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A REST/NRSF-dependent transcriptional remodeling governs GABAergic synaptic upscaling induced by chronic hyperactivity

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Table of Contents

Summary

REST (RE1-silencing transcription factor), also known as NRFS (Neuron-Restrictive Silencer Factor), has been initially identified as a negative transcription factor (Mori et al., 1992; Schoenherr CJ and Anderson DJ 1995; Chong et al., 1995). Its target genes encode synaptic proteins, ion channels and transporters, neurotrophins and neuropeptides (Bruce et al. 2005; Cargnin F et al., 2014; Baldelli P. and Meldolesi J. et al., 2015).

REST is highly expressed in pluripotent stem cells and neural progenitors while it becomes progressively downregulated in differentiating neurons (Ballas and Mendel, 2005; Ooi and Wood 2007; Gopalakrishnan, 2009). However, low levels of REST protein are found in adult neurons in the cortex, hippocampus and cerebellum (Palm K. et al., 1999; Calderone A. et al., 2003; Noh K.M. et al., 2012; Formisano L. et al. 2007; Formisano L. et al. 2013).

Emerging evidence shows that in mature neurons, REST can be upregulated and works as a master modulator of epigenetic processes, acting mostly as transcriptional repressor (McClelland et a., 2011, 2014; Ruano et al., 2012; Huang et al., 2017) and, occasionally, as a transcriptional activator (Kallunki P. et al., 1998; Perera et al., 2015; Bersten et al., 2014).

We have previously demonstrated that the increased transcription and synthesis of REST in response to prolonged electrical activity is critical for the downscaling of intrinsic excitability in excitatory neurons (Pozzi et al., 2013) and for the synaptic homeostasis of glutamatergic synapses that reduces their strength acting at the presynaptic level (Pecoraro-Bisogni et al., 2017).

Here we show that neuronal hyperactivity, obtained by treating for two days primary hippocampal neurons with 4-aminopyridine (4AP), induces a REST-dependent potentiation of the strength and number of somatic GABAergic synapses onto excitatory neurons, while the effect was lacking when the postsynaptic target cell was another inhibitory neuron.

A detailed temporal analysis of the transcriptional-profile changes induced by neuronal-hyperactivity, revealed a very fast (1 h) increase of REST mRNA, concurring with the increase of NPAS4 mRNA, a positive transcription factor, known for its capability to promote the hyperactivity-dependent functional differentiation of inhibitory synapses (Lin Y et al. 2008; Bloodgood et al., 2013; Spiegel I. et al., 2014).

It was previously reported that transient activation of NPAS4 upon hyperactivity, induced a delayed but similarly transient BDNF transcription, synthesis and release. It is very well known that BDNF is released in an activity-dependent way only by excitatory neurons (Hofer et al., 1990; Matsumoto et al. 2008; Dieni et al., 2012; Shinoda et al. 2014), thus its retrograde action onto GABAergic presynaptic contacts, is probably the key point for explaining the specific up-scaling of inhibitory synapses making contact onto excitatory neurons.

Indeed, we observed that the inhibition of REST activity, impaired NPAS4 induction by hyperactivity that in turn suppressed the activation of BNDF transcription, that normally follows NPAS4 activation with a delay of few hours. In accord with this findings, the block of TrkB receptors completely abolished the upscaling of eIPSC onto excitatory neurons.

Moreover, we also observed that the activation of NPAS4 and BNDF, normally transient (few hours), becomes long-lasting upon REST inhibition, persisting for 24/48 hours.

Finally, the inhibition of REST activity also suppressed the further delayed (12 h) hyperactivity-dependent increase of the transcription of vGAT, GAD67 and $GABA_{\varepsilon}Rs$, which represent the molecular fundaments of the functional upscaling of inhibitory synapses.

Our result strongly suggest that the hyperactivity-dependent activation of REST could exert a dual role: 1) an unexpected and immediate activation of the NAPS4-BDNF ”gene-program”, justifying why only somatic inhibitory synapses onto excitatory neurons are up-scaled by the hyperactivity, and 2) a delayed canonical

repressive action that makes transient the transcription of NAPS4 and BDNF, temporally confining their functional effects.

Altogether our data highlights the central role of REST in the complex remodelling of the neuronal transcriptional profile aimed to maintain the neural homeostasis strengthening inhibitory inputs onto the soma of excitatory target neurons and thus counteracting conditions of neural hyperactivity.

1. INTRODUCTION

Neurons in the brain are highly plastic, allowing an organism to learn and adapt to its environment. However, this ongoing plasticity could be unstable, potentially leading to aberrant levels of circuit activity that could contribute to severe pathologies such as epilepsy and ischemia. Indeed, even small changes in the balance between excitation and inhibition (E/I) can set off uncontrolled seizure-like activity. Despite the existence of many forces that constantly perturb the balance between E/I, such as learning-related or developmental changes in synapse number and strength, most of the time our brain is able to compensate for these changes and maintain stable function.

Neural circuits are subject to many forces that work to destabilize their activity. For example, Hebbian (or associative) synaptic plasticity is one of the most widely studied form of long-lasting activity-dependent changes in synaptic strength and includes both long-term potentiation (LTP) and its counterpart, long-term depression (LTD). Hebbian forms of plasticity typically function in an input-specific manner, are rapidly induced and long-lasting, and require correlated firing of the pre- and post-synaptic neurons (Malenka and Bear 2004; Luscher and Malenka 2012; Huganir and Nicoll 2013). LTP and LTD are widely thought to contribute to learning and information storage, but they can generate a powerful destabilizing force on network function (Miller and MacKay 1994; Abbott and Nelson 2000). Indeed, once LTP is induced, potentiated synapses are more excitable and can undergo further potentiation more easily, entering a cycle that, if unconstrained, eventually drives activity to a state prone to hyperexcitability (Turrigiano and Nelson 2000; Turrigiano 2008; Cooper and Bear 2012; Vitureira and Goda 2013).

Conversely, upon LTD induction, depressed synapses more easily undergo further LTD, which, if occurring in an unrestrained manner, could lead to pathological synapse silencing and elimination (Collingridge et al. 2010; Cooper and Bear 2012; Vitureira and Goda 2013).

Homeostatic plasticity (HP) is thought to balance Hebbian plasticity by modulating the activity of the synapse or the properties of ion channels.

Thus, homeostatic forms of plasticity are thought to provide a means of controlling neuronal activity by avoiding extremes and allowing network stability. The term homeostatic plasticity derives from two conflicting notions: 'homeostatic' (composed by “homeo” that stands for 'same' and “static” for 'state' or 'condition') and plasticity (or 'change'), thus homeostatic plasticity means "staying the same through change".

The first clear example of HP was characterized by Nelson’s research group (G. Turrigiano, 1998) (Fig. 1). Blockade for two days of spiking activity with tetrodotoxin (TTX), or of excitatory glutamagergic synapses with CNQX, generates a rebound phenomenon whereby the excitability of the network is increased when the drugs are removed (wash). A more direct test of the idea of firing rate homeostasis is to raise activity with bicuculline (acute bicuculline), and then to follow activity over time. After two days in bicuculline, activity has returned almost to control levels (2 days bicuculline). These experiments, for the first time defined a homeostatic form of plasticity aimed to stabilize the activity of a neuron or a neuronal circuit around a set-point value.

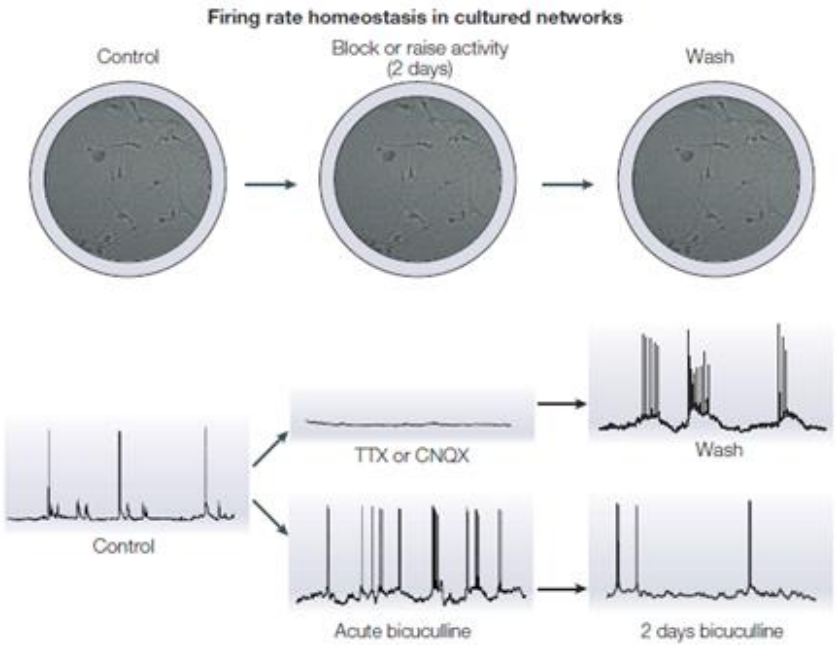


Figure 1 (from Turrigiano G., 1998) Evidence for firing rate homeostasis in cultured networks. Cultured cortical networks are composed of interconnected excitatory pyramidal and inhibitory interneurons, and develop spontaneous activity after a few days in vitro (control).

Currently a number of phenomena have been described that may contribute to stabilization of neuronal activity: the activity-dependent regulation of intrinsic neuronal firing (Marder and Prinz 2003; Zhang and Linden 2003); pre- and postsynaptic forms of excitatory synaptic plasticity, such as synaptic scaling, which is known to adjust all of a neuron's excitatory synapses up or down in the right direction to stabilize firing (Turrigiano and Nelson 2004; Davis 2006); the balancing of excitation and inhibition within neuronal networks (Maffei et al. 2004; Gonzalez-Islas and Wenner 2006); compensatory changes in synapse number (Kirov et al. 1999; Wierenga et al. 2006); homeostatic regulation of intrinsic excitability (Marder and Goaillard 2006; Turrigiano 2011).

In particular, each neuron has the ability to adjust synaptic or intrinsic excitability in a homeostatic manner to keep firing rates relatively constant (Fig. 2). This process is fundamental to maintain neuronal function (Davis and Bezprozvanny, 2001).

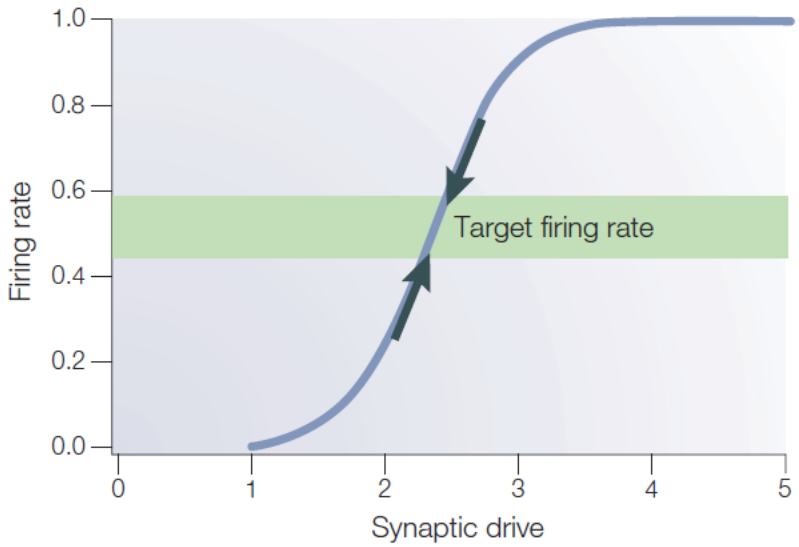


Figure 2: (Modified from Turriggiano and Nelson 2004) Illustration of the relationship between synaptic drive and firing rate for an individual neuron. As synaptic drive increases (through addition or increased strength of excitatory synapses, for example) and firing rate rises above the target level, homeostatic mechanisms (arrows) are engaged that reduce the strength of all inputs, thereby moving the neuron down the curve and back into the target zone. Conversely, if synaptic drive falls too low and firing rate falls below the target rate, the homeostatic regulatory process will increase the strength of all inputs and bring the neuron back within the target firing zone.

Then network activity will automatically settle towards some desired average level and will remain stable. These properties of tuning are proved to be dependent on the activity, on cells specific type, on circuitry type and on specific interaction between excitation and inhibition (Chang et al., 2010). Experimental evidence is rapidly accumulating that such homeostatic mechanisms exist and are important in circuit function. The two most relevant mechanisms of homeostatic plasticity in adult neurons are the intrinsic and synaptic homeostasis (Fig. 3).

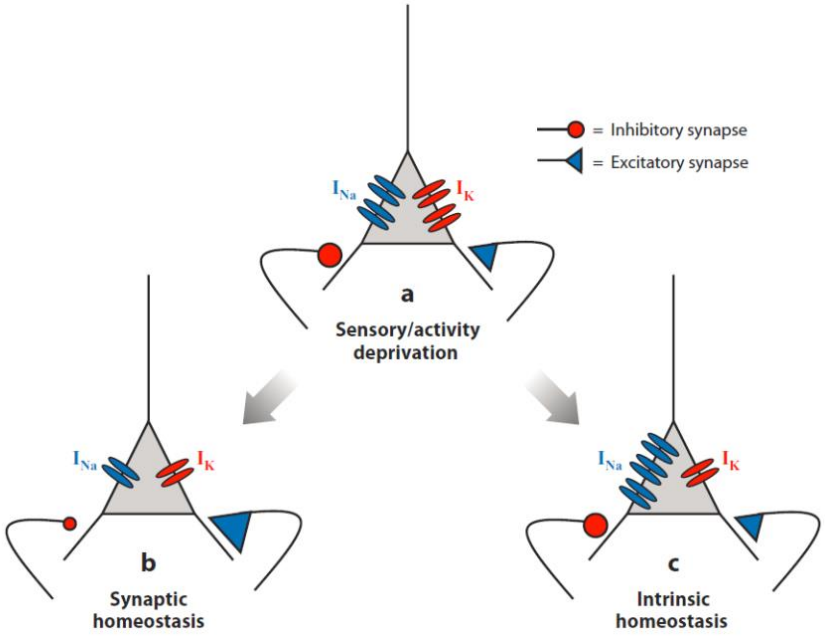


Figure 3: (From Turrigiano, Annu. Rev. Neurosci. 2011) Two fundamentally different mechanisms for the homeostatic regulation of neuronal firing. (a) Neuronal activity is determined both by the strength of excitatory and inhibitory synaptic inputs and by the balance of inward and outward voltage-dependent conductances that regulate intrinsic excitability, here illustrated as the relative number of Na^+ (blue) and K^+ (red) channels. Neurons can compensate for reduced sensory drive either by using synaptic mechanisms to modify the balance between excitatory and inhibitory inputs (b) or by using intrinsic mechanisms to modify the balance of inward and outward voltage-dependent currents (c).

Understanding the rules underling the interactions between intrinsic and synaptic network homeostasis is likely to shed light on some significant diseases such as epilepsy, schizophrenia, and autism all caused by an imbalance in excitation and inhibition.

1.1 Intrinsic homeostasis

Neurons express a large number of different classes of ion channels, each of which can be described in terms of voltage and time-dependence of activation and inactivation.

One of the consequences of the fact that neurons express many different ion channels is that similar intrinsic properties can arise from various combinations of conductance densities. These intrinsic properties are determined by a neuron's distribution of ion channels such as sodium (Na^+) channels, delayed-rectifier potassium (K^+) channels, L-type calcium (Ca^{2+}) channels (Desai NS, 2003). For example, it is well understood that the transient outward current, I_A, can influence a neuron's firing rate (Connor et al., 1977) and that Ca^{2+} dependent K^+ currents can contribute to after-hyperpolarizations (Sah & Faber, 2002; Pennefather et al., 1985).

The intrinsic homeostasis is based on the control of the membrane excitability (Zhang and Linden, 2003). Changes in intrinsic excitability that alter a neuron's input-output function can strongly affect network behaviour, and there is mounting evidence for activity-dependent plasticity of intrinsic excitability in a variety of neurons (Marder & Goaillard 2006; Zhang & Linden 2003). Homeostatic mechanism allows neurons to change their excitability in response to a fluctuation in neuronal activity. By modulating the magnitude and distribution of the channels the neurons can change neuronal excitability, synaptic integration, the pattern and rate of firing. Neurons can also change their excitability by modifying the balance between the inward and the outward currents density, depending on the identity and function of the target neuron (Breaton & Stuart 2009).

It is well known that changes in inward and outward current modulate the expression of channels and that a chronic deprivation of activity increases the excitability of pyramidal neurons (Fig. 4), becoming in this way more sensitive to the remaining inputs (Turrigiano, 1999).

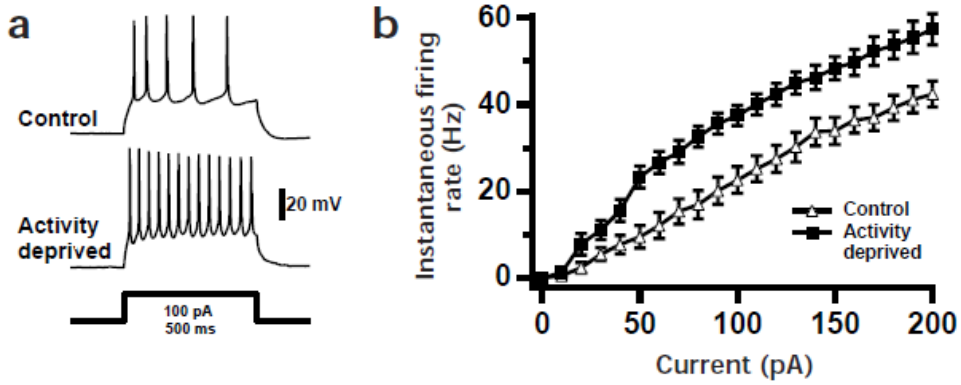


Fig. 4. (From Turrigiano et al., 1999) Chronic activity blockade increased the firing frequency and lowered the spike threshold of pyramidal neurons. (a) Sample spike trains evoked by a somatic current injection in neurons grown under control and activity deprived conditions. The neurons shown came from sister cultures and had very similar resting potentials and input resistances (control, –61 mV, 1.0 GW; activity deprived, –62 mV, 0.9 GW). (b) Average f–I curves for control (n= 18) and activity-deprived (n= 18) neurons. The plot shows initial instantaneous firing frequency (frequency of first spike interval) versus amplitude of current injection.

Another example of intrinsic homeostasis is shown by Brickley's research group (Brickley 2001). In this work they demonstrated that the extrasynaptic $GABA_A$ receptor-mediated tonic inhibition of granule cells (GCs) in the cerebellum is critical for normal cerebellar function and motor coordination. Loss of these receptors in transgenic mice triggers a form of homeostatic plasticity leading to a change in the magnitude of a voltage-independent K^+ conductance that maintains normal neuronal behaviour. The reason is that the neurons compensate for the loss of inhibition by increasing a potassium conductance active at rest.

Moreover, it was previously reported that the reduction of network activity produces a homeostatic response that increases the probability and the duration of firing rate and a significantly increases the inward Na^+ currents (Ergorov et al., 2002; Aptowicz et al., 2004). On the contrary, treatments enhancing the network activity (4AP) mostly decrease the firing frequency due to the reduction of the

density of voltage-gated Na^+ channels (Burrone et al., 2002; O'Leary et al., 2010; Pozzi et al., 2013).

It was recently shown that in addition to changes in inward and outward current densities, enhanced firing (by changes to Ca^{2+} influx) can regulate the location of the AIS so that it moves further from the soma (Grubb & Burrone 2010) (Fig. 5). Conversely Kuba's team reported that deprivation of auditory input in an avian brainstem auditory neuron leads to an increase in AIS length, thus augmenting the excitability of the neuron (Kuba et al. 2010).

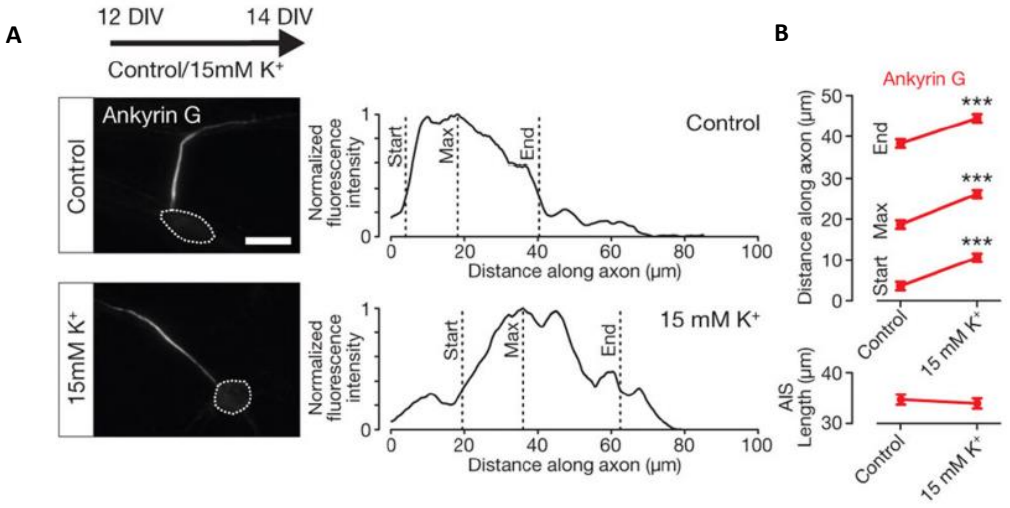


Figure 5. (From Grubb & Burrone 2010) Activity-dependent changes in AIS position. a, Ankyrin G label in control and 15 mM K+ conditions. Right, fluorescence intensity along the axon. Dotted lines indicate soma. b, Ankyrin G positions and length. In dissociated hippocampal neurons, global depolarization with 15 mM extracellular potassium from 12 to 14 days in vitro (DIV) produced a significant distal shift in AIS location (Fig. 1a-b). Labelling for the AIS scaffolding protein ankyrin G, showed that start, maximum, and end AIS positions were all significantly relocated away from the soma (Fig. 1a,b; start: Mann-Whitney U-test, $P < 0.0001$; maximum: $P < 0.0001$; end: $P < 0.0001$; $n = 885$ cells, 36 coverslips), leaving the length of the AIS unchanged (Fig. 1a,b; $P = 0.11$).

The exact contribution of these changes in AIS to neuronal excitability has not been determined, but they are predicted to alter firing threshold and so could play an important role in the homeostatic regulation of neuronal excitability.

1.2 Synaptic Homeostasis

Central neurons are set inside in complex networks and small changes in the E/I balance can have a major impact on ongoing activity, and important evidence indicates that the E/I balance is tightly regulated (Atallah & Scanziani 2009, Pouille et al. 2009, Shu et al. 2003).

Given this complexity, the ability of networks to compensate for external or internal perturbations and to maintain stable firing requires mechanisms that can adjust both excitatory and inhibitory synaptic strengths in a cell-type-specific manner. One of the best studied form of homeostatic plasticity at central excitatory synapses is called “synaptic scaling” acting on individual or small groups of synapses (Turrigiano 2008, Yu & Goda 2009; Pecoraro-Bisogni et al. 2017) (Fig. 6).

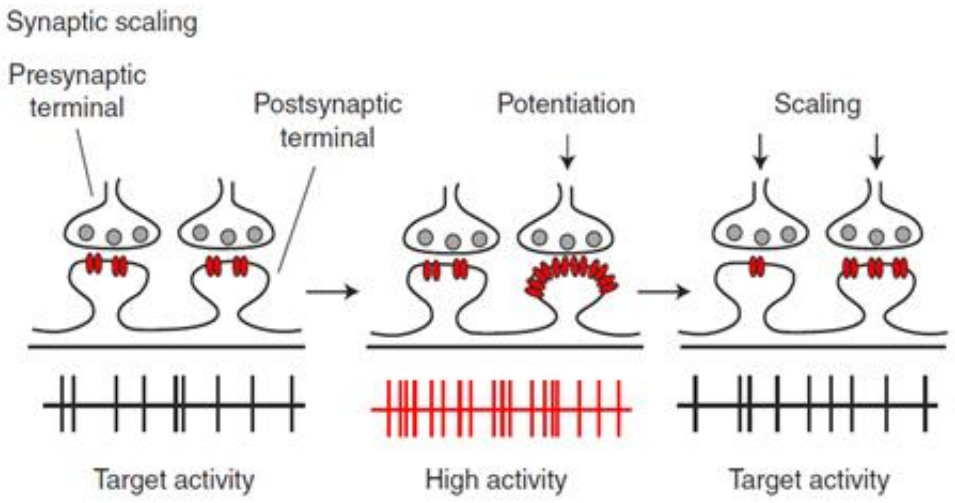


Figure 6: (from Turrigiano 2012) When activity is perturbed (illustrated here as the potentiation of some inputs through Hebbian mechanisms) this triggers synaptic scaling, which produces a proportional reduction in strength at all synapses of the right magnitude to return firing to baseline levels.Note that, because this mechanism scales synaptic strength up or down proportionally, the relative difference in synaptic strengths induced by Hebbian mechanisms is preserved.

By measuring miniature excitatory postsynaptic currents (mEPSCs; minis) researchers found that increased activity reduces the amplitudes mEPSCs onto cortical pyramidal neurons, whereas decreased activity has the opposite effect, indicating that quantal amplitude is regulated in a homeostatic manner by prolonged changes in activity. (Turrigiano 1999; Desai et al. 2002, Gainey et al. 2009). The mEPSCs represent the postsynaptic response to release of individual synaptic vesicle of neurotransmitter. A change of their amplitude suggests a postsynaptic alteration while a change in their frequency is typically interpreted as a presynaptic action or a change in the number of active synaptic contacts (Wierenga et al. 2005, Turrigiano and Nelson 2004) (Fig. 7). The neurons accomplish this global negative feedback control of synaptic strength by detecting changes in their own firing rates through a set of calcium-dependent sensors and their interaction with calcium/calmodulin dependent Kinase (CaMKK) and CaM Kinase IV (CaMKIV) (Ibata et al.,2008). The homeostatic regulation of synaptic strength concerns the pre and the post synaptic site: postsynaptically consist in the accumulation of glutamate receptors at synaptic sites (Wierenga et al., 2005) (Stellwagen and Malenka, 2006); presynaptically consist in change of synaptic vesicle polls size and change in release probability (Muller et al., 2012).

Several molecules are known to be involved in synaptic scaling such as the neurotrophin brain-derived neurotrophic factor (BDNF) (Rutherford et al. 1998), the immediate early gene Arc (Shepherd et al. 2006), the cytokine TNFα (Steinmetz & Turrigiano 2010, Stellwagen & Malenka 2006), the immune molecule MHC1 (Goddard et al. 2007), Beta3 integrin (Cingolani et al. 2008), the AMPAR binding protein PICK1 (Anggono et al. 2011), and the scaffold proteins PSD-95 and PSD-93 (Sun and Turrigiano 2011). Understanding the molecular actors that govern these processes could better clarify how neurons change their properties in response to altered activity.

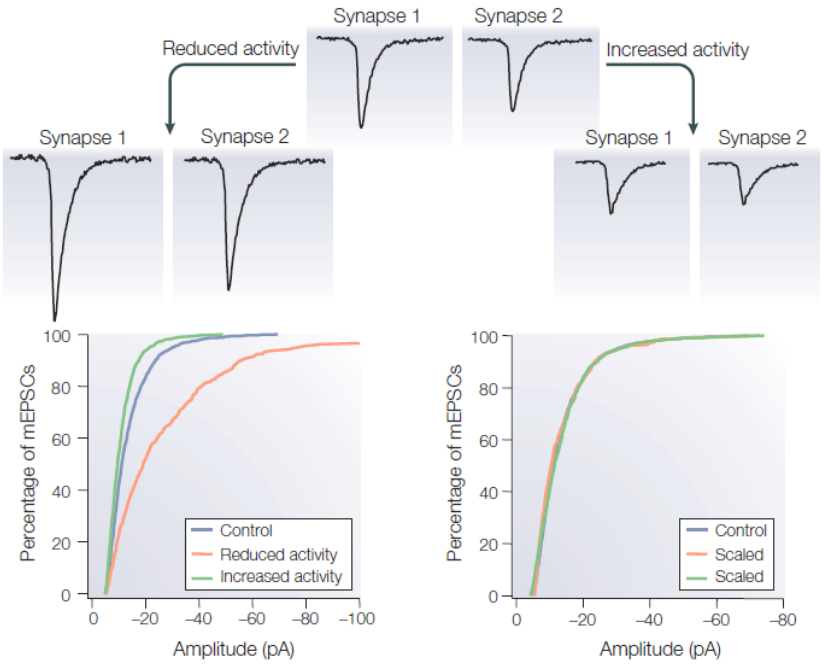


Figure 7: (Modified from Turrigiano and Nelson 2004) Synaptic scaling induces a multiplicative change in the distribution of synaptic weights. Increased activity reduces the amplitudes of miniature excitatory postsynaptic currents (mEPSCs) onto cortical pyramidal neurons, whereas decreased activity has the opposite effect, indicating that quantal amplitude is regulated in a homeostatic manner by prolonged changes in activity. Plotting mEPSC amplitudes as a cumulative histrogram (lower panels) shows that the entire distribution of amplitudes is increased (reduced activity) or decreased (increased activity). If these distributions are scaled up or down by multiplying each value in the experimental distribution by the same factor, they overlay the control distribution almost perfectly, indicating that all excitatory synapses onto pyramidal neurons are scaled up or down multiplicatively by prolonged changes in activity.

1.2.1 Postsynaptic expression of Homeostatic Synaptic Plasticity

The homeostatic modulation of the synaptic plasticity is characterized by two different but connected mechanisms: the pre- and the postsynaptic processes. The

postsynaptic modulation of synaptic scaling is correlated with changes in expression of synaptic glutamate receptors (Turriggiano, 2008; Shephard et al., 2006). Indeed, it was showed that the process of homeostatic plasticity is due to an increase or a decrease of activity mediated postsynaptically by a change in composition or accumulation of AMPA receptors (AMPARs) (Beneyto and Meador-Woodruff, 2004; Malenka and Bear, 2004) and NMDA receptors (NMDARs) (Bredt and Nicoll, 2003; Elias and Nicoll, 2007; Shephard and Hunganir, 2007; Triller and Choquet, 2008). In particular, AMPARs are enriched at excitatory glutamatergic synapses, where they are located at the postsynaptic membrane opposite the presynaptic active zone where glutamate-filled vesicles fuse with the plasma membrane and release their contents into the synaptic cleft. During the homeostatic plasticity process, AMPARs, adjust their number in a manner that opposes the external changes in activity (Wierenga et al., 2005).

