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Summary

Pre-clinical studies of fragile X syndrome (FXS) have focused on neurons with the role of glia remaining largely underexplored. We examined the astrocytic regulation of aberrant firing of FXS neurons derived from human pluripotent stem cells. Human FXS cortical neurons, co-cultured with human FXS astrocytes, fired frequent short duration spontaneous bursts of action potentials compared to less frequent, longer duration, bursts of control neurons co-cultured with control astrocytes. Intriguingly, bursts fired by FXS neurons co-cultured with control astrocytes are indistinguishable from control neurons. Conversely, control neurons exhibit aberrant firing in the presence of FXS astrocytes. Thus, the astrocyte genotype determines the neuronal firing phenotype. Strikingly, astrocytic conditioned medium, and not the physical presence of astrocytes, is capable of determining the firing phenotype. The mechanistic basis of this effect indicates that the astroglial-derived protein, S100β, restores normal firing by reversing the suppression of a persistent sodium current in FXS neurons.

Introduction

Fragile X syndrome (FXS), a leading genetic cause of intellectual disability and autism spectrum disorder, is caused by the absence of the Fragile X messenger ribonucleoprotein 1 (FMRP) produced by the *FMR1* gene [1-4]. Accumulating evidence from rodent models of FXS have given shape to a useful framework for investigating how the absence of FMRP contributes to various molecular, neuronal, and behavioral abnormalities [5-8]. As valuable as these findings have been in advancing our understanding of the mechanisms by which absence of FMRP leads to pathophysiology and for identifying potential therapeutic strategies for treating FXS, none has translated into effective clinical outcomes for patients [9-12]. This underscores the need for alternative strategies to model FXS using 'all-human' based platforms.

It is in this context that human pluripotent stem cell (hPSC)-derived neurons offer a useful model system [13-15]. The comparatively few studies that have used human *FMR1* null neurons to model FXS have reached different conclusions regarding the nature of the changes in cellular excitability and action potential firing properties. Nevertheless, they each share the common methodological approach of focusing only on neuronal function [16-21]. Such a neuro-centric approach ignores the pivotal role of glia on the development, maintenance and regulation of neuronal function and homeostasis [22,23], including neuronal rhythmogenesis [24]. In the context of FXS, co-culture of

hippocampal neurons from *Fmr1*$^{-/y}$ mice with wild-type astrocytes prevents the emergence of an altered dendritic morphology that is observed in co-cultures where neurons and astrocytes each do not express FMRP [25]. Furthermore, astrocyte-specific loss of FMRP expression has been reported to result in increased spine density in cortical neurons and impaired motor learning in mice [26]. Moreover, it has been proposed that molecules secreted by astrocytes are required for synapse formation and when absent in the astrocyte secretome from *Fmr1*$^{-/y}$ mice this leads to alterations in both spine morphology and synapse maturation [27]. Together, these rodent studies have contributed to steadily increasing evidence that astrocytes play essential roles in modulating the function of neurons and neural circuits. Nevertheless, studies examining the glia contribution to modulating neuronal electrophysiological phenotypes in a human model of FXS are lacking. Thus, in the present study we addressed this gap in knowledge by carrying out detailed electrophysiological analyses in co-cultures of cortical neurons and astrocytes each derived from human pluripotent stem cells (hPSCs) lacking FMRP. Our results identify an important cell non-autonomous contribution of human astrocytes in correcting aberrant electrical activity in human FXS neurons, thereby suggesting a framework for exploring new therapeutic strategies aimed at human neuron-glia interactions.

Results

Fibroblast-derived iPSCs were generated from one control (CON) and two FXS patient lines (FXS1, FXS2). Neural precursor cells (NPCs) and astrocyte precursor cells (APCs), generated from hPSCs, were terminally differentiated into cortical neurons [21] and astrocytes (Figure S1A), respectively. In addition, to ensure that the differences we observe in FXS patient-derived lines are due to the silencing of the *FMR1* gene and the absence of FMRP, and not due to differences in genetic backgrounds of the iPSC-derived neurons, we also analyzed a pair of isogenic embryonic stem cell (ESC) derived cells [28] that includes one in which the *FMR1* gene was genetically deleted (*FMR1*$^{-/y}$), and an otherwise genetically identical control line (*FMR1*$^{+/y}$). In each of these cell lines, the absence of FMRP (Figure S1B) and expression of pluripotent stem cell markers were confirmed (Figure S1C, Table S1).

Astrocyte genotype determines the electrophysiological phenotype of neurons

As a first step in our analysis, we used current-clamp recordings to characterize spontaneous bursts of action potentials fired in human cortical neurons, co-cultured with human astrocytes, each derived from induced pluripotent stem cells (iPSCs) generated from a healthy individual (CON, Figure 1A; Table S2). These control cortical neurons, co-cultured with control astrocytes, fired a low number of bursts, but the durations of individual bursts were long (Figure 1B, D; CON). However, human cortical neurons lacking FMRP, co-cultured with FXS astrocytes, derived from an iPSC line generated from a FXS patient (FXS1), exhibited a strikingly different pattern of activity consisting of a significantly higher number of bursts that were of a much shorter duration (FXS1, Figure 1C, D; Table S2) [21]. This same effect of the loss of FMRP on burst firing was also seen in a second patient iPSC-derived line (FXS2, Figure S2; Table S3). Furthermore, the same aberrant firing pattern was seen in neurons generated from a pair of isogenic embryonic stem cell lines (*FMR1*$^{+/y}$; *FMR1*$^{-/y}$), in which the *FMR1* gene was deleted using CRISPR/Cas9-mediated genome editing (Figure S2; Table S4). This aberrant pattern of activity in cortical neurons lacking FMRP, manifested as higher number of shorter bursts (Figure 1C) compared to those seen in control neurons (Figure 1B), will henceforth be referred to as the "FXS" firing pattern (Figure 1C). Surprisingly, the equivalent FXS neurons, when co-cultured with control astrocytes, exhibited normal burst firing consisting of a lower number of longer bursts (Figure 1E, G, H; Figure S2). Conversely, in the presence of FXS astrocytes, control neurons exhibited aberrant FXS firing patterns (Figure 1F, G, I). In other words, whether cortical neurons exhibit normal or FXS bursting activity is determined by the genotype of the astrocyte, not the genotype of the neuron (Figure 1H, I).

