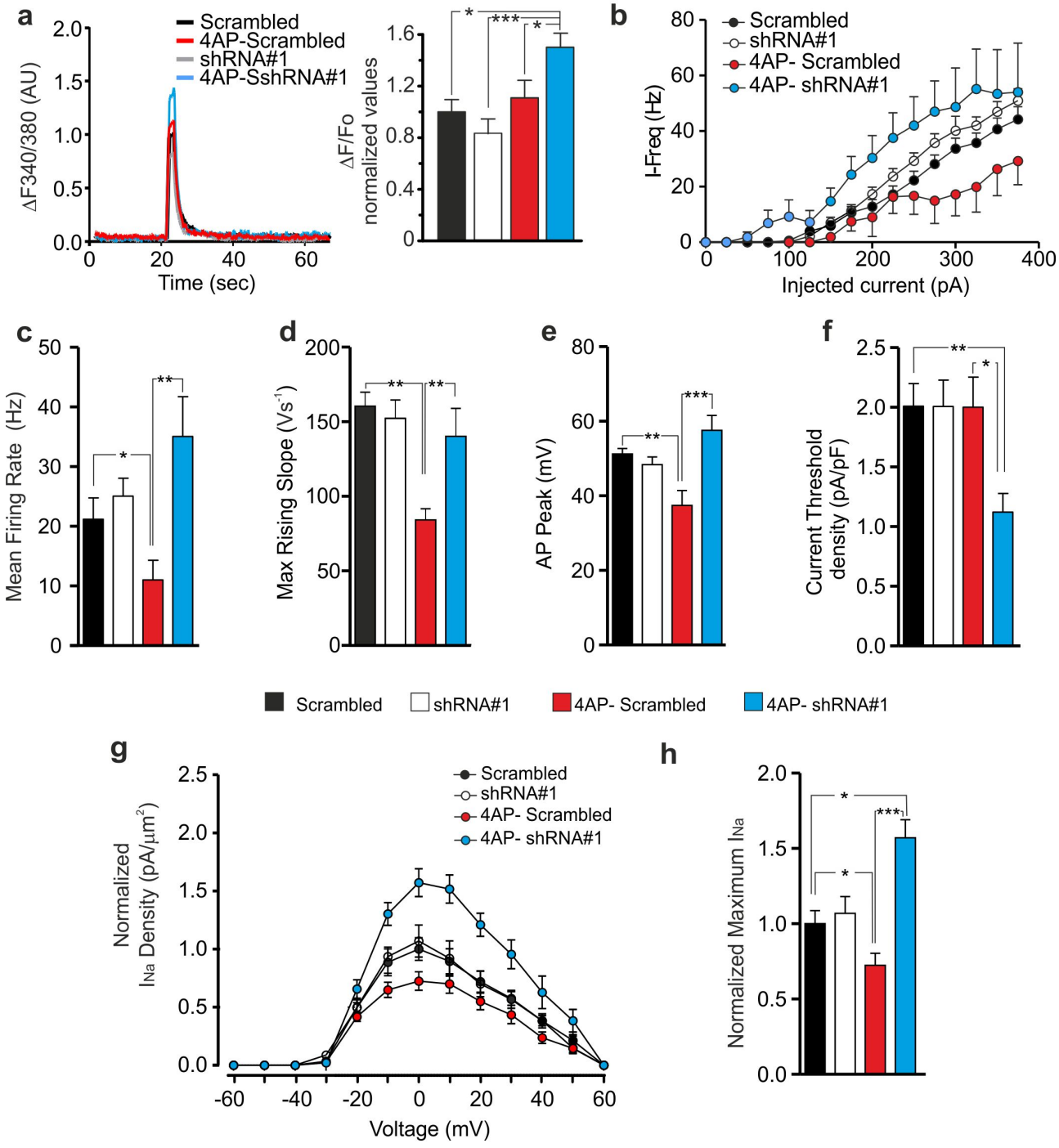
3. The effects of OE and KD of REST do not seem to fully account for the 4AP-induced changes in excitability. For example, the magnitude of the effect of OE on the FI curves is less extreme than the effects of 4AP, yet the effects on Na current are larger. This is not discussed.

We fully agree with the Referee. However, such differences are somewhat expected, considering that the two conditions are similar but not identical. Indeed, 4AP treatment increases REST/NRSF mRNA levels by 1.4 times, whereas REST OE through lentivirus infection is increased by almost 7-fold (added in "Results" section, p. 11). Furthermore, it is also known that RE1 sequences located

in the promoter region of several REST/NRSF target genes may vary considerably in terms of both repeat number and REST/NRSF binding affinity, suggesting that distinct RE1-containing genes interact differentially with REST/NRSF (Bruce et al. 2004; 2009). On the basis of such evidence, it is possible to speculate that the higher REST/NRSF mRNA levels achieved by OE could induce the repression of target genes with lower affinity for REST/NRSF. On the other hand, 4AP-mediated hyperactivity could activate a plethora of activity-dependent plasticity processes, in which other ion channels or proteins responsible for the control of excitability might be involved. Thus, this very complex scenario could explain why it is not possible to punctually reproduce the 4AP effects with REST OE. We have now discussed these important aspects in the "Discussion" (pp. 17-18).

