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By

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Abstract

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Homeostatic synaptic plasticity (HSP) is a form on non-Hebbian plasticity that allows neurons to sense their global level of activity and modulate their own function to keep firing rate within a working range. In particular, chronic elevation or reduction of network activity activates compensatory mechanisms that modulate synaptic strength in the opposite direction (i.e. reduced network activity leads to increased synaptic strength). Among the many mechanisms that mediate homeostatic synaptic plasticity, retinoic acid (RA) has emerged as a novel signaling molecule that is critically involved in homeostatic synaptic plasticity induced by blockade of activity. In neurons, global silencing of synaptic transmission triggers RA synthesis. RA then acts at synapses by a non-genomic mechanism that is independent of its well-known function as a transcriptional regulator, but operates through direct activation of protein translation in neuronal dendrites. Protein synthesis is activated by RA-binding to its receptor RARα, which functions locally in dendrites in a non-canonical manner as an RNA-binding protein that mediate RA's effect on translation.

In this dissertation I demonstrate the critical role that translational regulation operated by RA plays in synaptic scaling, a post-synaptic form of homeostatic plasticity which consists in the regulation of receptor abundance on the post-synaptic membrane in response to activity blockade. In this context, my work has focused on the regulation of both excitatory and inhibitory transmission operated by RA in order to elucidate whether this molecule, normally only associated with neural development, plays a pivotal role in the modulation of excitation and inhibition balance in the adult brain. Since, abnormalities in this balance have been linked to several pathological conditions such as autism and Fragile-X mental retardation, my work described here emphasizes the primary role of the dynamic regulation of the excitation/inhibition balance in neural networks mediated by RA.

To my twins, Giacomo and Tommaso:
the best experiment I have ever done.

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LIST OF ABBREVIATIONS

RA, retinoic acid

TTX, Tetrodotoxin

D-APV (also referred to as D-AP5), D-(-)-2-Amino-5-phosphonopentanoic acid, NMDA receptor blocker

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione is a competitive AMPA/kainate receptor antagonist

HSP, Homeostatic synaptic plasticity

AMPARs, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptors

NMDARs, N-methyl-D-aspartate receptors

GABARs, γ-Amino-butyric acid receptors

IPSCs, inhibitory post-synaptic currents

eIPSCs, evoked inhibitory post-synaptic currents

mIPSCs, miniature inhibitory post-synaptic currents

EPSCs, excitatory post-synaptic currents

eEPSCs, evoked excitatory post-synaptic currents

mEPSCs, miniature excitatory post-synaptic currents

FMRP, Fragile-X mental retardation protein

Chapter 1

INTRODUCTION

A dynamic range of network activity is essential for optimal information coding in the nervous system. It is thought that normal brain function requires neurons to operate at a constant overall activity level, and maintain a balance between the relative strength of individual synapses. In a neuron, activity levels are kept constant by a process collectively called homeostatic synaptic plasticity (HSP). HSP uniformly adjusts the strengths of a large portion if not all synapses in a neuron to maintain a particular activity level, and may be mediated by altered pre-synaptic transmitter release, synaptic vesicle loading properties, postsynaptic receptor functions, or neuronal membrane properties (Davis, 2006; Rich and Wenner, 2007; Turrigiano, 2012). Several signaling pathways are involved in various forms of HSP in the mammalian CNS and at the *Drosophila* neuromuscular junction (Turrigiano, 2008; Yu and Goda, 2009). In the mammalian nervous system, relative α/β CaMKII expression levels in neurons (Thiagarajan et al., 2002) , and CaMKIV-regulated transcriptional events (Ibata et al., 2008) mediate homeostatic compensation for the changes in neuronal firing and synaptic activity. Additionally, the level of Arc/Arg3.1, an immediate-early gene that is rapidly induced by neuronal activity (Guzowski et al., 2005), modulates homeostatic plasticity through a direct interaction with the endocytic pathway (Shepherd et al., 2006). Recent findings also implicated inactivity-induced postsynaptic synthesis and release of BDNF, which acts retrogradely to enhance presynaptic functions, in HSP (Jakawich et al., 2010). At the *Drosophila* neuromuscular junction, homeostatic synaptic plasticity is manifested mainly by changes in presynaptic release modulated by retrograde signaling mechanisms (Davis and Bezprozvanny, 2001). Multiple pathways were implicated involving molecules such as dysbindin (Dickman and Davis, 2009), Cav2.1 (Frank et al., 2006), the BMP ligand Gbb (Goold and Davis, 2007), Eph receptor and ephexin (Frank et al., 2009), and snapin (Dickman et al., 2012). In addition to these neuronal and muscle-derived molecules, glia-derived factors, such as the cytokine TNFα, have been demonstrated to control synaptic strength and to influence HSP (Beattie et al., 2002; Stellwagen and Malenka, 2006). Although a bewildering number of different pathways may thus contribute to HSP, recent progress has identified fundamental events that underlie many forms of HSP in mammalian nervous system, thereby suggesting that a limited number of basic molecular pathways can account for this important process.

Synaptic scaling is a form of HSP that was initially discovered in the visual cortex culture system where neuronal activity was either chronically blocked with tetradotoxin (TTX), or elevated by inhibition of GABAergic synaptic transmission (Turrigiano et al., 1998). The expression of synaptic scaling is thought to involve transcriptional events, which leads to changes in the abundance of AMPA-type glutamate receptors in the postsynaptic membrane (Turrigiano, 2012). An important property of synaptic scaling is that all synapses of a neuron are modified concurrently in a multiplicative fashion (i.e., stronger synapses are changed proportionally more than weaker synapses), thereby preserving the relative synaptic weights of the overall circuit (Turrigiano et al., 1998; Thiagarajan et al., 2005) (but see (Echegoyen et al., 2007). However, several recent studies

show that a fast adaptive form of HSP can be induced when excitatory synaptic transmission is blocked in addition to TTX treatment (Ju et al., 2004; Sutton et al., 2004; Sutton et al., 2006). Importantly, this rapid form of HSP is mediated by the local synthesis and synaptic insertion of homomeric GluA1 receptors, allowing adjustment of synaptic strength at spatially discrete locations within a neuron (Table 1). Although several biochemical signaling pathways that trigger dendritic protein synthesis upon increase in neuronal activity have been identified (Kelleher et al., 2004; Klann and Dever, 2004; Schuman et al., 2006), the signaling pathways involved in this type of inactivity-induced synaptic scaling remain largely unclear.

Even less evidence has been accumulated in the past years regarding the behavior of inhibitory transmission during HSP. In particular, no evidence has elucidated whether the same intracellular mechanisms mediating excitatory transmission can also modulate inhibitory transmission. Recent findings show that long-lasting changes in activity also modulate inhibitory transmission in the same cells or systems that express homeostatic changes at excitatory synapses (Kilman et al., 2002; Saliba et al., 2009). This modulation is mediated by changes in the number of post-synaptic $GABA_A$-receptors ($GABA_ARs$) in a reciprocal manner to the changes in excitatory receptors (Saliba et al., 2007). However, little is known about the mechanisms of homeostatic changes in synaptic inhibition and my thesis work has also focused on this question.

Therefore, this thesis will focus on recent progress in identifying retinoic acid (RA) as a novel synaptic signaling molecule that mediates activity-dependent regulation of neural function, in particular on the bidirectional modulation of both inhibitory and excitatory transmission.

1.1 Retinoid synthesis and signaling in the adult nervous system

Biological sources of retinoids include preformed Vitamin A from animal-derived food, or pro-Vitamin A carotenoids (e.g. β-carotene) from plant-derived foods. The majority of preformed Vitamin A and pro-Vitamin A are converted into all-trans-retinol by a series of reactions in the intestinal lumen and mucosa. Upon absorption into enterocytes, re-esterified retinol is transported to the liver, which is the major site for retinoid storage and processing in the body. Retinol is secreted from the liver in response to the body's needs and is transported in the blood bound to retinol binding protein (RBP). In target cells, a membrane receptor for RBP mediates cellular uptake of retinol (Kawaguchi et al., 2007), which is metabolized into its bioactive derivative, all-trans-retinoic acid (RA). RA then exerts its potent effects in a variety of biological systems.

RA is synthesized from retinol in a two-step oxidation reaction. First, cytosolic retinol dehydrogenase (ROLDH) or alcohol dehydrogenase (ADH) converts retinol to retinal (retinaldehyde). Second, retinal dehydrogenase (RALDH) oxidizes retinal to RA. These enzymes are expressed in the adult mammalian brain (Krezel et al., 1999; Zetterstrom et al., 1999). Local RA synthesis in adult brain has been demonstrated using transgenic mice expressing LacZ downstream of three canonical retinoic acid response elements (RAREs) (Thompson Haskell et al., 2002). Strikingly, in the forebrain, cerebellum and meninges, the rates of RA synthesis are comparable to, or exceed, the rates of RA synthesis in liver (Dev et al., 1993). Taken together, these studies unequivocally establish that RA synthesis occurs in the adult brain (Dev et al., 1993; Wagner et al., 2002).

Until recently, RA synthesis was assumed to only sub-serve regulation of gene expression, but as I will discuss below, recent studies revealed that RA has a second,

completely different function in controlling synaptic strength independent of transcription. During development, due to the powerful action of RA on gene transcription and nervous system patterning, the RA level and its spatial gradient are strictly regulated by the opposing action of the two main classes of RA metabolizing enzymes, the RALDHs and the CYP26, a cytochrome P450-related enzyme that oxidatively inactivates RA (McCaffery and Drager, 1993; Fujii et al., 1997; Niederreither et al., 1997; Berggren et al., 1999). Similarly, in mature CNS, RA is not uniformly available but is only present in discrete regions of the brain (Lane and Bailey, 2005; Bremner and McCaffery, 2008). In cultured hippocampal neurons, RA is not detectable when neurons are active (Aoto et al., 2008). RA synthesis is strongly induced by loss of synaptic activity and a decrease in dendritic calcium levels (Wang et al., 2011b), supporting its role as an important signaling molecule that modulates neuronal function in an active manner.

The action of RA is primarily mediated by nuclear retinoid receptor proteins called retinoic acid receptors (RAR-α, -β, -γ) and retinoid 'X' receptors (RXR-α, -β, -γ). Like other members of the steroid receptor family, RARs and RXRs are transcription factors. Although structurally similar, the ligand specificity differs between RARs and RXRs in that RARs bind RA with high affinity, whereas RXRs exclusively bind 9-cis-retinoic acid (Soprano et al., 2004). Because 9-cis-RA is undetectable *in vivo*, the effects of retinoids on gene transcription are presumed to be mediated by RA binding to RARs. In the adult mammalian brain, RARα is abundant in the cortex and hippocampus, RARβ is highly expressed in the basal ganglia, and RARγ is not detectable (Krezel et al., 1999; Zetterstrom et al., 1999). Although RARs are concentrated in cell nuclei, they shuttle in and out of the nucleus like other nuclear receptors. For example, when an RARα-GFP fusion protein was expressed in HeLa cells, 20% of the total protein was cytosolic, but rapidly moved into the nucleus upon RA-binding (Maruvada et al., 2003). RARα has also been shown to be both cytoplasmic and nuclear in mature hippocampal neurons (Aoto et al., 2008). Moreover, a recent study (Huang et al., 2008) reported that the subcellular localization of RARα exhibited a developmental shift from the nucleus to the cytosol. The expression levels of total RARα in postnatal hippocampus gradually decrease over time with increasing developmental maturity of the neurons. After postnatal day 29, equal or greater amounts of RARα were detected in the cytosol compared to the nucleus in both pyramidal and granule cells, consistent with the intriguing possibility that RARα may assume a function in the cytoplasm of mature neurons that differs from its function as a transcription factor.

In the developing nervous system, RA signaling is involved in neurogenesis and neuronal differentiation, and operates exclusively by regulating gene transcription. However, the evidence reviewed above, especially the continuing high levels of RA synthesis, indicates that RA signaling may also play an important role in the mature brain (Lane and Bailey, 2005). RA signaling has been implicated in activity-dependent long-lasting changes of synaptic efficacy that are thought to be the cellular mechanism underlying learning and memory. For example, impaired long-term potentiation (LTP) and long-term depression (LTD) have been demonstrated in mice lacking RARβ or both RARβ and RARγ (Chiang et al., 1998). The function of RARα in hippocampal plasticity remains unknown because targeted disruption of RARα results in early postnatal lethality (Lufkin et al., 1993). Additionally, both LTP and LTD are reduced in Vitamin A deficient mice, but the impaired plasticity can be reversed after administration of a Vitamin A supplemented diet, indicating that synaptic plasticity is modulated by retinoids in adult brain (Misner et

al., 2001). The involvement of RA in adult brain function is further supported by deficits in learning and memory tasks observed in RAR null or Vitamin A-deficient mice (Chiang et al., 1998; Cocco et al., 2002).