FMRP re-expression in the FXS astrocytes rescues the aberrant bursting in FXS neurons

To ascertain causality of this aberrant electrophysiological phenotype to FXS repeat expansion mutation and loss of FMRP expression in astrocytes, we removed CGG repeats in FXS2 iPSC line via two gRNAs flanking repeat expansion mutation using CRIPSR/Cas9 methodology [29,30]. The successful gene corrected iPSC clone, FXS2Δ, displayed re-expression of FMRP protein (Figure 1J, K) following the removal of CGG repeats (Figure S3) and associated reversal of the hypermethylation of *FMR1* promoter as shown via bi-sulfite sequencing (Figure 1L, Figure S3). Moreover, FXS2 neurons co-cultured with FXS2Δ astrocytes exhibited normal burst firing – less frequent bursts of longer duration (Fig. 1M, N).

Conditioned medium from control astrocytes by itself restores normal firing in FXS neurons, while medium from FXS astrocytes elicits aberrant firing in control neurons

Next, we asked if the astrocytes must be physically present in the same culture to elicit this cell non-autonomous effect on neuronal firing. Specifically, we asked if the astrocytic conditioned medium (ACM) alone, i.e., the cell supernatant from pure astrocyte cultures, can influence the burst firing properties of the cortical neurons even in the absence of the astrocytes themselves. To test this, we examined all four combinations of control/FXS neurons grown in the presence of the control/FXS ACM alone (Figure 2A; Table S5-7). Similar to the experiments described above, current-clamp recordings were carried out from cortical neurons during the 8th week of cell culture. For these experiments the neurons were grown in ACM, and not in the physical presence of astrocytes, for the preceding 5 weeks prior to electrophysiological analysis. Control neurons grown in control ACM exhibited normal burst firing (Figure 2B, D). Similarly, FXS neurons, grown in FXS ACM, also fired aberrant bursts as seen earlier in neurons grown in the presence of FXS astrocytes (Figure 1C, D). These results show that just the conditioned medium obtained from astrocytes of the same genotype is adequate for the neurons to exhibit the same firing patterns described in Figure 1B, C.

We next asked whether conditioned medium from astrocytes of one genotype can alter the bursting property of neurons of the *other* genotype? Strikingly, FXS cortical neurons, when grown in control ACM, displayed normal burst firing (Figure 2E, G). On the other hand, control neurons exhibited FXS bursting patterns when grown in FXS ACM (Figure 2F, G). Therefore, the physical proximity of astrocytes in co-cultures is not essential to switch the firing patterns of cortical neurons – the ACM alone is adequate to elicit the cell non-autonomous effects (Figure 2H, I). This was also seen in the isogenic pair and the second patient-derived lines (*FMR1*$^{+/y}$, *FMR1*$^{-/y}$; FXS2; Figure S4).

Persistent sodium currents are reduced in neurons exhibiting aberrant FXS firing

The results presented so far show that co-cultured control astrocytes and their conditioned media can correct the aberrant FXS bursting pattern. Conversely, co-cultured FXS astrocytes and their conditioned medium can induce aberrant FXS bursting in cortical neurons. The following question arises: what is the underlying electrophysiological basis of the alterations in firing caused by FXS, and its correction? To address this question, we turned to several earlier findings on rhythmic burst firing in neurons. For instance, analysis of altered neuronal excitability in the entorhinal cortex of

Fmr1 KO mice [31] implicated changes in the persistent sodium current (I_{NaP}). This current, which is active at subthreshold voltages, was also shown to determine the properties of neuronal burst firing in other studies [32-35]. More recently a role for I_{NaP} has been identified in bursting activity of human neurons lacking FMRP co-cultured with mouse astrocytes [21]. Hence, based on previously published experimental protocols [21,31], a slow depolarizing voltage ramp (20 mV/s; Figure 3A, *top*) was applied to control cortical neurons, co-cultured with control astrocytes, and the I_{NaP} was pharmacologically isolated (Figure 3A, *bottom, black trace*). Compared to control neurons, the magnitude of I_{NaP} was reduced significantly in FXS neurons co-cultured with FXS astrocytes (Figure 3A, *bottom, red trace*). Similarly, we observed a significant decrease in I_{NaP} in control neurons, co-cultured with FXS astrocytes (Figure 3B, *red trace*), compared to FXS neurons co-cultured with control astrocytes (Figure 3B, *black trace*). Therefore, in the presence of FXS astrocytes, cortical neurons exhibited a reduction in I_{NaP} (Figure 3C, D), and this reduced level of current is correlated with aberrant burst firing.

Enhancement of persistent sodium currents reverses aberrant FXS firing

Taken together, these results suggest a potential link between the reduction in the I_{NaP} current to the aberrant FXS bursting. This raises the possibility that a pharmacological reagent specifically designed to enhance I_{NaP} should also be effective in restoring normal bursting activity in FXS neurons. We tested this prediction by applying the I_{NaP} opener, veratridine, during our recordings from cortical neurons. First, after confirming the decrease in I_{NaP} in FXS neurons co-cultured with FXS astrocytes (Figure 4A, –veratridine), veratridine (0.5 μM) was bath applied for 20 minutes (Figure 3A, +veratridine). This treatment elicited the expected effect, i.e., an increase in I_{NaP} in the same neuron (Figure 4C). The same *in vitro* treatment with veratridine also reversed the impairment in I_{NaP} control neurons co-cultured with FXS astrocytes (Figure 4B, D).

Having established the efficacy of veratridine in enhancing I_{NaP} in two different human neuron-astrocyte combinations, we addressed the key question – will the same treatment also correct the aberrant FXS bursting pattern? To test this, we first established a baseline of aberrant bursting for 10 minutes (Figure 4E, "1") followed by bath application of veratridine for 20 minutes (Figure 4E, "2", "3") while continuing to record from the same neuron. Indeed, the same concentration of veratridine that reversed the reduction in I_{NaP} also restored normal burst firing in FXS neurons co-cultured with FXS astrocytes (Figure 4F, H, I). Furthermore, aberrant bursting in control neurons, co-cultured with FXS astrocytes, was also rectified by the same veratridine

treatment (Figure 4G, H, K). Thus, in both co-culture combinations, FXS neurons with FXS astrocytes, and control neurons with FXS astrocytes, that exhibit aberrant bursting patterns veratridine restores normal firing. In other words, this correction of aberrant activity can be achieved by just reversing the deficit in the neuronal I_{NaP}, despite the presence of FXS astrocytes in the co-culture.