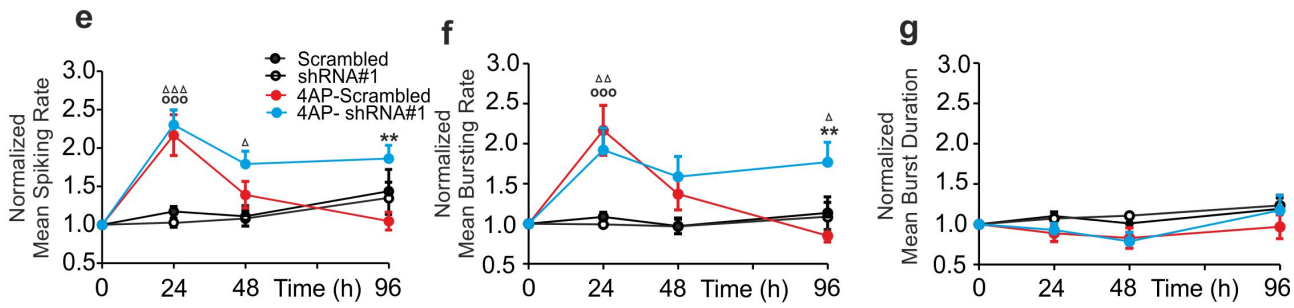
4. In figures 7 and 8 all conditions reported are in 4AP, and there is no comparison to matched control data. This makes it impossible to assess how fully KD of REST prevents the effects of 4AP. A comparison of the FI curves in Fig 7b with those in Fig. 2b or 5c suggests that there is only a partial block of the change in FI curve, consistent with the effects of OE. It is not surprising that 4AP might initiate changes that are not mediated by REST, but the authors side-step this issue and discuss the data as though changes in REST can fully account for the changes in excitability. Results should be presented relative to matched control data and this issue discussed in the manuscript.

The data reported in the old version of Fig. 7 are now presented in comparison to the correctly matched and appropriate controls (neurons transfected with either scrambled or REST/NRSF shRNA1 under resting conditions). To improve the clarity in panels 7b-h, the data relative to shRNA2-transfected cultures were removed and the comparison between shRNA2 and scrambled shRNA is now presented in Supplementary Fig. 8. Furthermore, with respect to Fig. 7a, we have also measured the evoked Ca^{2+} transients after 24 and 96 hrs of 4AP incubation. We found that, in line with spontaneous Ca^{2+} transients and firing rate shown in Fig. 1, the evoked Ca^{2+} transients were enhanced by 24 h treatment with 4-AP, and progressively reduced thereafter, returning to basal levels after 96 h. This new data has been added as Supplementary Fig. 6c. Moreover, as already mentioned in point #3, we have now inserted in the "Discussion" section a short paragraph, in which the possibility that 4AP stimulation initiates several REST/NRSF-independent homeostatic processes is considered (pp. 17-18).


a
Scrambled
4AP-Scrambled
shRNA#1
4AP-SshRNA#1
ΔF340/380 (AU)
Time (sec)
ΔF/Fo normalized values
b
Scrambled
shRNA#1
4AP- Scrambled
4AP- shRNA#1
I-Freq (Hz)
Injected current (pA)
c
Mean Firing Rate (Hz)
d
Max Rising Slope (Vs-1)
e
AP Peak (mV)
f
Current Threshold density (pA/pF)
Scrambled
shRNA#1
4AP- Scrambled
4AP- shRNA#1
g
Normalized INa Density (pA/μm2)
Voltage (mV)
h
Normalized Maximum INa

5. A similar comment holds for the network activity data in Fig 8; in Fig. e-f no control (non-4AP treated) data are shown, so it is not apparent how KD + 4AP compares to matched control condition.

We have added the time course of the firing rate in untreated neurons infected with either scramble or shRNA1 (see figure below). To improve clarity in Fig. 8, panels e-g, the traces of both shRNA2 infected and non infected culture were removed. A comparison between shRNA2 and scrambled shRNA is now presented in Supplementary Fig. 9. We concentrated on the effect of 4AP treatment at the 96 h time point when the REST/NRSF shRNA infected neurons still have an elevated neuronal activity.



6. My guess is the authors can make the case that REST mediates some of the effects of elevated activity on intrinsic excitability, but need to acknowledge clearly that changes in REST cannot account for all the changes in intrinsic excitability, nor all the network adaptation to 4AP treatment. Whether the effects represent a "central role" or a more minor role is not entirely clear at the moment given the lack of appropriate control comparisons.

We fully agree with this view. We have now clearly stated in the manuscript that the changes in REST/NRSF expression cannot account for all the homeostatic responses observed in our model. In particular we have added a new paragraph in the “Discussion” in which we clearly mention that REST/NRSF activity is critical for the intrinsic homeostatic plasticity, even though the entire process may not be entirely governed by this transcription factor (p. 17-18). A similar change was made in the last sentence of the "Abstract".