1.2 Retinoic acid signaling in synaptic scaling

Synaptic scaling is form of HSP that was initially discovered in cultured cortical neurons where neuronal activity was either chronically blocked with tetradotoxin (TTX), or elevated by inhibition of GABAergic synaptic transmission (Turrigiano et al., 1998). The expression of synaptic scaling is thought to involve transcriptional events that alter the abundance of AMPA-type glutamate receptors in the postsynaptic membrane (Turrigiano, 2012). An important property of synaptic scaling is that all synapses of a neuron are modified concurrently in a multiplicative fashion (i.e., stronger synapses are changed proportionally more than weaker synapses), thereby preserving the relative synaptic weights of the overall circuit (Turrigiano et al., 1998; Thiagarajan et al., 2005) but see (Echegoyen et al., 2007). However, several recent studies show that a fast adaptive form of HSP can be induced when excitatory synaptic transmission is blocked in conjunction with TTX treatment (Ju et al., 2004; Sutton et al., 2004; Sutton et al., 2006). Importantly, this rapid form of HSP is independent of transcription, and is mediated by the local synthesis and synaptic insertion of homomeric GluA1 receptors, allowing adjustment of synaptic strength at spatially discrete locations in a neuron. Although several biochemical signaling pathways can trigger dendritic protein synthesis upon increase in neuronal activity (Kelleher et al., 2004; Klann and Dever, 2004; Schuman et al., 2006), the signaling pathways involved in this type of inactivity-induced synaptic scaling remain largely unclear. However, several years ago Prof. Lu Chen's laboratory identified retinoic acid (RA) as a key mediator of transcription-independent HSP (Aoto et al., 2008).

1.3 RA as a potent regulator of dendritic protein synthesis and synaptic strength

The discovery of the role of RA in HSP was fortuitous. The group was intrigued by the potential involvement of RA signaling in Hebbian plasticity and hippocampal dependent learning, and investigated the direct effect of RA on excitatory synaptic transmission in hippocampal neurons. Aoto et al. found that acute RA application rapidly increased the amplitude of miniature excitatory postsynaptic currents (mEPSCs). The observed enhancement of mEPSC amplitude by RA was multiplicative in nature, reminiscent of synaptic scaling (Aoto et al., 2008). Additional experiments showed that the synaptic effect of RA was independent of the formation of new dendritic spines (Chen and Napoli, 2008), but instead operated by stimulating the synthesis and insertion of new postsynaptic GluA1-containing glutamate receptors into existing synapses (Aoto et al., 2008). Different from the known function of RA as a transcription factor, the effects of RA on synaptic transmission and surface GluR1 expression could not be blocked by transcription inhibitors, but were abolished by protein synthesis inhibitors. Moreover, RA directly stimulated GluA1 protein synthesis in synaptoneurosomes, a biochemical preparation that lacks nuclear components and therefore operates in a transcription-independent manner (Aoto et al., 2008; Poon and Chen, 2008).

The nature of RA-gated protein translation in neurons was further characterized by immunogold electron microscopy (Maghsoodi et al., 2008). Local protein synthesis in

dendrites requires mRNAs and the translation machinery, both of which are trafficked to dendrites through assembly of an electron-dense structure termed RNA granules, which are large RNA-protein complexes that serve not only as mRNA trafficking units but also as storage compartments for mRNAs and translation machinery (Krichevsky and Kosik, 2001; Anderson and Kedersha, 2006). Consistent with the rapid GluA1 synthesis induced by RA in synaptoneurosomes, brief RA treatments drastically increased the GluA1 labeling in dendritic RNA granules, a process that was blocked by inhibitors of protein synthesis, but not transcription (Maghsoodi et al., 2008). Thus, a novel non-genomic function underlies RA's action at the synapse.

The multiplicative increase in mEPSC amplitude and the activation of local protein synthesis by RA led us to explore its potential involvement in HSP. Indeed, previous research established that RA is both necessary and sufficient for HSP induced by blocking synaptic activity (i.e. TTX+APV treatment, note that TTX blocks voltage-gated sodium channels and APV blocks NMDA-type glutamate receptors), but not for HSP induced by chronic blockage of action potentials with TTX alone (Wang et al., 2011b). Inhibition of RA synthesis prevented up-regulation of synaptic strength induced by activity blockade. HSP induced by activity blockade occludes further increase in synaptic strength by RA, placing RA into the signaling pathway downstream of synaptic activity blockade.

1.4. RA as a synaptic activity sensor: the controversy of several HSP forms and their signaling components

The requirement of RA synthesis in HSP and the ability of RA to increase synaptic strength imply that RA is a critical link between synaptic activity levels and synaptic strength. Indeed, we found using an RA reporter system that RA synthesis was dramatically stimulated by activity blockade with TTX and APV (Aoto et al., 2008). However, a major source of confusion in understanding HSP is that many different experimental manipulations seem to lead to HSP (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006; Aoto et al., 2008; Turrigiano, 2008; Soden and Chen, 2010a). Thus, it was unclear whether RA mediates many forms of HSP, or whether RA simply mediates a single form of HSP. By analyzing different well-established HSP induction protocols, we found that stimulation of RA synthesis is required for all forms of HSP that are rapidly induced by blocking postsynaptic activity (e.g., treatments with TTX+APV, CNQX, or TTX+CNQX), whereas RA synthesis is not required for HSP induced by prolonged blockade of action potential firing alone (i.e., TTX-alone), which takes longer to develop (Wang et al., 2011b). This led us to speculate that at least two signaling pathways mediate HSP and operate in parallel with different time courses : an RA- and local protein synthesis-dependent rapid pathway and an RA- and local protein synthesis-independent slow pathway that requires transcription of new mRNAs, as was illustrated initially by Schuman and colleagues (Sutton et al., 2006).

We found that when synaptic activity and neuronal firing are both blocked (i.e. by treatment of neurons with TTX+APV) the immediate engagement of early RA-dependent cellular events is triggered by the resulting drop in dendritic calcium concentrations, which stimulates RA synthesis. RA thus leads to a rapid increase in excitatory synaptic strength produced by synaptic insertion of locally translated AMPA receptors that contain GluA1, lack GluA2, and are calcium-permeable (Aoto et al., 2008) (Fig. 2). The slow RA-independent pathway of HSP, conversely, is triggered by reduced neuronal firing, a drop in

somatic calcium influx, reduced activation of CaMKIV, and an increase in gene transcription; this pathway involves synaptic insertion of GluA2-containing AMPA receptors (Ibata et al., 2008). The fact that TTX-treatment alone can induce HSP, albeit with a delay, shows that the fast and slow pathways of HSP are mechanistically distinct and are independent of each other. Interestingly, the GluA2-lacking AMPA receptors that are inserted during the rapid pathway of HSP are transient, and are slowly replaced by GluA2-containing AMPA receptors (Sutton and Schuman, 2006). This observation suggests that the calcium-permeable AMPA receptors lacking GluA2 inserted by the rapid HSP pathway inactivate this pathway in a negative feedback loop while the slow pathway of HSP is beginning to operate.

Two molecular mechanisms have been proposed by which a drop in calcium concentration could induce the rapid HSP pathway. First, it was demonstrated that dendritic calcium serves as a critical inhibitor of RA synthesis in neurons, such that any decrease in dendritic calcium activates RA synthesis (Wang et al., 2011b). For example, when cellular calcium is buffered by BAPTA or EGTA, RA synthesis is turned on rapidly. Additionally, blockade of L-type calcium channels is as efficient as synaptic activity blockers in inducing RA synthesis and HSP in a cell-autonomous manner, strongly suggesting a negative coupling between dendritic calcium levels and RA synthesis (Wang et al., 2011b). The RA-induced synthesis and insertion of calcium-permeable AMPA receptors thus serves as a negative feedback signal to halt RA synthesis, thereby stabilizing synaptic strength. The downstream calcium-dependent signaling cascade that regulates RA synthesis remains to be determined in future work.

A second mechanism that may operate in parallel with the RA-dependent pathway to mediate the rapid HSP focuses on eukaryotic elongation factor-2 (eEF2). Sutton and colleagues (Sutton et al., 2007) showed elegantly that miniature synaptic transmission strongly promotes eEF2 phosphorylation, thereby inactivating it. Thus, blocking synaptic activity leads to rapid dephosphorylation and activation of eEF2 in a spatially controlled fashion, allowing local protein synthesis to occur. Because phosphorylation of eEF2 is catalyzed by a calcium/CaM-dependent protein kinase (Nairn et al., 1987), calcium entry through newly inserted calcium-permeable AMPA receptors serves as a negative feedback signal to slow down local protein synthesis, enabling the transition to the late phase HSP.

1.5 Dendritic RARα mediates synaptic RA signaling – the double life of a versatile protein

How does RA increase synaptic strength? As mentioned above, the transcriptional effects of RA are primarily mediated by RARs. Among the three isoforms of RARs, RARα is most abundantly expressed in the hippocampus, while RARβ is mostly found in the basal ganglia, and RARγ is not detectable in adult brain (Krezel et al., 1999; Zetterstrom et al., 1999). Targeted constitutive gene deletions provided evidence for a limited role of RARβ and RARγ in the adult brain, but the function of RARα in the adult brain remained unexplored due to early postnatal lethality of constitutive RARα knockout mice (Lufkin et al., 1993). To avoid confounding factors in constitutive RARα knockout mice, such as abnormal neuronal development and perinatal lethality (Lufkin et al., 1993; LaMantia, 1999; Mark et al., 1999), I studied the involvement of RARα in synaptic RA signaling using shRNA-dependent knockdowns and conditional knockout mice (Sarti et al., 2012a). Both acute knockdown and conditional knockout of RARα prevented RA-dependent HSP,

and blocked the RA-mediated increase in synaptic strength (Aoto et al., 2008; Sarti et al., 2012a). Moreover, we observed that RARα is transiently concentrated in actively translating dendritic RNA granules of neurons (Maghsoodi et al., 2008), suggesting an unanticipated involvement of RARα in local protein synthesis. Indeed, RARα in mature neurons acts as an mRNA-binding protein whose dendritic localization is evident in postnatal neurons (Aoto et al., 2008; Huang et al., 2008) and is dictated by a nuclear export signal (NES) (Poon and Chen, 2008).

Similar to other nuclear receptors, RARα consists of an N-terminal activation domain, a DNA-binding domain (DBD), a hinge region, a ligand binding domain (LBD) and a C-terminal F domain whose function was largely unknown (Evans, 1988; Green and Chambon, 1988; Tora et al., 1988b; Tora et al., 1988a; Tasset et al., 1990). Upon RA binding, RARα undergoes a conformational change which results in the release of corepressors, the recruitment of coactivators, and the stimulation of gene transcription. RARα contains a classical NES in the LBD, and mutation of the RARα NES leads to an accumulation of RARα in the nucleus (Poon and Chen, 2008). The c-terminal F domain of RARα acts as an RNA binding domain that interacts with mRNAs in a sequence-specific manner, and RARα binding inhibits translation of these target mRNAs (Poon and Chen, 2008). This inhibition is released by RA binding to RARα, thus de-repressing translation and accounting for the activation of GluA1 synthesis by RA.

The function of RARα in mRNA binding and translational regulation was further validated in the context of HSP. Acutely deleting RARα in neurons from RARα conditional KO mice eliminates RA's effect on excitatory synaptic transmission, and simultaneously inhibits activity blockade-induced HSP (Sarti et al., 2012a). By expressing the ligand-binding domain (LBD) and the mRNA-binding domain (F-domain) of RARα are both necessary - and are together sufficient - for the function of RARα in HSP.