Antibody inactivation of S100β alters neuronal firing patterns in control but not FXS co-cultures while addition of S100β restores normal firing by reversing the suppression of persistent sodium currents

Taken together, these results strongly suggest that an astrocyte derived soluble factor(s) determine if a neuron, irrespective of its genotype, will fire like a control or FXS neuron. This led us to ask what compound(s) in the secretome from the astrocytes may mediate this switch between the control and FXS burst firings patterns. In our search for a potential mechanism that mediates the cell non-autonomous effect of correcting aberrant activity in FXS neurons, we reasoned that such an astroglial-derived factor should be capable of eliciting the transition from FXS to control burst firing. Specifically, this factor should, similar to veratridine, be capable of restoring normal firing by reversing the reduction in I_{NaP}. Earlier findings suggested a promising candidate in S100β, a calcium-binding protein that is expressed primarily by astrocytes [36-38]. Importantly, rhythmic bursting patterns, similar to those seen here, were shown to be regulated by S100β released from astrocytes in mice by modulating the I_{NaP} current [39]. Thus, its glial specificity and previously established role in neuronal rhythmogenesis, led us to explore if S100β is capable of correcting aberrant FXS firing in neurons. If S100β were to fulfil such a role it would be predicted that its inactivation would have a differential effect on 'control neuron/control astrocyte' compared with 'FXS neuron/FXS astrocyte' co-cultures. As illustrated in Figure 5A, C the typical long-lasting but less frequent firing pattern of control neurons (co-cultured with control astrocytes) changes to one displaying shorter bursts of firing upon addition of an antibody to S100β. Importantly, the equivalent antibody treatment of FXS neurons (co-cultured with FXS astrocytes) did not alter the firing pattern of FXS neurons (Figure 5B, D). As illustrated in Figure 5E we also confirmed that the concentration of S100β was lower in ACM from FXS astrocyte cultures compared to that in ACM obtained from control astrocytes.

If the reduction in a potential astrocyte-derived candidate, S100β, is mediating the aberrant FXS firing patterns, then this raises the possibility that addition of S100β would reverse the decrease in I_{NaP} associated with this pattern. To test this, we first confirmed the reduction in I_{NaP} in FXS neurons co-cultured with FXS astrocytes was confirmed (Figure 6A, −S100β). Next, S100β (5 μM) was bath applied for 20 minutes while continuing to record from the same neuron (Figure 6A, +S100β). Similar to veratridine, this treatment elicited an increase in I_{NaP} in the same neuron (Figure 6C). Treatment with S100β also reversed the impairment in I_{NaP} seen in control neurons co-cultured with FXS astrocytes (Figure 6B, D). After confirming that S100β potentiates the I_{NaP}, we tested if it also reverses the aberrant FXS burst firing. We first confirmed that FXS neurons in the presence of FXS astrocytes exhibit the expected aberrant firing (Figure 6F, "1"). But, when S100β was bath applied after the baseline recording (Fig, 6F "2"), the same FXS neuron exhibited normal bursting patterns (Figure 6F, "3"). We also checked the efficacy of the same treatment on control neurons co-cultured with FXS astrocytes (Figure 6G). Here too the equivalent bath application of S100β switched FXS firing to normal firing (Figure 6G, "1" & "3'). Thus, under both conditions that give rise to aberrant FXS bursting in neurons, S100β succeeded in restoring normal firing despite the presence of the FXS astrocytes (Figure 6H-K).

Thus, in our human cell co-culture model to study FXS, the observation that whether burst firing activity in human cortical neurons is normal or aberrant depends on the genotype of the astrocytes present and not that of the neurons. Moreover, the physical presence of astrocytes is not required as astrocyte conditioned medium by itself is able to elicit the same cell non-autonomous effects. Finally, our data revealed that the concentration of the astroglial-derived protein, S100β, is reduced in the secretome of FXS astrocytes and that addition of S100β to co-cultures of either control or FXS neurons with FXS astrocytes restores normal firing patterns by reversing the suppression, in neurons, of a persistent sodium current.

Discussion

Here, we identify a pivotal role for astrocytes in correcting aberrant electrical activity in human FXS neurons, thereby shifting the focus to astrocytes in what was traditionally thought of as a developmental disorder involving neuronal dysfunction. Earlier analyses of deficits caused by

FXS, and strategies to reverse them, were aimed primarily at understanding synaptic and molecular signaling abnormalities [15,40,41]. More recent work has also examined alterations in intrinsic and network excitability, as well as modulation of ion channels by FMRP [42-45]. However, these analyses centered exclusively on neurons, and primarily were conducted in animal models of FXS. Recently, a handful of studies have characterized the impact of the loss of FMRP on excitability in human neurons. In one study, depolarizing current injections into human neurons lacking FMRP elicited at most single action potentials, not the more complex patterns of burst firing observed here [18]. Another study revealed higher spontaneous firing in neurons lacking FMRP, but used extracellular multi-electrode arrays, thereby lacking single-cell resolution [20]. We have also recently reported that human cortical neurons lacking FMRP display aberrant burst firing properties compared to control neurons[21]. However, and importantly for the present study, these earlier reports did not use human astrocytes in the co-culture but rather, if astrocytes were indeed present, these were of rodent origin. Specifically, in our previous study[21] the genotype of the co-cultured mouse-derived astrocytes had no effect on the electrophysiological phenotype observed in the human cortical neurons. We do not have an explanation as to why in our previous study[21] co-culture of human FXS neurons with (wild-type) mouse astrocytes did not result in FXS neurons displaying a control firing pattern. Consistent between our previous and current studies is the fact that the magnitude of the I_{NaP} predicts the firing pattern that is observed; it is greater in control neurons than FXS neurons and pharmacological interventions that enhance I_{NaP} in FXS neurons result in control-like firing patterns. Nevertheless, the presence of wild-type mouse astrocytes does not appear to influence the expression of I_{NaP} in human FXS neurons albeit that our earlier study show that, pharmacologically, these levels can be increased, at least functionally. Thus, while future studies will be needed to investigate if different mechanisms of regulation are at play when human FXS neurons are co-cultured with human versus rodent astrocytes, there is a convergence at the level of the functional alterations of I_{NaP}. The present study underscores the importance of the 'all-human' co-culture system in revealing new mechanistic insights into how astrocytes mediate the effects reported here. Our analyses in human neuron-astrocyte co-cultures revealed striking differences in the duration and number of spontaneous bursts of action potentials fired by FMRP-lacking cortical neurons. Importantly, we find the transitions between normal and aberrant bursting to be cell non-autonomous. This, in turn, enabled us to probe the mechanistic basis of neuron-glia interactions underlying alterations in excitability of human FXS cortical neurons.