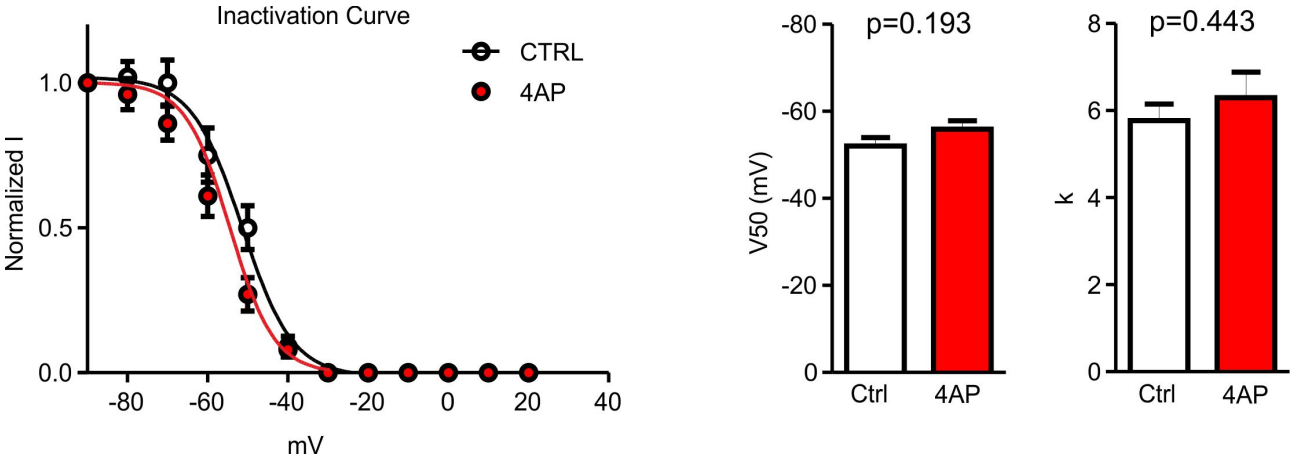
Minor points

7. This is not really a "new form of intrinsic homeostasis" as claimed in the abstract; the phenomenon is well described, but the role of REST is novel.

The abstract text has been now changed. The sentence: " *We characterized a new form of intrinsic homeostasis...*" has been changed as follows: "*We characterized the process of intrinsic homeostasis...*"

8. Is the change in Na inactivation significant?

We have re-analyzed the inactivation curve shown in Fig. 3d and found that the point at -50mV was significantly different between 4AP treated and untreated neurons (Student's unpaired two-tailed *t*-test; p<0.03). However, we have fitted the I/I_{max} *vs* Voltage for each cell (n=34 and n=20 for control and 4AP treated, respectively) by using a Boltzmann equation: $(I/I_{max}=[1+\exp(V_{50}-V_c)/k)]^{-1}$ (where V_{50} is the potential of half-maximal inactivation, V_c is the command voltage and k is the steepness constant). Mean V_{50} and k have been calculated and no significant changes have been observed (Student's unpaired two-tailed *t*-test, see figure below). This new analysis has been added to Supplementary Fig. 2.



9. Why does REST and Na channel expression return to baseline at 96 hr, when excitability is still depressed?

The observation is correct. However, it has to be noted that although both REST/NRSF and Nav1.2 mRNA levels (4a,b) return to baseline after 96 hr of 4AP incubation, the protein levels of these genes are still up-regulated at 96 hrs (Figs. 4c, 3e). This difference can be due to feedback mechanisms restoring resting state REST/NSRF transcription after an initial perturbation and/or to the longer half-life of REST/NSRF protein with respect to its mRNA. The data indicate that the transient activity-dependent modulation of REST/NRSF and Nav1.2 mRNA is sufficient to trigger a long-lasting homeostatic response. We have now added a short sentence to the "Discussion" section on this point (p. 17).

10. It is not meaningful to refer to these changes in excitability etc as "downscaling". That term was coined to refer specifically to proportional scaling of postsynaptic current amplitudes across synapses.

The sentence has been rephrased. We have substituted the word "*downscaling*" with "*down-regulation*" throughout the article.

Referee #2

The paper by Pozzi et al. identifies a novel and interesting link between transcriptional regulation mediated by REST/NRSF and homeostatic regulation of intrinsic excitability. This new finding contributes mechanistic insight into homeostatic regulation of neuronal firing properties and intrinsic excitability. Using Ca2+ imaging and electrophysiology, the authors show that the K+ channel blocker 4AP enhances overall network activity in cortical neuron cultures, but this effect slowly reverses over the next 48-96 hrs. These results support the idea that homeostatic adaptations renormalize network activity during sustained 4AP exposure, and are consistent with the previous literature using other pharmacological tools to enhance network activity (e.g., GABA-A receptor antagonists). The authors then show that a decrease in intrinsic neuron excitability likely contributes to this renormalization, an effect that is accompanied by decreases in voltage-gated Na+ currents and voltage-gated Na+ channel expression. Most significantly, the authors demonstrate that REST/NRSF plays an important role in these effects, as REST/NRSF overexpression reduces Na+ currents and excitability, whereas RNAi-mediated knockdown of REST/NRSF prevents the renormalization of firing activity during prolonged 4AP exposure. Overall, the data and manuscript are thorough, well-presented, and support these conclusions. However, I have several concerns that need to be addressed by the authors.