1.6 Involvement of synaptic RA signaling in mental retardation and autism-spectrum disorders (ASDs)

In the past decade, there has been an explosion of reports identifying genes related to synaptogenesis and synaptic function whose mutation, deletion or duplication was implicated in various forms of neuropsychiatric disorders (State and Levitt, 2011; Zoghbi and Bear, 2012). Among these genes, *Fmr1*, which encodes the protein FMRP, stands out because of the relatively high prevalence of its associated disorder. In human patients, impaired expression of Fmr1, most frequently due to expansion of CGG repeats in the *Fmr1* gene, causes Fragile-X syndrome (FXS), the most common inherited form of mental retardation that is also associated in some cases with symptoms characteristic of autism spectrum disorders (ASDs). FMRP knockout mice exhibit normal baseline synaptic transmission, but display impairments in certain forms of LTP (Li et al., 2002; Larson et al., 2005), and enhancements in mGluR-dependent LTD (Huber et al., 2002). Like RARα, FMRP is a dendritically localized RNA-binding protein that is believed to specifically bind to mRNAs and to regulate their translation (Laggerbauer et al., 2001; Li et al., 2001; Bassell and Warren, 2008). Dysregulated translation and elevated basal protein synthesis are found in *Fmr1* knockout neurons (Dolen et al., 2007; Muddashetty et al., 2007). However, whether FMRP is involved in translational regulation during homeostatic plasticity is currently unknown.

A potential involvement of FMRP in RA-mediated translational regulation was first proposed based on previous observation published by the labotaory that dendritic RNA granules, capable of actively translating GluA1 protein upon RA stimulation, are also enriched in FMRP. Indeed, in *Fmr1* knockout mice RA-dependent HSP was found to be completely absent whereas the RA-independent late phase of HSP was intact (Soden and Chen, 2010a). Since inactivity-dependent RA synthesis still occurs normally in *Fmr1* knockout neurons, cellular events downstream of RA were examined. Consistent with FMRP's role in regulating protein synthesis, RA-induced translational upregulation of various target mRNAs was abolished in the absence of FMRP (Soden and Chen, 2010a). These evidences taken together have motivated my experiments in the inhibitory transmission regulation where excitation/inhibition balance, another index of RA signaling in the brain, was analyzed in *fmr1* KO mice.

1.7 Contents of this dissertation

Though evidences show that RA is a critical factor in controlling translation and regulating synaptic plasticity, whether either of these systems is directly involved in controlling the strength of synaptic transmission, it still remains unclear. In this dissertation, I will describe my efforts to uncover the molecular mechanisms regulating local translation of the AMPA receptor subunit GluR1, particularly in the context of RA-mediated synaptic scaling.

Chapter 2 will describe the materials and methods used to conduct the experiments.

Chapter 3 will present my results regarding the role of RARα in the regulation of synaptic strength upon activity blockade. Here I have done a structure function analysis of RARα and determined that the Ligand Binding Domain is responsible for synaptic scaling. Results from this study have partially been published in the Frontiers in Molecular Biology.

Chapter 4 will show data from a study aimed at understanding whether RA plays a role in the regulation of inhibitory synapses. Here, RA plays a concomitant role in the up-regulation of excitatory synapses and in the down-regulation of inhibitory synapses. A version of this chapter was published on the Journal of Neuroscience.

Chapter 5 will present conclusions, perspectives, remaining questions, and future directions.

Chapter 2

EXPERIMENTAL PROCEDURES

2.1 Mouse husbandry and genotyping

The RARα floxed mouse (C57BL/6 background) is a generous gift from Drs. Pierre Chambon and Norbert Ghyselinck (IGBMC, Strasbourg, France) (Chapellier et al., 2002). Breeding colonies are maintained in the animal facility at Stanford Medical School. Genotyping of the mice was achieved by PCR with the following primers: Primer 1 Fwd 5'-GTGTGTGTGTGTATTCGCGTGC-3', Primer 2 Rev 5'-ACAAAGCAAGGCTTGTAGATGC-3' and compared with wild-type C57BL/6. Following infection with a lentiviral vector expressing CRE recombinase or a truncated and inactive version of CRE, successful *lox-P* mediated recombination in neuronal cultures was assayed by PCR with Primer 1 and Primer 3: 5'-TACACTAACTACCCTTGACC-3'. Conditions were 30 cycles for 30s at 92°C, annealing at decreasing temperatures in the range from 62°C to 56°C to increase product specificity and elongation for 30s at 72°C. Wildtype and $fmr1^{-/y}$ mice in the FVB background were obtained from Jackson Labs (Bar Harbor, Maine).

2.2 Primary hippocampal cultures and pharmacological manipulations

Mouse primary hippocampal cultures were prepared from brains at postnatal day 0 and maintained in serum and Neurobasal medium supplemented with Gem21 (Gemini Bioproducts, Sacramento, CA) and Glutamax (GIBCO-Brl, Grand Island, NY) for 2 weeks in vitro (Nam and Chen, 2005). Neurons were infected with a retroviral vector expressing proteins of interest at 4-7 DIV.

Rat primary hippocampal neuronal cultures were prepared as previously described (Aoto et al., 2008) from E21 rat pups. Dissociated cultures used for Dynamin-1 K44E overexpression (Gift from Dr. Mark Von Zastrow) and GluR1-C terminal overexpression were transfected using lipofectamine 200 (Invitrogen) at 10-11 DIV with a protocol previously described.

Manipulations used to induce synaptic scaling in dissociated mouse cultures include: TTX+APV (1 μM TTX+100 μM APV, 24 hours); TTX+APV+DEAB (1 μM TTX+100 μM APV+DEAB 10μM, 24 hours); RA (1 μM, 30 minutes followed by one hour of washout); TTX+CNQX (1μM TTX + 10 μM CNQX, 24 hours); Other drug treatments include: preincubation of neurons in Anisomycin (40 μM), Cyclohexammide (100 μM) or Actinomycin D (50 μM) for 30 minutes before incubation with RA.

2.3 Viral vectors and virus production

Lentivirus was produced and purified as described previously (Aoto et al., 2008). Briefly, human embryonic kidney 293T (HEK293T) cells were transfected using calcium phosphate with the transfer vector and three helper plasmids. After 48 h, supernatants were pooled, spun at 25,000 rpm through a sucrose cushion for 1.5 h, and resuspended in PBS. Virus expressing CRE recombinase or a truncated and inactive version of Cre (ΔCre) (gifts from Dr. Thomas Sudhof's lab, (Kaeser et al., 2011)) was applied overnight to the media of

dissociated neuronal cultures generated from RAR α floxed mice and washed out the following day. In rescue experiments, Cre Recombinase or ΔCre and RARα were expressed simultaneously from a bicistronic lentiviral transfer vector containing a GFP-Cre 5' open reading frame immediately followed by an IRES sequence fused to the RARα reading frame RARα or truncated versions of RARα expressing the different domains were inserted in the forward direction into the unique BstXI site located in the MCS region. Neurons were infected at 4-7 DIV and recorded from at 14-16 DIV.

2.4 Dissociated neuronal culture electrophysiology

Whole-cell patch-clamp recordings were made at room temperature from 14–16 DIV cultured neurons, with 4–6 MΩ borosilicate patch pipettes filled with an internal solution containing (in mM) 120 CsCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 0.3 Na_3-GTP, 4 Na_2-ATP (pH 7.35). Cultures were continuously superfused with external solution (in mM, 100 NaCl, 26 $NaHCO_3$, 2.5 KCl, 11 glucose, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.0 NaH_2PO_4). For mEPSC recording, tetrodotoxin (1 μM) and picrotoxin (100 μM) were included in the external saline. Cells were held at -60 mV. For mIPSCs, tetrodotoxin (TTX) (1μM), CNQX (10μM) and APV (50 μM) were included in the perfusion bath. Cells were held at −60mV. To test for the presence of calcium permeable AMPA receptors on the post-synaptic membrane, 10 μM 1-Naphtylacetylspermine trihydrochloride (NASPM) was bath perfused for 10 min before recording. EPSCs and IPSCs were collected using Clampex (Axon laboratory) and analyzed using Clampfit (Axon laboratory) and Mini Analysis Program (Synaptosoft).

2.5 Organotypic slices culture and electrophysiology

Hippocampal slice cultures were prepared from RARα floxed or $fmr1^{-/y}$ mice at post-natal day 7-8 as described previously (Soden and Chen, 2010b). At 1 DIV, CA1 of slices from RARα floxed mice were injected with lentiviral vectors expressing CRE recombinase or a truncated and inactive version of Cre (ΔCre) gifts from Dr. Thomas Sudhof's lab, (Kaeser et al., 2011) together with different rescue truncated versions of RARα. Lentivirus was produced and purified as described previously (Aoto et al., 2008; Sarti et al., 2012b). Pharmacological manipulations of cultured hippocampal slices include: Retinoic Acid (2 μM, 4 hours), TTX+CNQX (2 μM + 20 μM, 36 hours). Whole cell patch-clamp recordings from the CA1 region of mouse slice cultures were made at room temperature from 6–9 DIV slices with a 4–6 MΩ borosilicate patch pipette filled with an internal solution containing the following (in mM): 140 CsCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 0.3 Na_3-GTP, and 4 Na_2-ATP, pH 7.35. Slices were continuously superfused with external solution containing the following (in mM): 120 NaCl, 26 $NaHCO_3$, 2.5 KCl, 11 glucose, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 1.0 NaH_2PO_4. CNQX (10 μM) and APV (50 μM) were included in the external saline solution. The stimulating electrodes were placed over CA1 Starum Radiatum. Synaptic GABA-mediated responses were measured at −60 mV from two adjacent pair of cells where one was infected by the lentiviral vector expressing CRE or ΔCRE recombinase alone or in combination with RARα rescue constructs. Synaptic responses in cell pairs were averaged over 40-50 trials with an interval of 10s.

2.6 Statistical analysis

All graphs represent average values ± s.e.m. For each experimental group, the N and n numbers represent number of independent experiments and total number of neurons, and are indicated in the figures. Single-factor ANOVA or t-test were used for statistical analysis.

2.7 Drugs and Chemicals

Retinoic Acid, Picrotoxin, Naphtyl-acetyl-spermine trihydrochloride, Actinomycin D, Cycloheximide, Picrotoxin and 4-Diethylaminobenzaldehyde were purchased from Sigma-Aldrich. Tetrodotoxin was purchased from Ascent Scientific (Bristol, UK) and D-(-)-2-Amino-5-phosphonopentanoic acid was purchased from Tocris Biosciences (Ellisville, MO).

2.8 Subcellular localization of RARα domains

Rat neuronal cultures were co-transfected at 12 DIV using Lipofectamine 2000 with pmCherry-N1, a plasmid that expresses high levels of mCherry to allow neuronal visualization, and pEGFP-N1 expressing various truncated versions of RARα. Cells were then fixed at 14 DIV with 4% *para*-Formaldehyde, washed with PBS before mounting. Images were acquired using an Olympus FV1000 BX61WI laser-scanning confocal microscope.

2.9 Surface biotinylation assay

Biotinylation of surface proteins was carried out as previously described (Aoto et al, 2008). Briefly, cultured hippocampal cells were biotinylated with 1 mg/ml Ez-link sulfo-NHS-SS-biotin (Pierce, Madison, WI). Biotinylated cells were solubilized with lysis buffer (PBS with 1% Triton-X, 1% NP-40, 10% glycerol, 25 mM MgCl2, and a protease inhibitor cocktail). Lysate was bound for 3 hours at 4C using Ultralink-immobilized streptavidin beads to precipitate biotinylated proteins. Biotinylated surface proteins were eluted with denaturing buffer at 75^{o}C. Surface-expressed $GABA_A$ receptors were detected by western blot analysis using anti-β3 antibody (AbCam).

2.10 Immunocytochemistry, receptor internalization assay and image analysis

Procedures previously described (Tracy et al., 2011) were followed. For surface staining, coverslips were fixed in 4% PFA then incubated in blocking solution containing 2% Normal Goat Serum. Primary antibodies were added to the cells followed by flourophore-conjugated secondary antibodies. The primary antibody against the extracellular domain of GABA A receptors subunit β 2/3 was purchased from Millipore. For total receptor staining, cells were fixed with 4% PFA, then permeabilized with blocking solution containing Triton-X 100 0.3% and Normal Goat Serum 2%. Images were acquired using Olympus (Tokyo, Japan) FV1000 BX61WI laser-scanning confocal microscope, using an Olympus Plan Apochromat with sequential acquisition setting at 1024x1024 pixel resolution. Puncta staining was analyzed as described previously (Tracy et al., 2011). For the analysis of synaptic proteins, images from the same experiment were

thresholded identically by intensity to exclude the diffuse/intracellular pool. Synaptic colocalization was defined as a minimum 2-pixel overlap between the VGAT signal and the $GABA_AR$ β2/3 signal. Image quantification was performed blind to treatment group using MatLab.