Our results also add to accumulating evidence on how astrocytes modulate neuronal function in both health and disease. Disease models have shown that astrocytes can, depending on disease context, be either protective or injurious [46-50]. In the context of neurodevelopmental disorders, astrocytes have been implicated in the progression of Rett's syndrome in a mouse model wherein selective re-expression of methyl-CpG-binding protein 2 (*Mecp2*) in astrocytes restored normal dendritic morphology *in vivo* [51]. Studies using iPSCs from Down's Syndrome (DS) patients also reported a role for astroglia, as well as astrocytic conditioned medium, in abnormal neuronal phenotypes in DS pathology [52,53]. Consistent with our results, these studies also emphasized the role of S100β in the interaction between DS astroglia and DS neurons. Specifically, in rodent models of FXS, wild-type astrocytes, when co-cultured of hippocampal neurons lacking FMRP, reverse the altered dendritic morphology that is present when astrocytes also lack FMRP [25]. Indeed, loss of expression of FMRP from astrocytes alone leads to increased spine density in (FMRP-expressing) cortical neurons and impaired motor learning in mice demonstrating astrocyte modulation of neuronal morphology and behaviour [26]. Consistent with our current study showing that the physical presence of astrocytes is not required to impart changes in neuronal firing phenotype is the demonstration that the astrocyte secretome from *Fmr1*$^{-/y}$ mice influences spine morphology and synapse maturation [27]. It is also worth noting that the correction achieved by S100β in the present study does not completely re-capitulate the firing patterns seen in control neurons (Figure 6H, J), raising the possibility that other factors besides S100β may also contribute to this process. For instance, based on earlier studies, the efficacy of other promising candidate astroglial factors, such as IL6, remains to be explored. Our results provide an electrophysiological framework to examine these in future studies.

Finally, analysis of how S100β mediates its protective effect (i.e., switching FXS firing to control) revealed the persistent sodium conductance (I_{NaP}) as a key element. Specifically, we found a reduction in the I_{NaP} in neurons that exhibited the aberrant FXS firing pattern, irrespective of the genotype of the neuron in a range of combinations (i.e., FXS neurons, control neurons in the presence of FXS astrocyte or its conditioned medium; Figure 3A, B, *red traces*). Moreover, concentrations of S100β that reversed this aberrant FXS burst firing to control patterns, also restored suppressed I_{NaP} currents to control levels (Figure 6A, B, *grey traces*). Importantly, veratridine, a specific I_{NaP} opener, corrected the FXS burst firing to normal patterns, thereby confirming the mode of action through which S100β achieves its corrective action on aberrant

excitability seen in FXS neurons (Figure 4A-G). Interestingly, a similar mechanism was also seen earlier in the rat sensorimotor circuit wherein astrocytes were involved in generating neuronal rhythmic burst firing [39]. Furthermore, when inactivation of astrocytes prevented neuronal bursting in this study, it was restored by adding S100β to the extracellular space, which in turn was mediated by an enhancement in I_{NaP}. Similar to what we report here (Figure 6C, D), locally applied S100β also increased the peak amplitude of the isolated I_{NaP}. Thus, our findings also open up possibilities to investigate the *in vivo* functional consequences of these cell non-autonomous glial phenotypes in rodent models of FXS. The co-culture system described here offers a new framework for exploring mechanisms underlying neuron-glia interactions and their efficacy in correcting aberrations in neuronal excitability caused by FXS. Together, these findings elucidate previously undescribed mechanisms of FXS pathogenesis and identify potential therapeutic targets in astrocytes.

Limitations of the study

We have identified a key role for astrocytic regulation of neuronal firing phenotypes in an 'all-human' culture-based model of FXS. While our data support the notion that astrocytic-derived S100β restores control-like firing in FXS neurons by enhancing a persistent sodium current we cannot exclude the possibility that other factors in the astrocyte secretome also can act in a similar manner. Equally, while our study has highlighted that larger magnitude persistent sodium currents are present in control neurons and we can phenocopy control neuronal firing patterns in FXS neurons by co-culturing with control astrocytes or by adding control ACM, veratridine or S100β each of which enhances a persistent sodium current, we remain open to the idea that other ion channels could also play a pivotal role in generating the distinct firing patterns seen in control and FXS neurons. In other words, simply phenocopying the firing pattern does not imply that each are generated in the same manner, mechanistically. Nevertheless, if the temporal nature of neuronal output is central to circuit dysfunction present in models of FXS then 'correcting' aberrant firing to restore normal firing might be an approach, while agnostic to its mechanistic basis, that allows further identification of potential therapeutic targets.

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Inclusion and diversity: One or more of the authors of this paper self-identifies as an under-represented ethnic minority in their field of research or within their geographical location. We support inclusive, diverse and equitable conduct of research.

Figure Legends

Figure 1. Distinct patterns of bursting activity of hPSC-derived cortical neurons is determined by the genotype of the astrocyte and its secretome, not the genotype of the neuron. (A) Representative confocal images of hiPSC-derived cortical neurons (CON and FXS1) expressing Map2ab co-cultured with hiPSC derived astrocytes expressing GFAP. *Left to right*, neurons were co-cultured in four combinations: CON neurons with CON astrocytes, FXS1 neurons

with FXS1 astrocytes, FXS1 neurons with CON astrocytes, and CON neurons with FXS1 astrocytes. *Right*, legend for icons. Scale bar = 50 μm. **(B)** (i) Representative current-clamp recording (V_{HOLD} = −70 mV) from a CON neuron, co-cultured with CON astrocyte, firing spontaneous bursts of action potentials that occur at low frequencies but with long durations. (ii) Single burst shown in expanded time-base. **(C)** (i) Representative current-clamp recording of aberrant spontaneous activity in a FXS neuron, co-cultured with FXS1 astrocyte, containing a significantly higher number of bursts but of shorter duration. (ii) Single burst shown in expanded time-base. **(D)** Comparison of mean burst duration (CON neurons with CON astrocytes, 45.19 ± 4.065 s, n = 24, N = 4; FXS1 neurons with FXS1 astrocytes, 10.97 ± 1.126 s, n = 13, N = 3) and mean burst number per 10 min of recording (CON neurons with CON astrocytes, 6.167 ± 0.89; FXS1 neurons with FXS1 astrocytes, 25.92 ± 3.482). **(E)** Representative trace from a FXS1 neuron, co-cultured with CON astrocyte, exhibits a normal activity with low burst number and longer burst duration. **(F)** Conversely, a CON neuron, co-cultured with FXS1 astrocyte, shows aberrant activity containing higher number of bursts of shorter duration. **(G)** Comparison of mean burst duration (FXS1 neurons with CON astrocytes, 31.6 ± 4.42 s, n = 17, N = 3; CON neurons with FXS1 astrocytes, 9.37 ± 1.32 s, n = 18, N = 3) and mean burst number per 10 min of recording (FXS1 neurons with CON astrocytes, 5.18 ± 1.033; 33.39 ± 3.96). **(H)** Summary: control astrocytes correct aberrant burst firing in FXS neurons. **(I)** Summary: control neurons switch to aberrant firing in the presence of FXS astrocytes. (**J**) Immunoblot and (**K**) quantification depicting re-expression of FMRP (n=3) in FXS2 patient iPSC line and its isogenic corrected control (FXS 2Δ), expressed as % of an unrelated control (C). (**L**) Bisulfite sequencing of the FMR1 promoter showing its demethylation in the edited lines. (**M**) Representative current-clamp trace of spontaneous activity recorded from a FXS2 neuron co-cultured with FXS2Δ astrocytes exhibits normal firing consisting of a low number of longer bursts. (**N**) Comparison of mean burst duration (37.6 ± 3.84 s, n = 15, N = 3) and mean burst number per 10 min of recording (7.4 ± 0.80) in FXS2 neurons co-cultured with FXS2Δ astrocytes. All representative traces of aberrant activity patterns in cortical neurons are depicted in red, whereas normal spiking activity is depicted in black. On the other hand, for the genetic identity of the neuron and astrocyte, controls are depicted in black and FXS in red. ***p < 0.001, one-way ANOVA and post-hoc Sidak's test. All values are mean ± SEM.