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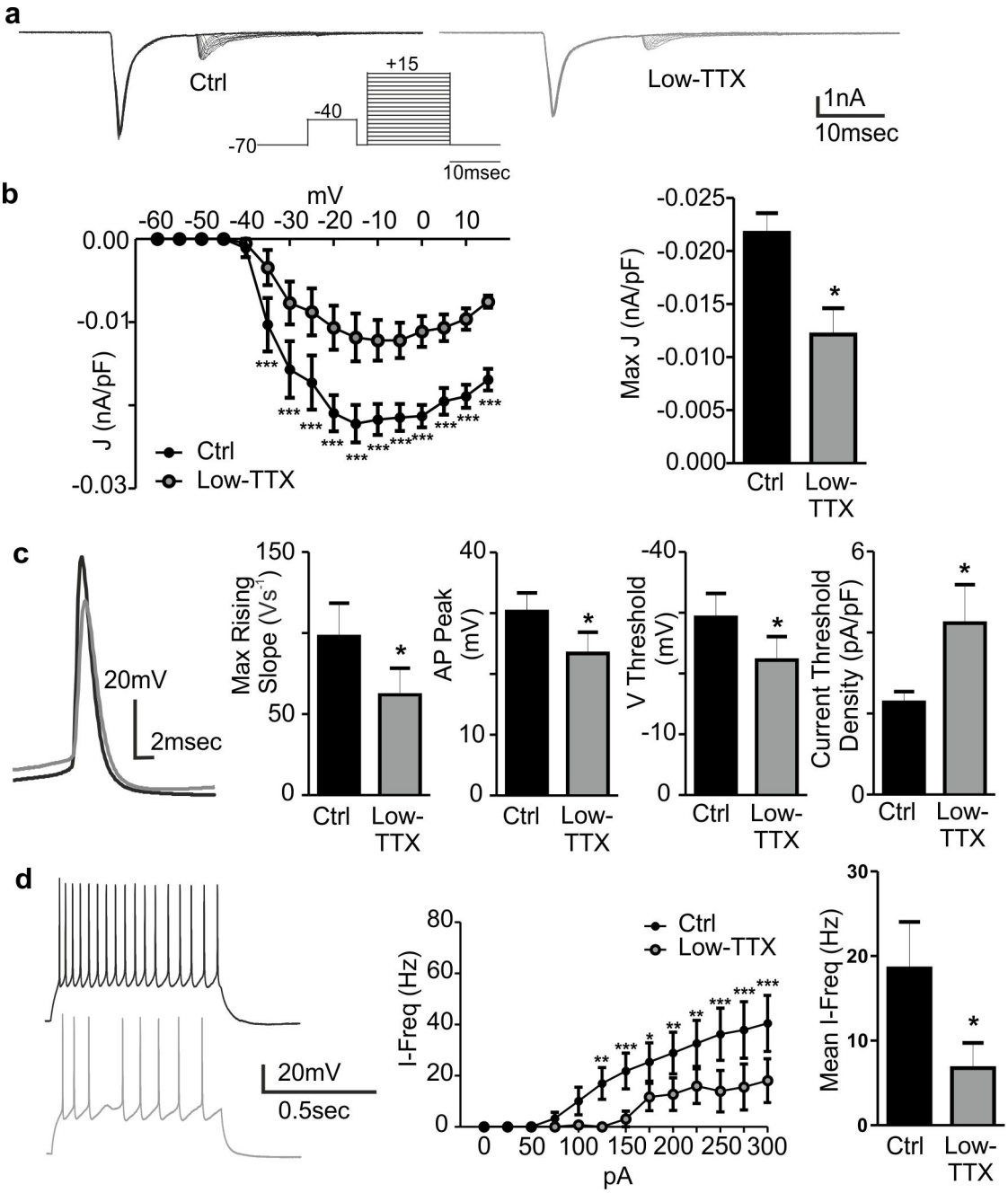
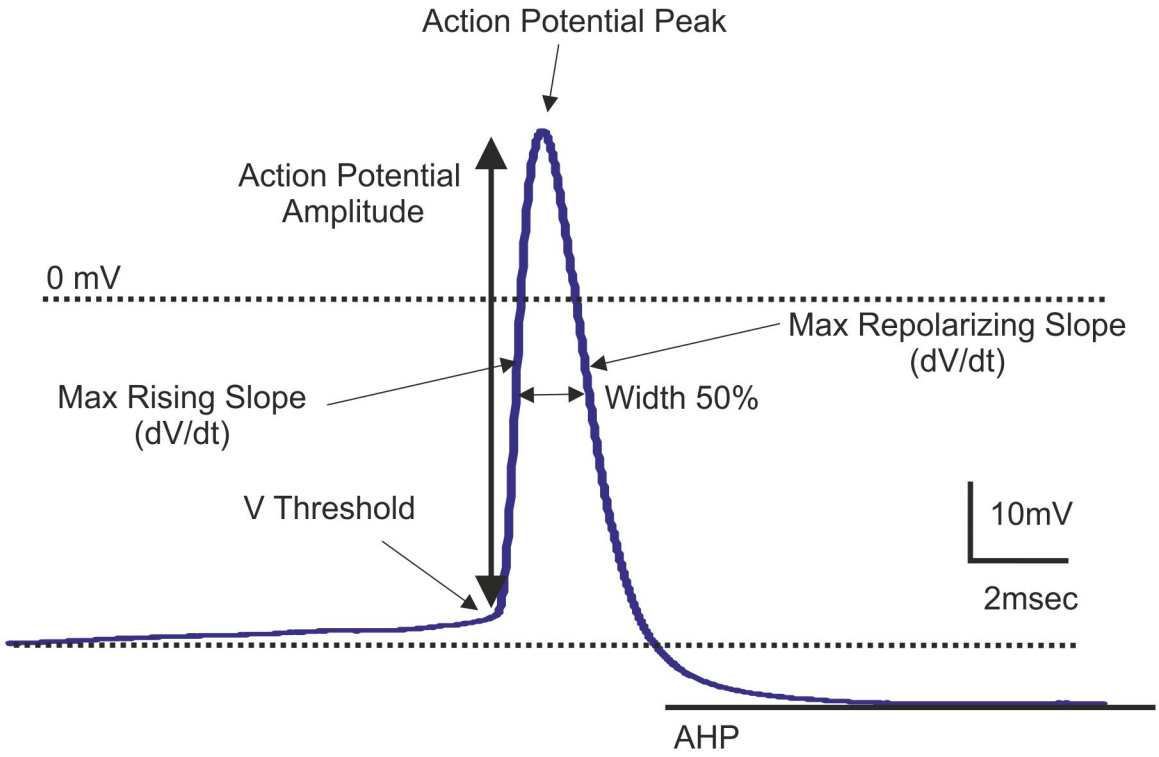
1) The authors correlate the changes they observe in Na+ currents with the decrease in excitability measured during prolonged current pulses, but they never actually demonstrate that the changes in Na+ currents are responsible for the excitability changes. Chronic 4AP treatment appears to augment spike-frequency adaptation, a parameter that has been traditionally associated more with recruitment of Ca2+-activated K+ currents than voltage-gated Na+ currents. The authors provide no data on a potential role for altered Ca2+-activated K currents nor do they demonstrate that a decrease in voltage-gated Na+ currents can reproduce the changes in spike-frequency adaptation that are observed. Can low doses of TTX reproduce the excitability changes they observe, i.e., a decrease in instantaneous firing frequency without a change in action potential threshold? Alternatively, it may be informative to examine post-burst after hyperpolarizations to gain some insight into whether Ca^{2+}-activated K+ currents are regulated. At the very least, some discussion of alternative mechanisms is necessary, which would include some rationale for how changes in Na channel expression can account for the changes in firing patterns observed.