AMPARs accumulation or depletion at the postsynaptic sites are mediated by Ca^{2}-dependent signalling pathways and its interaction with AMPARs subunits: GluA2 (Ibata et al., 2008) and GluA1 (Sutton et al., 2006). The scaling up or down is due to the interaction between calcium influx and proteins such as CaMKIV (Ibata et al., 2008), MeCP2 (Blackman et al., 2012), beta3integrine (Cingolani et al.,2008) that translate this signal in modulation of synaptic strength. The AMPARs are highly dynamic and their constant trafficking between extra synaptic and synaptic compartments allows the synaptic scaling-up or -down (Isaac et al., 2007). The scaling down in response to hyperactivity in the network is another cell-autonomous function of postsynaptic firing and involves enhanced Ca^{2+} influx (Fig. 8), gene transcription, the CaMKK/CaMKIV signaling pathway, and targets the GluA2 subunit (Goold and Nicoll 2010).

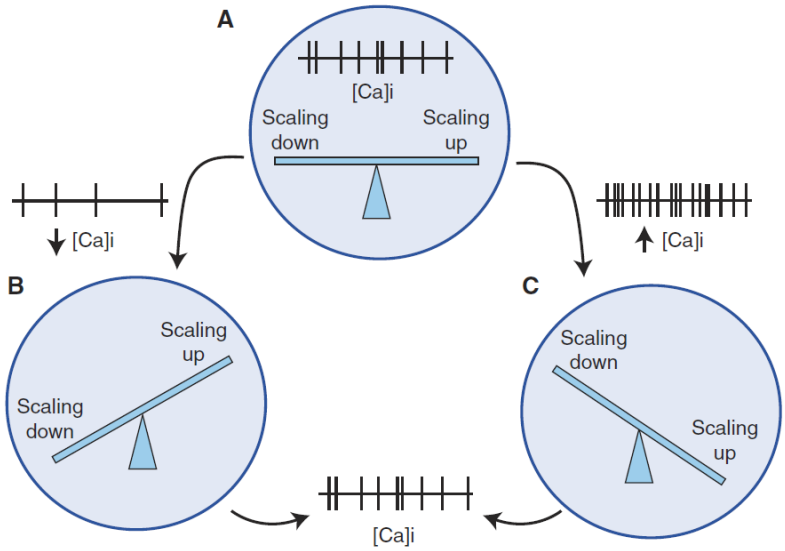


Figure 8: (modified from Turrigiano et al. 2012) Calcium-dependent pathways regulate both scaling up and scaling down. (B) If activity decreases (owing to sensory deprivation, learning-induced LTD, or other factors) then average somatic calcium will also decrease; this will enhance scaling up and reduce scaling down and restore firing to baseline. (C) Conversely, if firing increases and average somatic calcium increases, this will enhance scaling down and reduce scaling up, again restoring firing to baseline.

Two additional Ca^{2+}-dependent pathways have recently been identified. One involves the immediate early gene Homer1a, which is induced in a calcium-dependent manner by enhanced activity and is required for scaling down through a pathway that requires agonist-independent regulation of mGluRs and reduced tyrosine phosphorylation of GluA2 (Hu et al., 2010). The second involves the activation of Eph4A by elevated activity; Eph4A is also necessary for scaling down, and is thought to act by regulating the ubiquitin pathway to control AMPAR degradation (Fu et al. 2011).

1.2.2 Presynaptic expression of Homeostatic Synaptic Plasticity

The homeostatic regulation of the synaptic strength at the presynaptic level can involve, also, the homeostatic modulation of presynaptic neurotransmitters release machinery (Davis and Muller 2015).

Neurotransmitter is stored in synaptic vesicles (SVs), which can release their content by controlled fusion with a specialized region of the presynaptic membrane named active zone (AZ). Central synapses contain several SVs, which are not uniform but follow their functionality and localization. Different pools of vesicles have been described: the readily releasable pool (RRP), morphologically characterized by their physical contact with the AZ membrane; the recycling pool (RP) that contains SVs that can undergo exocytosis upon prolonged stimulation and resting pool (RestP) comprising vesicles that are incapable of exocytosis under physiological conditions. Therefore, the size of RRP is decisive for the synaptic release probability (Pr) often assessed as a parameter of presynaptic strength (Alabi and Tsien, 2012). The neurotransmitters release, at the presynaptic terminal is the product of several interaction between different proteins and molecules.

Recent evidences show E/I neighbouring synapses on the same dendritic branch have very similar release probabilities, and Pr is negatively correlated with the number of synapses on the branch. Increasing dendritic depolarization elicits a homeostatic decrease in Pr, and equalizing activity in the dendrite significantly reduces its variability, indicating that local dendritic activity is the major determinant of basal release probability (Branco et al. 2008). This is consistent with evidence that silencing a neuron before synapses are formed leads to a reduction in synaptic inputs to this less-excitable neuron, implying that axons, when faced with a choice of active or silent neurons, prefer to make synapses on more easily excited neurons. In contrast to the effect of early reduction of excitability, it was found that suppressing excitation after synapses are already in

place leads to a homeostatic increase in synaptic input strength, establishing that the homeostatic increase is cell autonomous. Burrone's findings indicate that decreased neuronal activity can have multiple and widely different effects depending on the developmental stage of neuronal networks and suggest that Pr can be bidirectionally regulated by changes in the amount of dendritic depolarization (Burrone et al. 2002). Neurons and networks use a family of homeostatic synaptic plasticity mechanisms to stabilize firing rates in the face of developmental or learning-induced changes in drive, and this contributes to the ability of central neuronal networks to maintain stable function and enables networks to maintain the specificity of synaptic changes that encode information.

1.3 Role of neuro-epigenetic in homeostatic plasticity

Although several molecular pathways underlying intrinsic and synaptic homeostasis have been identified (Davis G.W. 2013; Schanzenbacher C.T. et al., 2016), the scenario is becoming every day more puzzling and fragmented, and these two forms homeostasis are often seen as two distinct processes. Recent evidence suggest that HP could involve an activity dependent epigenetic remodeling of the neuronal transcriptional profile (Roberts TC et al., 2014; Hwang JY et al., 2017; Guzman-Karlsson MC et al., 2018). Neuronal activity may trigger transcriptional and epigenetic changes and is a critical aspect in development and in nervous system function (Ernfors, et al. 1991; West, et al. 2001, West, et al. 2002). Complex biological processes, such as organogenesis and homeostasis, are stringently regulated by genetic programs that are fine-tuned by epigenetic factors to establish cell fates and/or to respond to the microenvironment (Guzman-Karlsson MC et al., 2014). Indeed, gene regulatory networks that guide cell differentiation and function are modulated and stabilized by epigenetic modifications to DNA, RNA and proteins such as the neuro-epigenetics (Guzman-Karlsson MC et al., 2014). Neuro-epigenetics, in its most classical sense, encompasses a wide range of non-heritable changes in gene expression that occur

in response to environmental influences and that do not result from alterations in the DNA sequence. These alterations typically arise owing to DNA methylation or hydroxylmethylation, histone post-translational modifications and changes in nucleosome positioning; these processes are collectively referred to by the broad term ‘chromatin remodelling’. Recent studies have identified histone variants, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) as additional epigenetic mechanisms (Sweatt JD, 2013; Roberts TC et al., 2014). Epigenetic modifications are context- and cell type- dependent. Within a specific tissue, distinct cell types or even individual cells can possess unique patterns of DNA methylation and histone modifications to sustain functional homeostasis at a given time and/or spatial location. Indeed, the “single candidate gene”- based study of homeostatic plasticity processes has started to be substituted by a wider “multiple gene modification” prospective (Schanzenbacher CT et al., 2016; Meadows JP et al., 2015). The simultaneous regulation of thousands of genes and proteins to compensate for sustained alterations in network activity has been reported (Schanzenbacher CT et al., 2016; Meadows JP et al., 2015) and recent works point to neuro-epigenetics as the molecular mechanism underlying such regulation (Hwang JY et al., 2017; Guzman-Karlsson MC et al., 2014). Conceptually the epigenome, having the capacity to control the entire genomic output and sense pan-cellular signaling mechanism, might be the ideal control point for achieving coordinated orchestration of the readout of a plethora of ion channels, receptors, and trafficking mechanism in order to achieve homeostatic plasticity (Sweatt JD, 2013). Epigenetic regulation of gene expression in the nervous system represents an exciting area for future basic as well as translational research (Roopra et al., 2012).

2. REST - Repressor Element-1 Silencing Transcription factor

2.1 Overview

The Repressor Element-1 Silencing Transcription Factor (REST) also known as NRSF (Neuron Restrictive Silencing Factor), was first described in 1992 by Mori and co-workers who identified the transcription factor as a silencing factor for type II sodium channels (Mori et al., 1992) and as a key repressor of development and differentiation (Chong et al., 1995; Schroenner and Anderson, 1995). More recently REST was characterized not only as a negative transcription factor fundamental for the differentiation of progenitors cells but also active in differentiate neurons (Ballas and Mendel, 2005; Ooi and Wood 2007; Gopalakrishnan, 2009). Today REST is seen as an ideal candidate to fine-tune neuronal gene expression for shaping neuronal homeostasis following stressful experiences (Bithell, 2011; Noh et al., 2012). A consistent number of studies, conducted on neural stem cells and neural progenitors (Sun YM et al., 2005; Otto SJ et al., 2007), have led to a unified view in which a progressive downregulation of REST activity during neuronal development (Fig. 9) de-represses many neuronal genes (Tapia-Ramirez J et al., 1997).

REST was found to be implicated in the transcriptional regulation of more than 2,000 neuron-specific target genes, crucial for processes such as axonal growth, formation of synaptic contacts, and membrane excitability that trigger the morphological and functional differentiation of mature neurons (Mandel G et al., 2011; Su X et al., 2006; Cargnin F et al., 2014).

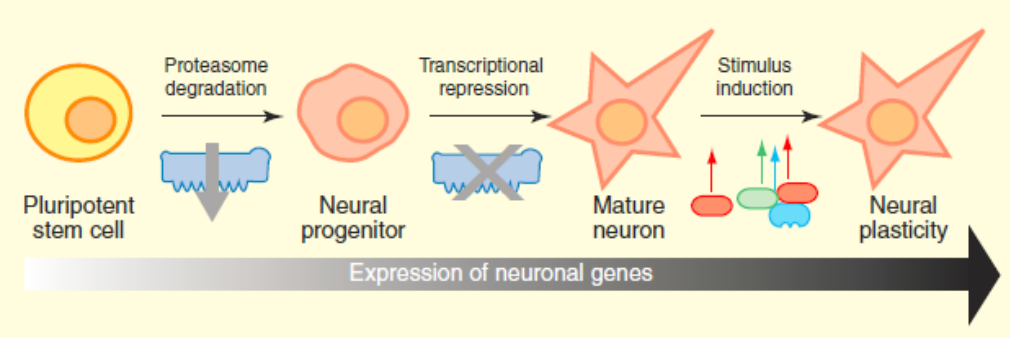


Figure 9: (Modified from Culson, 2005) The availability of REST/NRSF regulates neuronal gene expression in development and plasticity.

Whilst REST is normally quiescent in differentiated neurons, the transcriptional repressor can be transiently activated following various intrinsic and extrinsic neuronal insults such as seizures (Palm K. et al., 1999), global ischaemia (Calderone A. et al., 2003; Noh K.M. et al., 2012; Formisano L. et al. 2007) and stroke (Formisano L. et al. 2013).

In contrast, neuronal REST was also found to translocate to the nucleus during healthy ageing in the human brain (Mampay and Sheridan 2019), thereby repressing ROS-induced cell death genes, and was shown to protect ageing neurons from amyloid-β pathology (Kawamura et al., 2019; Lu et al., 2014). Furthermore, physical activity was shown to boost REST expression, which attenuated age-related neuro inflammation in the ageing rodent hippocampus (Dallagnol et al., 2017). Interestingly, in neuropathological states, such as Alzheimer's disease and Parkinson's disease, which are both hallmarked by protein misfolding and aggregation, REST fails to translocate to the neuronal nucleus (Kawamura et al., 2019; Lu et al., 2014) (Fig. 10).

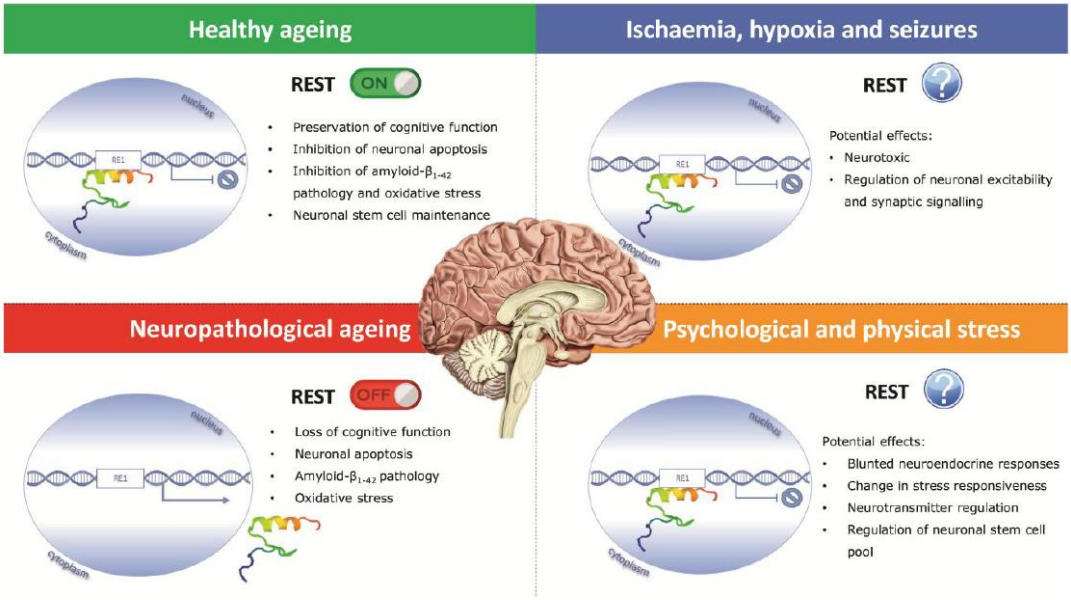


Figure 10: (from Myrthe Mampay, Graham K. Sheridan, 2019). Graphical abstract: REST role in different physiological and pathological conditions

Many genes (Fig. 11) expressed in adult neurons are governed or related to REST (Baldelli P. and Meldolesi J. et al., 2015). These include genes for transcription factors, dependent on REST for their repression, such as Sp1, Grin1, Ascl1, Isl1, and many others. Among the genes repressed by REST there are those encoding for channels, transporters and synaptic proteins controlling membrane excitability and synaptic transmission such as Na^+, Ca^{2+} and K^+ channels, as well as presynaptic and postsynaptic proteins (Uchida et al., 2010; van Loo et al., 2012).

The upregulation of the chloride transporter KCC2 in adult cortical neurons relies on low REST levels. This transporter is critical for the Cl^- switch that converts the function of GABA from excitatory to inhibitory (Uvarov et al., 2005; Yeo et al., 2009).

Moreover, REST modulates the expression of genes involved in various stress-related mediators, including CRH, BDNF and the serotonin (5-HT) 1A receptor (Chen et al., 2015; Singh-Taylor et al., 2018) as well as of genes encoding for

subunits of glutamatergic (NMDA and AMPA) receptors (Rodenas et al., 2012) and few G-protein-coupled receptors (Henriksson et al., 2014).

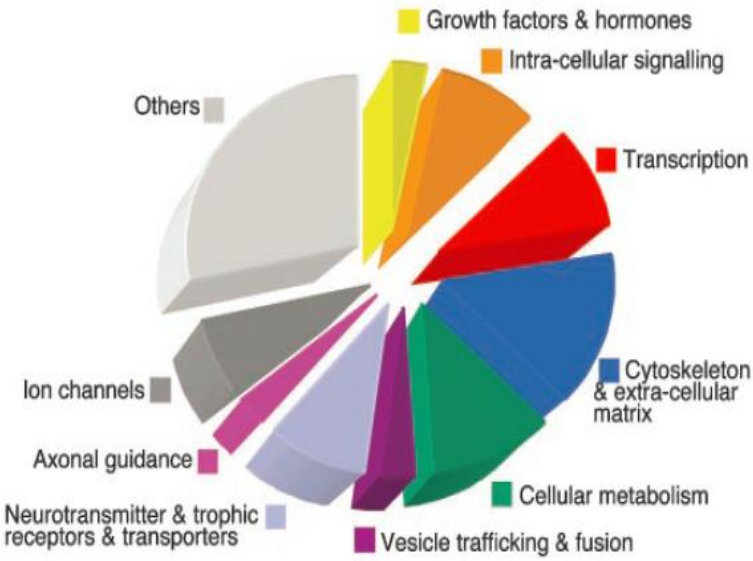


Figure 11: (from Bruce et al. 2005) Assignment of putative REST target genes within the RE1 sequence

2.3 Mechanism of Action:

REST initiates the repression of gene transcription by binding to restrictive element 1 (RE1), a 21–23 bp consensus sequence (Fig. 12), that is contained within the promoters of target genes (Ooi and Wood, 2007; Hwang J.Y. et al., 2017).

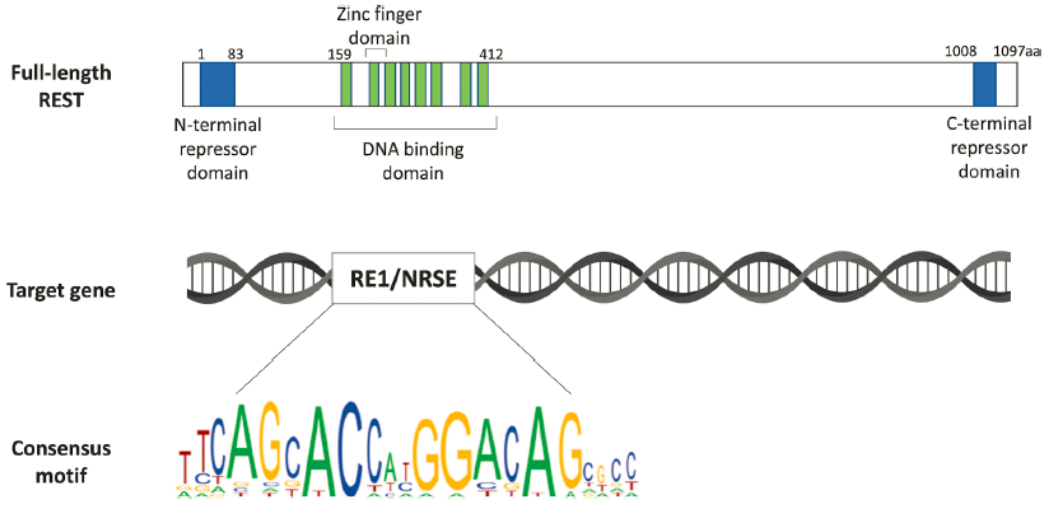


Figure 12: (from M. Mampay and G.K. Sheridan, 2019) The modular structure of full-length REST protein. The DNA binding domain contains eight C2H2 zinc fingers near the N-terminal repressor domain. The other repressor domain is located at the C-terminal. REST binds to its targets genes which contain the RE1/NRSE consensus motif, however other binding partners have been identified (Bruce et al., 2009; Ooi and Wood, 2007).

REST-mediated chromatin modifications require the assembly of a multimeric ‘‘repressosome’’ complex, as a matter of fact REST alone (Fig. 13) is not sufficient to repress gene expression (Yu et al., 2011).

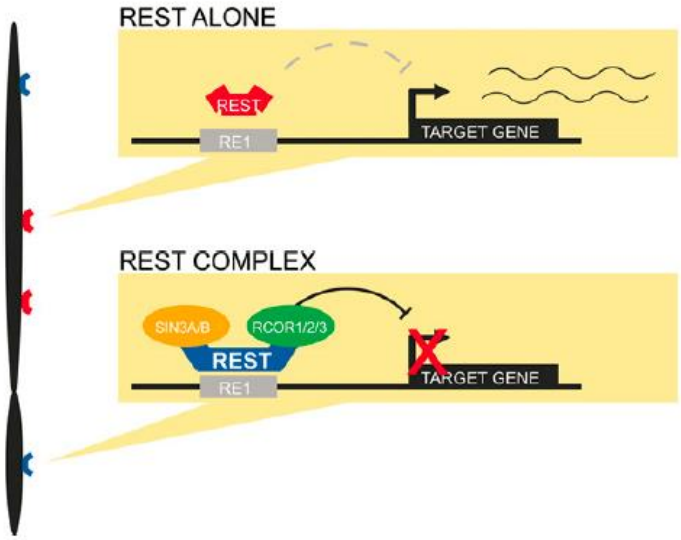


Figure 13: (from Yu et al., 2011). A general model of cofactor-dependent gene repression by REST in ESC. Gene repression by REST requires the assembly of a mulitmeric ‘‘repressosome’’ complex, and that binding by REST alone is not sufficient to repress gene expression.

REST-mediated gene repression is highly dependent on the recruitment of its epigenetic cofactors which are grouped in two separate corepressor complexes. The first one is mSin3A (at N-terminal) which serves as a binding site for histone deacetylases 1 and 2 (HDAC1/2) (Huang et al., 1999). The second one is co-repressors REST (CoREST) at C-terminal (Mampay M. and. Sheridan G.K., 2019), which in turn can recruit a number of chromatin remodelling proteins, such as (Fig. 14):

- HDAC1/2
- histone methyltransferase (G9A) which is a site-specific histone methyltransferase that adds a dimethylation mark to histone 3 at lysine 9 (H3K9me2)
- lysine-specific demethylase 1 (LSD1) which removes monomethyl and dimethyl moieties from H3K4, thus promoting gene repression
- methyl-CpG-binding protein (MeCP2) which is recruited to epigenetic marks and promotes epigenetic remodelling in a REST-dependent or independent manner
- carboxy-terminal binding protein 1 (CTBP1)
- various chromatin remodelling proteins such as Brg1, Braf35, Baf170, Baf57

Importantly, REST can recruit different combinations of corepressor complexes to mediate context specific gene expression modification.

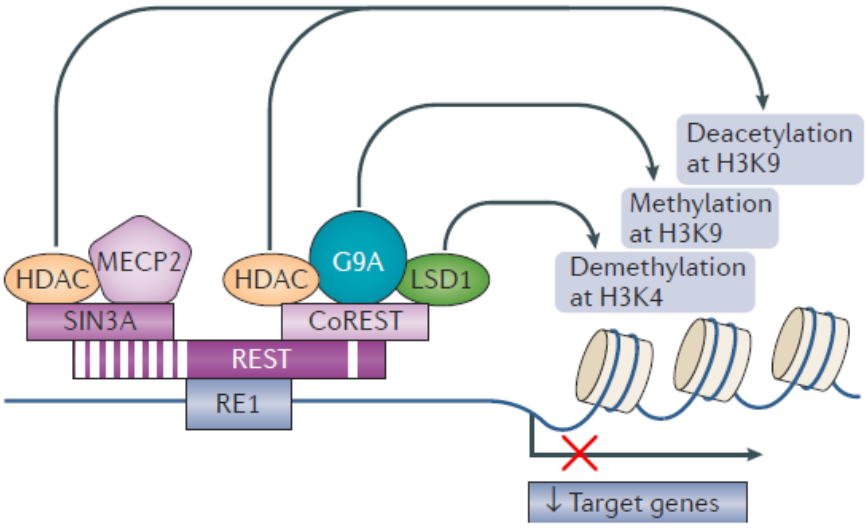


Figure 14: (from Hwang J.Y. et al. 2017) Graphical representation of REST activity and REST epigenetic cofactors.

2.4 Transcriptional regulation

It is crucial to understand the transcriptional regulation of REST to fully appreciate the complexity of REST-mediated repression of its target genes. The Wnt/β-catenin signalling pathway, known for its leading role in the differentiation of neuronal precursors, dendritic morphology and synaptic function, was shown to directly regulate REST to control the stem cell progenitor pool. REST, indeed, possesses the T cell-specific transcription factor (TCF) binding site that can be directly activated by the Wnt pathway (Inestrosa & Varela-Nallar, 2014; Nishihara et al., 2003). REST gene is also positively regulated by Oct4 and Nanog since the depletion of these transcription factors resulted in downregulation of REST expression (Loh et al., 2006). Furthermore, various epigenetic mechanisms are involved in the regulation of REST mRNA transcription, such as CpG methylation, MeCP2 binding and miRNA mediated processes (Kreisler et al., 2010; Wang et al., 2018). Interestingly genome wide analysis showed the presence of the consensus sequence (RE1 motif) within the genomic sequence of REST indicating an autoregulation (Johnson et al., 2007). (Fig. 15).

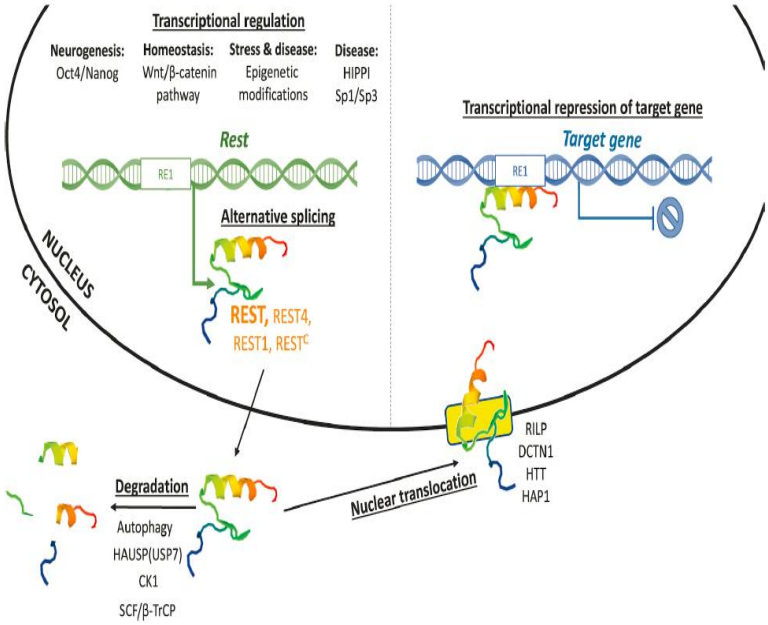


Figure 15: (from Mampay M. and Sheridan G.K. 2019). Brief overview of REST characteristics. Transcriptional regulation: The transcription factor itself is regulated through Wnt/β-catenin signalling, the embryogenic transcription factors Oct4 and Nanog, disease associated proteins such as the huntingtin interaction protein 1 (HIP1) protein interactor (HIPPI) and epigenetic modifications, which are likely induced by a self-regulatory feedback mechanism. Alternative splicing: upon REST transcription, alternative splicing can result in different isoforms with different functional regions, affecting their DNA binding affinities and repressive activity (e.g. REST4, REST1, RESTc). Degradation: In the cytosol REST can be targeted for proteasomal degradation through SCF/β-TrCP and HAUSP (USP7)-dependent de-ubiquitination, casein kinase-1 (CK-1) mediated phosphorylation and autophagy. Nuclear translocation: Nuclear trafficking of the protein is vital for its repressor activity and dependent on various nuclear localization signals, including REST/NRSF-interacting LIM domain protein (RILP) in neurons, dynactin 1 (DCTN1) in non-neuronal cells. Disease-associated proteins such as huntingtin (HTT), huntingtin-associated protein (HAP1) and likely amyloid-β can prevent REST's nuclear translocation. Transcriptional repression: As REST binds to its target genes, the protein acts as a scaffold to recruit a DNA-modifying complex of epigenetic cofactors. Through direct or indirect interaction with a variety of transcriptional and epigenetic cofactors REST exerts gene repression.

2.5 REST availability

During neurogenesis, REST is widely available. However, inducibility of REST protein expression after neurogenesis is tightly regulated through transcriptional repression and protein degradation via Skp1-Cul1-F-box protein (SCF)/β-TrCP-dependent, ubiquitin-based proteasomal degradation and HAUSP(USP7)-dependent de-ubiquitination (Ballas et al., 2005; Singh et al., 2011). In addition, various studies indicate that part of the degradation mechanism involves phosphorylation of two non-canonical degron motifs located in the C terminus of REST that require activity of beta-transducing repeat containing E3 ubiquitin protein ligase, βTrCP which primes REST for ubiquitin-based proteasomal degradation (Cheong and Virshup, 2011; Weissman, 2008; Westbrook et al., 2008). Nesti's group identified a proline-directed phosphorylation motif, at serines 861/864 upstream of the degron motifs, which is a substrate for the peptidylprolyl cis/trans isomerase, Pin1, as well as the ERK1/2 kinases. Mutation at S861/864

stabilizes REST, as does inhibition of Pin1 activity. Interestingly, they found that C-terminal domain small phosphatase 1 (CTDSP1), which is recruited by REST to neuronal genes, is present in REST immunocomplexes, dephosphorylates S861/864, and stabilizes REST. Expression of a REST peptide containing S861/864 in neural progenitors inhibits terminal neuronal differentiation. These results suggest that CTDSP1 activity stabilizes REST in stem cells and that ERK-dependent phosphorylation combined with Pin1 activity promotes REST degradation in neural progenitors (Nesti et al., 2014).

In pluripotent stem cells, neural progenitors and cancer cells, REST abundance is regulated by proteasomal degradation: in differentiated neurons under physiological conditions, casein kinase 1 (CK1) ensures that REST abundance remains at low basal levels (Hwang J.Y. et al., 2017). CK1 phosphorylates REST at serine residues within its two degron motifs and enables the recognition and binding of the E3 ligase β-TrCP. β-TrCP initiates the ubiquitylation of REST and primes it for ubiquitin-based proteasomal degradation (Fig. 16).