Figure 2. Astrocyte secretome is sufficient to determine the bursting activity patterns of hPSC-derived cortical neurons. (A) Representative confocal images of Map2ab-expressing hiPSC-derived cortical neurons (CON and FXS1) grown in astrocytic conditioned media (ACM). Left to right, neurons were grown in ACM in four combinations: CON neurons with CON ACM, FXS1 neurons with. FXS1 ACM, FXS1 neurons with CON ACM, and CON neurons with FXS1 ACM. Right, legend for icons. Scale bar = 50 μm. **(B)** Recording from a CON neuron, grown in CON ACM, exhibits normal firing consisting of a low number of longer bursts. **(C)** Recording from FXS1 neuron grown in FXS1 ACM shows a high number of bursts of shorter duration. **(D)** Comparison of mean burst duration (CON neurons in CON ACM, 42.29 ± 5.66 s, n = 16, N = 3; FXS1 neurons in FXS1 ACM, 8.501 ± 0.98 s, n = 12, N = 3) and mean burst number per 10 min of recording (CON neurons in CON ACM, 3.19 ± 0.46; FXS1 neurons in FXS1 ACM, 19.67 ± 2.13). **(E)** Representative recording from a FXS1 neuron, grown in CON ACM, exhibiting control burst firing, i.e., less frequent bursts of longer duration. Thus, control ACM exerts a non-cell autonomous effect in reversing aberrant FXS bursting. **(F)** Representative recording from a CON neuron, grown in FXS1 ACM, shows the aberrant FXS bursting. **(G)** Comparison of mean burst duration (FXS1 neurons in CON ACM, 51.66 ± 7.027 s, n = 12, N = 3; CON neurons in FXS1 ACM, 9.945 ± 1.026 s, n = 13, N = 3) and mean burst number per 10 min of recording (FXS1 neurons in CON ACM, 4.17 ±0.78 s; CON neurons in FXS1 ACM, 28.38 ± 3.61). **(H)** Summary: control ACM corrects aberrant burst firing in FXS neurons. **(I)** Summary: FXS ACM alone elicits aberrant firing in control neurons. ***p < 0.001, one-way ANOVA and post-hoc Sidak's test. All values are mean ± SEM.

Figure 3. **Neurons co-cultured with FXS astrocytes have reduced I_{NaP} densities. (A)** Persistent sodium currents (I_{NaP}) isolated by subtracting current traces evoked by a slow depolarizing ramp (*top*, -100 to -20 mV, 20 mV/s) in the absence and presence of TTX. Representative traces (*bottom*) are from a CON neuron co-cultured with CON astrocyte (*black*), and a FXS1 neuron co-cultured with FXS1 astrocyte (*red*). **(B)** Same as (A) but representative traces recorded from a CON neuron co-cultured with FXS1 astrocyte (*red*) and a FXS1 neuron co-cultured with a CON astrocyte (*black*). **(C)** Current density–voltage (I-V) curves plotted from the ramp-evoked persistent sodium current (I_{NaP}). Currents are normalized to the corresponding cell capacitance. *Inset:* Comparison of current densities at −20 mV reveals a significant reduction in I_{NaP} in FXS1 neurons co-cultured with FXS1 astrocytes (*red*; -0.32 ± 0.17, n = 6, N = 1) compared to CON neurons co-cultured with

CON astrocytes (*black*; -1.96 ± 0.6, n = 6, N= 1). **(D)** Current density–voltage (I-V) curves plotted from the ramp-evoked persistent sodium current (I_{NaP}). Currents are normalized to the corresponding cell capacitance. *Inset:* Comparison of current densities at −20 mV reveals a significant reduction in I_{NaP} in CON neurons co-cultured with FXS1 astrocytes (*red*; -0.322 ± 0.17, n = 6, N = 1) compared to FXS1 neurons co-cultured with CON astrocytes (*black*; -1.62 ± 0.28, n = 7, N = 2). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, Two-way, repeated measures ANOVA with post hoc Sidak's test (I-V plots), Mann-Whitney test. All values are mean ± SEM.

Figure 4. Pharmacological enhancement of the I_{NaP} corrects aberrant FXS firing. (A) Representative traces of persistent sodium currents (I_{NaP}) recorded from a FXS1 neuron co-cultured with FXS1 astrocyte (*red*). The I_{NaP} amplitude increases after addition of veratridine (*grey*). **(B)** Reduced I_{NaP} in a CON neuron, co-cultured with FXS1 astrocyte (*red*), is reversed by application of veratridine (*grey*). **(C)** I-V plots for FXS1 neurons with FXS1 astrocytes in before (*red*) and after veratridine (*grey*). *Inset:* Comparison of current densities at −20 mV reveals a significant increase in I_{NaP} in FXS1 neurons co-cultured with FXS1 astrocytes before (*red*; -0.44 ± 0.106, n = 8, N = 2) and after veratridine (*grey*; -1.15 ± 0.21). **(D)** I-V plots for CON neurons with FXS1 astrocytes in before (*red*) and after veratridine (*grey*). *Inset:* Comparison of current densities at −20 mV reveals a significant increase in I_{NaP} in CON neurons co-cultured with FXS1 astrocytes before (*red;* -0.293 ± 0.099, n=5, N=1) and after veratridine (*grey;* -1.635 ± 0.34). **(E)** Experimental protocol: baseline recording for 10 min (1), followed by bath application of veratridine (2), and then continuing to record from the same neuron for another 20 min; bursting properties from last 10 min were compared with first 10 min (3). **(F)** Baseline (*left trace, 1*) current-clamp recording (V_{HOLD} = −70 mV) from a FXS1 neuron, co-cultured with FXS1 astrocyte, shows more frequent bursts of shorter duration. Application of veratridine elicits a transition (*middle trace, 2*) that eventually culminates in control patterns of bursting (*right trace, 3*), i.e., less frequent bursts of longer duration. **(G)** As in (F) but traces recorded from a CON neuron co-cultured with FXS1 astrocyte. **(H)** Comparison of mean burst duration (7.4 ± 0.51 s; 20.36 ± 1.74 s, n=7, N=2) and mean burst number per 10 min of recording (44.14 ± 6.9; 26.71 ± 6.42) in FXS1 neurons with FXS1 astrocytes in the absence (*red*) and presence (*grey*) of veratridine (Vera). **(I)** Summary: veratridine reverses aberrant FXS burst firing to control firing. **(J)** Comparison of mean burst duration (8.05 ± 1.1 s; 36.74 ± 4.6 s, n=9, N=3) and mean burst number per 10 min of recording (29.22 ± 6.35; 9 ± 2.12) in CON neurons with FXS1 astrocytes in the absence (*red*) and presence

(*grey*) of veratridine. **(K)** Summary: veratridine reverses aberrant firing to control firing in CON neurons co-cultured with FXS astrocytes. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, Two-way, repeated measures ANOVA with post hoc Sidak's test (I-V plots), paired *t*-test, Wilcoxon test. All values are mean ± SEM.