As suggested, we have evaluated the effect of sub-saturating doses of TTX on neuronal excitability. We have used a voltage step protocol preceded by a brief pre-pulse (at -40 mV) that allows to inactivate axonal I_{Na} current, thus isolating the only somatic component (Milescu et al., 2010). This analysis was used to identify the concentration of TTX able to induce a partial block of voltage dependent Na^+ currents, thus reproducing the effects observed upon prolonged 4AP incubation. As shown in Suppl. Fig. 3 (see figure below), we found that 90-120 nM TTX, a concentration that significantly reduced Na^+ current density, exerted effects similar to those observed upon 4AP stimulation on the neuronal firing properties: augmentation of spike-frequency adaptation, reduction of action potential peak and maximum rising slope. However, both current and voltage threshold were reduced upon TTX treatment, although they were not affected during the 4AP treatment. Such a difference might be due to the fact that 4AP treatment (96 h) does not affect only Na^+ channels, as TTX does, but triggers a multitude of different molecular mechanisms, making the comparison of these two conditions very difficult.



As suggested, we have also measured the after hyperpolarization (AHP) and the maximal repolarizing slope upon 4AP stimulation to evaluate the possible contribution of K^+ conductances. We found that these parameters were unaltered between untreated and 4AP-treated (96 h) neurons (see Supplementary Table 1, below). It is possible, however, that the decreased AP peak and maximal rising slope impair the recruitment of voltage-gated Ca^{2+} channels, thus reducing the contribution of Ca^{2+}-activated K^+ currents. Finally, the possibility that other K^+ channels are modulated during long-term 4AP treatment cannot be ruled out.

All together these data point toward a predominant involvement of Na channels expression in the 4AP-mediated homeostatic responses, thus supporting our proposed model in which the transcriptional repressor activity of REST/NRSF is fundamental for such process.