In receptor internalization experiments, neurons were incubated with antibody against the extracellular domain of the β2/3 subunit at 37^oC for 15 minutes. Cells were successively washed thoroughly and incubated with either DMSO or RA and fixed at different time points. After the extracellular antibody was revealed with a secondary antibody coupled with Cy3, cells were permeabilized with blocking solution containing Triton-X 100 0.3% and Normal Goat Serum 2%. Internalized receptors were revealed by applying a secondary antibody coupled to Cy2. Map2 was used as a dendritic marker. Images were acquired and analyzed as described above.

Chapter 3

CONDITIONAL RARα KNOCKOUT MICE REVEAL ACUTE REQUIREMENT FOR RETINOIC ACID AND RARα IN HOMEOSTATIC PLASTICITY OF EXCITATORY TRANSMISSION

INTRODUCTION

During development, RA performs essential functions as a morphogen regulating gene expression. In the nervous system, RA signaling is involved in neurogenesis and neuronal differentiation. However, several recent lines of evidence support the idea that RA also performs postdevelopmental functions in the adult brain. First, RA can be rapidly synthesized in various regions of the adult brain (Dev et al., 1993). Second, compromised RA signaling (through either genetic knockout of RA receptors or vitamin A deficiency) leads to impaired long-term synaptic plasticity in the hippocampus, (Chiang et al., 1998; Misner et al., 2001). The involvement of RA in synaptic plasticity was further supported by deficits in learning and memory tasks observed in mice with a genetic deletion of RA receptors or with Vitamin A deficiency (Chiang et al., 1998; Cocco et al., 2002). Third, recent evidence established a role of RA in synaptic signaling in that RA directly potentiates glutamatergic synaptic transmission (Aoto et al., 2008). Blocking synaptic glutamate receptors activates RA synthesis, which in turn promotes local synthesis of various synaptic proteins, including GluA1 subunit of AMPA receptors. Synaptic insertion of homomeric GluA1 AMPA receptors increases synaptic transmission and therefore compensates the changes in synaptic activity homeostatically (Aoto et al., 2008). At least the latter action of RA is different from its role in development in that it does not require transcriptional activation (Aoto et al., 2008).

The expression and subcellular distribution of RARα in the hippocampus exhibit interesting developmental changes (Huang et al., 2008). Specifically, the expression levels of RARα in hippocampal neurons gradually decrease postnatally, and the subcellular localization of RARα protein shifts from the nucleus to the cytosol. While almost exclusively localized in the nucleus of developing neurons, at least equal amounts of RARα were detected in the cytosol and dendrites in mature hippocampal pyramidal and granule cells (Aoto et al., 2008; Huang et al., 2008; Maghsoodi et al., 2008). Functionally, dendritic RARα acts as an mRNA-binding protein that represses translation of target mRNAs, and thus performs a different role than the transcriptional function of RARα localized in the nucleus (Poon and Chen, 2008). In the dendrites, RA binding to RARα reduces RARα binding to mRNA, and de-represses translation (Poon and Chen, 2008).

The region of RARα that mediates mRNA binding has been determined (Poon and Chen, 2008), but it until recently it was unknown whether RA binding by RARα and its mRNA-binding ability were required for its role in regulating synaptic strength. For this reason, I have combined mouse genetics and electrophysiology to rigorously test the role of RA and RARα in homeostatic plasticity, and to systematically examine the contribution of various domains of RARα to synaptic signaling by RA. Consistent with earlier results, we found that RARα is essential for some forms of homeostatic plasticity, and that the carboxyl terminal half of the RARα, which contains the ligand binding domain (LBD) and the RNA-binding domain (F-domain), are both necessary and sufficient for the function of RARα in the homeostatic regulation of excitatory synaptic strength.

A version of this chapter was published in the Frontiers in Molecular Neuroscience.

RESULTS

3.1 Lentivirus-mediated conditional knockout (KO) of RARα in hippocampal neurons cultured from postnatal RARα$^{fl/fl}$ mice

The involvement of RARα in activity blockade-induced homeostatic synaptic plasticity (also called synaptic scaling) using shRNA-mediated knockdown was previously shown (Aoto et al., 2008). However, shRNA-mediated knockdowns suffer from a number of intrinsic limitations, such as potential off-target effects and the fact that no knockdown is ever complete. To test whether the shRNA-dependent results were reliable, we used a previously described conditional floxed RARα mouse (Chapellier et al., 2002). The RARα$^{fl/fl}$ mouse contains *loxP* sites flanking exon 4 (this exon was called exon 8 at the time of publication and was later corrected; Norbert Ghyselinck, personal communication) (**Figure 1A**). Cre-mediated recombination leads to excision of exon 4 that encodes a significant part of the DNA-binding domain of RARα (Zelent et al., 1989); after excision of exon 4, the exon 3/5 junction shifts of the reading frame, resulting in an RARα null allele (Chapellier et al., 2002) (**Figure 1A**). Because exon 4 is the first common exon shared by all RARα isoforms (Leroy et al., 1991), its excision leads to a complete deletion of all RARα isoforms.

The presence of the loxP sites was confirmed in conditional RARα KO mice by PCR analysis of tail DNA (**Figure 1B**). To obtain RARα KO neurons, I cultured hippocampal neurons from newborn RARα$^{fl/fl}$ mice and infected the neurons with lentiviruses expressing recently optimized versions of EGFP-tagged active or inactive cre-recombinase (Cre or ΔCre) (Kaeser et al., 2011). Rescue experiments were performed by co-expressing various RARα rescue proteins from the same vector as cre recombinase via an IRES sequence (**Figures 1C and 1D**).

3.2 Synaptic scaling is absent in RARα KO neurons

First, I tested whether the genetic knockout of RARα impairs homeostatic synaptic plasticity. Under control conditions, Cre-expressing conditional RARα KO neurons did not show changes in miniature excitatory post-synaptic current (mEPSC) amplitude or frequency, suggesting that RARα is not required for the maintenance of basal excitatory synaptic transmission (**Figures 2A - 2D**). To induce synaptic scaling, I applied a 24-hour treatment of tetrodotoxin (TTX) and D-2-amino-5-phosphonovalerate (APV), a well-established activity blockade protocol that activates homeostatic synaptic plasticity (Ju et al., 2004; Sutton et al., 2006; Aoto et al., 2008). Consistent with previous findings from the lab showing that RARα is required for this type of synaptic scaling, TTX+APV treatment induced a robust increase in mEPSC amplitude in uninfected and Cre-infected neurons (**Figure 2B**). Strikingly, deletion of RARα by Cre recombinase in RARα conditional KO neurons blocked this effect (**Figure 2B**).

Prof. Lu Chen's previous work established a role of RA in mediating activity blockade-induced homeostatic plasticity. Specifically, it was found that blocking synaptic activity rapidly induces RA synthesis in neurons, and that the newly synthesized RA increases excitatory synaptic transmission through activation of dendritic protein synthesis

(Aoto et al., 2008; Maghsoodi et al., 2008). Consistent with this observation, the conditional deletion of RARα eliminated the ability of RA to increase the mEPSC amplitude (**Figures 2C and 2D**), thus confirming the role of RARα in synaptic RA signaling using a genetic approach.

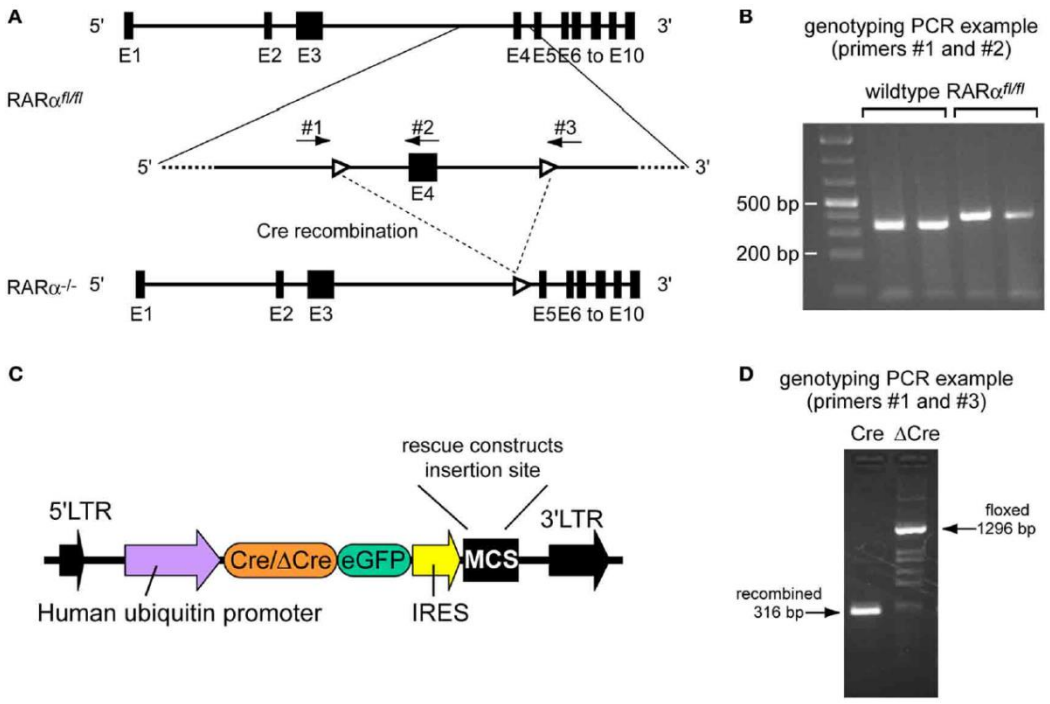

Figure 1: Characterization of the RARα conditional allele.
(A) Schematic drawing representing the RARα *loxP*-flanked allele (RARα $^{fl/fl}$), as well as the same allele following Cre-mediated excision of exon 4 (RARα $^{-/-}$). Black boxes represent exons 1-10 (E1-E10). White arrowheads represent *loxP* sites. The arrows indicate location of primers 1-3 used for characterization of the *loxP*-flanked allele and the recombined allele. Schematic is based on Gene ID: 19401. **(B)** Tail DNAs extracted from RARα $^{fl/fl}$ and wild-type C57BL/6 mice were genotyped by PCR using primers number 1 and number 2. Wild-type: 390 bp; floxed allele: 453 bp. **(C)** Schematic representation of the lentiviral transfer vector used to express Cre recombinase or ΔCre and RARα rescue constructs. **(D)** DNA from dissociated hippocampal neuronal cultures infected with either the active recombinase Cre or the inactive form ΔCre was harvested and genotyped by PCR using primers 1 and 3 to test for successful recombination indicated by the presence of a recombined allele (316 bp) or of a floxed allele (1296 bp).