Figure 5. Blocking S100β with an antibody alters the bursting pattern of neurons co-cultured with control astrocytes (A) Baseline (left) current-clamp recording (V_{HOLD} = −70 mV) from a CON neuron, co-cultured with CON astrocyte, shows less frequent bursts of longer duration. In presence of S100β antibody (right) the control burst firing changes to that of aberrant FXS burst firing. **(B)** Baseline (left) current-clamp recording (V_{HOLD} = −70 mV) from a FXS1 neuron, co-cultured with FXS1 astrocyte, shows more frequent bursts of shorter duration. The aberrant FXS burst firing is unaltered in the presence of S100β antibody (right). **(C)** Comparison of mean burst duration (38.69 ± 6.14 s; 7.48 ± 0.98 s, n=8, N=2) and mean burst number per 10 min of recording (10.13 ± 2.3; 12.5 ± 3.13) in CON neurons with CON astrocytes in the absence (black) and presence (red) of S100β Ab. **(D)** Comparison of mean burst duration (5.18 ± 0.76 s; 5.98 ± 0.93 s, n=7, N=2) and mean burst number per 10 min of recording (21.71 ± 2.17; 21.71 ± 1.82) in FXS1 neurons with FXS1 astrocytes in the absence and presence of S100β Ab. **(E)** S100β concentration is lower (2362 ± 230 pg/ml) in ACM derived from FXS astrocytes compared with that measured in ACM derived from control astrocytes (3301 ± 297 pg/ml; N=5). **$p < 0.01$, *$p < 0.05$ unpaired t-test, Mann-Whitney tests. All values are mean ± SEM.

Figure 6. S100β corrects aberrant FXS bursting in neurons by enhancing I_{NaP} (A) Persistent sodium currents (I_{NaP}) isolated by subtracting current traces evoked by a slow depolarizing ramp (*top*, -100 to -20 mV, 20 mV/s) in the absence and presence of TTX. Representative traces (*bottom*) are from a FXS1 neuron co-cultured with FXS1 astrocyte in the absence (*red*) and presence of S100β (*grey*). **(B)** Same as (A) but representative traces recorded from a CON neuron co-cultured with FXS1 astrocyte in the absence (*red*) and presence of S100β (*grey*). **(C)** Current density–voltage (I-V) curves plotted from the ramp-evoked persistent sodium current (I_{NaP}). Currents are normalized to the corresponding cell capacitance. *Inset:* Comparison of current densities at −20 mV reveals a significant increase in I_{NaP} in FXS1 neurons co-cultured with FXS1 astrocytes before (*red;* -0.44 ± 0.1, n = 8, N=3) and after S100β (*grey;* -1.41 ± 0.36). **(D)** Current density–voltage (I-V) curves plotted from the ramp-evoked persistent sodium current (I_{NaP}). Currents are

normalized to the corresponding cell capacitance. *Inset:* Comparison of current densities at −20 mV reveals a significant increase in I_{NaP} in CON neurons co-cultured with FXS1 astrocytes before (*red*; -0.41 ± 0.082, n = 8, N=3) and after S100β (*grey*; -1.64 ± 0.43). **(E)** Experimental protocol: baseline recording for 10 min (1), followed by bath application of S100β (2), and then continuing to record from the same neuron for another 20 min; bursting properties from last 10 min were compared with first 10 min (3). **(F)** Baseline (*left trace, 1*) current-clamp recording (V_{HOLD} = −70 mV) from a FXS1 neuron, co-cultured with FXS1 astrocyte, shows more frequent bursts of shorter duration. Application of S100β elicits a transition (*middle trace, 2*) that eventually culminates in control patterns of bursting (*right trace, 3*), i.e., less frequent bursts of longer duration. **(G)** As in (h) but traces recorded from a CON neuron co-cultured with FXS1 astrocyte. **(H)** Comparison of mean burst duration (9.58 ± 1.71 s; 25.51 ± 2.76 s, n= 10, N= 3) and mean burst number per 10 min of recording (22.4 ± 2.76; 14.9 ± 1.88) in FXS1 neurons with FXS1 astrocytes in the absence (*red*) and presence (*grey*) of S100β. **(I)** Summary: S100β reverses aberrant FXS burst firing to control firing. **(J)** Comparison of mean burst duration (8.406 ± 1.4 s; 30.76 ± 4.5 s, n= 8, N= 2) and mean burst number per 10 min of recording (31.63 ± 5.49; 11.13 ± 1.55) in CON neurons with FXS1 astrocytes in the absence (*red*) and presence (*grey*) of S100β. **(K)** Summary: S100β reverses aberrant firing to control firing in CON neurons co-cultured with FXS astrocytes. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, Two-way, repeated measures ANOVA with post hoc Sidak's test (I-V plots), paired *t*-test, Wilcoxon test. All values are mean ± SEM.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact:

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Materials Availability:

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sumantra (Shona) Chattarji (shona@ncbs.res.in).

Data and Code Availability:

- All data generated in this study will be made available upon reasonable request.
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this work paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell line source

ND30625 (apparently healthy control male), GM05848 (fragile X syndrome male) and GM07072 (fragile X syndrome male) fibroblasts and hereafter referred to as CON, FXS1, FXS2 lines, respectively, were obtained from the Coriell Institute for Medical Research under their consent and privacy guidelines as described on their website (http://catalog.coriell.org/). Induced human pluripotent stem cells were generated at Cedar-Sinai Medical Centre (Los Angeles, CA) using standard protocols as described previously [21]. Briefly, fibroblasts were re-programmed into non-integrating and virus-free hiPSC by nucleofection using episomal plasmids OCT4, SOX2, KLF4, L-MYC, LIN28, and shRNA to TP53.