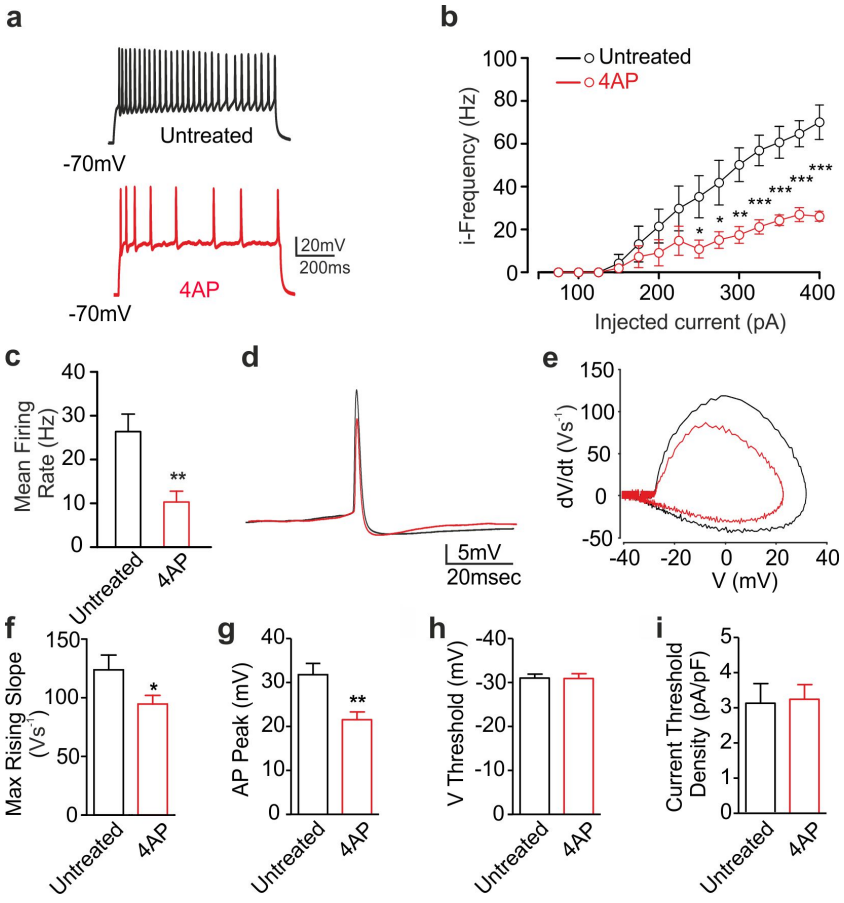
	Ctrl (n=15)	4AP (n=19)	p Value	Significance
Current Threshold Density (pA/pF)	3.11 ± 0.57	3.24 ± 0.42	0.85	**ns**
V Threshold (mV)	-31.01 ± 0.92	-30.92 ± 1.09	0.95	**ns**
Max Rising Slope (dV/dt)	123.8 ± 12.66	97.58 ± 7.527	0.039	*
Max Repolarizing Slope (dV/dt)	-38.88 ± 3.85	-32.37 ± 2.28	0.13	**ns**
AP Peak (mV)	31.79 ± 2.57	21.56 ± 1.74	0.001	***
AP amplitude (mV)	63.47 ± 2.82	52.48 ± 1.97	0.002	**
AHP (mV)	-40.91 ± 0.98	-42.41 ± 1.13	0.33	**ns**
Width at 50% of AP amplitude (msec)	1.66 ± 0.10	1.98± 0.16	0.16	**ns**
Capacitance (pF)	57.25 ± 6.16	47.89 ± 2.53	0.18	**ns**
R input (MΩ)	208.6 ± 17.87	209.2 ± 26.19	0.98	**ns**
Membrane potential (mV)	-61.16 ± 1.32	-56.00 ± 0.96	0.003	**

2) It is surprising to me that the prominent decrease in Nav expression (> 50%) observed following 96 hrs of 4AP (Figure 3) is not accompanied by changes in AP threshold or rheobase (Figure 2). By contrast, overexpression of REST leads to a significant increase in rheobase, while shRNA-mediated REST knockdown significantly reduces rheobase. This discrepancy between 4AP treatment and REST expression is puzzling and not discussed by the authors.
Assuming that this is not a measurement issue (see below), does this discrepancy simply reflect a more prominent reduction in Na+ currents after manipulation of REST expression? Or, does this potentially reflect regulation of other ion channels by chronic 4AP that act to offset the higher threshold one would expect from a decrease in Na+ channel density? A final possibility is that Na+ current measurements at the cell soma do not reflect Na+ channel density at the axon initial segment (AIS), with the AIS more intimately associated with threshold.

As correctly noted by the Referee, the lack of effect on both voltage and current thresholds upon 4AP stimulation is surprising.
As mentioned in the answer to Reviewer 1 (see point#3), several reasons could be at the basis of the discrepancies observed between REST OE and 4AP, namely:
1) the increase in REST mRNA levels achieved by OE is higher than that obtained upon 4AP treatment and, accordingly, REST OE induces a more prominent reduction in Na^+ currents with respect to 4AP treatment. Moreover, while REST OE only affects REST/NRSF target genes (not only Nav1.2, but possibly many others), 4AP treatment may trigger several REST-dependent and independent homeostatic responses, in which several other channels or proteins might be modulated;
2) as mentioned above, the regulation of other ion channels upon chronic 4AP incubation could act to offset the higher threshold. We and others (Driscol et al., 2013; Grubb et al., 2010) have observed more depolarized resting potentials in neurons chronically treated with activity-enhancers (4AP, $GABA_A$ receptor antagonists; high K^+);
3) as suggested, the different contribution exerted by somatic and axonal Na^+ channels is critical. Our data have been obtained with macro-patch recordings, a technique that allows revealing changes in the somatic Na^+ current.
It has been demonstrated that Nav1.2 is a specific REST target (Chong et al., 1995; Nadeau & Lester, 2002), and we clearly demonstrated that the 4AP-mediated REST mRNA increase well correlated with a down-regulation of Nav1.2 mRNA (Fig. 4). Recently, it has been demonstrated that high-threshold Nav1.2 channels are located both at the somatic level and at the proximal segment of the AIS, whereas the low-threshold Nav1.6 channels preferentially accumulate at the distal end of AIS, which represents the site for action potential initiation (Hu et al., 2009). The specific localization of Nav1.2 both at the soma and proximal AIS, together with its higher half-activation voltage with respect to Nav1.6, could explain why their reduced expression did not affect either voltage or current threshold. Some of these points above have been now pointed out in the "Discussion" section (p. 17-19).