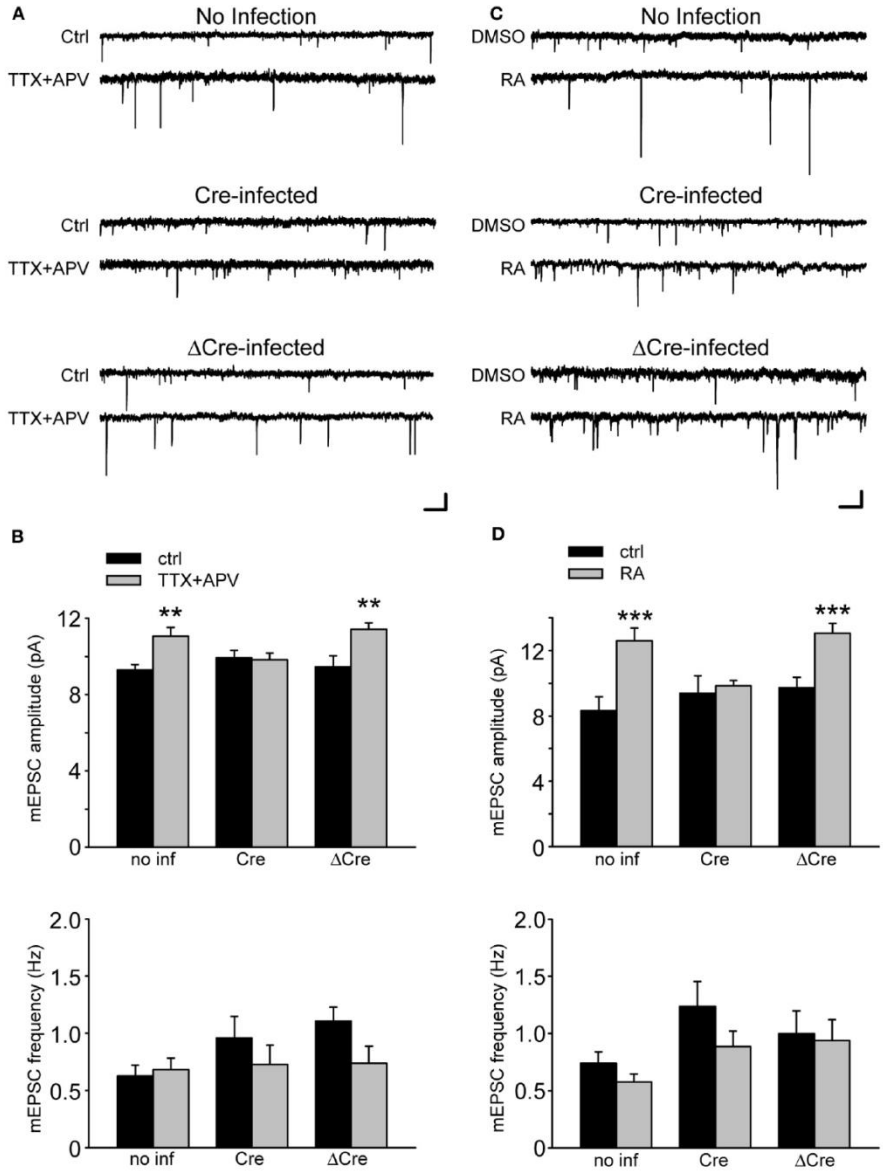

Figure 2: Retinoic Acid Receptor α is required in synaptic scaling induced by activity blockade and by retinoic acid. (A) Representative traces of mEPSCs from RARα $^{fl/fl}$ neurons infected with a lentiviral vector expressing Cre recombinase or the inactive ΔCre. Neurons have been treated with TTX and APV for 24 hours. Scale bars: 10 pA, 0.5 sec. **(B)** Quantification of average mEPSC amplitude and frequency from experiments shown in (A) (n = 12 – 19 from 3 independent experiments; **, $p < 0.005$). **(C)** Representative traces of mEPSCs from RARα KO neurons treated with retinoic acid (RA) for 30' followed by one hour of wash out. Scale bars: 10 pA, 0.5 sec. **(D)** Quantification of mEPSC amplitude and frequency from experiments shown in (C) (n = 12-19, from 3 independent experiments; ***, $p < 0.001$).

3.3 RARα mutants exhibit distinct subcellular localizations

The absence of synaptic scaling and RA signaling in conditional RARα KO neurons provides a basis for a structure-function analysis of RARα. I therefore generated RARα deletion mutants to investigate the contribution of various protein domains to RARα function. Similar to other members of the nuclear receptor superfamily, RARα protein has a modular domain structure, which is comprised of six regions: the N-terminal trans-activation domain (A/B region), the DNA binding domain (DBD or C region), a hinge region (D region), a ligand binding domain (LBD or E region), and a C-terminal F domain whose function is unclear (Krust et al., 1986; Green and Chambon, 1988; Zelent et al., 1989), but that we previously implicated in mRNA binding (Poon and Chen, 2008) (**Figure 3A**).

Three deletion mutants of RARα were generated: RARα DBD, RARα LBD/F (LBD and F-domain) and RARα F (F-domain only containing the terminal α-helix of LBD-H12) (**Figure 3A**). When expressed in cultured neurons as GFP-tagged proteins, the different RARα proteins exhibited distinct subcellular distributions. Compared to full-length RARα which was present in both the nucleus and the cytosol, RARα DBD was confined to the nucleus, presumably because of its two nuclear localization signals (NLS) (Hamy et al., 1991)(**Figure 3B**). By contrast, significant amounts of both the RARα LBD/F and the F-domain protein were present outside of the nucleus and present at high levels in dendrites (**Figure 3B**).

3.4 Rescue of synaptic scaling in RARα KO neurons

To directly examine the role of various RARα domains in synaptic scaling, I expressed RARα mutant proteins in neurons with RARα deletion. To achieve this, the various RARα mutants were expressed by inserting their coding sequences after the IRES sequence into the same lentiviral vector that was used for generating RARα KO neurons (**Figure 1C**). Co-expression of Cre and RARα in the same neurons allows me to examine the effect of rescue by the various RARα deletion mutants in the RARα KO background.

We examined the ability of various RARα mutants to restore RA-induced increase in synaptic strength and to rescue TTX+APV-induced synaptic scaling in RARα KO neurons by co-expressing RARα with Cre in $RAR\alpha^{fl/fl}$ neurons. As expected, expression of RARα FL completely rescued synaptic scaling, induced by RA or TTX+APV, in RARα KO neurons (**Figures 4B and 4C**). Expression of RARα DBD failed to rescue synaptic scaling by RA or TTX+APV, which is consistent with its role in DNA- but not RNA-binding (**Figures 4B and 4C**). Importantly, RARα LBD/F fully restored synaptic scaling (**Figures 4A, 4B and 4C**). This indicates that the LBD and F-domain, which convey the ligand-binding and RNA-binding ability respectively, works independently from the DBD in mediating RA's effect at the synapses and is sufficient to restore synaptic scaling when expressed at appropriate levels. This result also confirms the notion that RA and RARα function in a transcription-independent manner in synaptic scaling as the RARα LBD/F lacks the ability to bind DNA. Not surprisingly, when only the F domain is expressed in RARα KO neurons, RA failed to increase synaptic strength (**Figures 4A and 4B**). Likewise, synaptic scaling induced by TTX+APV was absent when only F domain is expressed (**Figure 4C**), demonstrating that activation of RARα by RA is a required step in

synaptic scaling. Moreover, when RA synthesis is blocked by 4-diethylamino-benzaldehyde (DEAB), an inhibitor for RALDH (Russo et al., 1988b), TTX+APV-induced synaptic scaling was completely blocked in RARα LBD/F-expressing neurons (**Figures 5A and 5B**).

Results from above establish that two RARα activities primarily mediate synaptic scaling: the RNA binding by the F domain, and RA binding by the LBD. While the former interaction silences translation of target mRNAs, the latter interaction acts as a switch to turn on translation of substrate mRNAs in neuronal dendrites (Poon and Chen, 2008). Previous work demonstrated that one of the essential targets of RA/RARα regulation of protein translation in the context of synaptic scaling is the AMPA receptor subunit GluA1 (Poon and Chen, 2008). As a result, the newly inserted synaptic AMPA receptors after TTX+APV treatment are calcium-permeable GluA1 homomeric receptors that are sensitive to polyamine blockers (Aoto et al., 2008). I therefore asked whether synaptic scaling rescued in RARα LBD/F-expressing neurons is also mediated by synaptic insertion of GluA1 homomeric receptors. 1-naphthyl acetyl spermine (NASPM), a synthetic analog of Joro Spider toxin in the polyamine family, completely reversed the increase in mEPSC amplitude in TTX+APV-treated neurons (**Figures 5A and 5B**), indicating that RARα LBD/F activates the signaling pathway identical to that of full length RARα upon activity blockade and is fully capable of mediating RA signaling in the context of synaptic scaling.

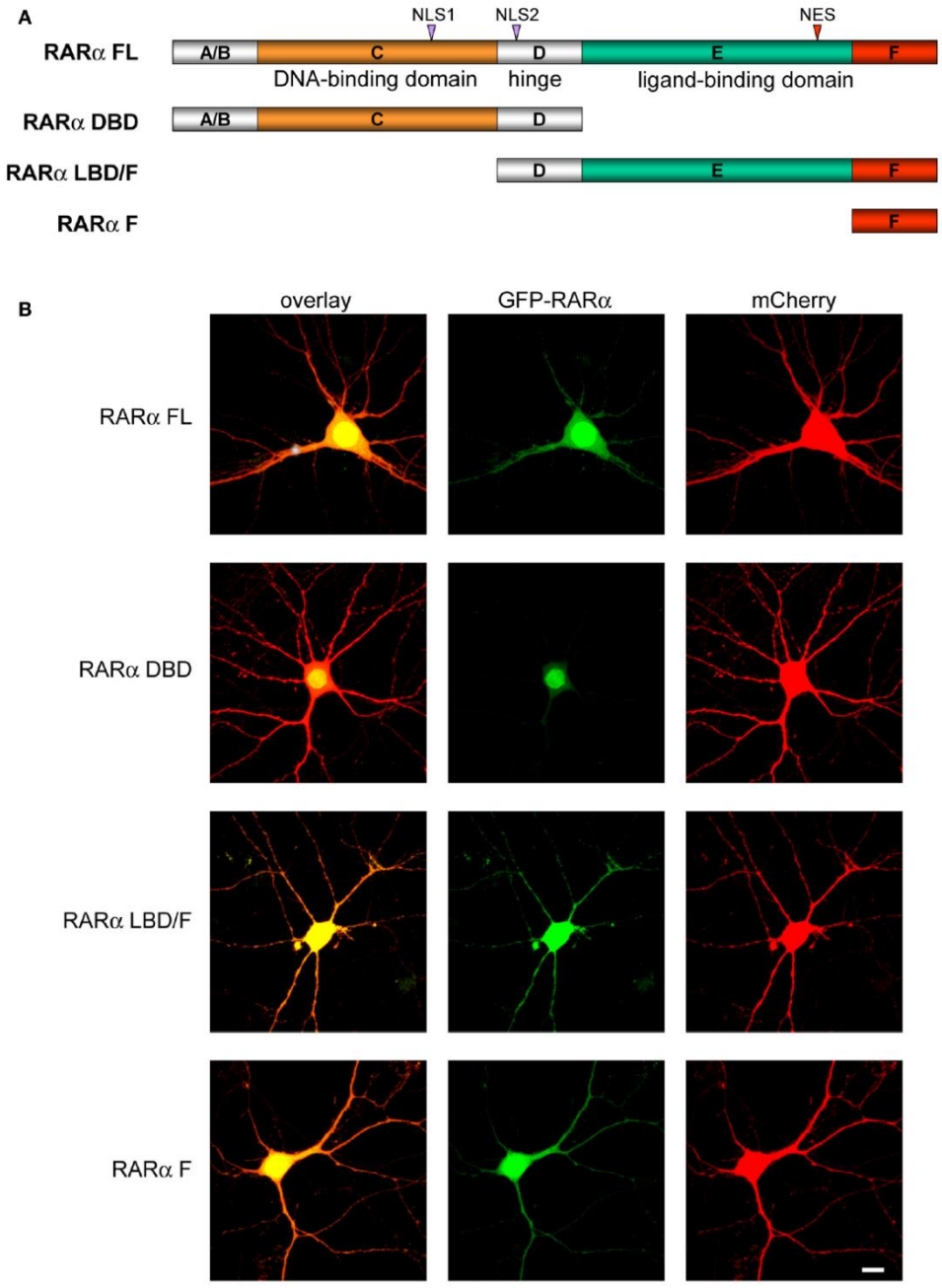

Figure 3: Subcellular localization of RARα and its deletion mutants. (A) Schematic of RARα deletion constructs. Recombinant full-length and truncated RARα domains were encoded as N-Terminal eGFP fusion proteins (FL, Full length; DBD, N-terminal DNA Binding Domain; LBD, Ligand Binding Domain; F, C-Terminal F Domain). NLS, Nuclear Localization Signal; NES, Nuclear Export Signal. **(B)** Subcellular distribution of full length or truncated RARα proteins (green). mCherry (red) was coepxressed to visualize neuronal morphology. Scale bar: 10 μm.