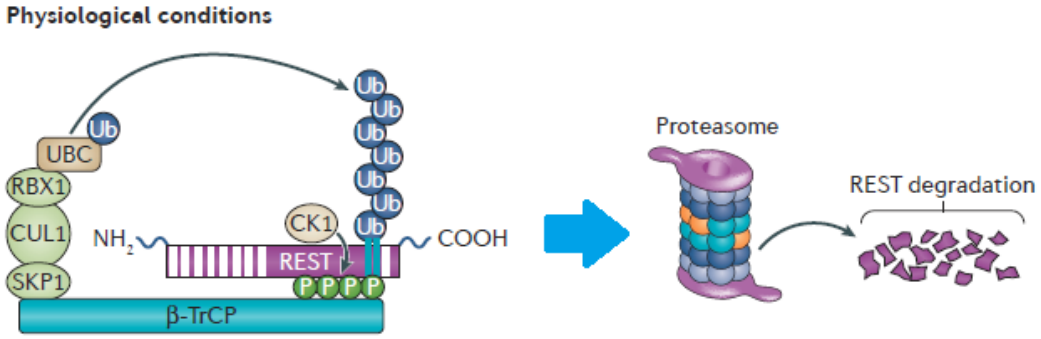


Figure 16: (from Hwang J.Y., et al., 2017) The regulation of restrictive element 1-silencing transcription factor degradation. Under physiological conditions, CK1 binds and phosphorylates REST at sites within two neighboring, but distinct, degron motifs. The phosphorylated degrons (phospho-degrons) are critical to recognition of REST by the E3 ubiquitin protein ligase β-TrCP, which ubiquitinates and targets REST for proteasomal degradation.

2.5 Homeostatic REST activity

Baram's group has recently shown (McClelland et al., 2014) that REST target genes are differently modulated by REST protein levels. According to their model (Fig. 17) a moderate increase of REST levels did not alter the transcription of genes with very low REST binding affinity. In parallel, in genes with high binding affinity for REST, the RE1 would likely be occupied even by the low REST levels. Increased REST levels would be unlikely to augment occupancy or promote suppression of these genes. In contrast, when REST binding is 'mid-range', then relatively small fluctuation of the transcription factor levels would result in major changes of repressor binding and gene repression. These genes are important for neuronal excitability, have a key role in neuronal homeostasis and include ion channels and their accessory subunits, neurotransmitter receptors, genes involved in Ca^{2+}-mediated cellular cascades etc. (McClelland et al., 2014; Baldelli P. and Meldolesi J., 2015.)

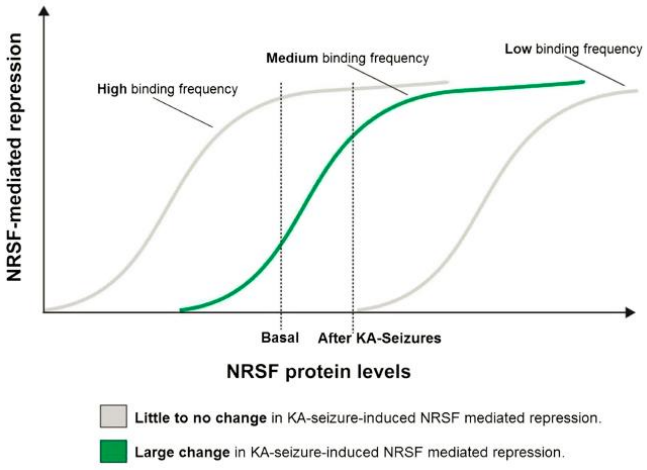


Figure 17: (from McClelland et al., 2014) A potential 'dynamic range' of repressor binding might enable gene regulation by moderate fluctuations of REST levels. Graphical representation of data observed showing that only a subset of RE1-containing genes are functionally repressed by seizure-induced increases in REST levels and that these genes appear to have moderate REST binding in the naive brain.

Neuronal excitation could be actively controlled by REST through its capability to negatively modulate voltage-gated Na^+ channels (Chong et al., 1995). REST also regulates expression of Ca^{2+} channels, K^+ channels and hyperpolarization-activated cyclic nucleotide-gated (HCN1) channels (McClelland et al., 2011).

Our group demonstrated that the use of 4-aminopyridine (4-AP), a K^+ channel blocker able to induce neuronal hyperactivity, causes a transient increase of REST mRNA and protein levels in excitatory neurons.

This is followed by a reduction of voltage-gated sodium channels (Nav) expression downregulating the sodium current density (I_{Na+}) at the single-cell level and reducing neuronal excitability and the overall neuronal network firing activity (Pozzi et al., 2013). These findings indicate that REST has the potential to maintain neuronal activity by restoring physiological firing activity and preserving intrinsic homeostatic plasticity. The transcriptional repression of Nav channels by REST/NRSF appears to be one of the mechanisms governing the homeostatic response engaged by hyperactivity (Fig. 18).

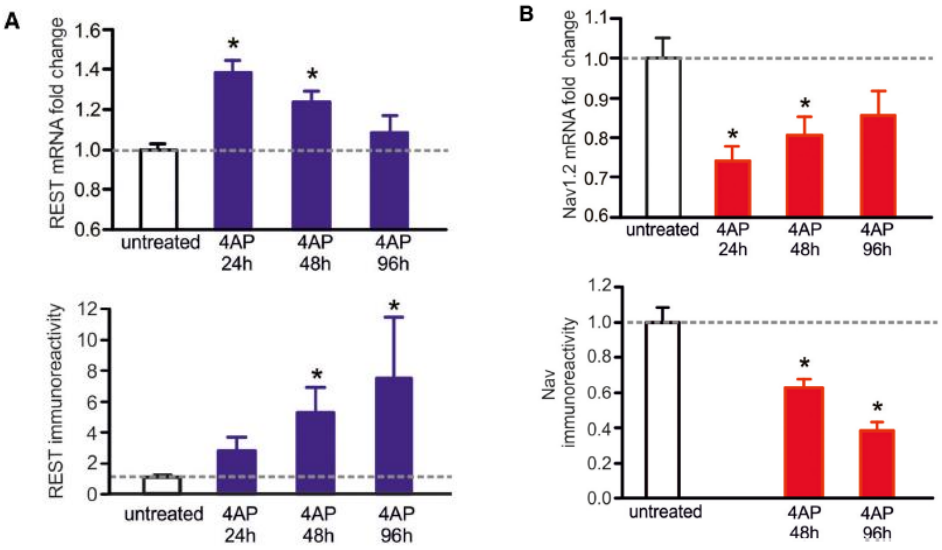


Figure 18: (Modified from Baldelli and Meldolesi 2015). 4AP induced cortical neuron hyperactivity increases REST expression and, in parallel, downregulates the expression of the Na^+ channel Nav1.2. (A) Analysis of REST (blue). Top, Quantitative RT-PCR analysis of REST mRNA levels in

cortical neurons that were either untreated or treated with 4AP (100 µM) for 24, 48, and 96 h. Bottom, Changes in the REST protein of cortical neurons treated as in the top panel, quantified by Western blotting. (B) Analysis of Nav1.2 (red). Top, Quantitative RT-PCR of the changes in Nav1.2 mRNA in cortical neurons, untreated or treated with 4AP. Bottom, Nav1.2 protein of cortical neurons, quantified by Western blotting. Notice that, for both mRNA and protein, the opposite changes were induced by 4AP: an increase in REST mRNA at 24 h, followed by a decrease back to the untreated level at 96 h, accompanied by a decrease of Nav1.2 mRNA, and followed by an increase at the same times; and a slow increase in REST protein (up to approximately eightfold at 96 h) accompanied by a slow decrease in Nav1.2.

REST also participates in synaptic homeostasis by reducing the strength of excitatory synapses at the presynaptic level. This involves a decrease in mEPSC frequency (Fig. 19) associated with a reduced intensity of VGLUT1-positive contacts and VGLUT-1 mRNA levels. Moreover, this REST-dependent presynaptic modulation leads to a reduction of the readily releasable pool (RRP), the recycling pool sizes, the mRNA and protein levels of Syn1, Syt2, and SNAP25, three fundamental molecular actors of the presynaptic machinery (Pecoraro-Bisogni et al. 2017). This experimental evidence adds new insights to the complex activity-dependent transcriptional regulation of the homeostatic plasticity processes mediated by REST.

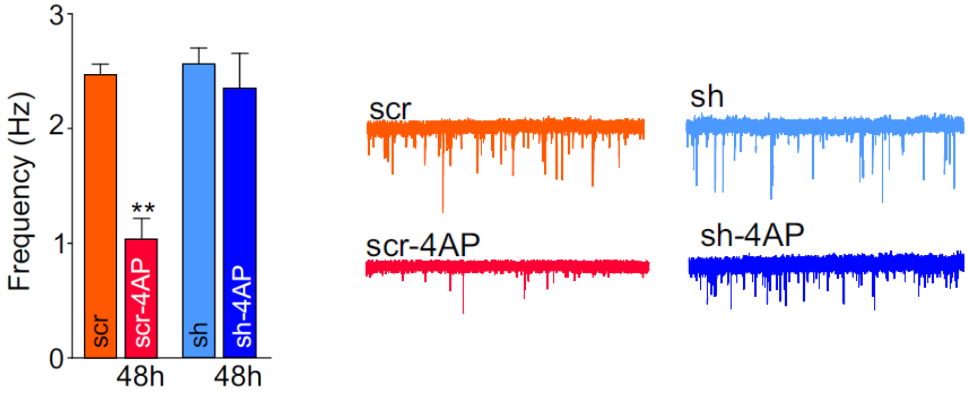


Figure 19: (modified from Pecoraro-Bisogni et al 2017). The frequency of mEPSCs is scaled-down by REST increase induced by neuronal hyperactivity. (right panel) Representative mEPSCs traces recorded at 18 div. (left panel) Mean ± SEM of mEPSC frequency.

2.6 REST, not only a transcriptional repressor

REST binding sites have been well described as silencing elements in numerous neuronal genes, however in some contexts it has been suggested to potentially act as neuron enhancer elements (Kallunki P. et al., 1998). For example, deletion of the REST binding site within the L1 cell adhesion molecule (L1cam) promoter not only causes gain of non-neuronal expression but also reduces expression of L1cam in the CA3 region of the hippocampus and cortex, suggestive of enhancer function (Fig. 20).

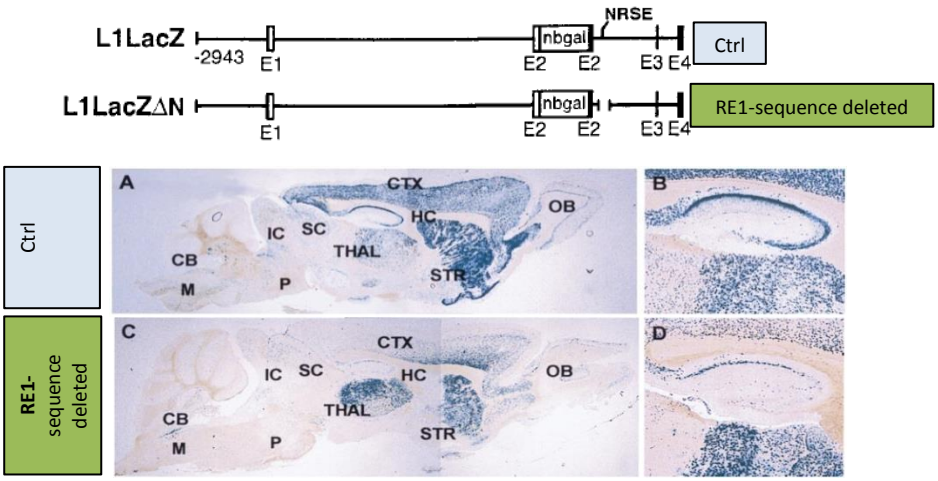


Figure 20: (modified from Kallunki et al., 1998) Expression of L1lacZ (A and B) and L1lacZDN (C and D) in sagittal sections of the adult (30 week old) mouse brain (A and C). (B and D) Higher magnification views of expression of the two transgenes in the hippocampus. CB, cerebellum; CTX, cortex; HC, hippocampus; IC, inferior colliculus; M, medulla; OB, olfactory bulb; P, pons; SC, superior colliculus; STR, striatum; THAL, thalamus. In the adult brain, intense expression of L1lacZ was observed within the cortex, striatum, thalamus, and hippocampus (A). Expression of the L1lacZDN transgene was consistently weaker than that of L1lacZ in the cortex, striatum, and hippocampus but not in the thalamus where it was more intense than that of L1lacZ (compare C to A). The reduction of lacZ expression upon deletion of the NRSE was particularly apparent within the CA3 region of the hippocampus (compare D to B).

Another research group suggested that REST plays a dual role as silencer and enhancer of gene expression in the nervous system and that its enhancer function

may depend on the proximity of the RE1 to the promoter (Bessis A. et al., 1997). In that study, elimination of the NRSE in the gene encoding the β2 subunit of the neuronal nicotinic acetylcholine receptor led to a loss of β-galactosidase expression in the spinal cord and dorsal root ganglia and a gain of expression in anterior brain regions. By preparing synthetic promoter constructs in which the NRSE was placed at different distances from a simian virus 40 promoter and testing the activity of such constructs in neuroblastoma cells, it was suggested that the NRSE needed to be close to a promoter to function as an enhancer.

A recent work (Perera et al., 2015) demonstrated that REST is able to recruit the ten-eleven translocation hydroxylases 3 (TET3, the major TET isoform in neurons) for context-specific hydroxymethylation and induction of gene expression (Colquitt et al., 2013; Hahn et al., 2013). Indeed, TET3 is recruited to the DNA by transcriptional regulators like REST for context-specific 5mC hydroxylation which positively correlates with gene expression (Hahn et al., 2013; Mellen et al., 2012). Subsequently, TET3 mediates H3K36 trimethylation by recruitment of histone writers such as NSD3 (Perera et al., 2015) (Fig. 21).

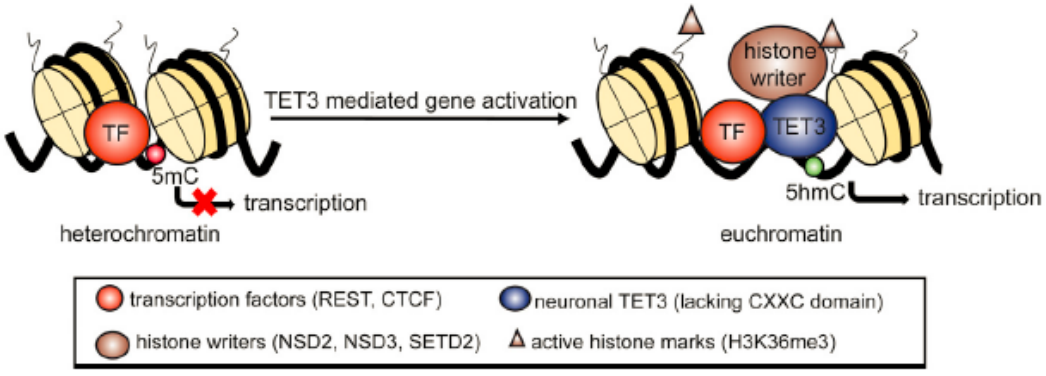


Figure 21: (from Perera et al., 2015) Model for neuronal TET3-mediated transcriptional activation and chromatin remodelling.

Finally, NPAS4, a known REST target gene, is a critical player in neuronal activity homeostasis. It has been demonstrated that the expression of NPAS4 is tightly

regulated (Lin Y. et al. 2008). NPAS4 is a brain restricted, activity-induced positive transcription factor able to regulate the expression of inhibitory synapse genes thus controlling homeostatic E/I balance in neurons. Bersten and colleagues showed that REST is able to strongly bind multiple locations within the promoter and Intron I of the NPAS4 gene repressing its transcription (Bersten et al., 2014). Interestingly they found that RE-1 element may play a role in aiding activation of the NPAS4 promoter as well as repression. This was explored by testing the ability of NPAS4/ARNT2 (ARNT2 is a partner protein of NPAS4) to activate the NPAS4 promoter when the promoter RE-1 site was deleted. They found that this deletion attenuates the ability of NPAS4/ARNT2 to activate the NPAS4 promoter (Fig. 22).

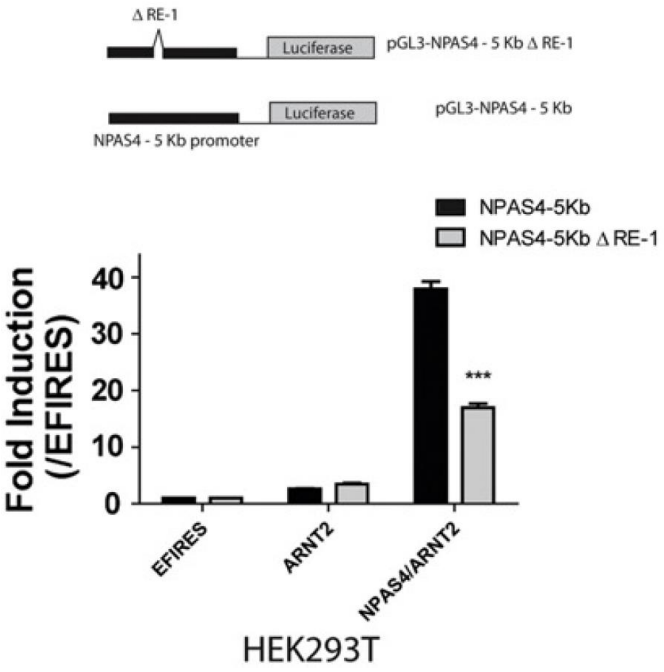


Figure 22: (from Bersten et al., 2014): NPAS4/ARNT2 activation of the NPAS4 promoter is reduced when the promoter RE-1 element is deleted. HEK293T cells were co-transfected with the indicated reporter genes and expression vectors and luciferase activities measured after 48 h.

2.7 Splice variant

The ability to mediate gene repression varies greatly between REST splice variants. Context-dependent pre-mRNA splicing creates alternate REST isoforms lacking key regulatory domains (Chen and Miller, 2013a).

Chen et al. (2017) recently proposed that the widespread variability in reported REST function is determined by alternative splice variants, which alter the proteins physiological requirements leading to differential data interpretation (Chen et al., 2017). For example, elevated nuclear REST was reported to be neurotoxic and harmful in ischaemia and Huntington's disease (HD), whereas Lu et al. (2014) demonstrated neuroprotective effects for increased nuclear REST during healthy ageing (Kaneko et al., 2014; Lu et al., 2014; Zuccato et al., 2007).

REST4 was the first reported alternative splice variant and is formed by inclusion of an additional exon (N3a, N3b, N3c, E4c or E5) which causes incorporation of a premature stop codon (Lee et al., 2000). Consequently, the translated REST4 protein contains only five of the eight original zinc finger domains, which reduces its binding affinities in competition with the full-length REST protein. At least 45 different predictive REST isoform variants can be produced by partially or complete skipping of the three constitutive exons (E2, E3, E4) (Fig. 23) (Chen and Miller, 2013a).

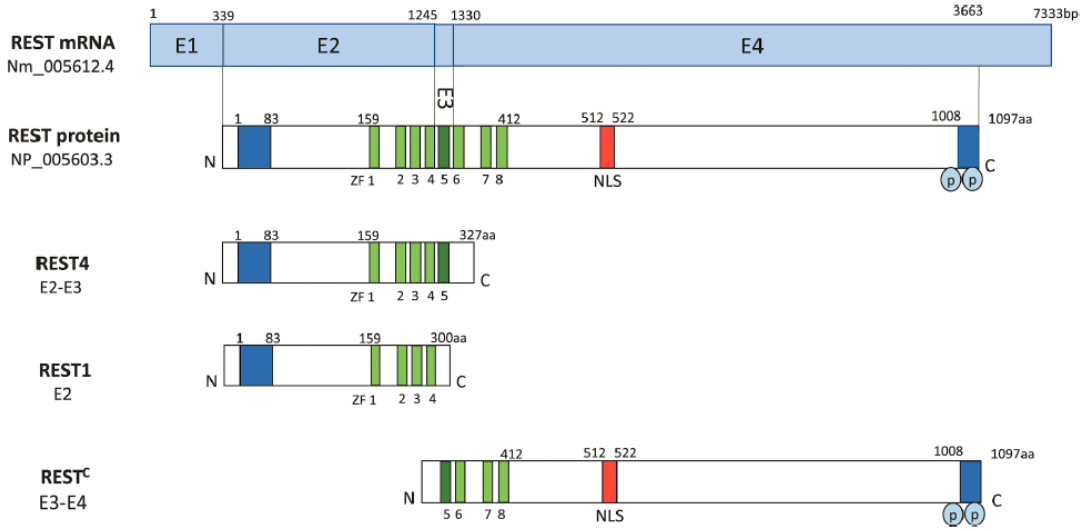


Figure 23: (M. Mampay and G.K. Sheridan, 2019) from Full-length REST and its splice variants. The major REST isoform has two repressor domains, eight zinc fingers (ZFs), a nuclear localisation signal (NLS) and a phosphodegron (P) motif, which is required for β-TrCP regulation (Westbrook et al., 2008). Alternative splicing of REST mRNA generates various REST isoforms which lack key regulatory domains, e.g. ZF5 and/or NLS which are thought to be required for nuclear localisation. Known isoform REST4 is read by exon E2-E3 and only contains the N-terminal repressor domain and ZFs 1–5. REST1 is translated through exon E2 and contains N-terminal repressor domain and ZFs 1–4. $REST^{C}$ is read by exon E3-E4 and only consists of the C-terminal repressor domain and ZFs 5–8 (Coulson and Concannon, 2016).

Accordingly, these alternatively spliced REST variants, which are mostly expressed in a cell-type/tissue-specific manner with individual differences, presumably contribute to the diverse, context-dependent regulation of REST gene expression. In neurons, REST frequently appears in its truncated, inactive form, REST4, which competes with full-length REST for the binding of target genes. Of note, the coexistence of the full length and the truncated forms attenuates the repression of REST target genes, and thus protects neuronal cells (Raj et al., 2011). Protection by REST4 was confirmed by studies of ethanol intoxication in control and REST knock-out mice (Cai et al., 2011).

2.8 REST activation: translocation from the cytosol to the nucleus

As a transcriptional modulator, the availability of REST and the translocation of the protein to the nuclear compartment is a key control point in regulating gene expression. Shimojo et al. (2001) originally identified the nuclear localisation sequence (NLS) to be responsible for the nuclear distribution signals (Shimojo et al., 2001). However, the splice variant REST4 lacks the NLS and still displays nuclear localisation. It was suggested that the essential domain for nuclear translocation was the zinc finger domain 5 (ZFD5), since REST1 lacks the corresponding amino acid sequence and is not targeted to the nucleus (Shimojo, 2006).

Furthermore, the same group identified the REST/NRSF interacting LIM domain protein (RILP). The LIM domain is a cysteine-histidine-rich, zinc-coordinating domain, consisting of tandemly repeated zinc fingers (Jurata L.W. and Gill G.N., 1998). LIM domains have been found to interact specifically with other LIM proteins (Feuerstein, R. et al., 1994; Schmeichel K.L. and Beckerle M.C., 1994). Such interaction is required for the nuclear translocation of REST/NRSF and REST4. Indeed, suppression of RILP expression by siRNA caused mislocalization of REST and REST4 to the cytosol, demonstrating that RILP controls REST and REST4 nuclear entry (Bassuk et al., 2008; Shimojo and Hersh, 2006; Shimojo et al, 2003).

REST nuclear translocation can be induced following psychological and physical stress where REST was reported to be upregulated in the neuronal nucleus in various rodent brain regions. In this context, the nuclear translocation was reported to blunt the glucocorticoid response towards future stressors (Korosi et al., 2010; Singh-Taylor et al., 2018). Furthermore, increased nuclear REST was found to decrease the survival of adult-born dentate granule cells (DGCs) and to accelerate the maturation of the remaining DGCs following psychological and physical stress (Chen et al., 2015). In contrast, neuronal REST was found to

translocate to the nucleus during healthy ageing in the human brain, thereby repressing ROS-induced cell death genes, and to protect ageing neurons from amyloid-β pathology (Kawamura et al., 2019; Lu et al., 2014). Furthermore, physical activity was shown to boost REST expression, which attenuated age-related neuro-inflammation in the ageing rodent hippocampus (Dallagnol et al., 2017). Interestingly, in neuropathological states, such as Alzheimer's disease and Parkinson's disease, which are both hallmarked by protein misfolding and aggregation, REST fails to translocate to the neuronal nucleus (Kawamura et al., 2019; Lu et al., 2014). However certain proteins also bind to REST to maintain its cytosolic location and prevent nuclear translocation, including huntingtin (HTT) and huntingtin-associated protein 1 (HAP1) (Shimojo, 2008; Zuccato et al., 2007). The role for the HTT had been elucidated by showing its modulation of the nuclear translocation of the transcriptional repressor REST/NRSF (Zuccato et al., 2003). Loss of this activity in mutant huntingtin contributes to the pathogenesis of Huntington disease (Thompson, 2003). Shimojo in 2008 identified dynactin p150Glued as a RILP interacting protein, similarly it was reported that dynactin p150Glued and huntingtin interact (Schroer T.A., 2004). Using in vitro expression of these proteins Shimojo demonstrated that huntingtin does not directly interact with REST/NRSF, but instead interacts with dynactin p150Glued, which in turn interacts with RILP, a protein shown to directly interact with REST/ NRSF. This data suggest that REST/NRSF, dynactin p150Glued, huntingtin, and RILP form a quaternary complex involved in the translocation of REST into the nucleus (Shimojo, 2008; Rigamonti et al., 2009). The complex additionally contains HAP1 that is the key regulator of REST/NRSF activity (Fig. 24). Indeed In the absence of HAP1, as in non-neuronal cells, this complex traffics REST/NRSF to the nucleus. In neuronal cells, HAP1 binds to huntingtin and causes the retention of the complex in the cytosol (Shimojo, 2008).

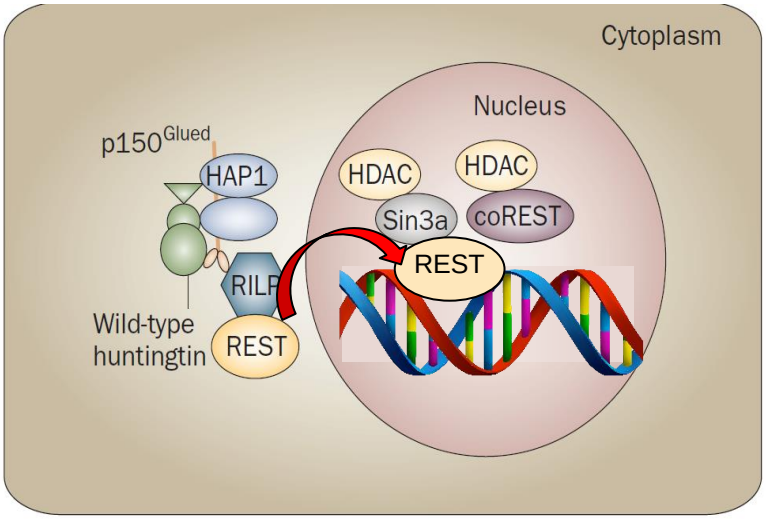


Figure 24: (modified from Rigamonti et al 2009) Schematic representation of the REST/NRSF translocation machinery.

2.9 REST cross talk with other transcription factors playing a role in the Homeostatic Plasticity

As previously mentioned, the level of REST in adult neurons is not always low. The transcriptional repressor can be transiently activated following various intrinsic and extrinsic neuronal insults such as seizures (Palm K. et al., 1999), global ischaemia (Calderone A. et al., 2003; Noh K.M. et al., 2012; Formisano L. et al. 2007) and stroke (Formisano L. et al. 2013). REST is a prime candidate to study in relation to stress-induced changes in neuronal gene expression and in the maintenance of neuronal function in the young and ageing brain including genes that regulate synaptic plasticity, neuronal differentiation, axonal growth, vesicular transport and ion conductance. For these reasons the transcription factor recently received an increasing amount of interest regarding its potential influence in modulating the neuronal stress response (Chen et al., 2015; Korosi et al., 2010; Lu et al., 2014; Otto et al., 2007; Singh-Taylor et al., 2018).

An overview of REST expression levels and its downstream effects on gene expression following different forms of cellular, neuropathological, psychological and physical stress in various brain regions is given in Table 1.

Table 1
Overview of REST expression levels and its downstream transcriptional role in modulating gene expression following different forms of cellular, neuropathological, psychological and physical stress in different regions of the human and rodent brain.