3) Related to the point above, I have some concerns regarding the threshold calculations, which were done with Minianalysis software. The threshold for firing is important information because the down regulation of sodium channels found following 4AP treatment might be expected to increase in threshold, but this is not observed by the authors. I wonder if the 5 kHz sampling rate is not high enough to detect the fast fluctuations in membrane potential that would potentially be revealed would with higher sampling, such as 20 kHz. Secondly, the threshold is reported as normalized, but it is unclear how this normalization was done. What does a number above or below 1 mean? Is greater than 1 an increase or decrease in threshold? It would be clearer to report the raw values in mV. Additionally, the action potential(s) chosen for analysis of threshold is not stated. Measuring threshold on top of a current injection is not ideal, but has been done using the rheobase action potential – the first action potential elicited by the current injection.

We thank the Referee for this observation. We would just like to point out that the previously adopted sampling frequency was actually 10 KHz, and not 5 KHz as erroneously stated in the text. As this frequency might be still not sufficient to accurately calculate the V-threshold, all the current-clamp experiments of Fig. 2 have been re-calculated in both control and 4-AP using 5-fold higher sampling rate frequency (50 KHz, see new Fig. 2 below). We also modified the "Methods" section by adding the new sampling rate (p. 32). V-threshold as well as other AP parameters have been re-calculated and are now reported in Supplementary Table 1. Moreover, the data in Fig. 2, 5 and 7 are now presented as absolute values, and not as normalized data, for better clarity. Finally, we have enriched the "Methods" section with a better description of the analysis used for current clamp recordings, explicitly stating that the threshold was not calculated on top of a current injection, but using the first action potential elicited by the minimal current injection.

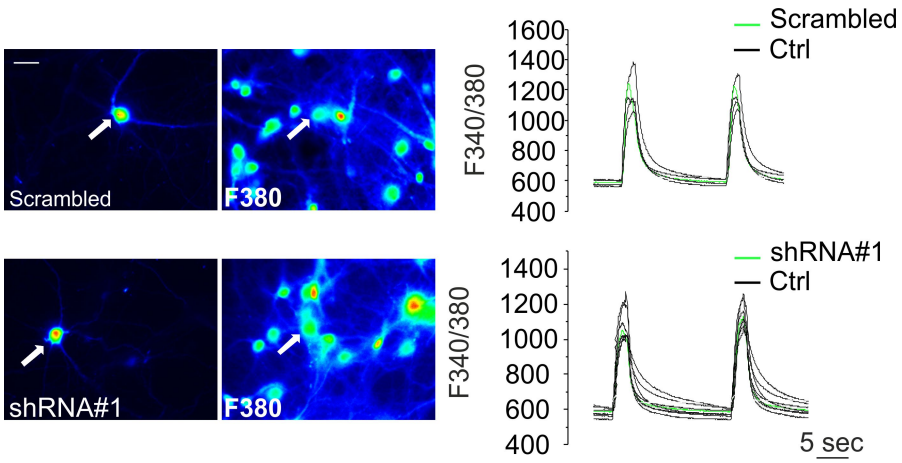


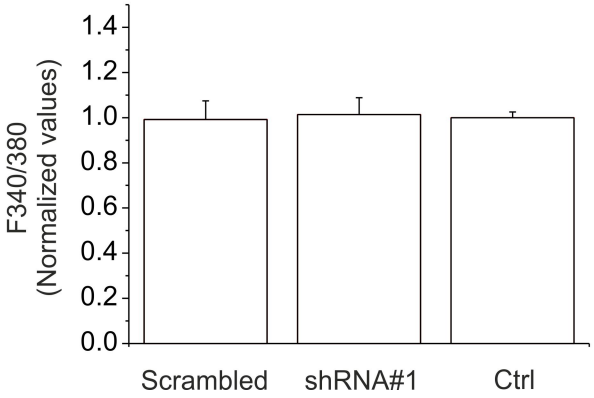
4) The results from the PI assay used to measure cell vitality is certainly consistent with the notion that the cells are not dying in the presence of chronic 4AP. However, it is not necessarily a sensitive measure of cell health, either. It would be useful to report the passive membrane properties of the neurons, as this is likely a better indicator of overall health than is the PI assay.

We have now reported the passive membrane properties of both control and 4AP-treated neurons (see Suppl. Table 1). No differences were detected between untreated neurons and neurons treated with 4AP, fully supporting our PI assay data. We have added such consideration in the revised manuscript (p. 9-10).

5) In figure 8, the authors use broad lentiviral expression of the shRNAs to measure the impact of REST/NRSF knockdown on renormalization of network activity. This data is very nice and convincing, but does not address the impact of these changes at the level of single cells in the network. Ultimately, the changes in intrinsic excitability should act to normalize firing patterns within individual cells. If the shRNA is expressed sparsely, do transfected neurons exhibit cell-autonomous changes in the firing rates/bursts of spontaneous activity, or do all neurons need to have shRNA expression to see the results in figure 8?