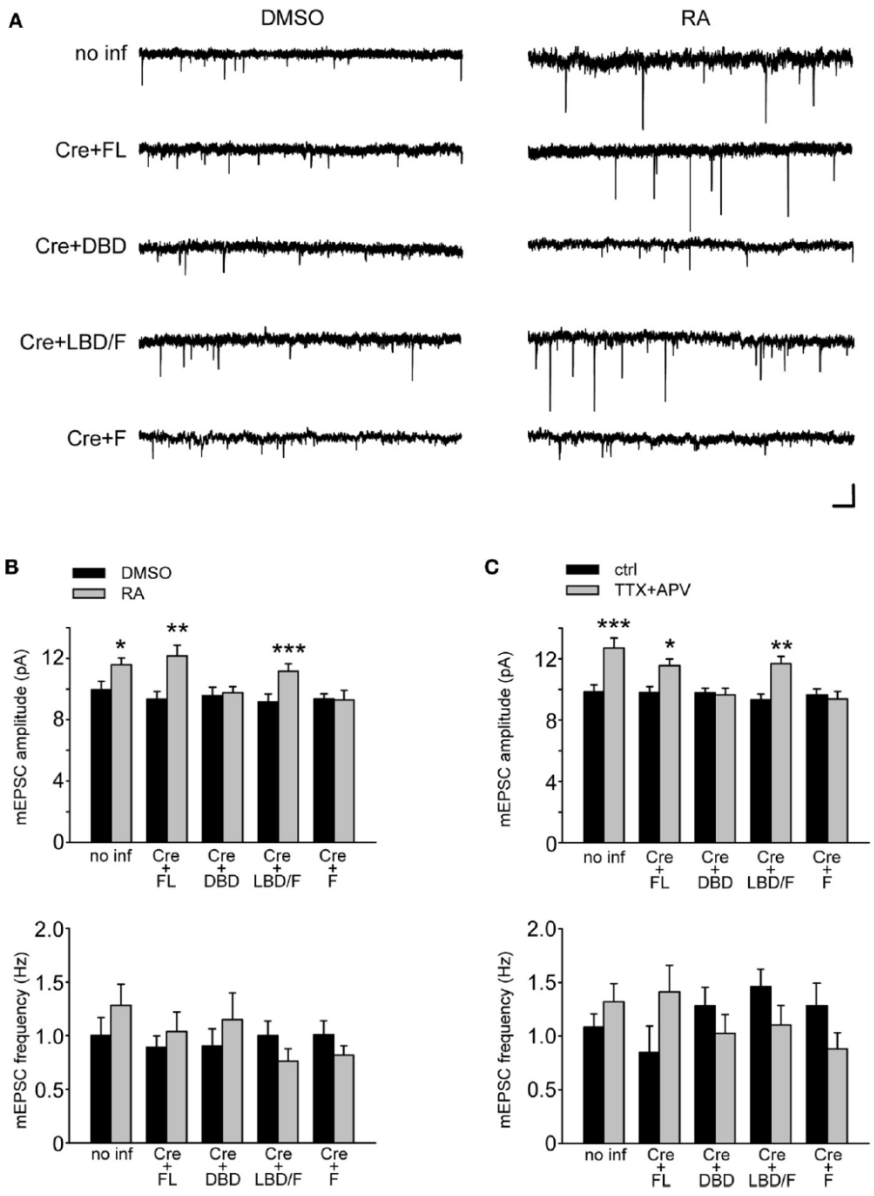

Figure 4: Functional rescue of synaptic scaling in RARα KO neurons RARα expression (A) Representative traces of mEPSCs from RARα$^{fl/fl}$ hippocampal neurons infected with a bicistronic lentiviral vector coding for Cre recombinase and either FL RARα or truncated RARα proteins. Neurons were treated with RA or with DMSO as control. Scale bars: 10 pA, 0.5 sec. **(B)** mEPSCs amplitude and frequency were quantified showing rescue of RA-induced synaptic scaling in neurons expressing FL or LBD/F RARα. **(C)** Synaptic scaling induced by activity blockade is rescued by LBD/F domain expression in neurons treated with TTX and APV for 24 hours. (for both B and D: n= 12-19, from 3 independent experiments; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$).

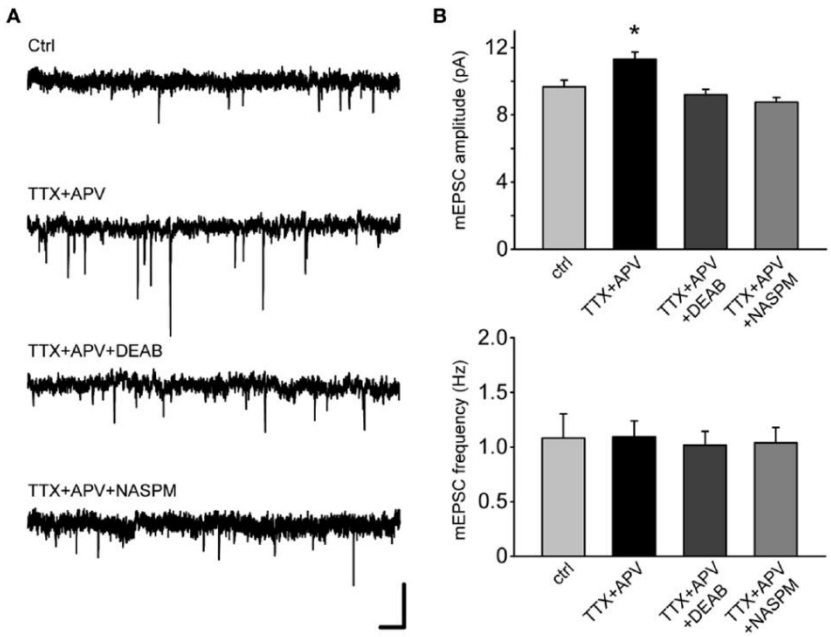

Figure 5: Synaptic scaling mediated by LBD/F Domain depends on RA synthesis and leads to synaptic insertion of calcium permeable AMPA receptors (A) Representative traces from RARα KO neurons expressing the LBD/F domain. To verify the dependence of synaptic scaling mediated by the LBD/F domain on RA synthesis, neurons were co-treated for 24 hours with TTX, APV and DEAB. In addition, to test whether synaptic scaling mediated by the LBD/F domain manifests as the insertion of calcium permeable AMPA receptors, neurons were pretreated with TTX and APV for 24 hours and NASPM was bath applied during mEPSCs recording. Scale bars: 10 pA, 0.5 sec. **(B)** mEPSCs amplitude and frequency analysis of (A) (n=12-15, from 2 independent experiments; **, $p < 0.005$).

3.5 Validation of shRNA-based knockdown and rescue approach to study RARα function

A critical involvement of RARα in homeostatic synaptic plasticity was previously suggested by experiments using a shRNA-based knockdown approach (Aoto et al., 2008). Although accepted as a useful and less time-consuming method in understanding the function(s) of a particular protein, the RNAi method also faced skepticism given the potential off-target effects inherent to the approach. I therefore decided to go back to this system and compare the results obtained with the knockdown-rescue approach with that of the genetic approach. Standard knockdown-rescue experiments were performed by transfecting cultured hippocampal neurons with plasmids expressing both a shRNA against RARα and a truncated version of the RARα that is also resistant to the shRNA (rescue), and subject these neurons to 24-hr TTX+APV treatment. The results thus obtained completely corroborate those obtained with the genetic knockout-rescue experiments (**Figure 6A-C**), demonstrating that the shRNA approach is a valid approach for studying RARα functions in mature neurons. This section was generated in collaboration with Jason Aoto, a former student in Prof. Chen laboratory.

3.6 Synaptic scaling in neurons overexpressing various RARα mutants

Results from the rescue experiments done in RARα KO or KD neurons provided important functional information regarding the contribution of different RARα domains to synaptic RA signaling. I was curious whether any of the RARα deletion mutants could act dominant negatively in neurons expressing normal levels of endogenous RARα. To achieve this, I simply co-expressed ΔCre with various RARα fragments, using the same lentiviral vector that delivered Cre and RARα in the rescue experiments.

Interestingly, when challenged with RA treatment, neurons overexpressing RARα full-length protein failed to up-regulate mEPSC amplitude to a similar level as the uninfected neurons (**Figures 7A and 7B**) suggesting that increasing expression levels of RARα compromises a neuron's ability to undergo up-regulation of synaptic strength by RA. This result is somewhat unexpected as it cannot be explained by a simple dominant negative scenario, and is not likely due to general side effects associated with viral infection because RARα FL rescued synaptic scaling when expressed in RARα KO neurons.

Then, I examined RA-induced scaling in neurons overexpressing the RARα DBD. The RARα DBD localizes primarily to the nucleus and does not participate in RNA binding or RA binding. Overexpression of RARα DBD did not affect RA-induced synaptic scaling because endogenous RARα is sufficient to mediate RA's action (**Figures 7A and 7B**). This rules out the possibility of viral infection-induced side effects in general, and suggests instead that the expression levels of RARα affect its function in synaptic signaling. Indeed, similar to RARα full-length overexpression result, overexpression of RARα LBD/F also impaired synaptic scaling by RA (**Figures 7A and 7B**), although this mutant RARα is able to bind to mRNA and respond to RA (Poon and Chen, 2008). Interestingly, overexpression of F-domain in neurons also prevented the increase in mEPSC amplitude after RA treatment (**Figures 7A and 7B**). Because F-domain mediates RNA-binding, but does not mediate RA-induced translational switch, it can act dominant negatively when expressed alone to compete with endogenous RARα in RNA-binding and sequester RNA substrates.
Given the critical role of RA in activity blockade-induced synaptic scaling, I next examined the effect of overexpression of various RARα mutants in TTX+APV-induced synaptic

scaling with the prediction that those mediate by normal RA-induced synaptic

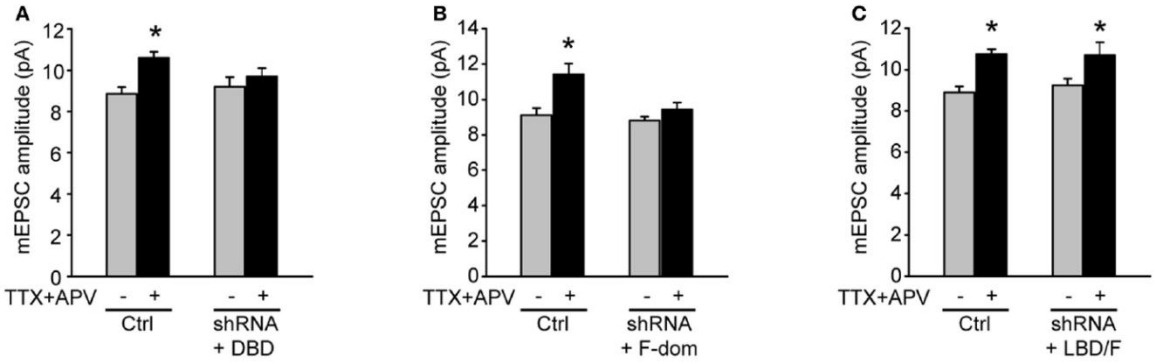

Figure 6: LDB/F Domain is required for activity blockade induced synaptic scaling. Rat primary hippocampal cultures were co-transfected with shRNA against RARα and three different truncated forms of RARα spanning **(A)** the DBD, **(B)** the F Domain and **(C)** the LBD/F Domains. Synaptic scaling induced by activity blockade with TTX and APV for 24hours was rescued by co-expression of an RNAi resistant form of the LBD/F domain as shown by quantitative analysis of mEPSCs amplitude (n= 9 - 12, from 2 independent experiments; *, $p < 0.05$).