Context	REST	Source	Target gene expression	Epigenetic markers	Reference
Ischaemia, hypoxia and seizures					
Global ischaemia	REST ↑	Hippocampal CA1 neurons Rat	CK↓	N/A	Kaneko et al. (2014)
Global ischaemia	REST ↑	Hippocampal CA1 neurons Rat	GluR2 ↓	N/A	Calderone et al. (2003)
Ischaemic stroke	REST ↑	Hippocampal CA1 neurons Rat	Gria2, Grin1, Chrb2, Nefh, Trpv1, Chrm4, Syt6 ↓ GluA2, GluN1, GluN2B ↓	H3K9ac ↓ H3K14ac ↓ H3K9me2 ↑	Noh et al. (2012)
Hyperthermia-induced epilepsy	REST ↑	Hippocampal neurons Rat	Hcn1 ↓	N/A	Patterson et al. (2017)
Kainic acid-induced epilepsy	REST ↑	Hippocampus Rat	Calb1, Glra2, Grin2a, Hcn1, Kcnc2, Klf9, Lrp11, Myo5b, Stmn2 ↓	N/A	McClelland et al. (2014)
Neurodegenerative diseases					
Alzheimer's Disease	*Rest* ↓	PFC neuronal nuclei Mouse	Bcl2, Sod1, Foxo1 ↓	H3K9ac ↑	Lu et al. (2014)
Alzheimer's Disease	*Rest* ↓	Neuron-derived extracellular vesicles Human	N/A	N/A	Ashton et al. (2017)
Parkinson's Disease	REST↓	Dopaminergic neurons Human	N/A	N/A	Kawamura et al. (2019)
Huntington Disease	REST ↑	Cerebral cortex Human	Bdnf ↓	N/A	Zuccato et al. (2007)
Prion Diseases	REST ↑	Primary cortical neurons Rat	FOXO1, cytochrome *c*, Caspase 3 ↑	N/A	Song et al. (2016)
Psychological and physical stress					
Chronic social defeat	*Rest* ↑	Dentate granule cells Mouse	GluN2B↓	N/A	Chen et al. (2015)
Chronic traumatic stress	*Rest* ↑	Prefrontal cortex Rat	CCR5 ↑	N/A	Mou and Zhao (2016)
Augmented maternal care	*Rest* ↑	Neuronal Rat	Crh ↓	MeCP2 ↑ H3K27me3 ↑ H3K9me2 ↑	Singh-Taylor et al. (2018)
Augmented maternal care	*Rest* ↑	Hypothalamic PVN Rat	Crh ↓, vGlut2 ↓	N/A	Korosi et al. (2010)
Maternal Separation	*Rest4* ↑	mPFC Rat	Glur2, Nr1, Chr, CamKIIα, L1, Adcy5, 5Htr1a, Kcnc1 ↑ Nav1 ↓	Mir132, -124, -9-1, -9-3, -212, and -29a ↑	Uchida et al. (2010)

Table 1 (from Mampay M. and Sheridan G.K, 2019) Owerview of REST expression levels following following different forms of cellular, neuropathological, psychological and physical stress.

REST interacts with also other transcription factor, such as NPAS4 (Bersten et al., 2014). NPAS4 is a transcription factor present in both excitatory and inhibitory neurons, plays a role in the development of inhibitory synapses by regulating the expression of activity-dependent genes, which in turn control the number and the strength of GABA-releasing synapses that form on excitatory neurons (Lin et al., 2008).

2.10 The NPAS4–BNDF pathway in the activity-dependent up-scaling of inhibitory inputs

NPAS4 is an early gene, rapidly activated by neuronal hyperactivity, able to induce the expression of many other genes crucial for the formation and maintenance of inhibitory synapses onto excitatory neurons (Hong EJ et al., 2008; Lin Y et al. 2008). Greenberg and colleagues have reported that neuronal hyperactivity, (evoked by treatments with KCl or bicuculline,) increased NPAS4 expression (Lin Y et al., 2008) which in turn induced increased BNDF expression and release from excitatory neurons (Lin et al., 2008; Bloodgood et al., 2013; Spiegel et al., Cell. 2014) thereby promoting an increased number of inhibitory synapses on excitatory neurons.

In particular, BDNF expression is consistently reduced by almost twofold in cultures expressing Npas4-RNAi compared with control cultures and primary cultures from NPAS4$^{-/-}$ mice. These data showed a similar decrease in depolarization-induced BDNF expression compared with their wild-type littermates (Lin Y et al., 2008).

Brain-derived neurotrophic factor (BDNF) stood out because it had previously been shown to regulate GABAergic synapse maturation and function (Chattopadhyaya et al., 2004; Marty S. et al., 2000; Huang Z.J. et al. 1999; Rutherford L.C. et al., 1997; Seil F.J. et al., 2000). The BDNF gene has many promoters and the activity-dependent BDNF mRNA transcripts are mainly controlled by promoters I and IV (Aid T. et al., 2007; Tao X. et al. 1998). BDNF is well known for its capability to be expressed and released only by excitatory neurons (Rutherford Lana C. et al., 1997; Spiegel I. et al., 2014;) and for its effectiveness in inducing functional up-scaling of GABAergic synapses (Fredrick J. Seil and Rosemarie Drake-Baumann, 2000; Huang ZJ et al., 1999; Marty S. et al., 2000). BNDF is one of the main target genes of NPAS4; it is produced and released by excitatory neurons but not by inhibitory neurons (Tyler W.J. and Pozzo-Miller, J. Neurosci 2001; Lessmann et al., 1994; Lessman and Heumann, 1998; Schinder et al., 2000) and is well known to regulate the number and the function of inhibitory synapses (Marty S et al., 2000; Genoud

C. et al., 2004; Baldelli P et al., 2005). However, BDNF release dysregulation, which generates an excessively intense or prolonged BDNF stimulation, was reported to play a causative role in the process of epileptogenesis, probably due to the BDNF capability of potentiating the strength of excitatory glutamatergic transmission (Garringa-Canut et al., 2006).

Transcription of BDNF mRNA is robustly induced by neuronal activity in a manner that is highly correlated with synaptic development and plasticity, while BDNF protein has numerous effects on synapses during development and in adult animals, where it is capable of modulating both functional and morphological aspects of excitatory and inhibitory synapses (Lu, 2003). Moreover, neuronal activity-dependent transcription of BDNF has been shown to be essential for development of cortical inhibition (Hong et al., 2008). BDNF gene is located on chromosome 11 and is transcribed from multiple promoters located upstream of distinct 5' noncoding exons to produce a heterogeneous population of BDNF mRNAs (Aid et al., 2007). According with the results of Timmusk's group both rat and mouse BDNF genes consist of eight 5' untranslated exons and one protein coding 3' exon (Aid et al., 2007). BDNF mRNA transcripts are characterized by alternative exon usage at the 5' untranslated region (UTR) and a common coding sequence (CDS) (Parrini et al., 2017). The expression of these alternative BDNF transcripts is controlled by multiple promoters that differentially contribute to activity-dependent BDNF induction (Aid et al., 2007). Transcription can be initiated by at least nine promoters, each of which is regulated in a developmental, tissue-specific, and activity-dependent manner. For example, neuronal depolarization enhances levels of exons I and IV (Aid et al., 2007). BDNF is an important mediator of activity-dependent functions of the nervous system and its expression is dysregulated in several neuropsychiatric disorders. The investigation of the regulation of BDNF transcription by membrane depolarization allowed to identify an asymmetric E-box-like element, PasRE (basic helix-loop-helix (bHLH)-PAS transcription factor response element), in BDNF promoter I and demonstrated that

binding of NPAS4/ARNT2 is crucial for neuronal activity-dependent transcription from promoter I (Pruunsild P. et al., 2011). The authors also showed that binding of CREB (cAMP response element-binding protein) to the cAMP/Ca^{2+} response element (CRE) in BDNF promoter IV is critical for its activity-dependent transcription but its full induction is also dependent on ARNT2 and NPAS4 binding to a PasRE in promoter IV.

Greenberg's team show that in the mouse hippocampus behaviorally driven expression of NPAS4, coordinates the redistribution of inhibitory synapses onto CA1 pyramidal neuron, simultaneously increasing inhibitory synapse number on the cell body while decreasing the number of inhibitory synapses on the apical dendrites. This rearrangement of inhibition is mediated in part by the NPAS4 target gene brain derived neurotrophic factor (BDNF), which specifically regulates somatic, and not dendritic, inhibition. These findings indicate that sensory stimuli, by inducing NPAS4 and its target genes, differentially control spatial features of neuronal inhibition in a way that restricts the output of the neuron while creating a dendritic environment that is permissive for plasticity. (Bloodgood et al., 2013).

3. Aim of the project

The aim of my project was to investigate the role played by the negative transcription factor, REST/NRSF, in the synaptic homeostasis evoked by neuronal hyperactivity onto inhibitory GABAergic synapses.

I used various technical approaches: patch-clamp recordings, immunocytochemistry, biochemistry, molecular biology and functional imaging.

I investigated at functional and structural level the hyperactivity-dependent up-scaling of the inhibitory GABAergic synapses.

I observed that this process is characterized by a strong postsynaptic target specificity, it is strictly REST-dependent and involves other transcriptional regulators such as NPAS4 and the neurotrophin, BNDF. Thus, I investigated in detail the transcriptional and proteomic changes of REST and REST-target genes relevant for the mechanism of action underlying the synaptic homeostasis of the inhibitory inputs.

4. RESULTS

4.1 The experimental model of neuronal hyperactivity

Homeostatic plasticity of GABAergic interneurons was studied in cultured hippocampal and cortical neurons in vitro. We evoked a condition of chronic neuronal hyperactivity by treating primary hippocampal or cortical neurons (18 div) with the non-selective potassium channel blocker, 4-aminopyridine (4AP; 100 µM).

Application of 4AP is an effective method to boost the rate of spontaneous firing and synaptic activity that is already present in functionally mature (>17 div) cultured neuronal networks (Fig. 25). On the contrary, 4AP was ineffective in immature neurons where spontaneous activity is still moderate, and mainly TTX-insensitive (<10-12 div) (Pecoraro et al., 2017).

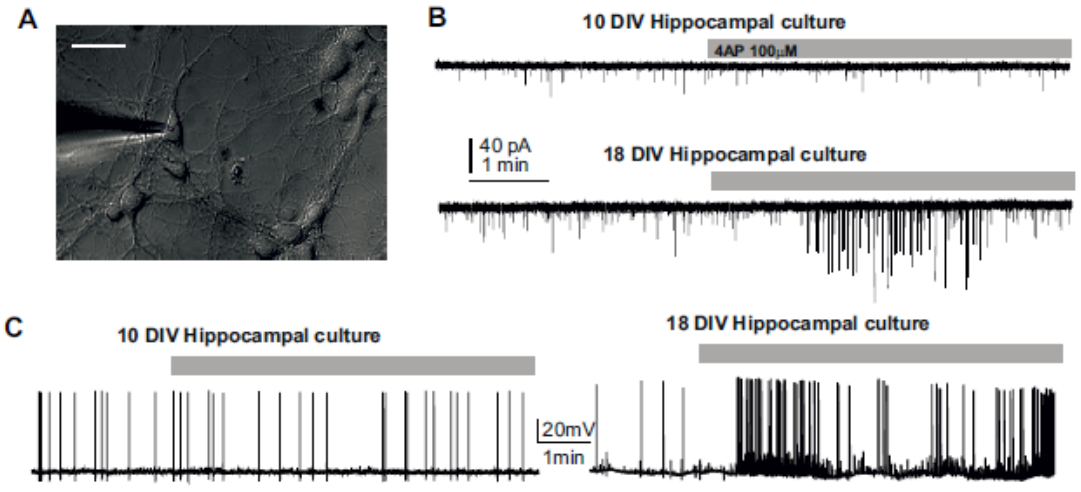


Figure 25: (from Pecoraro-Bisogni et al. 2017): Acute 4AP treatment induces hyperactivity only in mature cultured neurons A). Representative microphotograph of a patched neuron in a low-density culture. Scale bar, 100 m. B) Voltage-clamp recordings (Vh = -70mV) of spontaneous postsynaptic currents in 10 div (upper trace) and 18 div (lower trace) hippocampal cultures. C) Current-clamp recordings of spontaneous action potentials in 10 div (left trace) and 18 div (right trace)

hippocampal cultures. The horizontal gray bar in panels B,C represents the treatment with 4AP (100 μM).

To study homeostatic plasticity induced by chronic (24, 48 and 96 h) neuronal hyperactivity in inhibitory neurons we used the "GAD67-GFP" transgenic mice (Tamamaki N. et al., 2003).

In these mice, the fluorescent protein reporter, GFP, was specifically expressed in GABAergic neurons, under the control of the endogenous GAD1 promoter. GFP substitute the introns in the 5' and 3' flanking region in one of the two GAD67 alleles. Using these knock-in mice we were able to clearly distinguish GAD67-GFP positive interneurons from GAD67-GFP negative excitatory neurons (Fig. 26 A, B).

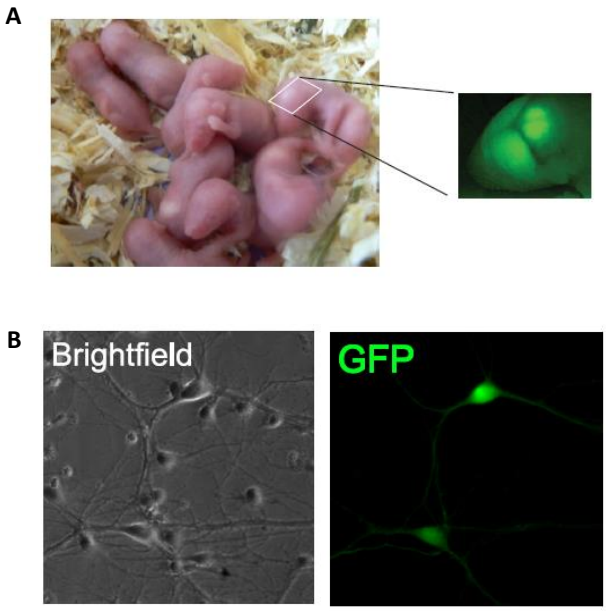


Figure 26: Knock-in GAD67-GFP mice. (A) Newborn pups at P0-P1. (B) Bright field (left) and fluorescence (right) microphotographs of hippocampal cultured neurons (16 div).

4.2 Validation of the experimental strategy used for investigating the role of REST in the process of homeostatic plasticity

In our previous works (Pozzi et al., 2013; Pecoraro-Bisogni et al., 2017), we successfully used shRNA to block the increase of REST mRNA level induced by neuronal hyperactivity. shRNA was introduced inside the cells through a lentiviral vector, that required 6/7 days after infection to reach maximal expression level.
However, such time delay, necessary to obtain an expression level of shRNA able to inhibit new REST transcription, introduce some limitations.
In particular, the reduction of REST protein takes time (6/7 days) and when we compared the effect of 4AP on neurons treated with scramble-RNA or shRNA, basically we are working in two different neuronal populations that are not easily comparable. In other words, the increased hyperactivity that we observed in neurons pre-treated with shRNA and then stimulated for 48 h with 4AP probably cannot be exclusively ascribed to the suppression the hyperactivity-dependent increase of REST transcription, but it could be partially depend on lower basal level of REST that in turn induces higher level of REST targets genes making neurons more responsive to the 4AP-stimulation.
To avoid such limitations, in this new set of experiments, we adopted a different approach aimed to block as much faster REST activity induced by neuronal hyperactivity without interfering with the REST expression level.
We used a decoys oligo-deoxynucleotides (ODN) that sequesters both basal REST and new REST synthetized in response to neuronal hyperactivity, thus ODN will permit a faster block of the activity of REST and consequently a better dissection of the effects of REST activation and expression change in response to hyperactivity.

Cultured neurons were treated with (200nM) decoys oligo-deoxynucleotides (ODNs), that act as surrogate binding sites for transcription factors and sequester the native transcription factor from its genomic binding sites (Fig. 27). Decoy ODNs

have been successfully used to rapidly inhibit REST activity but not its expression (Soldati et al., 2011). ODNs are double-stranded oligo-deoxynucleotides corresponding to the DNA-binding element (RE1) of REST and act sequestering it, thereby abrogating its transcriptional activity. ODNs were designed with phosphorothiolate modification on the first three nucleotides in order to avoid their degradation (Lee et al. 2003; Osako et al. 2007).

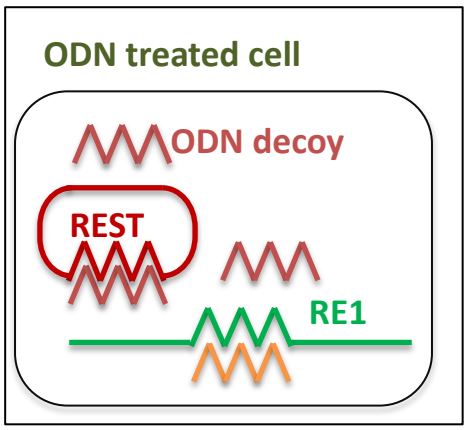


Figure 27: Schematic representation of the ODN activity. Oligo-deoxynuclotides (ODNs) with a complementary sequence to RE1. ODN binds REST abrogating its capability to modulate the transcriptional activity.

In order to verify ODN capability to efficiently permeate through the neuronal plasma membrane we incubated for 12 h and 24 h cultured hippocampal neurons at (17div) with a ODN tagged to a fluorescent probe Cyanine-3, Cy3 (Cy3-ODN) at two different concentrations 100nM and 200nM. After 12 h of incubation the 60% of the cultured neurons were loaded with ODN-Cy3 while after 24 h the fraction of loaded cells reached the 80% (Fig. 28).

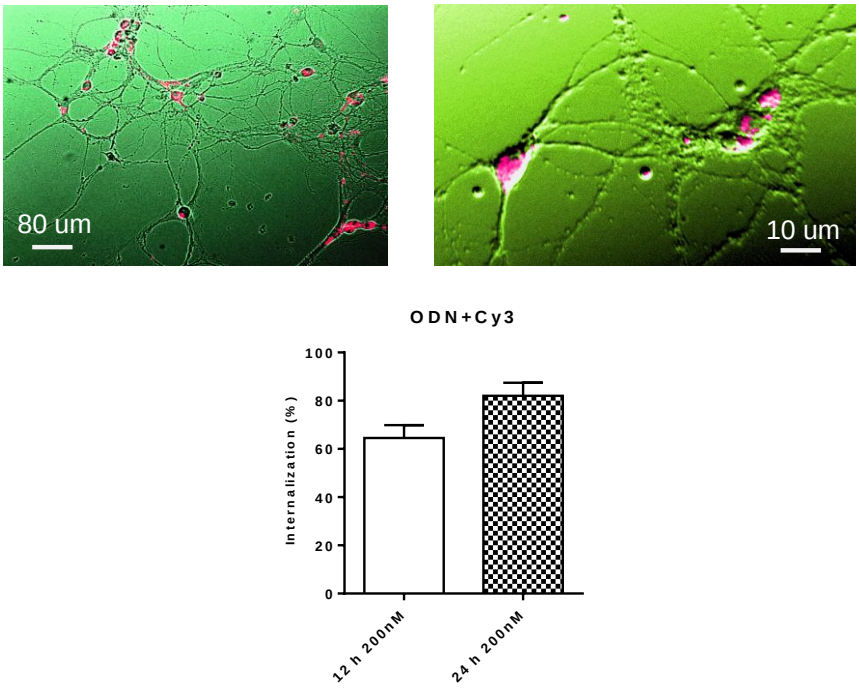


Figure 28: Cy3-ODN permeates through the membrane. Bars show internalization (%) of hippocampal low density neurons at div 17 treated with ODN-Cy3 (200nM) for 12 h and 24 h .

When the subcellular distribution of Cy3-ODN was analyzed at high magnification, in unstimulated (ctrl) hippocampal neurons we detected the most of the Cy3-ODN in the cytosol while only a small fraction of the Cy3-ODN reached the nucleus (Fig. 29). This result is in accord with the capability of ODN to bind both REST protein at its DNA binding site and the RE1 sequence in the promoter region of the REST target genes. This distribution demonstrates that in un-stimulated neurons REST is inactive because of it is manly resident into the cytoplasm.

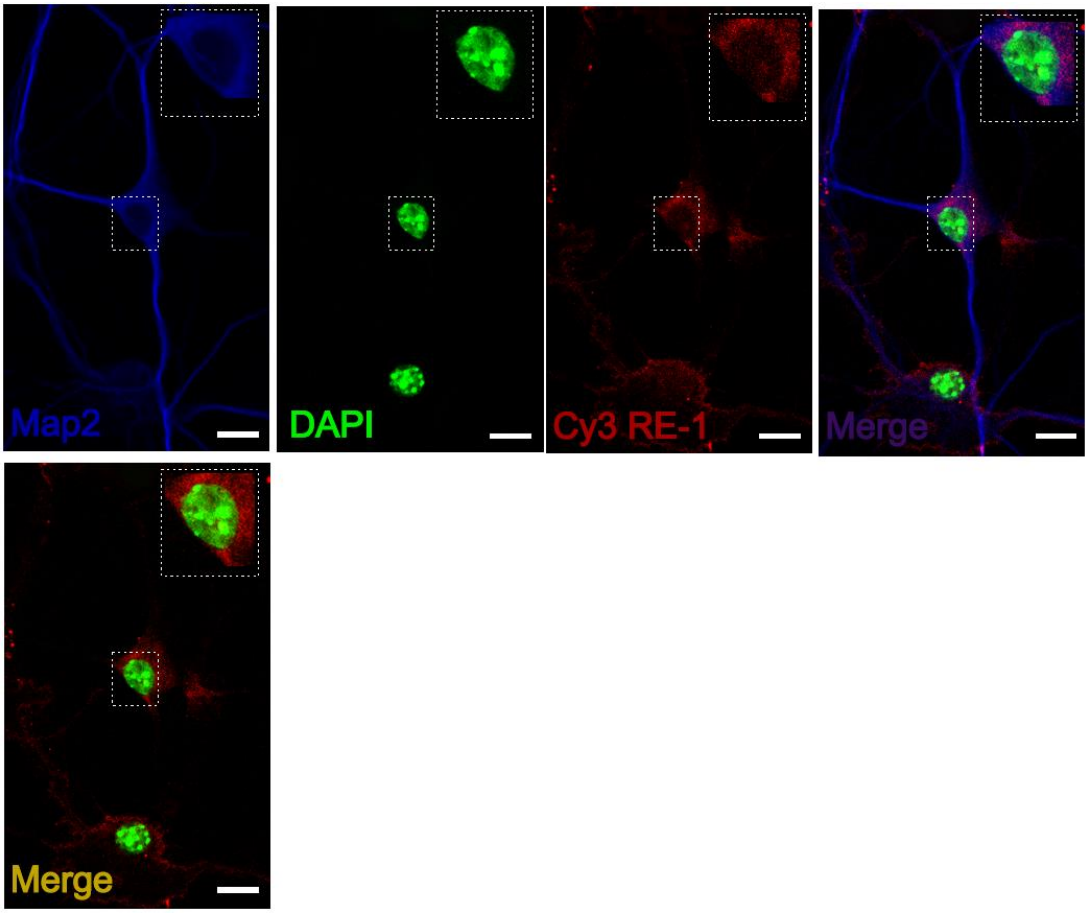


Figure 29: REST localization in unstimulated (ctrl) condition. Rrepresentative image acquired using a ×63 objective in a Leica SP8 confocal microscope.

However, it is well known (Hwang J.Y. et al., 2017; Kaneko et al., 2014; Rigamonti et al., 2009) that neuronal insults or hyperactivity enhance both the translocation from the cytosol to the nucleus (Shimojo et al., 2001) of the low of level of REST already present in the cell, and in parallel increase REST expression favoring REST transcription and synthesis (Pozzi et al., 2013; Pecoraro-Bisogni et al., 2017).

Thus, Cy3-ODN was also used for further verifying whether neuronal hyperactivity was able to induce REST translocation from the cytosol to the nucleus and REST over-expression.

This analysis was performed on low density hippocampal neurons (17 div) obtained from GAD67-GFP transgenic pups to dissect a possible change of REST localization/expression in excitatory and inhibitory neurons (Fig. 30 A, B).

We initially compared REST partition ($AreaCy3_{nucleus}/AreaCy3_{cyto}$) between the cytosol and the nucleus in control condition (ODN-Cy3 24 h; empty black bars) and in neurons maintained for 1 h in a condition of hyperactivity (4AP 1h followed by ODN-Cy3 24 h; empty red bars).

This comparison revealed that in both excitatory and inhibitory neurons REST efficiently translocate from the cytosol to the nucleus upon a stimulation of 1 h with 4AP ($AreaCy3_{nucleus}/AreaCy3_{cyto}$ = 68 %) whilst it remains mainly localized in the cytosol ($AreaCy3_{nucleus}/AreaCy3_{cyto}$= 30 %) in control (unstimulated) condition (Fig. 30 C).

Moreover, stimulating with 4AP neurons that were previously pretreated with ODN-Cy3 (ODN-Cy3 24 h followed by 4AP 1 h; gray bars), we validated the capability of ODN to efficiently abrogated REST translocation to the nucleus in response to neuronal hyperactivity, that indeed showed a % of partition $AreaCy3_{nucleus}/AreaCy3_{cyto}$= 30 %) comparable to the unstimulated condition (Fig. 30 C).

Finally, we also compared the percentage of Cy3-positive area in respect of the total area, in the cytosol ($Aera_{Cy3}$ cyto/=$Area_{TOT}$ cyto) and in the nucleus ($Aera_{Cy3}$ nucleus/=$Area_{TOT}$ nucleus) in both excitatory end inhibitory neurons un-stimulated (ODN-Cy3 24 h; empty black bars), stimulated with 4AP (4AP 1 h followed by ODN-Cy3 24 h; empty red bar) or stimulated with 4AP after the ODN-pre-treatment (ODN-Cy3 24 h followed by 4AP 1 h; gray bars) (Fig. 30 D).

This analysis revealed that in excitatory and inhibitory neurons, hyperactivity evoked by 4AP (1 h) increased the area positive to REST in both the nucleus (Fig. 29 D) and the cytoplasm (Fig. 30 E). While this effect was expected in the nucleus, because of the nuclear translocation of REST, the increase in the cytoplasm clearly demonstrated that neuronal hyperactivity enhanced REST expression level in both excitatory and inhibitory neurons. Notably, the pre-treatment with ODN, did not inhibit the increase of REST expression, suggesting the possibility of an auto-

inhibitory action of basal-REST on itself (Fig. 30 D, E), that indeed we have successively confirmed with immunoblot experiments (see Fig. 31 and 45 A).

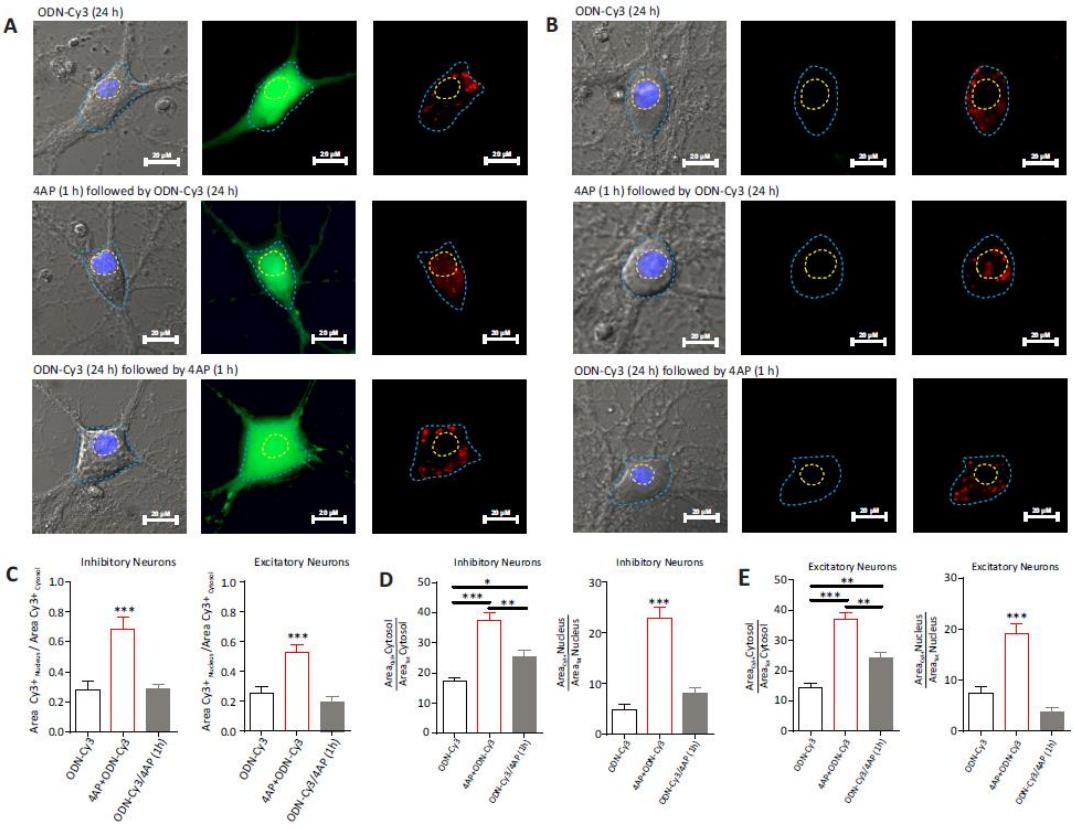


Figure 30. ODN-Cy3 staining, reveals cytoplasmic and nuclear localization of REST. **(A, B)** Representative fluorescence images of hippocampal inhibitory (A) and excitatory (B) neurons (18 div) treated with: 1) ODN for 25 h (1st row), 2) with 4AP (1 h) followed by ODN-Cy3 for 24 h (2nd row) or with 3) ODN-Cy3 for 24 h followed by 1h in 4AP (3rd row).

(C) Bar plots represents mean ± SEM of REST partition between the cytosol and the nucleus ($AreaCy3_{nucleus}/AreaCy3_{cyto}$) of excitatory (lefts; n=33) and inhibitory (right; n=33) neurons. One way ANOVA followed by Kruskal-Wallis multiple comparison test. *P<0.05; **P<0.01; ***P<0.001.

(D, E) Bar plots represents mean ± SEM of the percentage of Cy3-postive area in respect of the total area of the cytosol (D; $Aera_{Cy3}cyto/=Area_{TOT}cyto$; n=33) and of the nucleus (E; $Aera_{Cy3}nucleus/=Area_{TOT}nucleus$; n=33) in both excitatory end inhibitory neurons. One way ANOVA followed by Kruskal-Wallis multiple comparison test. *P<0.05; **P<0.01; ***P<0.001.

Finally, we also validated ODN capability to block REST transcriptional regulation. We and other research groups (Pozzi et al., 2013; Pecoraro et al., 2017;

McClelland et al. 2011; 2014) have previously shown that some REST target genes: HCN1, Nav1.2 and Syn1 undergoing a REST-dependent down-regulation upon neuronal hyperactivity. Here we treated cultured hippocampal neurons (18 div) with 4AP for 24 h observing the expected down-regulation of the mRNA level of the three REST target genes (Fig. 31). Such effect was fully suppressed when neurons were co-treated with 4AP+ODN, demonstrating its capability to inhibit REST-mediated transcriptional inhibition activated by neuronal hyperactivity. Notably, we observed that ODN treatment significantly increased the level of REST mRNA, confirming that the basal level of REST exerted a negative control on REST transcription (Fig. 31).