The human embryonic stem cell (hESC) line Shef 4, referred to as *FMR1*$^{+/y}$, was obtained from the UK Stem Cell Bank [54]. The Shef 4 *FMR1* null line, referred to as *FMR1*$^{-/y}$, was generated using CRISPR-Cas9 technology as described previously [28]. Further details about each of the cell lines are provided in Table S1.

Generation of FXS isogenic control iPSC line

The FXS-2 iPSC line carrying the CGG repeat expansion mutation (Figure S3) was gene corrected using CRISPR/Cas9 genome editing. In brief, two guide RNAs (gRNAs; gRNA-1: 5'. GACGGAGGCGCCGCTGCCAG - 3' and gRNA-2 5'- GCCCGCAGCCCACCTCTCGG-3') flanking the repeat expansion mutation were cloned in px458 vector using published protocols [29]. Gene editing in iPSCs was performed as per previously published protocol [30]. Briefly, iPSCs were dissociated into single-cell suspension with 1X Accutase (Sigma-Aldrich). 8 x 10^5 cells were transfected with 2 µg Cas9-gRNA-1 plasmid, 2 µg Cas9-gRNA-2 plasmid and 1 µg eGFP-puromycin resistance plasmid using the Amaxa 4D-Nucleofector system (Lonza) according to the manufacturer's instructions with a pulse load of CA137. Cells were plated down onto Matrigel™ (Corning) coated plates in E8 medium with 10 µM ROCK inhibitor (Tocris). After 24 hours, transfected cells were selected for with addition of 1 µg/ml puromycin (Sigma-Aldrich) for a further 24 hours. Clonal analyses were performed on manually picked individual clones and

screened for successful gene-editing via immunofluorescence for FMRP and PCR flanking repeat expansion mutation. Successful gene editing clone was determined by re-expression of FMRP and presence of positive PCR amplicon. Successful gene-edited clones were further validated using repeat primed PCR, using AmplideX FMRI PCR kit from Asuragen, which showed negative for gene-edited clone (Figure S3).

Maintenance and expansion of hPSCs

hiPSC and hESC colonies were cultured and propagated in xeno-free and feeder-free conditions using Essential 8 medium (Thermo Fisher Scientific) in reduced growth-factor Matrigel^{TM}-coated 6-well plates (Nunc Nunclon delta surface, Thermo Fisher Scientific) at 37 °C, 5% CO_2 in a humidified incubator. Colonies were grown to 90% confluence; enzymatically passaged using 2:1 ratio of collagenase (ThermoFisher Scientific, USA) and dispase (ThermoFisher Scientific, USA) for further propagation and cryopreservation [55]. All cells showed normal G banding karyotype (Table S1) and were free from mycoplasma. All cell lines and experimental procedures used in the present study (Study number: 22/SCR/VI-29.4.16) were approved by the Institutional Committee for Stem Cell Research (IC-SCR), InStem, Bangalore, India.

Derivation of Neural Progenitor Cells (NPCs)

NPCs were derived from each of the cell lines mentioned above using previously published protocols [21,55]. Briefly, hPSCs were enzymatically lifted and cellular aggregates were placed on to non-adherent dishes forming a suspension culture. The medium was supplemented with LDN 193189 (Stratech), SB431542 (Tocris) and basic fibroblast growth factor (FGF) (PeproTech) to form neural spheres and rosettes at 37°C, 5% CO_2. Rosettes were dissociated using accutase and plated as a monolayer at 37°C, 3% O_2 and 5% CO_2, which were then maintained, characterized, expanded, and cryopreserved as NPCs.

Generation of Astrocytic Progenitor Cells (APCs) and astrocyte differentiation

hPSCs were neuralized and then converted to spheres as described earlier [21,56]. Next, to induce astrogliogenesis spheres were subjected to glial enrichment medium (EF20) containing Advanced DMEM/F12 with 1% Anti-anti, 1% N2, 1% Glutamax, 0.1% B27, (each from ThermoFisher Scientific) 20 ng/ml epidermal growth factor (EGF; R&D Systems), 20 ng/ml FGF, 5 mg/ml heparin (Sigma-Aldrich) for 2 weeks before being cultured in glial maturation medium. For

maturation of early gliospheres, the medium (EL20) was supplemented with 20 ng/ml leukemia inhibitory factor (LIF) (Sigma-Aldrich) and 20 ng/ml EGF for 4 weeks. After maturation, spheres were propagated in EF20 medium with mechanical dissociation every fortnight to prevent aggregation and loss of viability. The gliospheres were dissociated into monolayers of APCs using papain dissociation kit (Worthington Biochemical Corporation) and plated onto Matrigel^{TM} (1:80 dilution) coated plates. Further, APCs were propagated in EF20 medium until confluent and enzymatically passaged using accutase or cryopreserved.

A population of APCs were differentiated into astrocytes for 14 days using astrocyte differentiation media containing Neurobasal medium (Thermo Fisher Scientific, USA), 1% Anti-anti, 1% Glutamax, 1% N2, 0.2% B27, 1% non-essential amino acid medium (NEAA) (ThermoFisher Scientific) and 10 ng/ml ciliary neurotrophic factor (CNTF) (R&D Systems) at 37^0C, 5% CO_2 and maintained for co-culture experiments or harvest of conditioned medium.

Astrocyte Conditioned Media (ACM)

After 14 days of differentiation of astrocytes, the astrocyte differentiation medium was completely replaced with fresh media at 48 hrs intervals. The cell supernatant was carefully harvested at 48 hrs intervals and stored at -20^0C. Henceforth, this has been referred to as astrocytes conditioned media (ACM).

Astrocyte and neuron co-culture

Astrocytes were plated on to a 13-mm plastic coverslip (Thermo Fisher Scientific) coated with poly-L-ornithine, laminin, fibronectin (each from Sigma-Aldrich) and Matrigel^{TM} at 35,000 cells/coverslip; cultured for 48 hrs at 37°C, 5% CO_2 using CNTF. After 48 hrs NPCs (35,000 cells/coverslip) were plated on to astrocytes and maintained in hypoxic conditions i.e., 37°C, 5% CO_2 and 3% O_2 in medium containing Advanced DMEM-F12 supplemented with 1% Anti-anti, 0.5% Glutamax, 0.5% N2, 0.2% B27, and 2 µg/ml heparin for 1 week, followed by 10 µM/ml forskolin (R&D Systems) for 2 weeks and maintained in neuronal differentiation medium containing 5 ng/ml each of BDNF and GDNF (R&D Systems) for 5 weeks or up to recording. Immunophenotype and electrophysiological recordings were carried out at 6 - 8 weeks [21].