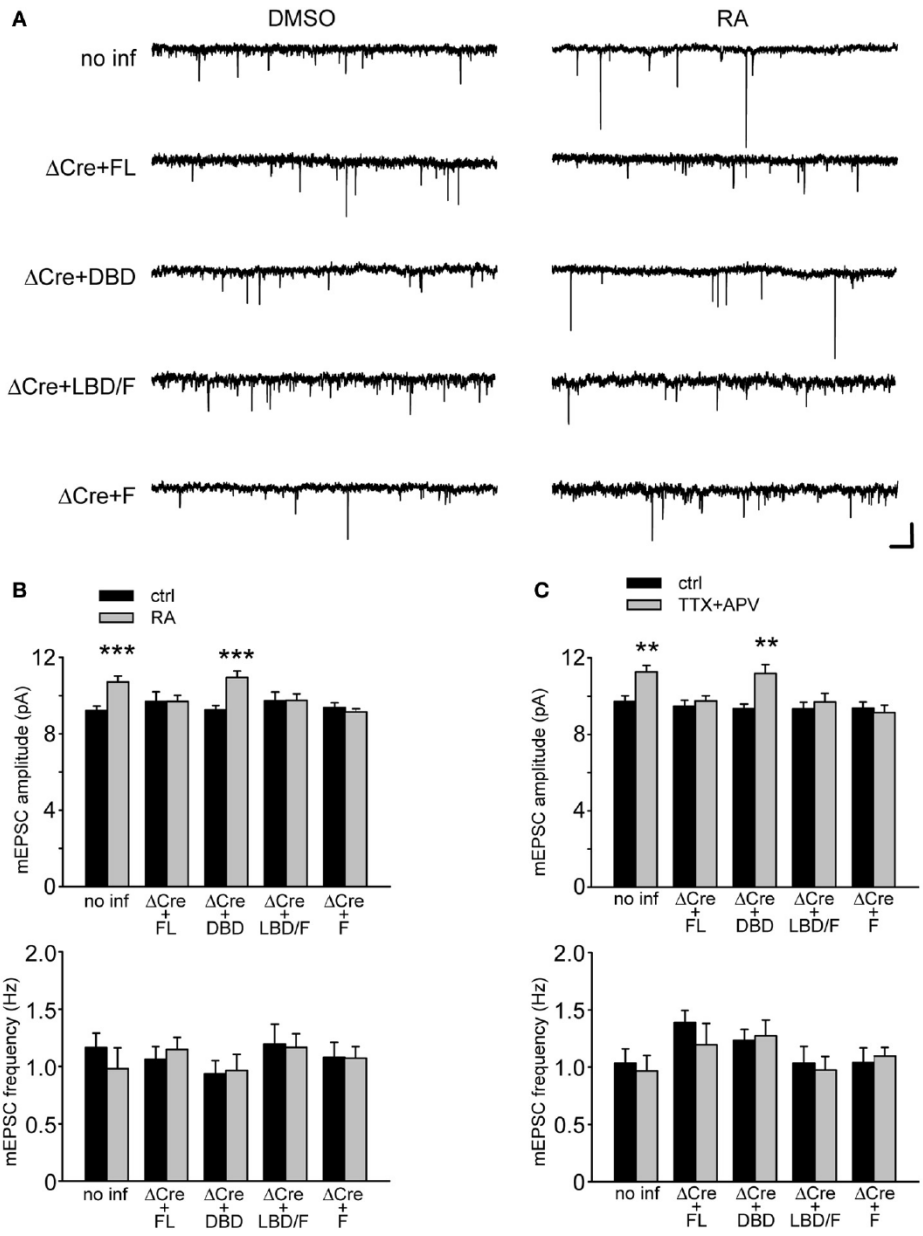

Figure 7: Synaptic scaling in neurons overexpressing RARα domains (A) Representative traces from RARα$^{fl/fl}$ hippocampal neurons infected with lentiviral vectors co-expressing Cre and either full length or deletion mutants of RARα. Neurons were treated with RA or with DMSO as control and mEPSCs recorded at -60mV. Scale bars: 10 pA, 0.5 sec. **(B)** Quantitative analysis of mEPSCs amplitude and frequency in neurons treated with RA. **(C)** Quantitative analysis of mEPSCs amplitude and frequency recorded in RARα KO hippocampal neurons upon treatment with TTX and APV for 24 hours. (for both B and C: n= 12-13, from 2 independent experiments; *, $p < 0.05$; **, $p < 0.005$).

scaling should also support TTX+APV-induced synaptic scaling. Indeed, neurons overexpressing RARα DBD did not interfere with the function of endogenous RARα in TTX+APV-induced synaptic scaling, but overexpression of RARα FL, LBD/F and F-domain blocked synaptic scaling (**Figure 7C**). These results suggest that RARα expression levels in neurons are tightly coupled to its function, and may be strictly regulated in both developing and mature neurons.

DISCUSSION

The involvement of RARα in RA-mediated homeostatic synaptic plasticity was demonstrated previously using an shRNA-based knockdown method (Aoto et al., 2008). Although rescue experiments with an shRNA-resistant version of full-length RARα was done in that study, the complexity of RNAi experiments does not allow us to completely exclude the possibility of an off-target effect. I intended to achieve two goals in the current study: to validate the shRNA results using a genetic approach, and to perform structure-function analysis of RARα. Indeed, results from the RARα knockout neurons confirmed our previous findings, validating the conclusion that RARα is required for homeostatic up-regulation of synaptic strength. Importantly, co-expression of full length RARα together with the Cre-recombinase successfully rescued synaptic scaling, making this an ideal system for subsequent structure-function analyses.

These results provide strong evidence for a non-genomic role of RARα in regulating excitatory synaptic strength. Specifically, I show that knocking out RARα in mature neurons acutely blocks homeostatic up-regulation of synaptic strength, a process mediated by RA. The rescue experiments with various forms of mutant RARα revealed that individual RARα domains perform differential functions in RA-mediated homeostatic synaptic plasticity – the DNA-binding activity of RARα was dispensable, while the RNA-binding activity of the F-domain and the RA-binding activity of the ligand-binding domain were both required. The LBD and F domains of the receptor do not participate in DNA-binding, and therefore are not known to be directly mediating the transcriptional regulation by RARα. Instead, previous work from the laboratory demonstrated that the F domain has mRNA binding abilities and that binding occurs in a sequence specific manner. The consensus sequences for binding are potentially present in many dendritically localized mRNAs, in particular the ones encoding proteins known to be involved in synaptic scaling, such as the mRNA coding for the GluA1 subunit of AMPA receptors (Poon and Chen, 2008). *In vitro* studies suggested that binding of the LBD/F domain to mRNA regulates translation in an RA-dependent manner so that addition of RA leads to translational de-repression and increased GluA1 protein levels (Poon and Chen, 2008). Recent evidence from our lab and others indicate that RARα protein is not restricted in the nucleus in mature neurons, but can be found also in neuronal dendrites (Huang et al., 2008; Maghsoodi et al., 2008), further supporting a possible non-genomic function of RARα in mature brains. Taken together, it can be hypothesized that direct binding of RARα to GluA1 mRNA through the F Domain and the ability of RARα to localize to dendrites provides a repertoire of dendritic GluA1 mRNA that is translationally dormant under basal synaptic activity. Another important piece of the puzzle is the regulation of RA synthesis by changes in synaptic activity. It was previously shown that blocking excitatory synaptic transmission leads to rapid up-regulation of RA synthesis (Aoto et al., 2008), a process that is tightly controlled by dendritic calcium levels (Wang et al., 2011b). The RA thus made binds to the

LBD domain of RARα and reduces its mRNA-binding affinity, allowing rapid increase of dendritic GluA1 synthesis through translational derepression (Poon and Chen, 2008). Synaptic insertion of newly synthesized GluA1 homomeric AMPA receptors compensates the decrease in excitatory synaptic transmission. Additionally, the calcium-permeable nature of these receptors sends a negative feedback signal to reduce and eventually halt RA synthesis, thus stabilizing synaptic strength. In this context, findings from this study on the critical role of the RARα LBD/F domains in synaptic scaling provides an important functional correlate to the observations previously made *in vitro*, and also suggest a basic mechanism for translational regulation that allows neurons to quickly respond to changes in activity with increased protein levels.

One of the somewhat unexpected findings in my work is the impact of RARα expression levels on synaptic scaling – synaptic scaling is fully rescued when the full length or the LBD/F domain of the RARα is expressed in RARα KO neurons, but is blocked by expression of full-length LBD/F domains in WT neurons containing endogenous RARα (Figure 4 and 5). I reasoned that there may be two limiting factors in dendrites for synaptic RA-signaling: the amount of RA produced during activity blockade-induced synthesis, and the amount of mRNAs in dendrites that serve as RARα substrates. When RARα is expressed at higher levels, these two limiting factors can limit synaptic scaling through two non-mutually excluding mechanisms. If the amount of GluA1 mRNA is limiting, there will be a fraction of RARα that is not mRNA-bound. Additionally, a large portion of GluA1 mRNA may fail to localize to dendrites because of their association with somatically localized RARα due to high RARα expression levels. If the amount of RA is limiting, a portion of dendritic RARα that is GluA1 mRNA-bound may not be activated by RA produced during activity blockade. Additionally, the mRNA-free RARα will further exacerbate the situation by competing with GluA1-bound RARα for RA binding. One or both limiting factors could explain the lack of synaptic scaling under TTX+APV treatment. The fact that synaptic scaling induced by direct application of exogenous RA is impaired in RARα full length and LBD/F domain expressing WT neurons argues that simply supplying more RA is not enough, and that dendritically localized GluA1 mRNA may be the other limiting factor. The developmentally regulated reduction of RARα expression as neurons mature (Huang et al., 2008) supports this notion and suggest that protein expression levels can be tightly coupled with switches of function during development.

Chapter 4

RAPID SUPPRESSION OF INHIBITORY SYNAPTIC TRANSMISSION BY RETINOIC ACID

INTRODUCTION:

Optimal information processing requires that changes in the inputs of a neural network efficiently and faithfully translate into changes in their outputs, an ability that demands both network stability and appropriately balanced synaptic connectivity. In this regard, much attention has focused on excitatory synaptic transmission, as it is the direct driving force for generating postsynaptic action potentials and for propagation of information. However, inhibitory synaptic transmission is critically involved in gating, sculpting, and tuning the output generated by excitatory inputs, and in some cases, even in instructing excitatory synaptic plasticity (Fagiolini et al., 2004; Haider et al., 2006; Sibilla and Ballerini, 2009; Levy and Reyes, 2011). It is therefore likely that synaptic inhibition also undergoes plastic changes in response to altered inputs (i.e. reduced or blocked synaptic excitation), thus shifting the excitation/inhibition ratio and achieving balanced synaptic excitation and inhibition.

The efficient modulation of excitation and inhibition underlies the homeostatic adaptations observed in different systems, such as the developing visual cortex (Komatsu, 1994; Hensch et al., 1998; Morales et al., 2002; Maffei et al., 2004; Hensch and Fagiolini, 2005), the auditory cortex (Kotak et al., 2005; Sun et al., 2010; Yang et al., 2011), and the barrel cortex (Higley and Contreras, 2006; House et al., 2011). In addition, numerous pathological conditions, such as autism, schizophrenia, epilepsy and intellectual disability, may arise from the inability of neural networks to homeostatically adjust to external inputs (Ramocki and Zoghbi, 2008). In particular, in *fmr1* KO mice, a mouse model of Fragile-X syndrome (FXS), absence of homeostatic adjustment of synaptic excitation (Soden and Chen, 2010) and evidence for an altered excitation/inhibition balance (Gibson et al., 2008) have both been demonstrated, emphasizing the primary role of the dynamic regulation of the excitation/inhibition balance in neural networks and normal brain function.

Here, I investigated whether synaptic retinoic acid (RA) signaling plays an essential role in regulating inhibitory synaptic transmission in response to reduced synaptic excitation, and whether this action of RA involves its function in regulating protein synthesis or whether transcriptional regulator is required as well. My results suggest that RA acts as a central organizer to alter synaptic excitation/inhibition (E/I) balance through its ability to directly modulate both excitatory and inhibitory synaptic strength. I further show that in the absence of FMRP, RA fails to regulate inhibitory synaptic strength, and suggest that the resulting impact on the synaptic E/I ratio may contribute to FXS pathogenesis.

RESULTS

4.1 Activity Blockade-Induced Downscaling of Inhibitory Synaptic Transmission is mediated by RA

Similar to previous reports (Kilman et al., 2002; Saliba et al., 2009), I observed that suppressing neuronal activity with TTX+APV effectively downscales synaptic inhibition

(**Fig. 8A**). Treating cultured hippocampal neurons with TTX+APV for 24 hours significantly reduced the average amplitude of miniature inhibitory postsynaptic currents (mIPSCs) without affecting the mIPSC frequency. Importantly, DEAB (4-diethylamino-benzaldehyde), a blocker of the RA synthesizing enzyme retinal dehydrogenase (Russo et al., 1988a; Wang et al., 2011a), prevented the downscaling of mIPSCs induced by TTX+APV treatment (**Fig. 8A**), suggesting that RA synthesis is required for the downscaling of synaptic inhibition in addition to upscaling of synaptic excitation.

I next asked whether RA directly modulates inhibitory synaptic responses. Brief RA treatment (30 min RA + 60 min wash) in cultured hippocampal neurons led to a robust decrease in mIPSC amplitude without affecting the mIPSC frequency (**Fig. 8B**). Addition of RA to neurons that had been treated with TTX+APV for 24 hours did not further decrease the mIPSC amplitude (**Fig. 8B**), indicating that prior chronic TTX+APV treatment occludes RA-induced downscaling of synaptic inhibition.

These results, taken together with previous studies showing that reduced excitatory synaptic activity leads to rapid synthesis of RA in neurons (Wang et al., 2011a), establish a direct role of RA in the downscaling of synaptic inhibition upon activity blockade.