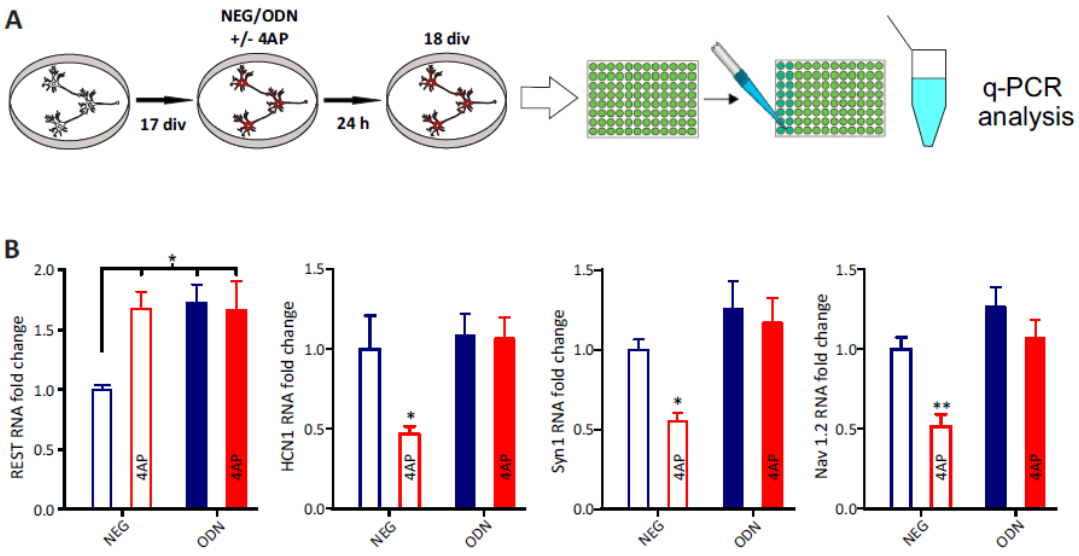


Figure 31: ODNs decoy validation: **(A)** Experimental scheme of the treatment that precedes q-PCR analysis **(B)** Bar plots show Means ± SEM fold change for HCN1, Syn1 and Nav1.2 mRNA transcript in cortical neurons treated with NEG; NEG+4AP; ODN and ODN+4AP at 24 h. Two-way anova followed by Tukey multiple comparison test *P<0.05. n=9 from three independent neuronal preparations.

4.3 Opposite effect of neuronal-hyperactivity on the spontaneous EPSCs and IPSCs

Spontaneous synaptic activity recorded in the absence of any pharmacological blocker of ligand- and voltage-gated conductance and in absence of external input is a robust feature of cultured neuronal networks *in vitro* between 14 and 30 DIV. Indeed, cultured neurons typically reach their full functional maturation at 14 DIV and maintain such activity for the subsequent 2 weeks (Pinheiro P.S. and Muller C., 2008).

Spontaneous synaptic activity can be defined as a mixture of inhibitory and excitatory synaptic currents of variable amplitude. The largest synaptic currents (I>30pA) are generated by spontaneous action potentials (APs), while the smallest currents (6pA<I<30pA), correspond to the miniature postsynaptic events due to spontaneous synaptic vesicle fusion independent on the calcium influx due to the AP activation. Such spontaneous synaptic currents can be used to monitor the overall electrical activity of the whole neuronal network (Luhmann et al., 2016).

In order to investigate the effect of neuronal hyperactivity on the spontaneous electrical activity of the network and in order to identify the possible role of REST in the homeostatic control of such condition of hyperactivity, we investigated spontaneous postsynaptic currents (sPSCs; I>30pA) in low density (120 cells/mm^2) cortical cultured neurons, prepared from GAD67-GFP knock-in mice postnatal day 0-2 at (P0/2) and recorded at 19 days in vitro (DIV). Neurons were treated for 48 h with: (i) either NEG (the negative control decoy ODN; NEG) or NEG and 4AP (NEG; NEG+4AP) and (ii) ODN (corresponding to the DNA-binding element, RE1) or ODN and 4AP (ODN; ODN+4AP) (Fig. 32 A).

The analysis of the spontaneous excitatory synaptic events, revealed that the frequency of sEPSCs was not affected while the amplitude of the sEPSCs was significantly decreased by the treatment for 48 h with 4AP (Fig. 32 B, C). On the contrary sEPSCs decay, rise and area were not affected (Fig. 32 E, F, G). Notably,

when the 4AP treatment was performed in presence of ODN, the decrease of the sEPSCs amplitude was suppressed (Fig. 32 B, C). This results fully confirmed the REST dependent down-scaling of the strength of glutamatergic excitatory synapses that we have previously demonstrated in hippocampal neurons maintained for 48 h in 4AP (Pecoraro et al., 2017).

In order to investigate whether also inhibitory inputs could be involved in such REST-dependent process of homeostatic plasticity, we focused our attention onto sIPSCs amplitude and frequency. We focused our analysis to the largest sIPSCs (>30pA), that are generated by spontaneous action potential, disregarding the smallest (6<I<30) sIPSCs that correspond to the miniature postsynaptic currents, that we have here subsequently isolated thanks to recordings in presence of TTX and specifically analyzed.

Notably, both the mean amplitude and frequency of the sIPSCs were increased by neuronal hyperactivity and these effects were both lost when 4AP was applied in presence of ODN, testifying the crucial role played by REST in the upscaling of the strength of the inhibitory inputs (Fig. 32 A, C).

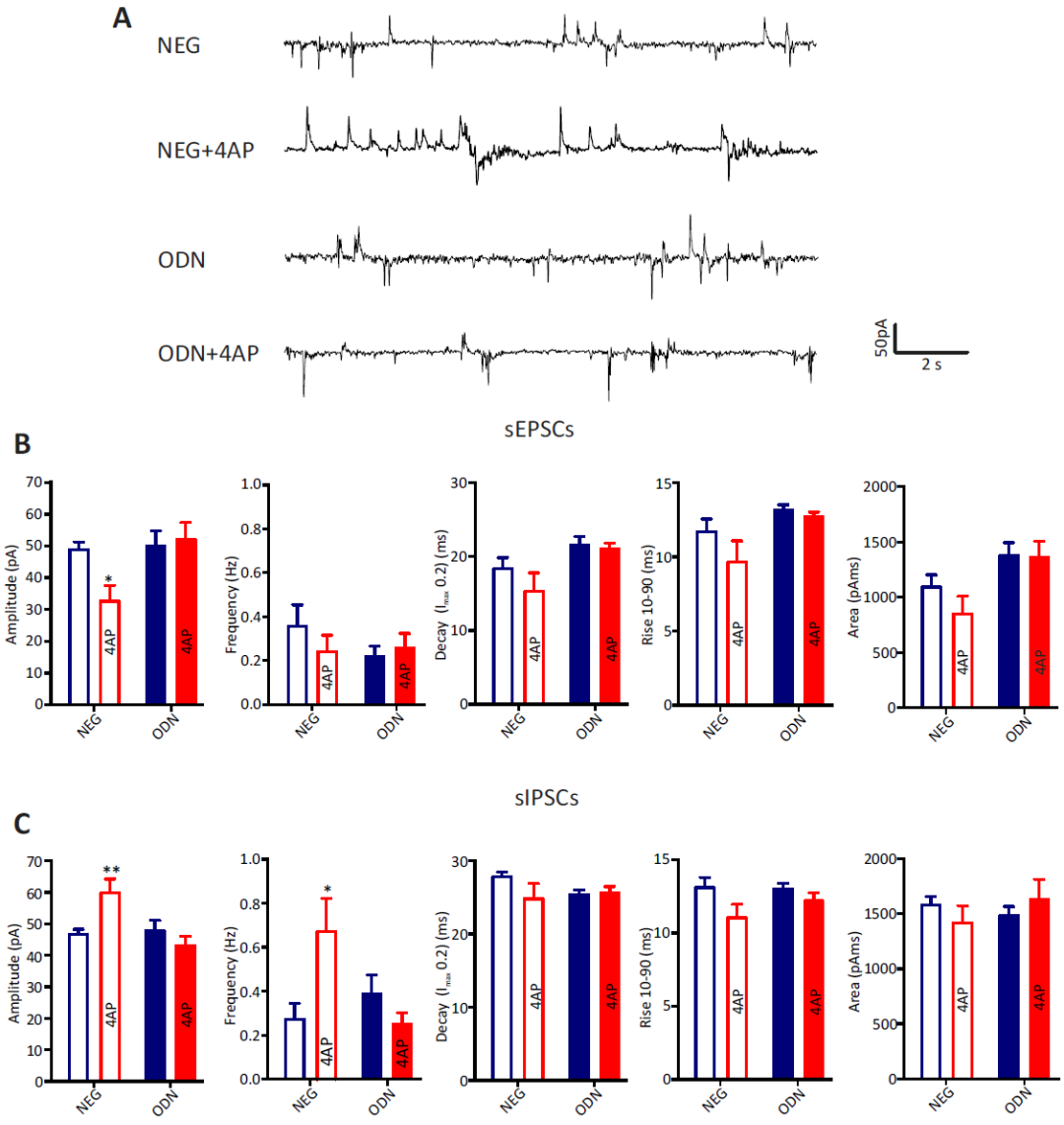


Figure 32. sPSCs in low density cultured cortical neurons (19 div). **(A)** Representative whole-cell voltage-clamp traces of sPSCs recorded at -40mV, in voltage clamp continuous, in neurons treated with NEG, NEG+4AP, ODN and ODN+4AP (upper panel). Note that at this holding potential sIPSCs are outward currents while sEPSCs are inward currents. **(B)** Bar plots represent mean ± SEM of sEPSCs amplitude, frequency, rise decay area of NEG (n=16), NEG+4AP (n=14), ODN (n=14) and ODN+4AP (n=17) treated cells. **(C)** Bar plots represent mean ± SEM of sIPSCs amplitude, frequency rise decay area of NEG (n=16), NEG+4AP (n=14), ODN (n=14) and ODN+4AP treated cells (n=17). SEM ***P<0.001 (Two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups)

4.4 Neuronal hyperactivity induces a REST-mediated increase of eIPSCs onto excitatory neurons

To investigate more precisely the mechanism underlying the enhancement of the amplitude of sIPSCs we studied evoked (e)IPSCs in cultured hippocampal neurons (18-21 div) obtained from GAD67-GFP knock-in mice (Fig. 33).

Extracellular stimulation in loose-patch configuration was used to evoke action potentials (Aps) in presynaptic inhibitory interneurons (GAD67-GFP positive) and patch-clamp recordings was obtained from both postsynaptic excitatory (GAD67-GFP negative) and inhibitory (GAD67-GFP positive) neurons.

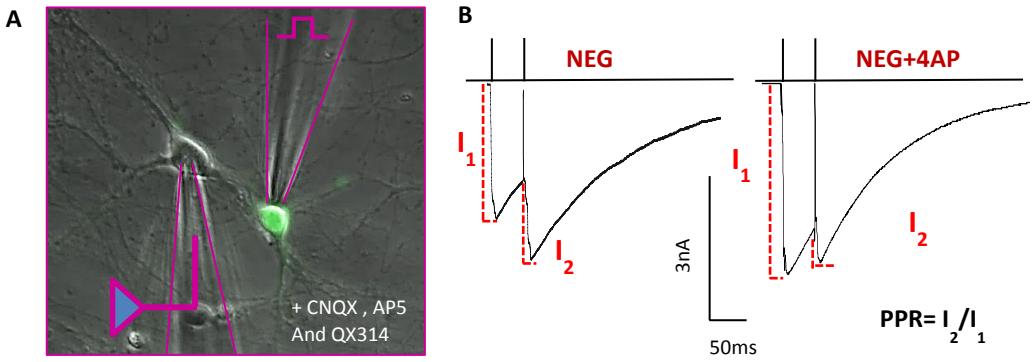


Figure 33: The experimental configuration of the eIPSCs onto excitatory neurons. (A) The stimulation electrode is located on the GABAergic neuron (GAD67-GFP+) and a recording electrode patching the excitatory cell (GAD67-GFP-). (B) A paired pulse protocol stimulation is used to record eIPSCs in paired cells (IPI = 50ms).

When eIPSCs were recorded from an excitatory neuron (Fig. 34 A, B), we observed an increase in the mean amplitude upon 4AP treatment (Fig. 34 A). Interestingly this effect was suppressed upon 4AP+ODN treatment. These data suggest an active process of REST-mediated synaptic homeostasis in the GABAergic synapses making contact onto excitatory target neurons.

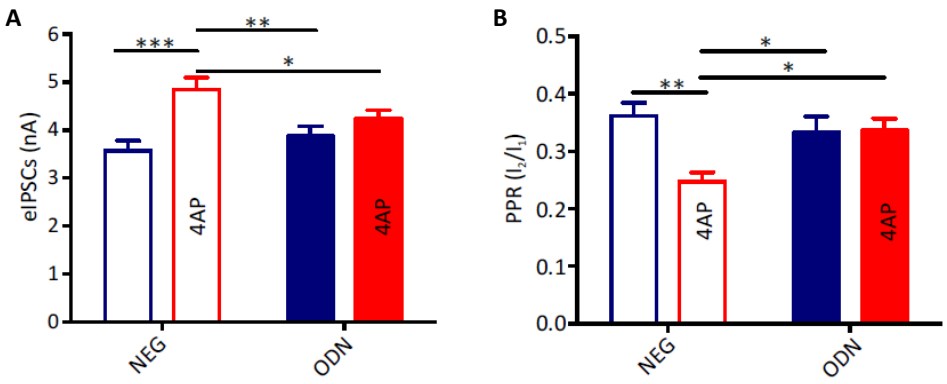


Figure 34: REST-mediated increase of the eIPSCs in 4AP treated neurons is accompanied by an increase of the paired pulse depression. (A) The amplitude of the first eIPSC in the pair recordings protocol and the PPR (B) of NEG (n=35), NEG+4AP (n=33), ODN (n=33) and ODN+4AP (n=32) treated cells are shown as means ± SEM *P<0.05, **P<0.01, ***P<0.001 (Two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups).

To fully exploit the enhancement of the eIPSCs evoked by 4AP, we investigated paired-pulse depression, an exquisitely presynaptic form of short-term plasticity that mainly depends on the initial release probability. We observed that the enhancement of the eIPSCs evoked by 4AP, was accompanied by an increase of the paired pulse depression (Fig. 34 B), revealed by the decreased paired pulse ratio (PPR) in NEG+4AP-treated neurons and again this effect was suppressed in neurons treated with 4AP and ODN. As an increase in the initial release probability (Pr) is the main drive of depression, these data indicate that the increased amplitude of the eIPSCs could arise from a REST-dependent increased Pr.

To better define to what extent neuronal hyperactivity modulates the quantal parameters of synchronous GABA release, we estimated the readily releasable pool for synchronous release (RRP) and the probability of release of any given SV in the RRP (Pr) using cumulative amplitude analysis.

When neurons were challenged with a train of 2s at 20 Hz (40 action potentials), a significant depression of eIPSCs became apparent during the stimulation period irrespective of the amplitude of the first current in the train (Fig. 35 A-C).

Accordingly, the cumulative profile of the eIPSC amplitude displays a rapid rise followed by a slower linear increase reflecting the equilibrium between depletion and constant replenishment of the RRP (Fig. 35 C). The graphical extraction of the RRPsyn and Pr from the cumulative curves of each individual neuron showed that the increase in the single eIPSC amplitude induced by 4AP is due to an increase of both the mean Pr and RRPsyn (Fig. 35 D-E).

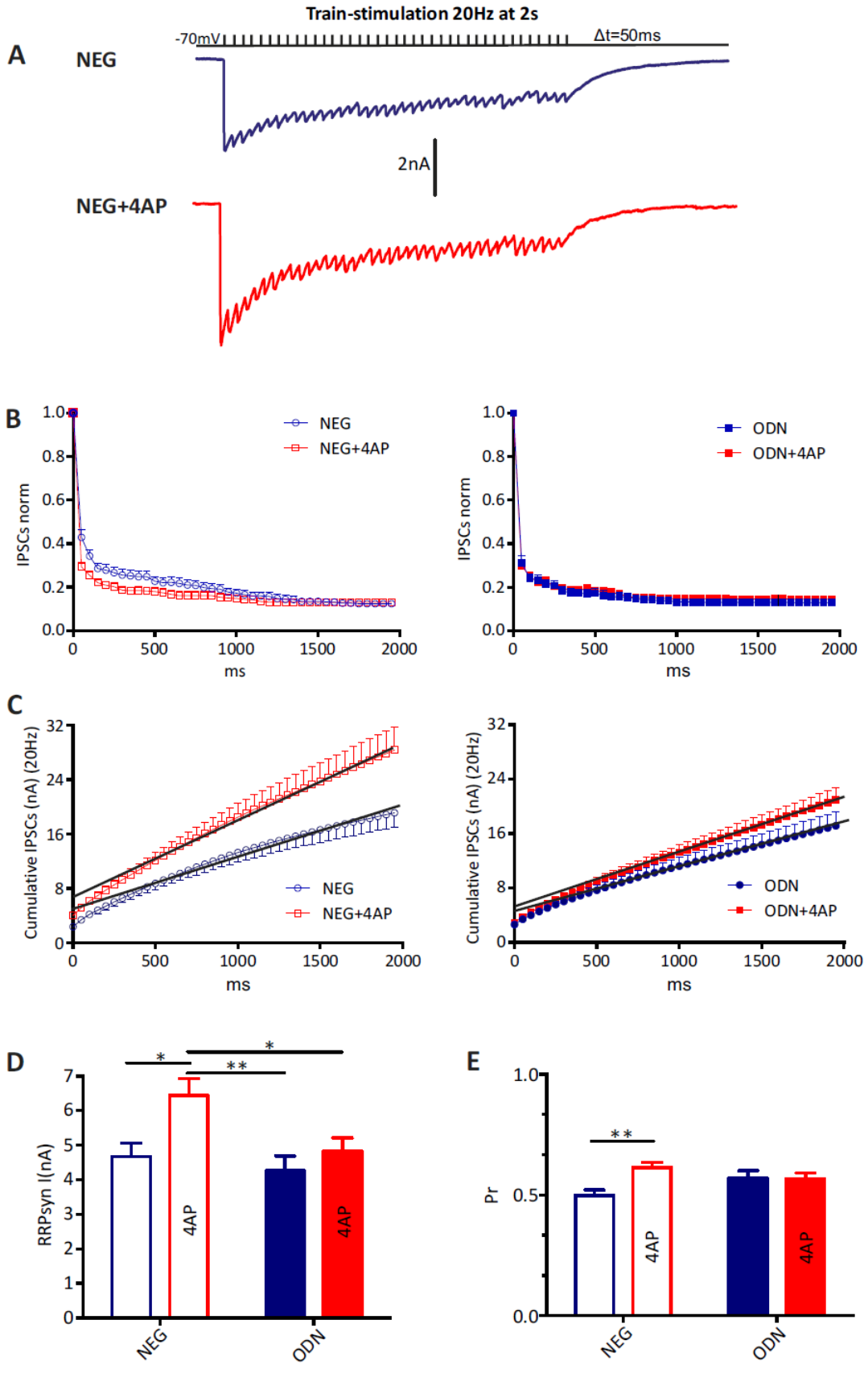

A
Train-stimulation 20Hz at 2s
-70mV
Δt=50ms
NEG
2nA
NEG+4AP
B
IPSCs norm
NEG
NEG+4AP
ODN
ODN+4AP
ms
C
Cumulative IPSCs (nA) (20Hz)
D
RRPsyn I(nA)
4AP
NEG
ODN
E
Pr

Figure 35: Estimation of RRPsyn and Pr by using the cumulative amplitude profile analysis. **(A)** The upper traces show the applied stimulation protocol used to evoke a tetanic stimulation lasting 2 sec at 20Hz in NEG (Blu) and NEG+4AP (Red) treated neurons. The lower trace shows representative eISPCs evoked by the tetanic stimulation. **(B)** Plot of normalized eIPSCs amplitude versus time during a tetanic stimulation at 20Hz for 2 sec. **(C)** Cumulative eIPSC amplitude profile. To calculate the RRPsyn, data points in the range of 1-2 s were fitted by linear regression and back-extrapolated to time 0. **(D, E)** Bars show RRPsyn and Pr of NEG (n=35), NEG+4AP (n=33), ODN (n=33) and ODN+4AP (n=32) treated cells as mean ± SEM,*P<0.05, **P<0.01, ***P<0.001 (Two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups).

Notably, this effect was blocked when REST activity was inhibited by ODN treatment, suggesting a hyperactivity-induced REST-mediated synaptic homeostasis of GABAergic synaptic boutons that make contacts onto excitatory postsynaptic target neurons (Fig. 35 D, E).

4.5 Neuronal hyperactivity doesn't affect eIPSCs onto inhibitory neurons

With the purpose of evaluating whether the neuronal hyperactivity also affects GABAergic transmission onto inhibitory neurons, we extracellularly stimulated in loose-patch configuration a presynaptic inhibitory interneuron (GAD67-GFP positive), recording eISPCs from another inhibitory (GAD67-GFP positive) neuron (Fig. 36 A-B).

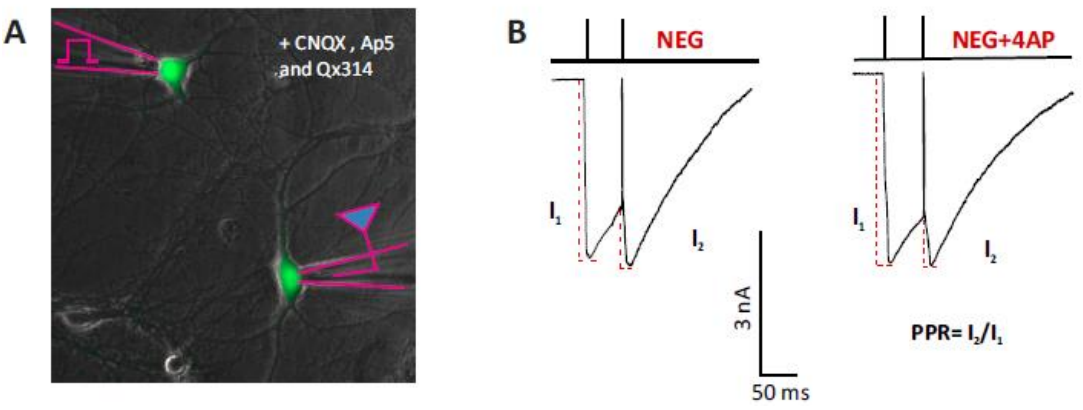


Figure 36: eIPSCs recorded from inhibitory neurons. **(A)** The stimulation electrode (on the left) is located on a GABAergic neuron (GAD67/GFP+) and a recording pipette (on the right) patches another inhibitory (GAD67/GFP+) neuron. **(B)** Representative paired pulse protocol stimulation (upper traces) and the corresponding eIPSCs voltage clamp (Vh=-70mV) recordings in paired cells.

The analysis of the mean eIPSCs amplitude and PPR recorded in neurons treated with 4AP, clearly showed that GABAergic synapses onto inhibitory neurons are not homeostatically modulated by chronic neuronal hyperactivity (Fig. 37 A, B).

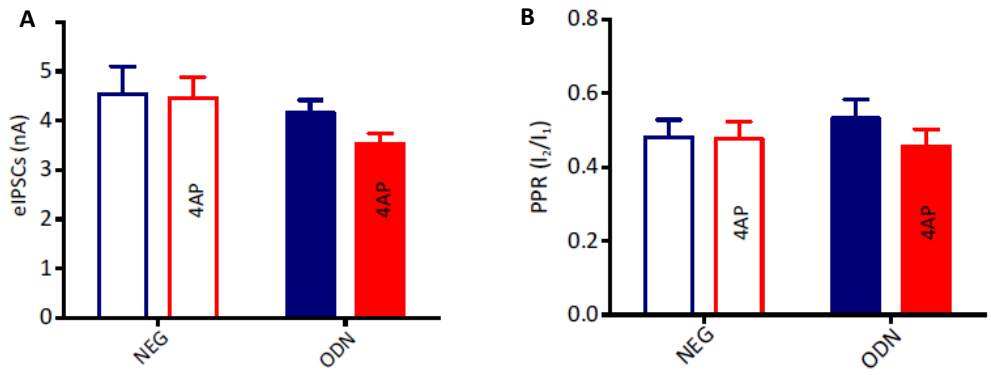


Figure 37: Lack of effect of 4AP on eIPSCs recorded from inhibitory neurons (A) The amplitude of the first eIPSC in the pair recordings protocol and the PPR (B) of NEG (n=14), NEG+4AP (n=14), ODN (n=16) and ODN+4AP (n=16) treated cells are shown as means ± SEM (Two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups).

The absence of a process of homeostatic plasticity was further confirmed by the analysis of the quantal parameters of release: Pr and RRPsyn (Fig. 38 A-D).

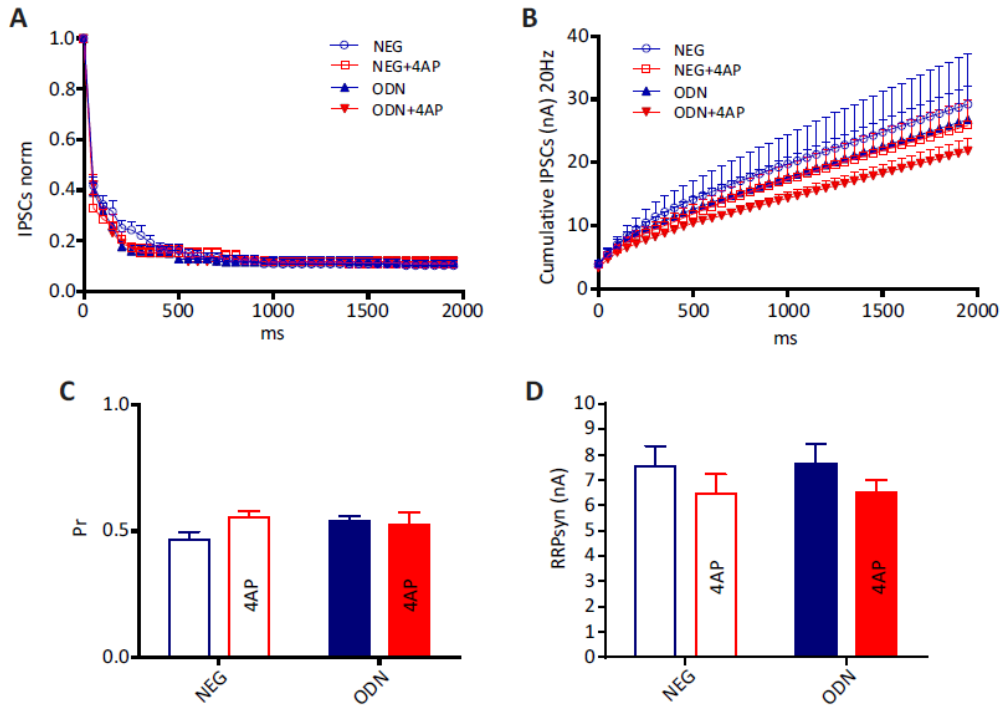


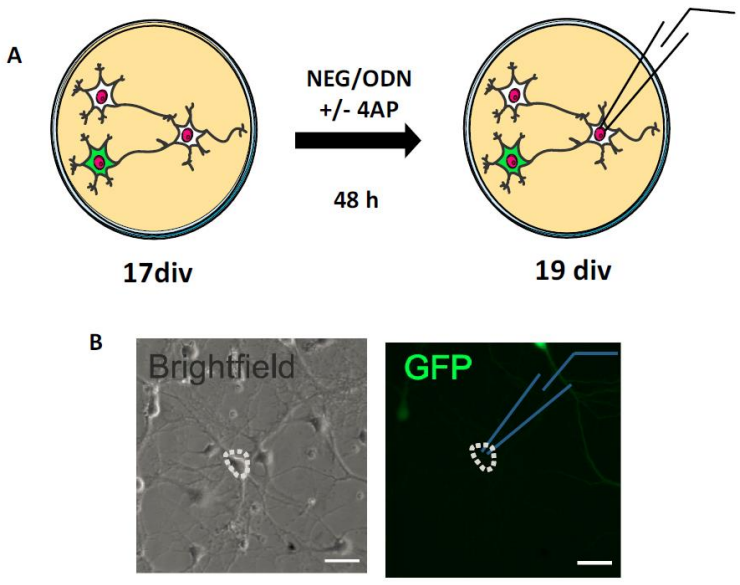
Figure 38. Analysis of RRPsyn and Pr by the use of cumulative amplitude profile analysis. (A) Plot of normalized eIPSCs amplitude versus time during a tetanic stimulation at 20Hz for 2 sec. (B) Cumulative eIPSC amplitude profile. To calculate the RRPsyn, data points in the range of 1-2 s were fitted by linear regression and back-extrapolated to time 0. (C,D) Bars show RRPsyn and Pr of NEG (n=14), NEG+4AP (n=14), ODN (n=16) and ODN+4AP (n=16) treated cells as mean ± SEM,*P<0.05, **P<0.01, ***P<0.001 (Two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups).

4.6 Neuronal hyperactivity induces a REST-mediated increase of mIPSCs frequency recorded from excitatory neurons

Miniature postsynaptic currents (mPSCs) are observed in the absence of presynaptic action potentials and are caused by action potential-independent release of neurotransmitters (i.e., quantal release) (Edwards et al. 1990) from the presynaptic terminals of both excitatory or inhibitory neurons. mPSCs are widely used to get information about synaptic properties (Turrigiano, 1999; Turrigiano, 2008).

Changes in mPSC amplitude are consistent with higher/lower density/conductance of postsynaptic receptors at individual synapses (O'Brien R.J. et al., 1998). Moreover, changes in mPSCs frequency is determined by the total number of presynaptic terminals onto postsynaptic neuron and the quantal-release probability at the individual synapses (Shao LR and Dudek FE., 2005). Indeed, an elevated mPSCs frequency is usually interpreted as an increase in either the presynaptic release probability at existing sites, due to an increment in the vesicular pool or in the vesicular turnover rate (Murthy V.N. et al., 2001), or in the number of functional synaptic sites (Malenka R.C. and Nicoll R.A., 1997).