Neuron-ACM co-culture

Neurons were differentiated from NPCs as described earlier [21]. Briefly NPCs were plated on to 13-mm glass coverslips (VWR, Radnor, PA) coated with poly-L-ornithine, laminin, fibronectin (Sigma, St. Louis, MO) and Matrigel™ at 35,000 cells/coverslip; maintained in hypoxic environment for 8 weeks. Cells were fed with 50% of neuronal maturation medium and 50% of ACM from 3rd week to 8th week, followed by immunostaining and electrophysiological characterization.

METHOD DETAILS

Immunofluorescence/immunocytochemistry and imaging

Cells were stained using standard immunofluorescence techniques as previously described [21]. Briefly cells were fixed using 4% paraformaldehyde (PFA; Sigma-Aldrich) for 10 minutes, washed three times (5 minutes each) with phosphate buffered saline (PBS; Sigma-Aldrich) 0.1% Tween 20 solution (PBS-T; Sigma-Aldrich), followed by permeabilization using 0.3% Triton X-100 (USB Corporation) for 10 minutes. Further, cells were washed with PBS-T and blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich) for 1 hour and incubated with primary and secondary antibodies (see Key Resource Table) for 1 hour each at room temperature. The nuclei were counterstained with DAPI (Sigma-Aldrich), and mounted onto glass slides using FluorSave™ (Merck Millipore). The images were captured at 512 x 512 pixels using a confocal laser scanning microscope (Fluoview 3000 Olympus, Japan), and were acquired using either 40X (1.3 NA) or 60X (1.4 NA) oil immersion objectives and diode lasers 405 nm, 488 nm and 561 nm. Z step size was set at 0.5 µm with 1 airy unit of pinhole diameter.

Western blot and ELISA

Cell pellets were lysed using lysis buffer (50 mM Tris-HCL, pH 7.4, 2 mM EDTA, 0.1% SDS, 1% Triton-X 100, 0.5% Na-deoxycholate, 150 mM NaCl, protease and phosphatase inhibitor cocktails), followed by protein estimation performed using BCA assay kit (ThermoFisher Scientific). 20 µg/sample protein was loaded on a precast NuPAGE 4-12% Bis-Tris Protein gel (ThermoFisher Scientific) followed by protein transfer onto a 0.45 µm nitrocellulose membrane (GE Healthcare). Blocking was done using 1:1 TBST (0.2 M Trizma base, 0.15 M NaCl, 0.1% Tween-20) and Odyssey blocking buffer (Li-COR Bioscience, USA) for 1 hr at room temperature. The membranes were incubated with primary antibody FMRP (1:1000, AbCam, Cambridge, UK) and β-actin (1:6000, Merck), in blocking buffer overnight at 4°C. Next day blot was washed with

TBST and incubated in secondary antibodies with IRDye 680RD and IRDye 800CW (Li-COR Bioscience, USA) respectively for 1 hr at room temperature. Following further washing, the blot was dried and imaged using Li-COR Odyssey FC infrared system. S100β levels were measured in ACM using human S100β DuoSet ELISA (R&D Systems, Minneapolis) in combination with the respective ancillary reagent kit (#DY008, R&D Systems) according to the manufacturer's instructions. Briefly, wells were coated with capture antibody (4µg/ml in PBS) overnight at room temperature (RT); followed by washing (0.05% Tween 20 in PBS) and blocked in 1% BSA in PBS. A further 100 µl of sample or standard in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (TBS)) was added, followed by detection antibody (100 ng/ml in reagent diluent, 2 hours at RT each) and incubated with Streptavidin-HRP B (20 min at RT), followed by washing and addition of substrate solution (20 min at RT). The final reaction was stopped using stop solution and optical density was determined at 450 nm with the correction of 540 nm.

Electrophysiology

Whole-cell patch clamp recordings were performed using similar protocols as described previously [21,55,57]. All the recordings were performed at room temperature. Briefly, coverslips containing human cortical neurons and astrocytes co-cultured for 8 weeks in vitro were transferred to the recording chamber perfused with an external recording solution comprising of (in mM): NaCl 152, KCl 2.8, HEPES 10, $CaCl_2$ 2, glucose 10, pH 7.3 – 7.4 (300 – 320 mOsm) with a flow rate of approximately 1.35 ml per minute. Recordings were performed using a MultiClamp 700B amplifier (Molecular Devices, San Jose, CA). Data were sampled at 10 kHz, and digitized, via a Digidata 1550, at 20 kHz. Patch-pipettes, fabricated from thick-walled borosilicate glass, were filled with an internal recording solution containing (in mM): K-gluconate 155, $MgCl_2$ 2, HEPES 10, Na-PiCreatine 10, Mg_2-ATP 2, and Na_3-GTP 0.3, pH 7.3 (280 - 290 mOsm) and had resistances of 3-4 MΩ. Spontaneous network recordings were performed in the current- clamp mode with bridge balance mode and pipette capacitance neutralized. Membrane voltage was held at −70 mV. The duration of action potential bursts was measured from the start of the first action potential to the time of the last action potential with bursts being defined as two or more action potentials occurring during a period of depolarization. Stimulation protocols were generated using pClamp 10.5 software and subsequent offline analysis was conducted using Clampfit 11.1 software.

S100β (Sigma-Aldrich) and veratridine (Hello Bio) baseline bursts were recorded for 10 min. Veratridine (0.5 µM) and S100β (5 µM) were bath applied and bursts were further recorded from the same cell for 20 min. The last 10 min were taken for analysis. For experiments examining

the effects of S100β antibody application on the bursting profile, an initial baseline (10 min) of activity was rec followed by bath application of S100β antibody (1:100). Bursts of action potentials were then recorded (for a further 10 min) and their durations and frequencies compared to pre-antibody recordings.

To record the persistent sodium current (I_{NaP}), neurons were clamped at –80 mV, then stepped to –100 mV and a depolarizing voltage ramp to –20 mV (20 mV/s) was applied [31]. I_{NaP} was isolated by subtracting the current recorded in the presence of tetrodotoxin (TTX; 1 µM, Hello Bio) from current recorded immediately prior in the same neuron. S100β and veratridine were bath applied to assess their effects on the I_{NaP} in the presence of TTX.

Quantification and Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM) and each data set was assessed for normality. Students t-tests were used for data sets that passed the normality test, and Mann-Whitney and Wilcoxon tests were used for data sets that failed the normality test. Paired statistical tests were used for data sets before and after drug application. Two –way repeated measures ANOVA, followed by post-hoc Sidak's test were used for I_{NaP} current – voltage relationships. GraphPad Prism 7 (GraphPad software Inc., La Jolla, CA, RRID: SCR_002798) was used for all the statistical tests. In all the experiments, 'n' denotes the number of cells while 'N' represents number of de novo preparations of batches of culture from which 'n' is obtained. For all the pharmacology experiments, only one coverslip per drug application was used.

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