To specifically address this issue, cultured neurons were transfected with a vector coding for either scrambled or REST shRNA#1 affecting REST expression only in sparse neurons. As MEA recordings do not allow measuring the spontaneous activity at single cell level under standard culturing conditions, we used Ca^{2+} imaging of single transfected neurons. The analysis of the spontaneous Ca^{2+} transients showed that there were no significant differences between non-transfected, scrambled shRNA-transfected or shRNA#1-transfected neurons after 96 h of 4AP treatment (see figure below, where data are normalized to the average value of non-transfected control neurons within the same recorded field; scrambled n= 21; REST/NRSF shRNA n= 23; non-transfected neurons n=220; 3 independent cultures).
It should be noted that the extent of spontaneous Ca^{2+} transients measured at the level of a single transfected cell is dependent on the synaptic inputs that such neuron receives from the others surrounding non-transfected neurons, whose homeostatic response to 4AP is taking place. Thus, although the intrinsic homeostasis is blocked by shRNA#1 in the transfected neuron, its spontaneous Ca^{2+} signals are not different from scrambled-transfected neurons because the synaptic inputs come from neurons that are still modulated by the homeostatic response. This consideration, together with the evidence that the modulation of excitability in individual neurons alters the number of contacting synapses (Peng et al., 2010; Burrone et al., 2002), makes any clear conclusion very difficult to draw.
For this reason, we are inclined not to include this new finding in the revised paper. However, the presence of a cell autonomous effect of REST/NRSF activity might be partly inferred by the results shown in Fig. 7, where REST/NRSF specific shRNAs or scrambled sequences thereof were expressed sparsely at the single cell level before the 4AP treatment. Despite the evoked, and not the spontaneous, excitability was evaluated in this experiment, the data shown in Fig. 7 clearly indicate that only the neurons transfected with REST/NRSF shRNA exhibited a higher (evoked) excitability compared to untreated or scrambled-transfected neurons.


Scrambled
F380
shRNA#1
F380
F340/380
1600
1400
1200
1000
800
600
400
Scrambled
Ctrl
shRNA#1
Ctrl
5 sec


F340/380
(Normalized values)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Scrambled
shRNA#1
Ctrl

Specific Comments

1) The authors report that the maximal rising slope of the AP is diminished following chronic 4AP. Some traces that exemplify this effect would be nice to include.
We have now added to Fig. 2:
i) representative traces of the first action potential elicited by current injection (new Fig. 2d), from which all the parameters concerning AP shape have been obtained;
ii) the plot of the time derivative of the voltage (dV/dt) versus voltage (the so-called phase-plane plot, new Fig. 2e) relative to the first action potential which was used to better identify Voltage threshold, the maximal rising and repolarizing slopes (Bean, 2007).

2) In figure 2, plotting the Normalized Threshold in Fig.2e next to example traces in Fig.2a implies that the analysis was done on multiple action potentials??. This should be clarified.
The voltage threshold, as well as others parameters regarding AP properties, were calculated on the first AP elicited by minimal current injection (5 pA steps) lasting 1 sec. In the new Fig. 2, all the normalized values of the threshold have been substituted by absolute values.

3) Figure 2a should include a voltage scale bar.
The voltage scale bar has been added to Fig. 2a.

4) In the Western Blot shown in Figure 3e, the Na/K pump band is overexposed. Is this the exposure used for the quantification? The figure legend indicates the methods are described in "MM" however this information seems to be omitted from the manuscript. A lower exposure of this band should be provided and the authors should clarify how densiometric measurements were made.
We have changed the image by choosing a lower exposure sample of Na/K pump from the fluorograms used for the quantification. The following information has now been added to the "Methods" section (p. 29-30): *"The quantification has been made by normalizing the optical density of the proteins of interest (PanNav, Na/K pump, REST) to that of the loading control (βIII tubulin, GAPDH) within the same experiment. The analysis was performed by averaging the results of three independent preparations. Each lane corresponds to a single culture coverslip from a single experiment. The immunoreactive bands were analyzed by using the ImageJ software.* "

5) In the "Protein extraction and western blotting" section on page 27 the authors indicate that the proteins were quantified by DC Protein Assay and "80 mg were separated by 10% SDS-PAGE." I am assuming that this is formatting issue with greek letters, and the loading material was 80 micrograms - is this correct?
We apologize for the formatting error and thank the Reviewer for having picked it up. Clearly, the total protein amount used for western blot analysis was 80 micrograms (80 μg).

Acceptance 24 September 2013

Thank you for submitting your revised manuscript to The EMBO Journal. I am terribly sorry for the delay in getting back to you with a decision. I have now heard back from referee #1. I still have not heard back from referee #2 and at this stage I don't think I will. I will therefore go ahead and take the decision based on the report that I have on hand.

As you can see below, referee #1 is happy with the introduced changes. I am therefore very pleased to accept the paper for publication in The EMBO Journal.

Please see below for important information on how to proceed and necessary forms to us as soon as possible.

Thank you for contributing to the EMBO Journal

REFEREE REPORT

Referee #1

The authors have satisfied my concerns.