Then, to further investigate the role of REST role on the synaptic homeostasis of the inhibitory synapses we recorded miniature inhibitory postsynaptic currents (mIPSCs) from excitatory neurons (GAD67-GFP negative) in cultured hippocampal networks treated with NEG, NEG+4AP, ODN and ODN+4AP at 19 div (Fig 39 A). We observed that chronic treatment with 4AP (48 h) significantly increased the frequency of mIPSCs onto excitatory neurons in NEG+4AP treated neurons. This effect is fully blocked by ODN (Fig. 39 D, left). On the other hand, the treatment with 4AP did not affect the amplitude of mIPSCs (Fig. 39 D, right).



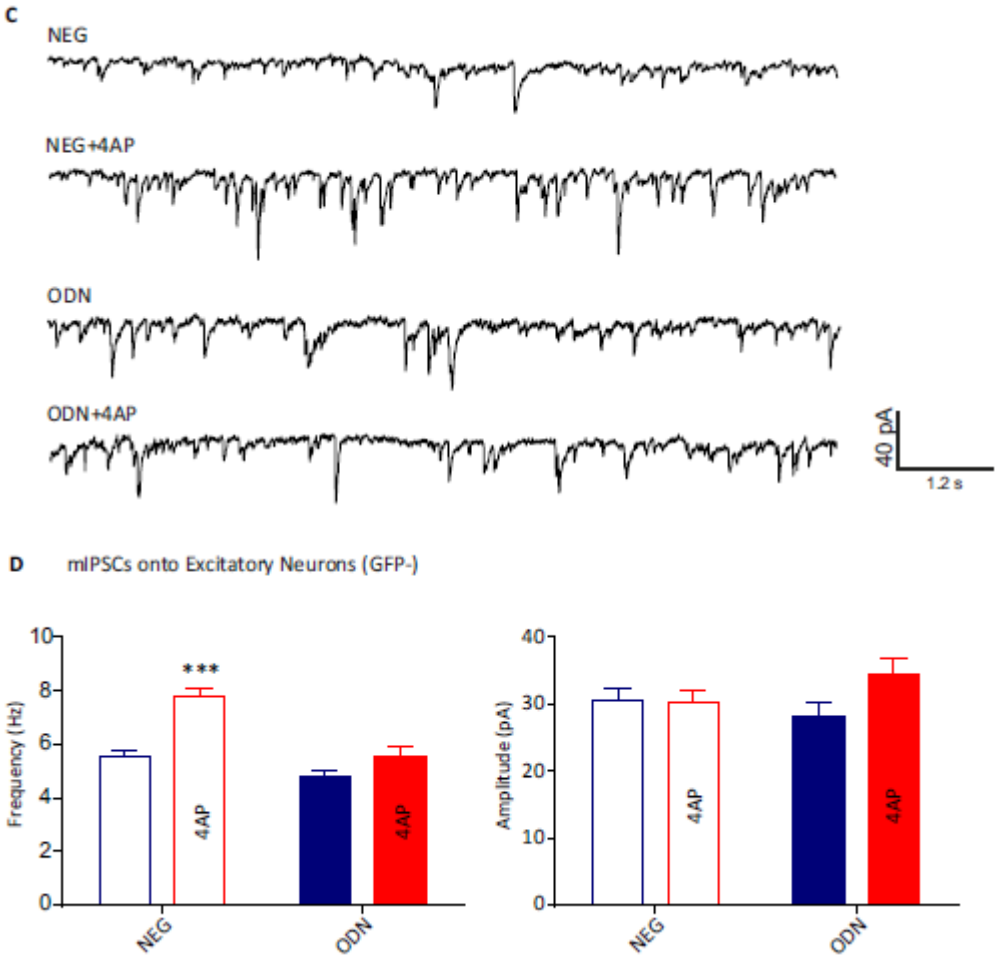


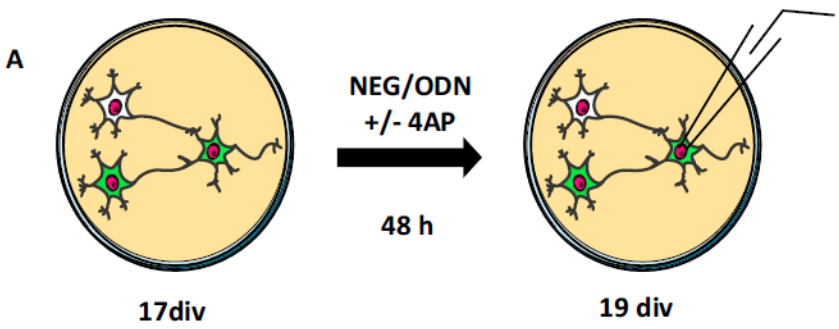
Figure 39. The frequency of mIPSCs recorded from excitatory neurons is increased by chronic hyperactivity in hippocampal neurons. (A-B) Graphical representation of the experimental procedure. (C) Representative mIPSCs traces recorded at 19 div. Mean ± SEM of frequency (D, left) and amplitude (E, right) of NEG (n=25), NEG+4AP (n=21), ODN (n=21) and ODN+4AP (n=20) treated neurons. ***P<0.001 Two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups.

As it is well accepted that the amplitude of mIPSCs reflects postsynaptic effects, whereas mIPSCs frequency is dependent on presynaptic properties, our data suggest a role of REST at the presynaptic site. As mentioned, a such REST-dependent effect could be ascribed to changes at presynaptic terminals in terms of the number of active synapses and/or to a modulation of the presynaptic release machinery.

4.7 Neuronal hyperactivity doesn't affect mIPSCs recorded from inhibitory neurons

In order to investigate the effect of neuronal hyperactivity onto mIPSCs recorded from inhibitory neurons, we used the same experimental setting already described in the previous paragraph. Briefly neurons were treated with 4AP at 17 div and mIPSCs were recorded after 48 h (19 div). We observed that chronic treatment with 4AP did not affect neither the frequency (Fig. 40 D) nor the amplitude (Fig. 40 E) of mIPSCs recorder from inhibitory neurons.

These results confirm a peculiar behaviour already highlighted by the eIPSCs analysis: a REST dependent up-scaling of inhibitory GABAergic transmission occurs in response to neuronal hyperactivity only when the postsynaptic target cell is an excitatory neuron, while is lacking when the postsynaptic target neuron is inhibitory.



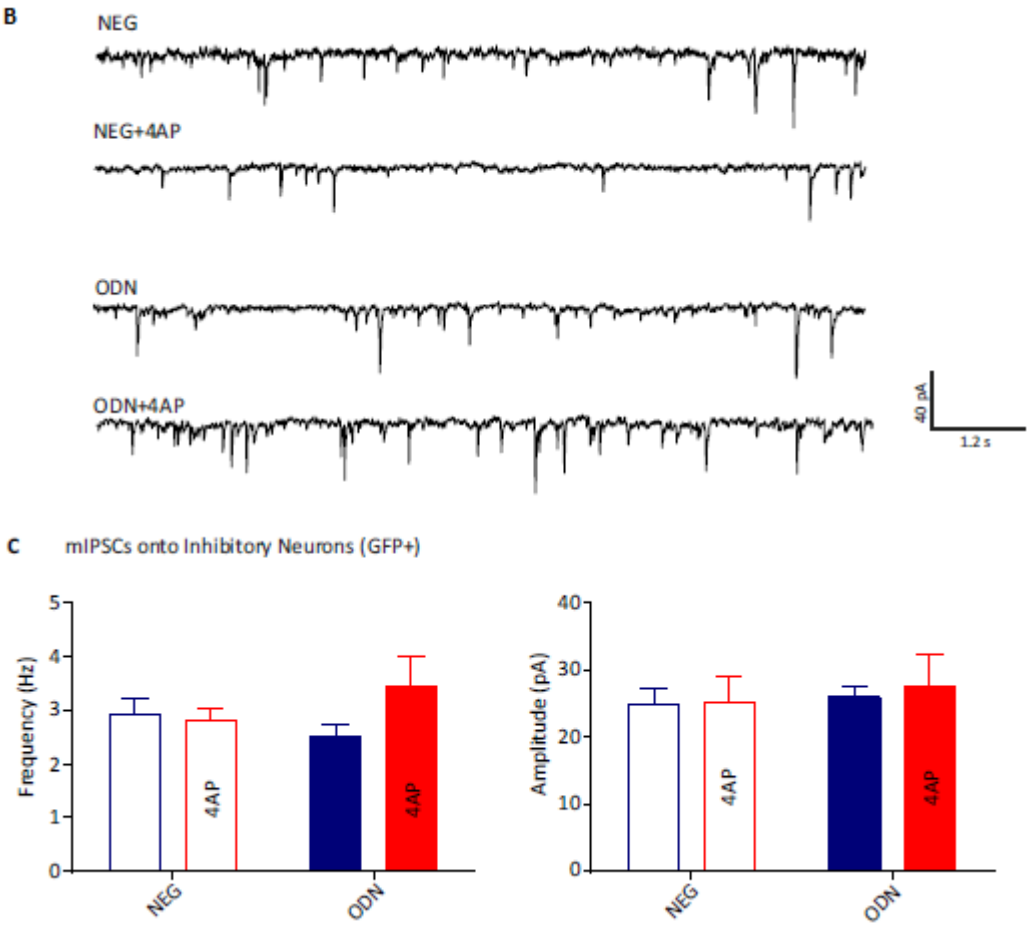


Figure 40: Neuronal hyperactivity did not affect mIPSCs recorded from inhibitory neurons (A) Graphical representation of the experimental procedure. (B) Representative mIPSCs traces recorded at 19 div. Mean ± SEM of frequency (C, left) and amplitude (C, right) of NEG (n=13), NEG+4AP (13), ODN (n=12) and ODN+4AP (n=11) treated neurons. Two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups.

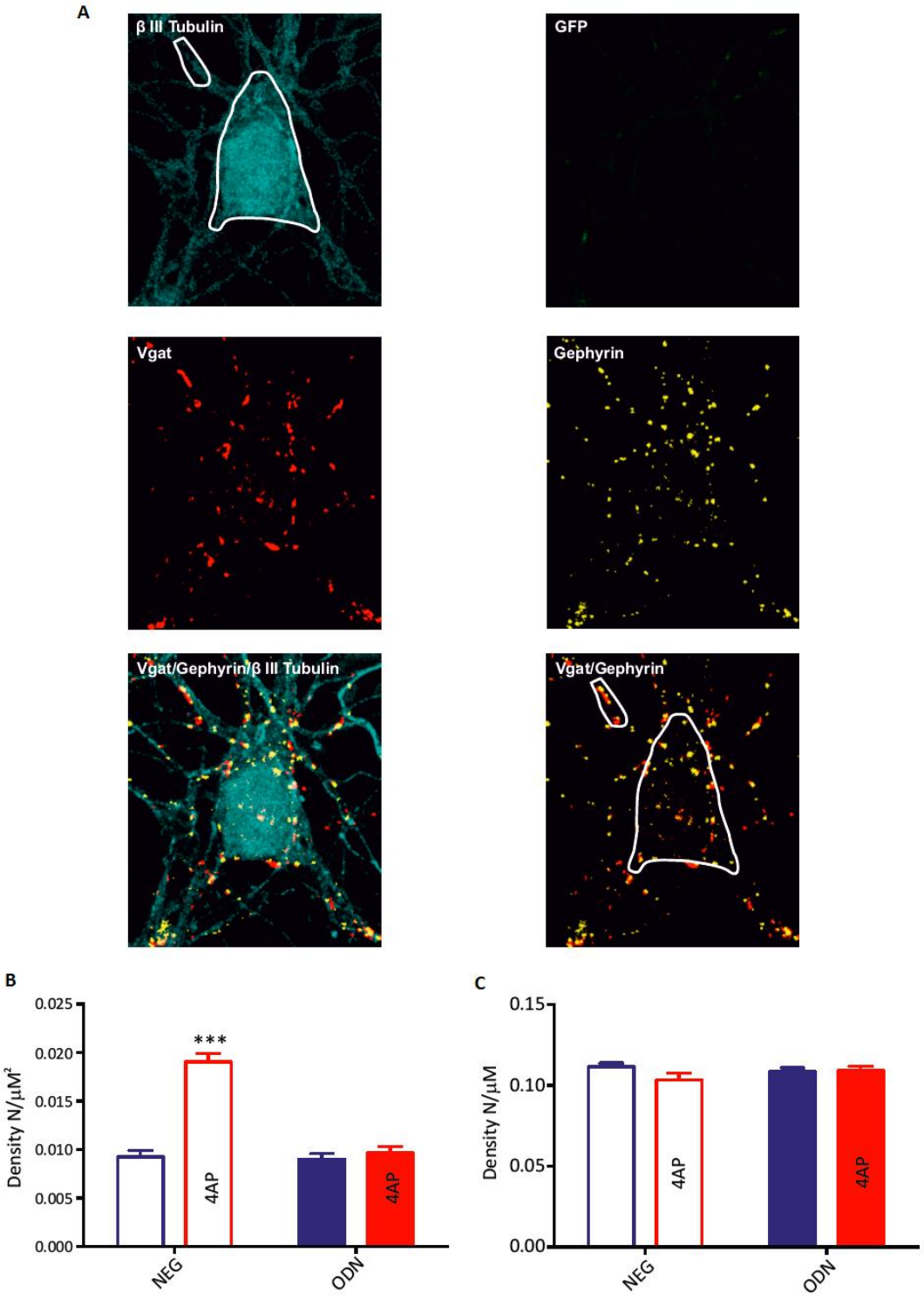
4.8 REST-dependent increase of Somatic GABAergic synapses onto excitatory neurons

It has been shown that the formation of functional inhibitory synapses are marked by the appearance of stably apposed Gephyrin and vGAT clusters at sites previously lacking either component (Dobie and Craig, 2011).

To evaluate whether the process of REST mediated synaptic homeostasis could also affect the number of GABAergic synaptic contacts we performed an immunocytochemistry analysis (Fig. 41) of vGAT and gephyrin positive puncta, representing putative GABAergic synapses.

Immunostaining approach was performed onto hippocampal cultured neurons, 17 div, obtained from knock-in GAD67-GFP mice treated at with ODNs ± 4AP for 48 h and then stained as follows: (i) an anti-vGAT antibody was used to identify presynaptic inhibitory terminals (Martens et al., 2008); (ii) an anti-gephyrin antibody was used to reveal inhibitory postsynaptic scaffolding proteins (Fritschy et al., 2008) (iii) an anti-beta-tubulin antibody was used to visualize the entire neuronal structure (Fig. 41).

Chronic hyperactivity (4AP 48 h) induced an increase of the density of axo-somatic inhibitory synapses but not of the axo-dendritic synapses when the postsynaptic target was an excitatory neurons (GAD67-GFP negative) (Fig. 41 B, C). This effect was blocked when neurons were co-treated with 4AP+ODN, suggesting a REST involvement. Interestingly, we did not observe any effect on both axo-somatic and axo-dendritic synapses when the post-synaptic target was another inhibitory neuron (GAD67-GFP positive) (Fig. 41 E, F).


A
β III Tubulin
GFP
Vgat
Gephyrin
Vgat/Gephyrin/β III Tubulin
Vgat/Gephyrin
B
Density N/µM²
0.025
0.020
0.015
0.010
0.005
0.000
***
4AP
4AP
NEG
ODN
C
Density N/µM
0.15
0.10
0.05
0.00
4AP
4AP
NEG
ODN

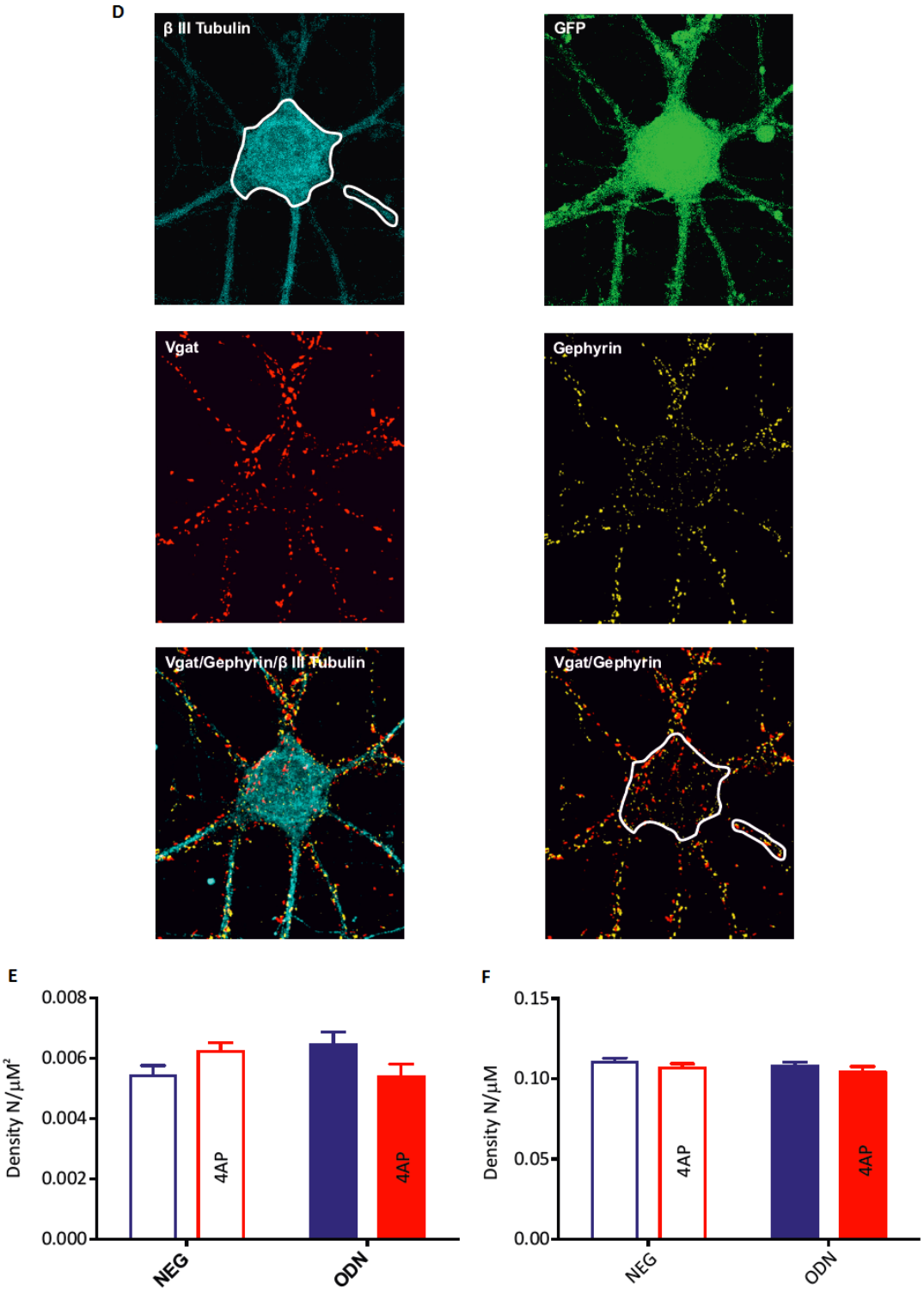


Figure 41: Analysis of the number of putative GABAergic synapses onto excitatory/inhibitory neurons. (A-D) Representative mouse hippocampal excitatory and inhibitory neuron (from Knock-in GAD67-GFP mice). Axons and dendrites are immuno-stained (at 20 div) with antibodies against β3-tubulin (white), vGAT (yellow), gephyrin (red) with magnification of somatic and dendritic area (n=3

different preparation). Quantification of the normalized density of inhibitory synapses onto excitatory (B, C) and inhibitory neurons (E, F) at somatic and dendritic area (n=3 different preparation). ***p<0.001 two-way ANOVA followed by Sidak multiple comparisons test between treatments and groups.

4.9 REST-dependent up-scaling of GABAergic synapses: possible involvement of "NPAS4-BDNF gene program"

Our results demonstrated that REST, a negative transcription factor, induced an upscaling of the inhibitory synaptic transmission characterized by a marked postsynaptic target specificity. With the intention of finding a molecular fundament explaining such effect, we investigated the possibility that REST could play a role in the activity-dependent synthesis and secretion of BDNF.

This neurotrophin is indeed well known for its capability to be expressed and released in an activity-dependent way only by excitatory neurons and for its effectiveness in inducing functional up-scaling of GABAergic synapses (Fredrick J. Seil and Rosemarie Drake-Baumann, 2000; Marty S. et al., 2000; Lin Y. et al., 2008; Baldelli et al., 2005; Huang ZJ et al., 1999).

We initially evaluated the possible crosstalk between REST and BDNF performing a detailed time course analysis of the change in the mRNA levels of REST and total coding sequence (cds) BNDF in neurons maintained in a condition of hyperactivity with 4AP for 6, 24, 48 and 96 h (Fig. 42 A, B). Both REST and cds-BNDF showed an immediate increase in response to hyperactivity, showing a significant enhancement at 6 h, more relevant for the BNDF (Fig. 42 A, B). Notably, REST increment persisted at 24 and 48 h of stimulation recovering its control level only after 96 h (Fig. 42 A), while the fast and strong increase of cds-BDNF was short-lasting and recovered its control level already after 24 h (Fig. 42 B). Notably, P1-BNDF, one the two splicing variants of BNDF, that together with the P4 variant, are particularly sensitive to neuronal hyperactivity (Pruunsild et al., 2011; Lin Y. et al., 2008) and Syt-4, a synaptotagmim variant playing a role in the release of SVs containing BDNF (Dean et al., 2009), showed a similar behaviour, a very fast

increase followed by slower decrease, in response to chronic neuronal hyperactivity (Fig. 42 C, D).

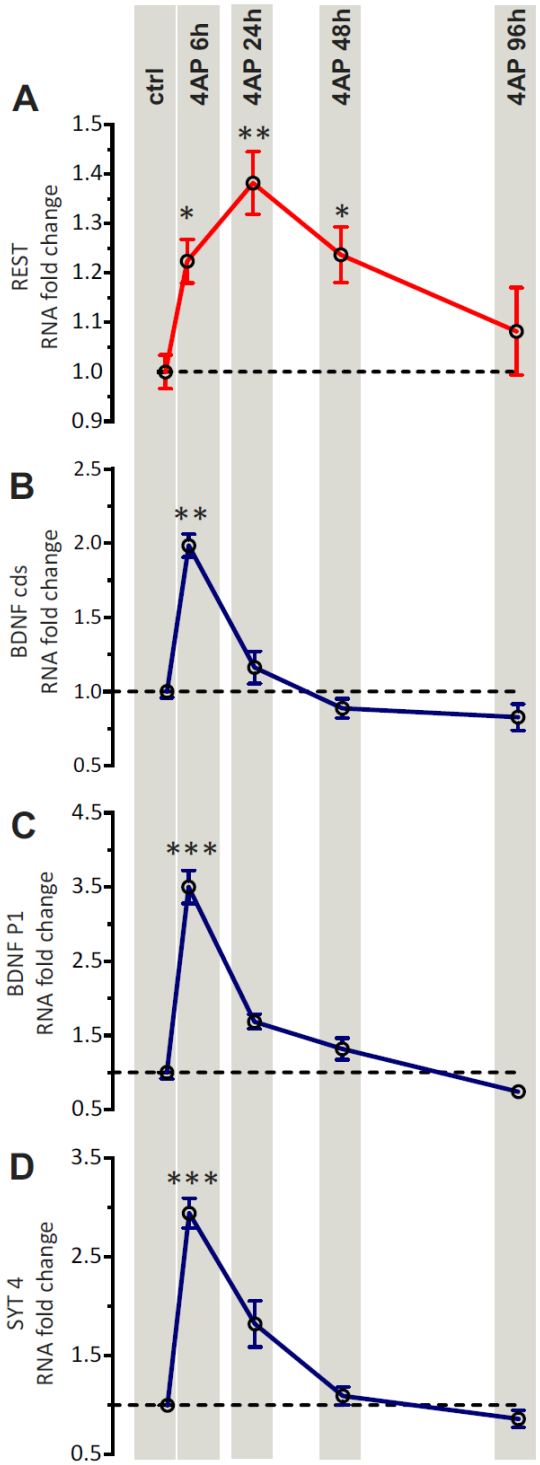


Figure 42: Time dependent up-regulation of mRNA levels of transcription factors upon 4AP treatment. RT-qPCR analysis of changes (means ± SEM) in BDNF cds (coding sequence), BDNF P1

(promoter 1) and Syt-4 and REST mRNA transcript levels in untreated or 4AP-treated at different time points (3, 8, 24, 48 and 96h) cortical neurons. One way anova followed by Kruskal-Wallis multiple comparison test . * p<0,05, ** p<0.01, *** p<0.0001, untreated vs 4AP-treated neurons. For each time point, n=11 from 3 independent neuronal preparations.

With the aim to evaluate whether BNDF played a functional role in the 4AP-induced strengthened of inhibitory synapses, we studied the effect of 4AP on the eIPSCs amplitude and PPR in cultured hippocampal neurons (17 div) treated for 48 h with TrkB/fc, a scavenger recombinant protein able to efficiently suppress BDNF binding to its TrkB receptor. Notably, the increase of the eIPSC amplitude and the decrease of the PPR, that are normally observed in response to 4AP treatment were completely suppressed (Fig. 43), demonstrating the BNDF involvement.

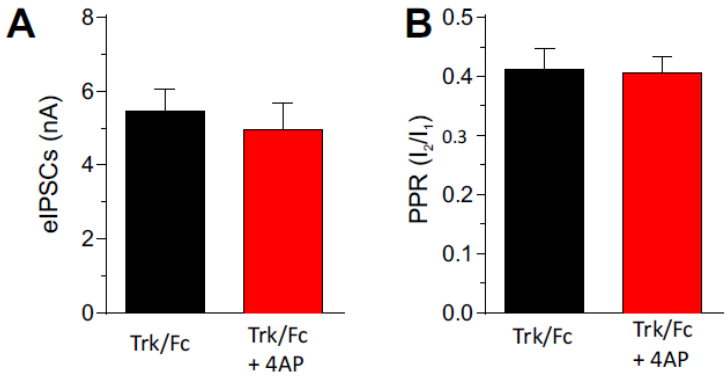


Figure 43. Bar plots represent the amplitude (A) of the first eIPSC in the pair recordings protocol and the (B) PPR (I_2/I_1) of TrkB/fc (n=16) and TrkB/fc + 4AP (n=17) treated cells are shown as means ± SEM (unpaired Student's t test)

During the last decade the research group of Greenberg (Lin et al., 2008; Bloodgood et al., 2013; Spiegel et al., 2014) elegantly demonstrated that NPAS4, an immediate early gene, rapidly activated by neuronal hyperactivity, is able to promote and increase the number and strength of inhibitory synapses onto excitatory neurons, thanks to the activation of a complex NAPS4-BDNF gene program. Indeed, NAPS4 is a positive transcription factor that increases the expression of many target genes and among them BNDF is one of its main targets.

In order to dissect with high temporal resolution the crosstalk between the activation of REST and BDNF involvement, we analyzed in parallel mRNA changes of REST, NPAS4, cds-BDNF, P1-BNDF, P4-BNDF and Syt-4 in cultured neurons (17 div) treated with NEG, NEG+4AP, ODN and ODN+4AP for 1, 3, 6, 12 and 24 h (Fig. 44 A-F).

Unexpectedly, REST mRNA increased already after 1 h of incubation in 4AP and the increment of REST persisted with a moderate decrease until 24 h. As previously highlighted in this work (Fig. 32, 33), ODN increased REST mRNA level, demonstrating that its basal level in unstimulated (control) neurons exerts an auto-inhibitory action (Fig. 44 A).

As previously reported (Lin Y. et al., 2008), NPAS4 mRNA increased already after 1 h, but this increment was transient, starting to decrease after 12 h and recovering its control value after 24 h. Notably, inhibition of REST activity by ODN, fully suppressed the 4AP-dependent NPAS4 increase at 1 h, while subsequently (3-6 h) ODN lost its inhibitory action on NPAS4-mRNA and notably, in neurons treated with ODN+4AP, NPAS4 reached after 24 h a value higher than in 4AP alone (Fig. 44 B).

This result, strongly suggest that the activation of REST already present in unstimulated neurons is implicated in the immediate (1 h) activity-dependent induction of NPAS4, while the persistency of the REST mRNA increment plays a crucial role in the recovery of NPAS4 mRNA level after 24 h.

In parallel, we analyzed 4AP-induced change of the P1- and P4-BNDF mRNA, the two BNDF splice variants particularly sensitive to the activity-dependent regulation (Aid et al., 2007).

In particular, it was previously shown that NPAS4 is crucial for the activity-dependent activation of P1-BDNF transcription, while the activation P4-BNDF depends on the bind of CREB to CRE in the P4 promoter, however its full induction is obtained with the interaction with NPAS4 (Pruunsild et al., 2011).

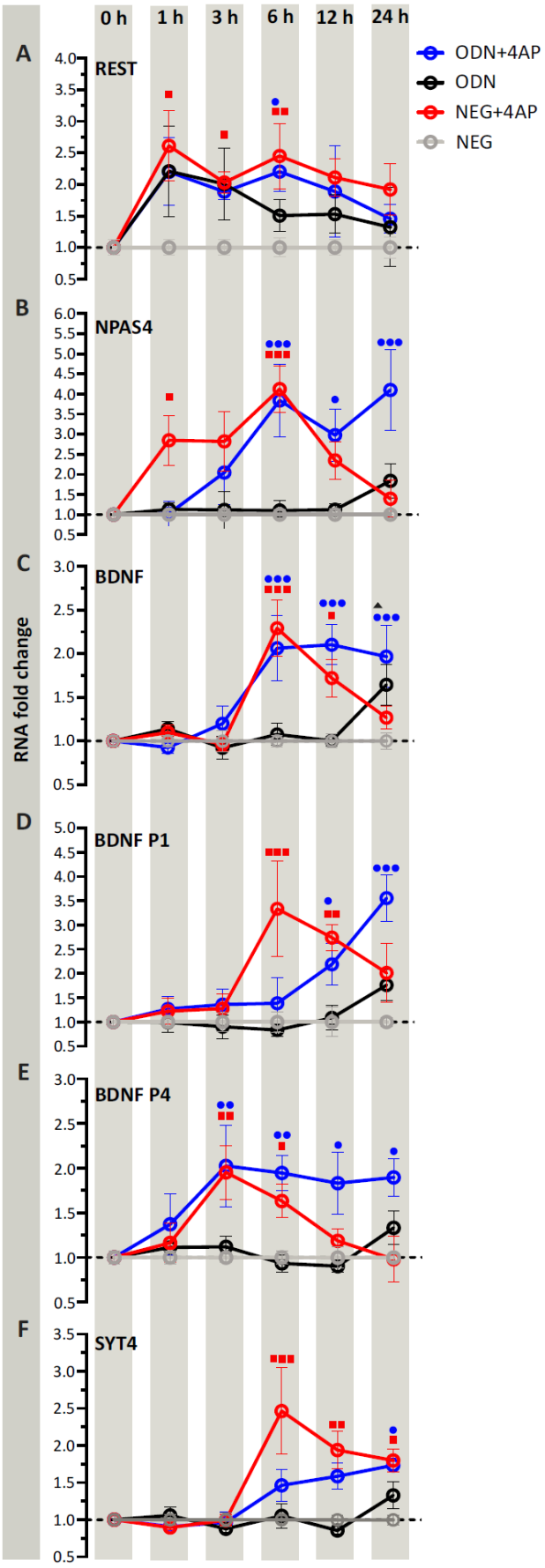

0 h
1 h
3 h
6 h
12 h
24 h
ODN+4AP
ODN
NEG+4AP
NEG
A
REST
B
NPAS4
C
BDNF
RNA fold change
D
BDNF P1
E
BDNF P4
F
SYT4

Figure 44: Time-course analysis of the mRNA levels in order to dissect the crosstalk between the activation of REST and BDNF involvement. RT-qPCR analysis of changes (means ± SEM) in REST (A), NPAS4 (B), BDNF cds (C), BDNF P1 (D), BDNF P4 (E) and Syt-4 (F) mRNA transcript levels in NEG, NEG+4AP, ODN and ODN+4AP cortical neurons treated at different time points (0, 1, 3, 6, 12, and 24 h). All values are normalized to the NEG level. For each time point, n = 9 from 3 independent neuronal preparations. Two-way ANOVA followed by Sidak multiple comparisons test ■ NEG vs NEG+4AP; ▲ NEG vs ODN; ● NEG vs ODN+4AP; *p < 0.05, **p < 0.01, ***p < 0.001.

However, NAPS4 is not the unique transcription factor able to modulate BDNF transcription, for example it is well known that BDNF is one of the many target genes of the negative transcription factor REST (Hara et al., 2009; Otto et al., 2007; Bruce et al., 2004) and previous reports showed that activation of REST induced a down-regulation of BDNF transcription and expression (Otto et al., 2007; Zuccato et al., 2007).

The time course of cds-BNDF, P1-BDNF and P4-BDNF (Fig. 44 C, D, E) mRNA change revealed that 4AP-treatment increased cds- and P1-BNDF with a time delay of 6 h while the increase of P4-BDNF was faster, peaking after 3 h.

The increments of cds-, P1- and P4-BDNF in response to 4AP-stimulation were transient, and all of them recovered their control value after 24h. Interestingly, the block of REST with ODN did not exert any effect on the cds-BNDF change in response to 4AP. ODN did not interfere with the increase of P4-BNDF but it suppressed its ability to recover the control value, while ODN dramatically affects the time course of the change of P1-BNDF mRNA that increased more slowly and was not able to recover its control value after 24 h of treatment with 4AP (Fig. 44D).

These results clearly suggest that REST exerts two distinct effects on BNDF: 1) it is involved in the NPAS4-depedent activation of P1-BDNF and 2) it plays a fundamental role in the temporal confinement of the both cds-, P1- and P4-BNDF increment, allowing its full recovery to control value after 24 h.

In order to translationally confirm the REST-dependent transcriptional changes induced by neuronal-hyperactivity, we are using immunoblot assay in cortical neurons (17 div) treated with NEG, NEG+4AP, ODN and ODN+4AP for 24 h. The

analysis, that is actually still in progress, confirmed a parallel and significant increase of REST, NPAS4 and SYT4 protein level upon NEG+4AP treatment (Fig. 45 A-D). When 4AP was applied in presence of ODN the increase of NPAS4 protein was suppressed while ODN was not able to inhibit the increase Syt4.

Notably ODN treatment increased REST protein level further confirming an auto-inhibitory action of the basal level of REST on itself (Fig. 45 B).

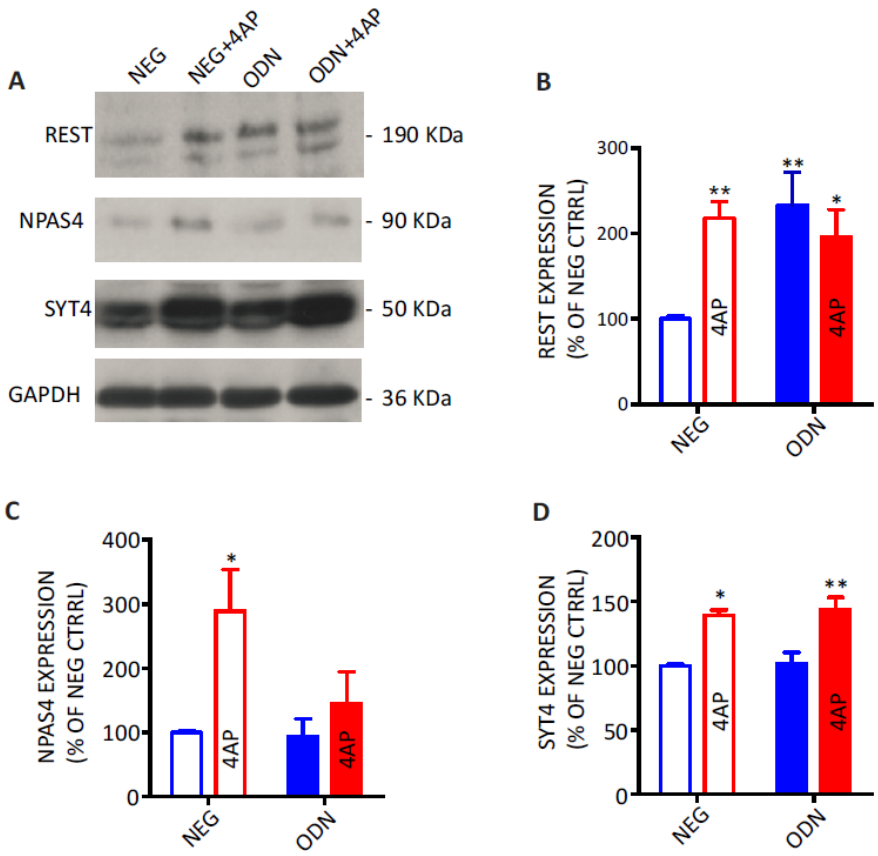


Figure 45: Western blot analysis of the proteins levels in order to dissect the crosstalk between the activation of REST and NPAS4/SYT4 involvement. (A) Representative immunoblots. (B-D) Analysis of changes (means ± SEM) in REST (B), NPAS4 (C) and Syt-4 (D) protein levels in NEG, NEG+4AP, ODN and ODN+4AP cortical neurons treated for 24 h. All values are normalized to the NEG level. GAPDH immunoreactivity was included as control of equal loading. For each protein, n = 5 from 3 independent neuronal preparations. Two-way ANOVA followed by Sidak multiple comparisons test. *$p < 0.05$, **$p < 0.01$.

In order to investigate whether the REST-dependent activation of the NAPS4/BNDF gene program is responsible of the hyperactivity-dependent upscaling of the strength of the inhibitory inputs, we analyzed the changes of mRNAs coding for GAD65, vGAT, GAD67 and the epsilon subunit of the GABAA receptor (Fig. 46 A-D), in the same time-scale (1, 3, 6, 12, 24 h) adopted for the investigation of REST, NAPS4 and BDNF mRNAs changes.

GAD65, the enzyme synthetizing GABA that mediates transient GABA synthesis (Kanaani et al., 2004), was not modulated by the neuronal hyperactivity induced by 4AP treatment. On the contrary GAD67, that regulates the basal level of GABA (Raju et al., 2005), vGAT and epsilon GABA R subunit mRNAs showed a significant increase in response to 4AP that was blocked by inhibition of REST with ODN. Notably, while the "presynaptic" vGAT and GAD67 mRNA showed a transient increase of mRNA fully blocked in neurons treated with 4AP+ODN, the epsilon GABAA-R mRNA showed a slower increase not fully blocked by ODN (Fig. 46 A-D).

Transcriptional analysis well be very soon completed with immunoblot assays of vGAT, GAD67 and epsilon subunit of the GABAA receptor.

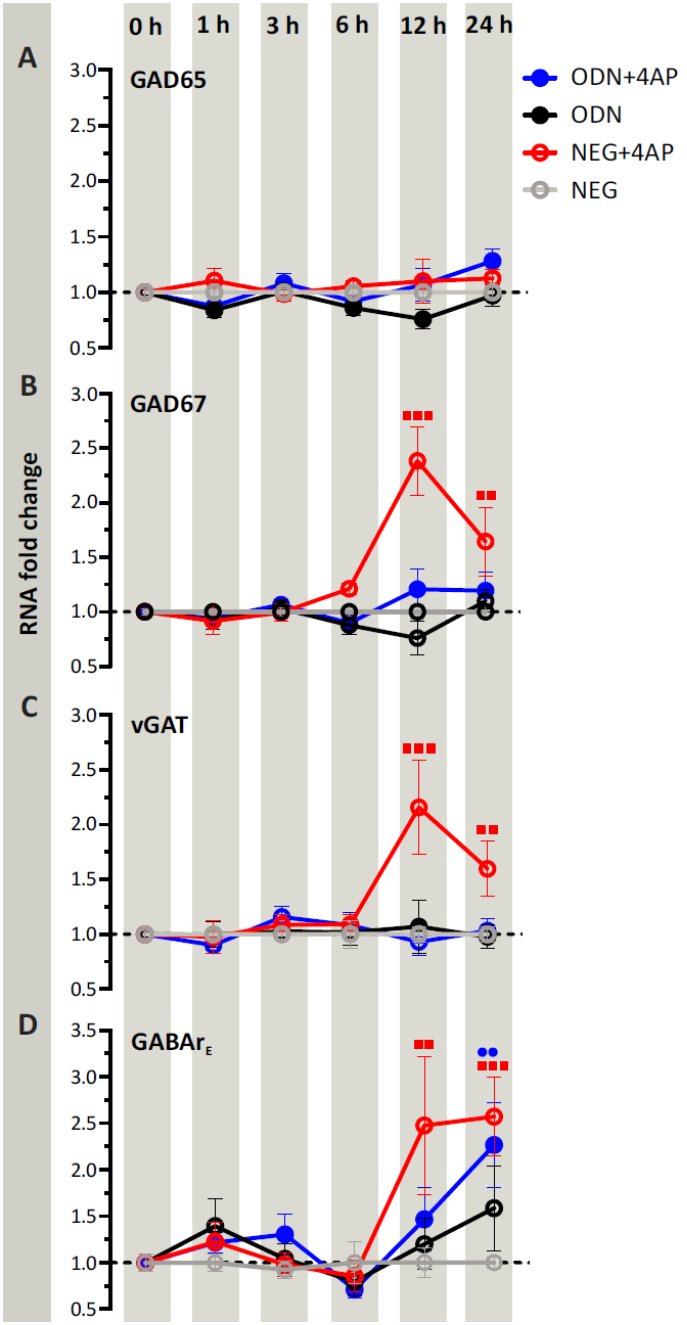


Figure 46: The expression levels of REST target genes that are modulated upon 4AP treatment in a REST-dependent manner. RT-qPCR analysis of changes (means ± SEM) in GAD65 (A), GAD67 (B), vGAT (C) and GABArε (D) mRNA transcript levels in NEG, NEG+4AP, ODN and ODN+4AP cortical neurons treated at different time points (0, 1, 3, 6, 12, and 24 h). All values are normalized to the NEG level. For each time point, n = 9 from 3 independent neuronal preparations. Two-way ANOVA followed by Sidak multiple comparisons test ■ NEG vs NEG+4AP; ▲ NEG vs ODN; ● NEG vs ODN+4AP; *p < 0.05, **p < 0.01, ***p < 0.001.

5. Discussion and Conclusion

Homeostatic plasticity adjusts neuronal membrane excitability and the strength of excitatory and inhibitory inputs in order to maintain in the brain circuits an appropriate level of electrical activity (Davis and Bezprozvanny, 2001; Turrigiano and Nelson, 2004). The two main mechanisms adopted by neuronal networks to accomplish to this alteration are intrinsic and synaptic homeostasis (Turrigiano, 2011). The first experimental evidence of the capability of brain circuits to react to a condition of perturbation of their electrical activity with a process of HP have been produced by Turrigiano and collaborators in 1998, showing that cultured cortical neurons maintained for 48 h in a condition of chronic-deprivation of activity (TTX) or -hyperactivity (bicuculline), are able to recover from the altered condition in order to restore a "normal" neuronal activity.

In these last 2 decades a judge amount of data has been accumulated revealing multiple signaling pathways underlying Homeostatic Plasticity, HP (Turrigiano 2011; Davis 2006; Turrigiano and Nelson 2004; Marder and Prinz 2003; Zhang and Linden 2003). However, recent advances on the epigenetics basis of neural plasticity, could shed a little bit of light on a such puzzling scenario depicted by a such wide number and variety of the transcriptional and post-transcriptional signaling pathways underlying HP. Epigenetic mechanisms are broadly defined as processes that regulate gene expression through the alteration of chromatin structure without changing nucleotide base sequences. Five major epigenetic mechanisms that cells utilize are histone modification, histone variant exchange, nucleotide modification, non-coding RNA-mediated regulation and chromatin remodeling (Kouzarides, 2007). With the exception of non-coding RNAs, these mechanisms alter chromatin structure and function, adding a very complex layer of regulation to gene expression. These mechanisms are best known for their actions during cell differentiation and cell division (Reik W., 2007), including processes involved in the transgenerational passage of gene-regulatory

information and the integration of environmental signals for the coordination of transcriptional responses in fully differentiated cells (Goldberg et al., 2007).

In the last few decades, an increasing amount of recent experimental advances are showing that the brain is strongly influenced and affected by epigenetic factors (Stroud et al., 2017). Many intrinsic factors can alter neural functions during the lifetime indeed several epigenetic mechanisms have been shown to regulate learning-induced gene expression in post-mitotic neurons and to establish persistent behavioral responses (Swank & Sweatt, 2001; Levenson & Sweatt, 2005; Guan et al., 2002; Barrett & Wood, 2008). Moreover, also extrinsic factors such as diet, exercise, environmental variations, stressors can play crucial events in the regulation of various forms of neuronal plasticity, including HP (Hwang JY et al., 2017; Heyward et al., 2016; Chen H. et al., 2012).

REST (RE1-silencing transcription factor) has been initially identified as a negative transcription factor. Its target genes encode postsynaptic receptors, ion channels and transporters, neuropeptides and synaptic proteins (Bruce et al., 2004). However, more recent evidences showed that in mature neurons, REST can be upregulated by neuronal hyperactivity (Calderone et al., 2003; Palm et al., 1998) and works as a master epigenetic modulator (Huang et al., 1999), acting mostly as transcriptional repressor (Bersten et al., 2014; Yu et al., 2011) and, occasionally, as a transcriptional activator (Perera et al., 2015; Kallunki et al., 1998).

We have previously demonstrated that REST is critical for the downscaling of intrinsic excitability in neurons subjected to prolonged elevation of electrical activity. Indeed, REST can modulate the intrinsic properties on excitatory hippocampal neurons highlighting a novel function of REST in the physiology of mature neurons, going beyond its well-known role as a master regulator of neuronal differentiation (Pozzi et al., 2013).

More recently, we found that it participates to the synaptic homeostasis of glutamatergic synapses by reducing their strength at the presynaptic level. Indeed, chronic hyperactivity triggers a REST-dependent decrease of the size of RRP

through the transcriptional and translational repression of specific presynaptic REST target genes (Pecoraro-Bisogni et al., 2017).

To complete the picture on the role of REST on the HP induced by neural hyperactivity, here we investigated its contribution in synaptic homeostasis of GABAergic synapses in response to chronic hyperactivity, triggered by the long-term treatment (48 h) with a well-known convulsive agent, 4AP, an unselective K^+ channels inhibitor.

For this study we have taken advantage from a knock-in GAD67-GFP mice that allow to visually identify GABAergic neurons. In these mice, the fluorescent protein reporter, GFP, was specifically expressed in GABAergic neurons (Fig. 26), under the control of the endogenous GAD67 promoter (Tamamaki N. et al., 2003).

Here, we did not use shRNA strategy, that we have adopted in our previous studies (Pozzi et al., 2013; Pecoraro-Bisogni et., 2017) but we blocked REST activity by using oligo-deoxynuclotides (ODNs) that act as surrogate binding sites for REST and sequester the native transcription factor from its genomic binding sites. This approach allows to block REST activity in few hours blocking not only the effects of REST expression change in response to hyperactivity but also the effect of basal level of REST already present in unstimulated neurons.

The use of ODN tagged with a fluorescent probe allowed us to demonstrated that REST is present in the cytoplasm of both excitatory and inhibitory unstimulated neurons and the treatment with 4AP induces a significant translocation of REST to the nucleus that was efficiently inhibited when ODN was applied before 4AP treatment (Fig. 30).

Spontaneous synaptic activity in the absence of external stimulation, is a robust feature of neuronal networks occurring both *in vivo* and *in vitro*. Such spontaneous synaptic are ascribed to synaptic currents that are partially generated by action

potentials (APs) with amplitude larger than 30 pA and partially APs-independent (miniature events) with amplitude lower than 30pA (Pinheiro P.S. and Muller C., 2008). This approach allowed us to obtain a rough estimation of the effects exerted by 2 days of hyperactivity (48 h in 4AP) had on the strength of both excitatory and inhibitory inputs. The analysis confirmed that 4AP treatment induced a REST-dependent downscaling of the amplitude of the EPSCs but interestingly we also identified an REST-dependent upscaling in both frequency and amplitude of the inhibitory postsynaptic currents (Fig. 32).

To investigate in more detail the mechanism underlying the upscaling of the inhibitory inputs, we performed minimal extracellular stimulation in loose-patch configuration in order to analyze the eIPSCs onto excitatory and inhibitory postsynaptic target neurons. 4AP treatment leads to an increase of the amplitude of the eIPSCs only when the postsynaptic target neuron was excitatory and such effect was abolished by REST inhibition with ODN. Moreover, this effect was accompanied by a decrease of PPR, suggesting a presynaptic mechanism of action (Fig. 34).

To further investigate this aspect, we studied the quantal parameters of the neurotransmitter release using the cumulative amplitude analysis, showing an increase of both the RRPsyn and the Pr of GABAergic synapses (Fig. 35 D, E). This effect was fully blocked when REST activity was inhibited by ODN treatment, demonstrating that the REST-mediated up-scaling of GABAergic synaptic contacts in response to neural hyperactivity acts through a presynaptic mechanism of action. Notably, eIPSCs on inhibitory neurons was not affected, demonstrating the strong postsynaptic target specificity of this REST dependent synaptic homeostasis (Fig. 37, 38).

To investigate whether the REST-dependent synaptic homeostasis also involved a change at the postsynaptic level we analyzed miniature inhibitory postsynaptic currents (mIPSCs) in cultured hippocampal neurons. Indeed, miniature events represent the postsynaptic response due to the fusion of individual vesicles and

the analysis of mIPSCs amplitude allows to estimate the postsynaptic properties of neurotransmission while changes mIPSCs frequency could be ascribed to presynaptic effects or changes in the number of functional synaptic contacts. While amplitude of mIPSCs was not affected by 4AP, the frequency was significantly enhanced by hyperactivity and inhibition of REST suppressed this effect confirming a REST role at presynaptic site (Fig. 39). Interestingly, in accord with the effect of 4AP on the evoked IPSCs, when mIPSC were recorded from inhibitory neurons we did not observe any effect (Fig. 40).

The increase of the frequency of mIPSCs could be explained by an increase in the number of SVs ready for release in the active zone, but also by an increase in the number of functional inhibitory synapses. To evaluate this last possibility, we performed immunocytochemistry assays (Fig. 41 A-C) observing that 4AP treatment induced an increase in density of axo-somatic inhibitory synapses but no changes on the axo-dendritic synapses. This effect was blocked by ODN treatment demonstrating the involvement of REST. Moreover, as previously observed also in this case the effect was present only when the target neuron was excitatory (GAD67 negative) (Fig. 41 D-F).

The strong target specificity that characterizes the REST-dependent HP, suggested us to investigate the possible involvement of BNDF. Indeed, it was previously shown that BNDF is expressed and released in response to hyperactivity only by excitatory neurons (Hofer et al., 1990; Matsumoto et al. 2008) and it is well known that the chronic treatment with BDNF strengthen GABAergic inhibitory inputs mainly acting at the presynaptic site increasing (Baldelli et al., 2002, Baldelli et al., 2005). We performed a time course analysis of the change in the REST and BDNF mRNA levels of cortical neurons maintained in 4AP for 6, 24, 48 and 96 h (Fig. 42). Both REST and BDNF showed a time dependent up-regulation of mRNA levels upon 4AP treatment. While the mRNA levels of cds-BDNF, BDNF-P1 and Syt4 (Fig. 44 B-D) recovered their control level after 24 h, REST mRNA returned to control levels after 96 h (Fig. 42 A). In order to evaluate the role of BDNF in the potentiation off

inhibitory synapses, we studied the effect of 4AP on the eIPSCs amplitude and PPR in cultured hippocampal neurons (17 div) treated for 48 h with a TrkB/fc, a recombinant chimeric protein that works as a BDNF-scavenger able to efficiently suppress its binding to the endogenous TrkB receptor.
Interestingly TrkB/fc fully suppressed the increase in the amplitude of the eIPSCs and the decrease of the PPR previously observed in presence of 4AP (Fig. 34), demonstrating the BDNF involvement (Fig. 43).

As previously mentioned, REST/NRSF is known as a negative transcription factor (Mori et al., 1992), for this reason it is difficult to explain how REST could induce an up-scaling of inhibitory synapses onto excitatory neurons. Nonetheless, recent experimental evidences showed that REST could be not only a repressor (Perera et al., 2015; Bersten et al., 2014; Kallunki P. et al., 1998). During the last decade it has been demonstrated that NPAS4 supports the development of inhibitory synapses through a NPAS4-dependent BNDF release, which is synthetized only by excitatory neurons (Hofer et al., 1990), upon hyperactivity (Lin et al., 2008; Bloodgood et al., 2013; Spiegel et al., 2014). Therefore, REST could be involved in the "NPAS4-BDNF gene program".
In order to dissect the possible crosstalk between the activation of REST and NPAS4-BDNF involvement, we analysed in parallel the mRNA changes of REST, NPAS4, cds-BDNF, P1-BNDF, P4-BNDF and Syt-4 in cultured neurons (17 div) treated with NEG, NEG+4AP, ODN and ODN+4AP for 1, 3, 6, 12 and 24 h (Fig. 44 A-F). Interestingly, REST mRNA increased after 1 h and persisted with a moderate decrease at 24 h. NPAS4 mRNA increased after 1 h, in agreement with the Greenberg work (Lin Y. et al., 2008), and started to decrease at 12 h recovering its control value after 24 h. When 4AP was applied in presence of ODN, the fast (1 h) and transient (recovery after 24 h) increase of NPAS4 was substitute by a delayed and persistent NPAS4 increase.

These results suggest that the basal level of REST is implicated in the immediate (1 h) activity-dependent induction of NPAS4, while the persistent increase of REST is at the basis of the NPAS4 mRNA return to its basal level.

Notably the ODN not only deleted the 4AP-dependent NPAS4 increase (1 h) but also suppressed P1-BDNF mRNA increase (3 h), the BNDF splice variant directly controlled by NPAS4 (Lin Y et al., 2008; Pruunsild et al., 2011). Moreover, ODN also repressed the capability of cds-, P4- and P1-BDNF mRNA to recover its control level after 24 h of hyperactivity, demonstrating that the sustained REST increase also plays a crucial role as temporal constrain of the BNDF activity (Fig. 44). Previous reports have indeed clearly demonstrated the epileptogenic role played by an excessive BDNF increase due to its dysregulation (Garriga-Canut et al., 2006) and accordingly our results demonstrated the homeostatic control exerted by REST on BNDF activity.

In order to investigate the gene underlying the functional changes induced by the REST-dependent activation of the NPAS4/BNDF gene program, we used rtPCR to analyse changes in mRNA coding for REST target genes relevant for the function of GABAergic synapses, such as GAD65 (Frederikse and Kasinathan, 2015), vGAT Saritas-Yildirim et al., 2015) , GAD67 (Lunyak et al., 2002) and the epsilon subunit of the GABAA receptor (Bersten et al., 2014). While GAD65 was not modulated by the neuronal hyperactivity GAD67, vGAT and epsilon GABA R subunit mRNAs showed a significant increase in response to 4AP that was blocked by ODN treatment (Fig. 46 A-D).

In these years, we focused our attention onto the role of REST in the process of homeostatic plasticity evoked by neuronal hyperactivity with a well-known convulsive agent, the unspecific K^+ channel inhibitor, 4AP (Avoli et al., 2002; Pozzi et al., 2013). In 2013, we demonstrated that REST modulates the intrinsic properties on excitatory hippocampal neurons. The increased expression of REST,

induced by neuronal hyperexcitation, decreases VGNa channel expression downregulating I_{Na} at the single-cell level and reducing neuronal excitability and the overall neuronal network firing activity (Pozzi et al., 2013). More recently we demonstrated the existence of a REST-dependent transcriptional and translational rearrangement able to scale down the strength of excitatory synapses by modulating the transcriptional profile of multiple REST target genes involved in the structural and functional regulation of the presynaptic release machinery (Pecoraro-Bisogni et al., 2017).

The experimental evidences that I have collected in my PhD thesis-project strongly suggest that REST-induction by hyperactivity also affects the inhibitory transmission onto excitatory neurons, discovering a form of homeostatic upscaling of GABAergic synapses depending on the nature of the postsynaptic target neuron. This postsynaptic target specificity is probably due to a REST-dependent induction of a downstream transcription factor, NPAS4, known for its capability of activating BDNF release from excitatory neurons upon hyperactivity. The retrograde action of BDNF, released from the soma of excitatory neurons onto GABAergic presynaptic contacts, could explain the observed postsynaptic target specificity. Altogether, these results define a picture where REST works as master transcriptional regulator able to interact with other transcription factors protecting neuronal circuit from seizure-like activity (Fig. 47).

In summary, REST links the enhancement of neuronal circuit excitability to the activation of both intrinsic and synaptic homeostatic processes in order to restore a more physiological electrical behaviour. Probably REST/NRSF is only one of the many transcription factors that act as key drivers of a complex neuro-epigenetic program aimed to maintain neuronal homeostasis responding to various forms of stressors that perturb the physiological level of activity of the brain circuits.

The role of these epigenetic effectors is to determine which genes must be actively expressed and which genes must be repressed. The dysregulation of these transcription factors is thought to contribute to neurological diseases. Indeed, dysregulation of REST activity was reported in pathological states such as ischemia

(Calderone et al., 2003; Formisano L et al., 2007; Noh KM et al., 2012) and epileptic seizures (Palm K et al., 1998; Spencer EM et al., 2006; McClelland S et al., 2011). Despite such evidences, as mentioned, it is also known that REST has a protective role. High REST levels were found in the normal aging brain and were associated with the downregulation of potentially toxic genes involved in oxidative stress and amyloid β-protein toxicity, while low REST levels were present in Alzheimer's brains (Lu T, et al., 2014).

Overall these data demonstrated that under physiological conditions REST plays a predominant homeostatic and neuroprotective action (Pozzi et al., 2013; Lu et al., 2014; Baldelli P and Meldolesi J., 2015; Pecoraro-Bisogni et al., 2017). On the other hand, when REST is dysregulated, it may contribute to pathological conditions, contributing to the loss of the neuronal homeostasis and thus worsening the progression of the disease (Calderone et al., 2003; Zuccato et al., 2007; Formisano L et al., 2007 Noh KM et al., 2012; Hwang J.Y., et al., 2017).

For these reasons it is important to characterize more in detail the molecular basis underlying homeostatic plasticity, in order to find new strategy for modulating the transcription factors involved in HP and to avoid any dysregulation preventing the development of severe pathologies. An interesting point that we are actually attempting is the development of new efficient and innovative therapeutic approaches not based on drugs, but on the use of specific diets, aimed at enhancing the REST-mediated neuro-epigenetic processes, driving homeostatic plasticity.

Indeed, it is very well known that ketogenic diet (KD) has been employed as a treatment for drug-resistant epilepsy for over 90 years (Kossoff and Hartman, 2012). Despite the substantial efficacy of the KD, its use remains limited because of difficulties in implementation and tolerability but an effective alternative dietary approach is the low-glucose diet (LGD), which has a comparable efficacy with respect to the classic KD, but it is much better tolerated (Pfeifer et al., 2008).

Although these diets clearly exerted anti-epileptic effects, their mechanism of action are still obscure. In the last decade, distinct mechanisms of action

underlying the anti-seizure properties of LGD have been postulated (Garriga-Canut et al., 2006; Lutas A and Yellen G, 2013; Forte N. et al., 2016). KD and LGD have also been shown to interfere with transcriptional processes. Indeed, it has been shown that REST activity is also efficiently activated by the reduction of intracellular concentration of NADH induced by the inhibition of glycolysis (Garriga-Canut et al., 2006). Such metabolic recruitment of REST can induce the transcriptional repression of the brain-derived neurotrophic factor (BDNF) and its receptor TrkB (Garriga-Canut et al., 2006), two REST target genes well known for their strong pro-epileptogenic effects. Furthermore, the antiepileptic effects of glycolysis inhibition were abolished in conditional REST knockout, indicating that REST is required for the antiepileptic effect of LGD (Hu et al., 2011).

Thus, we are actually going to investigate whether a low-glucose diet (LGD) is capable to activate REST-mediated homeostatic mechanisms that protecting from the development of hyper-excitability, it could prevent or counteract epileptogenesis in a mice model of hereditary human epilepsy.

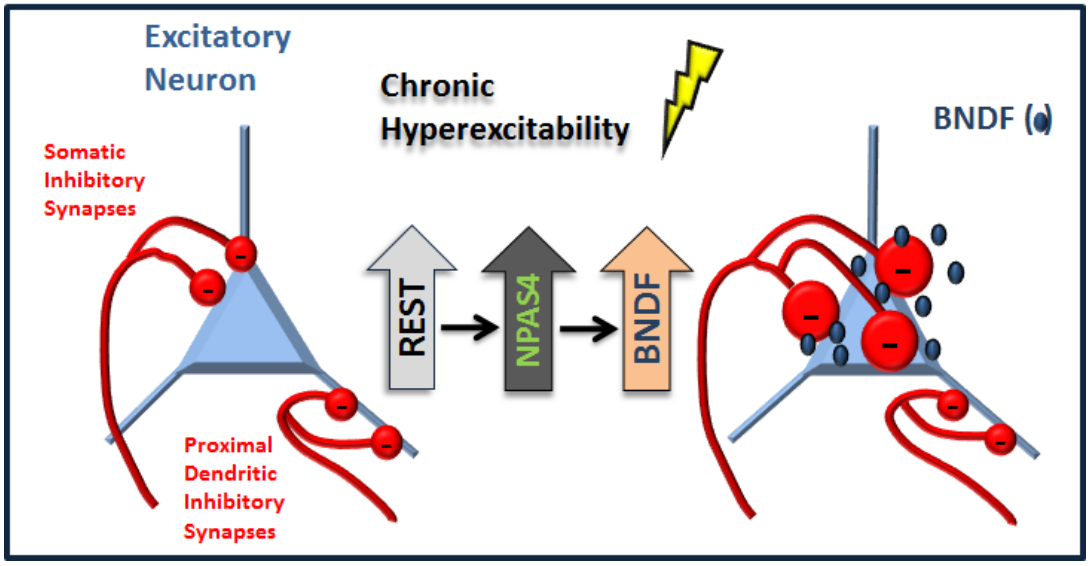


Figure 47: Schematic sketch showing the mechanism of action of the REST dependent pathway on the inhibitory synapses. Activity-dependent increase of REST induces a NPAS4-BDNF pathway that scaling-up the strength and the number of somatic inhibitory synapses onto excitatory neurons.

6. Material and Methods

Animals

GAD67-GFP knock-in mice were generated by inserting the cDNA encoding enhanced GFP into the GAD67 locus in TT2 embryonic stem cells, as described in (Tamamaki et al., 2003). Heterozygous GAD67-GFP males were mated with wild-type C57BL6/J females, and GFP-positive pups were identified at birth through a Dual Fluorescent Protein Flashlight (DFP-1, NIGHTSEA, Lexington, MA USA) and confirmed by genotyping, performed by PCR with the following primers: TR-1b: GGCACAGCTCTCCCTTCTGTTTGC; TR-3:GCTCTCCTTTCGCGTTCCGACAG; TRGFP-8:CTGCTTGTCGGCCATGATATAGACG. All animals were provided by our institutional animal breeding facility in accordance with the guidelines approved by the local Animal Care Committee of the University of Genova. Experiments used 0–2-day-old pups of either sex. Some control experiments were done using wild-type C57BL6/J mice. All experiments were carried out in accordance with the guidelines established by the European Communities Council (Directive 2010/63/EU of 4 March 2014) and were approved by the Italian Ministry of Health (authorization 73/2014-PR and 1276/2015-PR).

Cell cultures

Primary hippocampal neurons were prepared from postnatal GAD67-GFP knock-in mice (P0–P1), as previously described (Beaudoin GM et al. 2012, Valente et al., 2016). In brief, hippocampi were dissociated by enzymatic digestion in 0.25% trypsin for 6 min at 37 °C and then triturated with a fire-polished Pasteur pipette (Banker G and Goslin K, 1998). No antimitotic drugs were added to prevent glia proliferation. The following solutions were used for cell culture preparations: HANKS solution, prepared from HBSS (GIBCO 14170-088; red) supplemented with 10 mM HEPES, 30 mM D-glucose, 5 µg/ml Gentamycin, pH 7.4 with KOH; dissection solution, prepared from HANKS solution supplemented with 10% bovine serum albumin and 6mM MgSO4*7H2O. Primary hippocampal neurons were

plated at density of 120 cells/mm2 on 3.5-cm-diameter Petri dishes (Falcon® 35 mm, 353001) treated for 24 h with poly-L-lysine (0.1 mg/ml; Sigma-Aldrich) in borate buffer (0.1 M). Cells were grown in a culture medium consisting of Neurobasal A (Gibco™) supplemented with 2% B-27 (Invitrogen, Italy), 1 mM Glutamax, and 5 µg/ml Gentamycin and maintained at 37 °C in a humidified incubator with 5% CO2.

ODN decoy

Single stranded oligonucleotides were synthesized by Sigma Genosys (St. Louis, MO, USA). Annealing was performed in 10X annealing buffer (100 mM Tris-HCl, pH8.0, 10 mM EDTA, 1 M NaCl) by heating to at least 5–10°C above their Tm and cooling slowly using a heat block. A ODN decoy (RE1) was designed corresponding to a canonical REST-binding site (Johnson et al. 2006). A negative decoy control (NEG) was generated using a sequence corresponding to a non-canonical RE1 that has been shown not to bind REST (Bruce et al. 2004). The decoy ODN sequences, including a phosphorothiolate modification on the first three nucleotides were: ODN (Positive decoy): (Top) 5'-GpPpCpPTPTTCAGCACCACGGACAGCGCCAGC-3', (Bot) 3'-GpPpCpPTPGGCGCTGTCCGTGGTGCTGAAAGC-5'; NEG (Negative decoy) (Top) 5'-GpPpCpPTPTCCAGCACAGTGGTCAGACCC-3', (Bot) 3'-GpPpCpPTPTCTGACCACTGTGCTGGAAGC-5'; ODN-Top-Cy3: 5'-GpPpCpPTP TTCAGCACCACGGACAGCGCCAGC-Cy3.

Patch-Clamp Recordings

Neuronal extracellular recordings was studied using loose-patch configuration, on a presynaptic interneuron (GAD67-GFP+) and the response was analyzed in a postsynaptic excitatory cell (GAD67-GFP-) or in a postsynaptic inhibitory cell (GAD67-GFP+). Whole-cell patch-clamp recordings were conducted on hippocampal neurons at density of 120 cells/mm2. Electrophysiological experiments were performed at 19 div after treatment with NEG ± 4AP and ODN ±

4AP for 48 h. Patch pipettes, prepared from thin borosilicate glass (Kimble, Kimax, Mexico), were pulled and fire-polished to a final resistance of 3–4 MΩ.

The eIPSCs were recorded in Tyrode extracellular solution to which D-()-2-amino-5-phosphonopentanoic acid (D-AP5; 50 µM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 µM) and QX314 (10 mM) were added to block NMDA, non-NMDA, and to block the voltage-activated Na+ channels, respectively. We adopted an internal solution (Cl-eq) containing (in mM) 140 KCl, 4 NaCl, 1 MgSO4, 0.1 EGTA, 15 glucose, 5HEPES, 3 ATP, and 0.1 GTP (pH 7.2with KOH). All recordings were performed at 22–24 °C. The external solution composition was (in mM) 140 NaCl, 2 CaCl2, 1 MgCl2, 4 KCl, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). Under this condition, internal and external chloride concentrations were equimolar, shifting the chloride reversal potential from a negative value to 0 mV. This experimental configuration, that transforms eIPSCs in inward currents, is typically used for increasing the amplitude of the eIPSCs, evoked at negative holding potentials. Patch-clamp recordings with leak currents > 200 pA or series resistance > 20 MΩ were discarded. Data acquisition was performed using PatchMaster program (HEKA Elektronik). Series resistance (Rs) was compensated 80% (2 µs response time) and the compensation was readjusted before each stimulation. The shown potentials were not corrected for the measured liquid junction potential (9 mV). Voltage clamp was used to record evoked inhibitory postsynaptic currents (eIPSCs), clamping the neuron at −70 mV with the patch pipette recording the response from postsynaptic excitatory cell (GAD67-GFP-) and stimulating the presynaptic interneuron (GAD67-GFP+) with two short (0.5 ms) voltage steps to +40mV applied at 50 ms interval by using specific blockers of excitatory (CNQX, 10 µM), D-(−)-2-amino-5-phosphonopentanoic acid (D-APV; 50 µM; Tocris), synaptic transmission. For each couple of eIPSCs, the ratio I2/I1 (paired-pulse ratio, PPR) was calculated, where I1 and I2 are the amplitudes of the eIPSCs evoked by the conditioning and test stimuli, respectively. The amplitude of I2 was determined as the difference between the I2 peak and the corresponding value of I1 calculated by

mono-exponential fitting of the eIPSC decay. Only cells with resting membrane potentials between 57 and 64mV were considered for analysis.

The same experimental and analysis protocols were used to evaluate whether BNDF played a functional role in the 4AP-induced strengthened of inhibitory synapses. Indeed, to block the activity of BDNF (Sakuragi et al., 2013), cultured neurons were incubated at 17 div with the TrkB-Fc (1µg/mL, T8694-SIGMA) a BDNF scavenger, which is a chimeric molecule of TrkB and IgG-Fc a cDNA sequence encoding the extracellular domain of human TrkB1 fused to the Fc region of human IgG1. The eIPSCs and PPR were recorded at 19 div.

Cumulative eIPSC amplitude analysis to estimate Pr and RRP size

The size of the RRP of synchronous release (RRPsyn) and the probability that any given SV in the RRP will be released (Pr) were calculated using the cumulative amplitude analysis (Marconi et al., 2012; Baldelli et al., 2005; Schneggenburger et al., 2002). High frequency stimulation (2 s at 20 Hz) was applied to presynaptic fibers with the extracellular electrode. The RRPsyn was determined by summing up peak IPSC amplitudes during 40 repetitive stimuli applied at a frequency of 20 Hz. The analysis assumes that the depression during the steady-state induced by the train is limited by a constant recycling of SVs and that equilibrium is present between released and recycled SVs. The number of data points to include in the fit of the steady-state phase was evaluated by calculating, for each cell, the best linear fit which included the maximal number of data points starting from the last one. According to this procedure, the intercept with the y-axis gave an estimation of the size of the synchronous readily releasable pool (RRP) and the ratio between the first eIPSC amplitude (I1), evoked by the stimulation train, and RRPsyn yielded an estimation of Pr.

Whole-cell patch-clamp recordings of mIPSCs and sPSCs

Patch pipettes, prepared from thin borosilicate glass (Kimble, Kimax, Mexico), were pulled to a final resistance of 4–5 MΩ when filled with the internal solution (Cl-eq) containing (in mM) 140 KCl, 4 NaCl, 1 MgSO4, 0.1 EGTA, 15 glucose, 5HEPES, 3 ATP, and 0.1 GTP (pH 7.2with KOH). Neurons were treated at 17 div, with NEG ± 4AP and ODN ± 4AP for 48 h and recorded at 19 div. mIPSCs were recorded from cultured hippocampal excitatory (GAD67-GFP-) and inhibitory (GAD67-GFP+) neurons, using a double EPC-10 amplifier (HEKA Electronic, Lambrecht, Germany). Cells were maintained in a standard external solution (Tyrode) containing (in mM): 140 NaCl, 2 CaCl2, 1 MgCl2, 4 KCl, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). Unless otherwise indicated, D-(-)-2-amino-5-phosphonopentanoic acid (D-APV; 50 µM Tocris); CNQX (10 µm, Tocris); CGP58845 hydrochloride (10 µM Tocris) and TTX (1 µM), were added to the Tyrode external solution. Experiments were performed at 22–24 °C and mIPSCs were acquired at 20 kHz sample frequency and filtered at half the acquisition rate with an 8-pole low-pass Bessel filter. Recordings with leak currents >100 pA or series resistance >20 MΩ were discarded. Data acquisition was performed using PatchMaster program (HEKA Elektronic). The mIPSCs analysis was performed by using the Minianalysis program (Synaptosoft, Leonia, NJ and the Prism software (GraphPad Software, Inc.). The amplitude and frequency of mIPSCs were calculated using a peak detector function with a threshold amplitude set at 4 pA and a threshold area at 50 ms*pA.

sPSCs were recorded from cultured hippocampal neurons treated at 17 div, with NEG ± 4AP and ODN ± 4AP for 48 h and recorded at 19 div. Cells were maintained in an external solution containing (in mM): 140 NaCl, 1.6 CaCl2, 0 MgCl2, 4 KCl, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). Patch pipettes, prepared from thin borosilicate glass (Kimble, Kimax, Mexico), were pulled to a final resistance of 3–4 MΩ when filled with the internal solution containing (in mM) 126 K gluconate, 4 NaCl, 1 MgSO4, 0.02 CaCl2, 0.1 BAPTA, 15 glucose, 5 HEPES, 3 ATP, and 0.1 GTP

(pH 7.2 with KOH). No blockers were added to the external solution. Recordings with leak currents >100 pA or series resistance >20 MΩ were discarded. Data acquisition was performed using PatchMaster program (HEKA Elektronic). The sPSCs analysis was performed by using the Minianalysis program (Synaptosoft, Leonia, NJ and the Prism software (GraphPad Software, Inc.).

Immunocytochemistry

Immunocytochemistry of primary hippocampal neurons (from Knock-in GAD67-GFP mice) after treatment with NEG ± 4AP and ODN ± 4AP for 48 h, were fixed at 19 div with 4% paraformaldehyde/4% sucrose for 12 min at room temperature and then washed with phosphate buffered saline (PBS). Cells were then permeabilized with methanol (-20°C; 10 min on ice) followed by 0.2% Triton X-100 for 10 min (Liao et al., 1999) and blocking with 5% FBS/0.1% BSA for 30 min in PBS, before their incubation for 2h with primary antibodies in PBS/5% FBS/0.1% BSA, then washed with PBS and final incubation for 2h with secondary antibodies. For immunofluorescence analysis, neurons were immunostained with antibodies against chicken β3-Tubulin (1:500, Synaptic System 302306), rabbit vGAT (1:500, Synaptic System 131011) and mouse Gephyrine (1:200, Synaptic System 147011). Secondary antibodies were Alexa405 goat anti-chicken (abcam, ab175674), Alexa546 goat anti-rabbit and Alexa647 goat anti-mouse (1:500 in all cases, Thermo fisher Cat. A11010, A32728). Images of cultured neurons were acquired with a Leica SP8 confocal microscope using a 63× objective and 1024×1024 pixels (1pixel=0.24 µm). Confocal images were analyzed using ImageJ. Each single stack was filtered at the maximal fluorescence intensities of stacks were Z-projected. Analysis of fluorescence intensity was performed on dendritic ROIs (3-5 per image) of 60-160 µm in lengths and somatic ROIs (2-3 per image) of 1000-2000 µm2 in blind condition. The intensity of each ROIs has been calculated as the plot profile of average intensity of each puncta that circumscribes the vGAT and Gephyrine positive synaptic signal. The synaptic densities on dendrites were obtained by counting the total number of positive puncta divided by the length of the tubulin

positive segment (in µm). The synaptic densities on somas were obtained by counting the total number of positive puncta divided by the Area of the tubulin positive segment (in µm2). The number of samples (n) represents the number of coverslips from at least three neuronal preparations conducted in parallel. From each coverslip, at least 10–15 images were collected.

Live cell-imaging (Cyanine-3 treatment)

Images of cultured neurons were acquired using an Olympus IX71 microscope with a 40× objective (Olympus LCPlanFI 40X/0.60 Ph2) with an Hamamatsu (ORCA-ER) camera. Neurons fluorescence were recorded by Leica EL6000 fluorescence lamp.
Cultured Hippocampal neurons, from GAD67-GFP knock-in mice, at (17div) were incubated for 12 h and 24 h with a ODN tagged to a fluorescent probe Cyanine-3, (Cy3-ODN) and then washed 2 times with warm solution (Tyrode) containing (in mM): 140 NaCl, 2 CaCl2, 1 MgCl2, 4 KCl, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). Cells were then treated for 5 minutes with staining solution (freshly prepared) containing Tyrode solution added with Hoechst-333342 (3 µl/ml) and washed with warm Tyrode and immediately acquired. Images were analyzed using ImageJ. For each images we perform a somatic and nuclear ROIs of CY3 positive area in order to compare the REST partition (AreaCy3nucleus/AreaCy3cyto) between the cytosol and the nucleus in excitatory and inhibitory neurons. We also analyzed REST expression comparing the percentage of Cy3-positive area versus the total area, in the cytosol (AeraCy3 cyto/=AreaTOT cyto) and in the nucleus (AeraCy3 nucleus/=AreaTOT nucleus) in excitatory and inhibitory neurons.

Real-time PCR (RT-qPCR)

Quantitative RT-PCR was performed in accordance with MIQE guidelines (Bustin et al., 2009). RNA was extracted with TriaZol reagent and purified on RNeasy spin columns (Qiagen). RNA samples were quantified at 260nm with an ND1000 Nanodrop spectrophotometer (Thermo Scientific). RNA purity was also determined by absorbance at 280 and 230 nm. All samples showed A260/280 and A260/230

ratios greater than 1.9. Reverse transcription was performed according to the manufacturer's recommendations on 1 µg of RNA with the QuantiTect Reverse Transcription Kit (Qiagen), which includes a genomic DNA–removal step. SYBR green RT-qPCR was performed in triplicate with 10 ng of template cDNA using QuantiTect Master Mix (Qiagen) on a 7900-HT Fast Real-time System (Applied Biosystems) as previously described (Pozzi et al. 2013; Deidda et al. 2015), using the following universal conditions: 5 min at 95 °C, 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 30 s. Product specificity and occurrence of primer dimers were verified by melting-curve analysis. Primers were designed with Beacon Designer software (Premier Biosoft) to avoid template secondary structure and significant cross homology with other genes by BLAST search. The PCR reaction efficiency for each primer pair was calculated via the standard curve method with four serial-dilution points of cDNA. The PCR efficiency calculated for each primer set was used for subsequent analysis. All experimental samples were detected within the linear range of the assay. Gene-expression data were normalized via the multiple-internal-control-gene method (Vandesompele et al. 2002) with the GeNorm algorithm available in qBasePlus software (Biogazelle).

The control genes used were GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and PPIA (peptidylprolyl isomerase), the expression of these genes was found not to be affected by the 4AP treatment. Primers sequences (5'-3') were: GAPDH-F: GAACATCATCCCTGCATCCA; GAPDH-R: CCAGTGAGCTTCCCGTTCA; PPIA-F: CACTGTCGCTTTTCGCCGCTTG; PPIA-R: TTTCTGCTGTCTTTGGAACTTTGTCTGC; REST-F: GAACCACCTCCCAGTATG; REST-R: CTTCTGACAATCCTCCATAG; Bdnf cds-F: GATGCCGCAAACATGTCTATGA; Bdnf cds-R: TAATACTGTCACACACGCTCAGCTC; Bdnf (P1) F: TGGTAACCTCGCTCATTCATTAGA; Bdnf (P1) R:CCCTTCGCAATATCCGCAAAG; Bdnf (P4) F: CAAATGGAGCTTCTCGCTGAAGGC; Bdnf (P4) R:GTGGAAATTGCATGGCGGAGGTAA; NPAS4-F: AGGGTTTGCTGATGAGTTGC; NPAS4-R: CCCCTCCACTTCCATCTTC; GABArƐ-F: TCAATGCGAAGAACACTTGG; GABArƐ-R: AGAAGGAGACCCAGGAGAGC; GAD67 -F: CTAGGGACCCAGGGAAAG; GAD67 -R: GTACATCTGTCATCCATCATCC; GAD65-F:

ACCAATTATGGAGCGTCACAGG; GAD65-R: CTGAGGAGCAGCACCTTCTC; vGAT-F: TTCAGTGCTTGGAATCTAC; vGAT-R: TTCTCCAGAGTGAAGTCG; Syt4-F: CCTCACTCATCGCCATCCA; Syt4-R: GACCGCAGCTCACTCCAT.

Protein Extraction and Western Blotting Analysis

Total cell lysates were obtained from cortical neurons at 18 or 19 DIV treated as previously described. Neurons were lysed in lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA and 1 % Triton X-100) supplemented with protease inhibitor cocktail (Cell Signaling, Danvers, MA, USA). After 10 min of incubation, lysates were collected and clarified by centrifugation (10 min at 10,000 g). Protein concentrations were determined by the Bradford assays (Biorad, Hercules, CA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), and equivalent amounts of protein were subjected to SDS polyacrylamide gel electrophoresis on 8 % polyacrylamide gels and blotted onto nitrocellulose membranes (Whatman, Sigma-Aldrich, St. Louis, MO). Membranes were blocked for 1 h in 5 % non-fat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 8.0) plus 0.1 % Triton X-100 and incubated overnight at 4 °C or for 2 h at room temperature with the following primary antibodies: anti REST (1:500, 07-579 Millipore, MA, USA), anti NPAS-4 (1:500, S408-79 Thermo Scientific, Waltham, MA, USA), anti Synaptotagmin 4 (1:2000, 105043 Synaptic System, Goettingen, Germany, EU), anti-GAD67 (1:2000, MAB5406, Sigma-Aldrich, St. Louis, MO, USA), anti-GABA(A) ε Receptor (1:500, AGA-015 Alomone, Jerusalem, Israel), anti v-GAT (1:1000, 131002 Synaptic System, Goettingen, Germany, EU), anti GAPDH (1:2000, sc-25778 Santa Cruz Biotechnology, Inc., Texas, U.S.A). After several washes, membranes were incubated for 1 h at room temperature with peroxidase-conjugated anti-mouse (1:3000; BioRad, Hercules, CA) or anti-rabbit (1:3000; BioRad, Hercules, CA) antibodies. Bands were revealed with the ECL chemiluminescence detection system (Thermo Scientific, Waltham, MA, USA). Immunoblots were quantified by

densitometric analysis of the fluorograms (Quantity One software; Bio-Rad, Hercules, CA) obtained in the linear range of the emulsion response.

Statistical Analysis

All experiments were replicated at least three times. Data are shown as means ± SEM. To compare two normally distributed sample groups, the unpaired Student's two-tailed t test was used. To compare three experimental group not normally distributed we used one way ANOVA followed by Kruskal-Wallis multiple comparison test. To compare more than experimental groups (NEG ± 4AP and ODN ± 4AP) we used a two-way ANOVA followed by Sidak's post hoc test for functional analysis. Alpha levels for all tests were 0.05% (95% confidence intervals). Statistical analysis was carried out using OriginPro-8 (OriginLab Corp., Northampton, MA, USA) and Prism (GraphPad Software, Inc.) software.

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9. Appendix:

Articles published by Cosimo Prestigio during the PhD course.

- **Spike-Related Electrophysiological Identification of Cultured Hippocampal Excitatory and Inhibitory Neurons.** Mol Neurobiol. (2019) 56(9):6276-6292.

Cosimo Prestigio, Ferrante D., Valente P., Casagrande S., Albanesi E., Yanagawa Y., Benfenati F., Baldelli P.

Abstract: Cultured hippocampal neurons represent the most widely used experimental substrate for in vitro electrophysiological studies. Nevertheless, in most cases, the nature of neuron under study is not identified as excitatory or inhibitory, or even worse, recorded neurons are considered as excitatory because of the paucity of GABAergic interneurons. Thus, the definition of reliable criteria able to guarantee an unequivocal identification of excitatory and inhibitory cultured hippocampal neurons is an unmet need. To reach this goal, we compared the electrophysiological properties and the localization and size of the axon initial segment (AIS) of cultured hippocampal neurons, taking advantage from GAD67-GFP knock-in mice, which expressing green fluorescent protein (GFP) in gamma-aminobutyric acid (GABA)-containing cells, allowed to unambiguously determine the precise nature of the neuron under study. Our results demonstrate that the passive electrophysiological properties, the localization and size of the AIS, and the shape and frequency of the action potential (AP) are not reliable to unequivocally identify neurons as excitatory or inhibitory. The only parameter, related to the shape of the single AP, showing minimal overlap between the sample-point distributions of the two neuronal subpopulations, was the AP half-width. However, the estimation of the AP failure ratio evoked by a short train of high-current steps applied at increasing frequency (40-140 Hz) resulted to be indisputably the safer and faster way to identify the excitatory or inhibitory nature of an unknown neuron. Our findings provide a precise framework for further electrophysiological investigations of in vitro hippocampal neurons.

- **REST-Dependent Presynaptic Homeostasis Induced by Chronic Neuronal Hyperactivity.** Mol Neurobiol. (2018) 55(6):4959-4972.

Pecoraro-Bisogni F., Lignani G., Contestabile A., Castroflorio E., Pozzi D., Rocchi A., **Prestigio C.**, Orlando M., Valente P., Massacesi M., Benfenati F., Baldelli P.

Abstract: Homeostatic plasticity is a regulatory feedback response in which either synaptic strength or intrinsic excitability can be adjusted up or down to offset sustained changes in neuronal activity. Although a growing number of evidences

constantly provide new insights into these two apparently distinct homeostatic processes, a unified molecular model remains unknown. We recently demonstrated that REST is a transcriptional repressor critical for the downscaling of intrinsic excitability in cultured hippocampal neurons subjected to prolonged elevation of electrical activity. Here, we report that, in the same experimental system, REST also participates in synaptic homeostasis by reducing the strength of excitatory synapses by specifically acting at the presynaptic level. Indeed, chronic hyperactivity triggers a REST-dependent decrease of the size of synaptic vesicle pools through the transcriptional and translational repression of specific presynaptic REST target genes. Together with our previous report, the data identify REST as a fundamental molecular player for neuronal homeostasis able to downscale simultaneously both intrinsic excitability and presynaptic efficiency in response to elevated neuronal activity. This experimental evidence adds new insights to the complex activity-dependent transcriptional regulation of the homeostatic plasticity processes mediated by REST.

- **PRRT2 controls neuronal excitability by negatively modulating Na+ channel 1.2/1.6 activity.** Brain. (2018) 141(4):1000-1016.

Fruscione F., Valente P., Sterlini B., Romei A., Baldassari S., Fadda M., **Prestigio C.**, Giansante G., Sartorelli J., Rossi P., Rubio A., Gambardella A., Nieus T., Broccoli V., Fassio A., Baldelli P., Corradi A., Zara F., Benfenati F.

See Lerche (doi:10.1093/brain/awy073) for a scientific commentary on this article.Proline-rich transmembrane protein 2 (PRRT2) is the causative gene for a heterogeneous group of familial paroxysmal neurological disorders that include seizures with onset in the first year of life (benign familial infantile seizures), paroxysmal kinesigenic dyskinesia or a combination of both. Most of the PRRT2 mutations are loss-of-function leading to haploinsufficiency and 80% of the patients carry the same frameshift mutation (c.649dupC; p.Arg217Profs*8), which leads to a premature stop codon. To model the disease and dissect the physiological role of PRRT2, we studied the phenotype of neurons differentiated from induced pluripotent stem cells from previously described heterozygous and homozygous siblings carrying the c.649dupC mutation. Single-cell patch-clamp experiments on induced pluripotent stem cell-derived neurons from homozygous patients showed increased Na+ currents that were fully rescued by expression of wild-type PRRT2. Closely similar electrophysiological features were observed in primary neurons obtained from the recently characterized PRRT2 knockout mouse. This phenotype was associated with an increased length of the axon initial segment and with markedly augmented spontaneous and evoked firing and bursting activities evaluated, at the network level, by multi-electrode array electrophysiology. Using HEK-293 cells stably expressing Nav channel subtypes, we demonstrated that the expression of PRRT2 decreases the membrane exposure and Na+ current of Nav1.2/Nav1.6, but not Nav1.1, channels. Moreover, PRRT2

directly interacted with Nav1.2/Nav1.6 channels and induced a negative shift in the voltage-dependence of inactivation and a slow-down in the recovery from inactivation. In addition, by co-immunoprecipitation assays, we showed that the PRRT2-Nav interaction also occurs in brain tissue. The study demonstrates that the lack of PRRT2 leads to a hyperactivity of voltage-dependent Na+ channels in homozygous PRRT2 knockout human and mouse neurons and that, in addition to the reported synaptic functions, PRRT2 is an important negative modulator of Nav1.2 and Nav1.6 channels. Given the predominant paroxysmal character of PRRT2-linked diseases, the disturbance in cellular excitability by lack of negative modulation of Na+ channels appears as the key pathogenetic mechanism.

- **PRRT2 Is a Key Component of the Ca2+-Dependent Neurotransmitter Release Machinery.** Cell Rep. (2016) 15(1):117-131.

Valente P., Castroflorio E., Rossi P., Fadda M., Sterlini B., Cervigni RI., **Prestigio C.**, Giovedì S., Onofri F., Mura E., Guarnieri FC., Marte A., Orlando M., Zara F., Fassio A., Valtorta F., Baldelli P., Corradi A., Benfenati F.

Abstract: Heterozygous mutations in proline-rich transmembrane protein 2 (PRRT2) underlie a group of paroxysmal disorders, including epilepsy, kinesigenic dyskinesia, and migraine. Most of the mutations lead to impaired PRRT2 expression, suggesting that loss of PRRT2 function may contribute to pathogenesis. We show that PRRT2 is enriched in presynaptic terminals and that its silencing decreases the number of synapses and increases the number of docked synaptic vesicles at rest. PRRT2-silenced neurons exhibit a severe impairment of synchronous release, attributable to a sharp decrease in release probability and Ca(2+) sensitivity and associated with a marked increase of the asynchronous/synchronous release ratio. PRRT2 interacts with the synaptic proteins SNAP-25 and synaptotagmin 1/2. The results indicate that PRRT2 is intimately connected with the Ca(2+)-sensing machinery and that it plays an important role in the final steps of neurotransmitter release.