4.2 Downscaling of mIPSCs is not dependent on upscaling of mEPSCs

Next, I asked whether RA downscales inhibitory synaptic strength indirectly via upscaling of excitatory synaptic transmission. I first tested whether downscaling of mIPSCs still occurred when AMPA receptor (AMPAR) activity was blocked by a selective antagonist (CNQX). I used an activity blockade protocol (TTX+CNQX) that robustly induces synaptic upscaling and blocks both pre-existing and newly inserted AMPARs, with the latter as a result of homeostatic scaling (Jakawich et al.; Wang et al., 2011a). After 24-hr TTX+CNQX treatment, cultured neurons were transferred to ACSF containing TTX+CNQX+APV for mIPSC recordings. I found that the mIPSC amplitude, but not the mIPSC frequency, was again significantly decreased (**Fig. 9A**). Because CNQX was present throughout the treatment and recording period, increases in synaptic AMPAR responses could not have caused the downscaling of synaptic inhibition.

It is possible that physical insertion (and not activity *per se*) of AMPARs into synapses mediates RA-dependent downscaling of synaptic inhibition through an unknown interaction. To test this possibility, I transfected a GFP-tagged C-terminal fragment of GluA1 (GluA1C) into cultured hippocampal neurons. Overexpression of GluA1C blocks activity-dependent synaptic trafficking of AMPARs (Shi et al., 2001; Haas et al., 2006). I first asked whether expression of GluA1C blocked synaptic upscaling of mEPSCs. Indeed, compared to GFP-transfected neurons which exhibited a significant increase in mEPSC amplitude in response to acute RA treatment, GluA1C-expressing neurons failed to respond to RA treatment (**Fig. 9B**). By contrast, in the same experiments the RA-dependent downscaling of mIPSCs was not affected by GluA1C overexpression (**Fig. 9C**). The frequency of mEPSCs and mIPSCs was not affected by GluA1C overexpression or by RA treatment (**Fig. 9B and 9C**).

Together, these data indicate that upscaling of excitatory synaptic transmission and downscaling of inhibitory synaptic transmission are independent parallel processes triggered by RA.

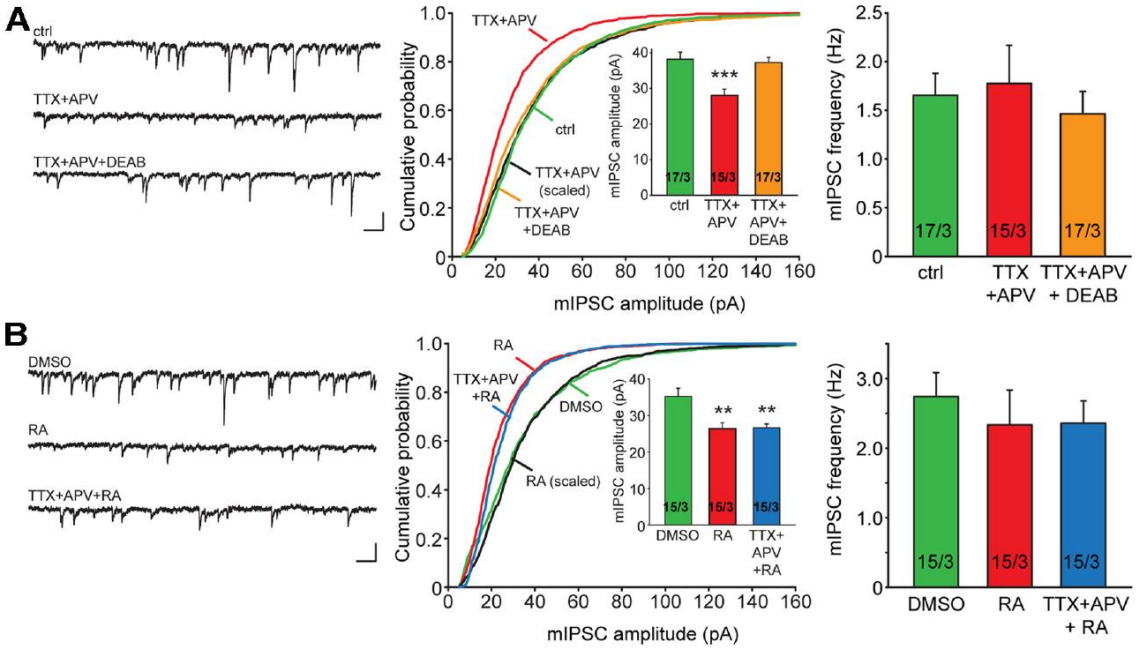

Figure 8: Retinoic acid mediates homeostatic down-scaling of inhibitory synaptic transmission (A) Representative traces (left) and quantification of mIPSC amplitude (middle) and frequency (right) recorded from cultured rat hippocampal neurons treated with control vehicle, TTX+APV (24 hr), or TTX+APV+DEAB (24 hr) (***, $p < 0.001$). (**B**) Representative traces (left) and quantification (middle and right) of mIPSCs amplitude and frequency recorded from cultured neurons treated with DMSO, RA (1 μM, 0.5 hr), or TTX+APV (24 hr) followed by RA (0.5hr) (**, $p < 0.01$).

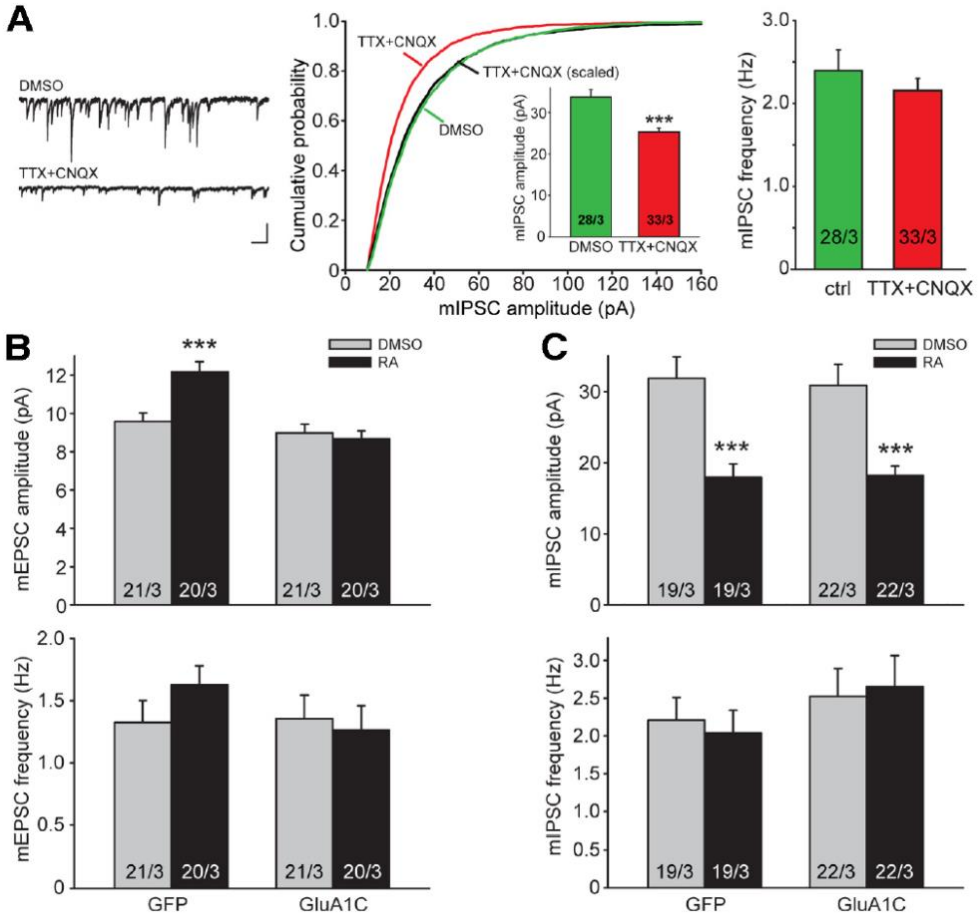

Figure 9: Downscaling of synaptic inhibition is independent of upscaling of synaptic excitation (**A**) Inhibitory synaptic scaling induced by TTX+CNQX (24 hr) (***, $p < 0.001$). (**B**) Average mEPSC amplitude and frequency from dissociated neurons over-expressing the C-terminal domain of GluR1 and treated with DMSO or 1 μM RA (***, $p < 0.001$). (**C**) Average amplitude and frequency of mIPSCs from neurons overexpressing GluR1 C-terminal domain and treated with DMSO or 1 μM RA (***, $p < 0.001$).

4.3 Reduced $GABA_AR$ abundance underlies homeostatic downscaling of synaptic inhibition

How does RA reduce synaptic inhibition? The lack of a change in mIPSC frequency suggests a postsynaptic mechanism (i.e. reduced postsynaptic $GABA_AR$ abundance). Therefore Prof. Lu Chen performed surface protein biotinylation experiments to examine the surface abundance of $GABA_ARs$ after RA treatment or activity blockade. Both acute RA and chronic activity blockade with TTX+APV significantly reduced the amount of $GABA_ARs$ on the neuronal surface (**Fig. 10A and 10B**).

To further examine whether the synaptic abundance of $GABA_ARs$ is affected by RA- or activity blockade-induced downscaling of synaptic inhibition, she performed immunocytochemistry experiments that measured the abundance of synaptic surface-exposed $GABA_ARs$. Here, she used an antibody to $GABA_AR$ β2/3 subunits that recognizes an extracellular epitope to probe surface receptors in non-permeabilized neurons, employing vesicular GABA transporter (vGAT) immunolabeling as a general marker for inhibitory synapses. Both acute RA treatment and chronic activity blockade significantly reduced the synaptic abundance of $GABA_AR$, manifested as reduced integrated puncta intensity, average puncta intensity, and puncta size (**Fig. 10C**, **10D**). The same observations were made when the staining was performed under permeabilized condition to examine the total abundance of synaptic $GABA_ARs$ (data not shown). The RA-induced reduction in synaptic abundance of $GABA_ARs$ corroborates our observation that RA decreases mIPSC amplitudes (**Figure 8B**), and also leads to a decrease in evoked IPSC (eIPSCs) amplitudes (see **Fig. 12** below).

Surface $GABA_ARs$ are constantly recycled (Kittler et al., 2000). Therefore, the reduced synaptic abundance of $GABA_ARs$ could be caused by enhanced endocytosis or reduced exocytosis of $GABA_ARs$. To distinguish between these two possibilities, she directly measured surface $GABA_AR$ endocytosis using antibody labeling for $GABA_ARs$ in live neurons. After 15-min incubation at 37^oC with a primary antibody recognizing the β2/3 subunit of $GABA_ARs$, neurons were treated with DMSO or RA for additional 15, 30, 45 and 60 minutes. The remaining surface localized and internalized β2/3receptors were labeled in the neurons after fixation with and without permeabilization. Consistent with the results from the immunostaining of synaptic $GABA_ARs$ (**Fig. 10C, 10D**), RA treatment significantly reduced surface $GABA_ARs$ at the 60 min time point (**Fig. 11A and 11B**). Importantly, although surface $GABA_ARs$ are fairly stable upon DMSO treatment, RA treatment significantly increased the proportion of endocytosed $GABA_ARs$ at the earlier time points of less than 60 min (**Fig. 11B and 11C**). We observed a significant decrease in the endocytosed $GABA_AR$ pool in the RA group at the 60 min time point (**Fig. 11C**), probably because of lysosomal degradation of internalized receptors (Kittler et al., 2004).

Next I asked whether blocking clathrin-mediated endocytosis by overexpression of a dominant negative mutant of dynamin I (the K44E substitution) (Chu et al., 1997) blocks RA's effect on mIPSCs. Dynamin WT or mutant constructs were co-transfected with GFP in 10-11 DIV cultured neurons and recording were performed three days later. Dynamin I K44E overexpression has been previously reported to increase $GABA_AR$ surface density in a mammalian cell line (Herring et al., 2003), but no effect of mutant dynamin on basal inhibitory transmission in cultured neurons has been described previously. Here, we found that basal transmission was not affected by overexpressing the dominant negative K44E (**Fig. 11D**). However, dynamin I K44E-expressing neurons failed to respond to RA

treatment (**Fig. 11D**). Thus, endocytosis of $GABA_AR$ is required for synaptic downscaling.

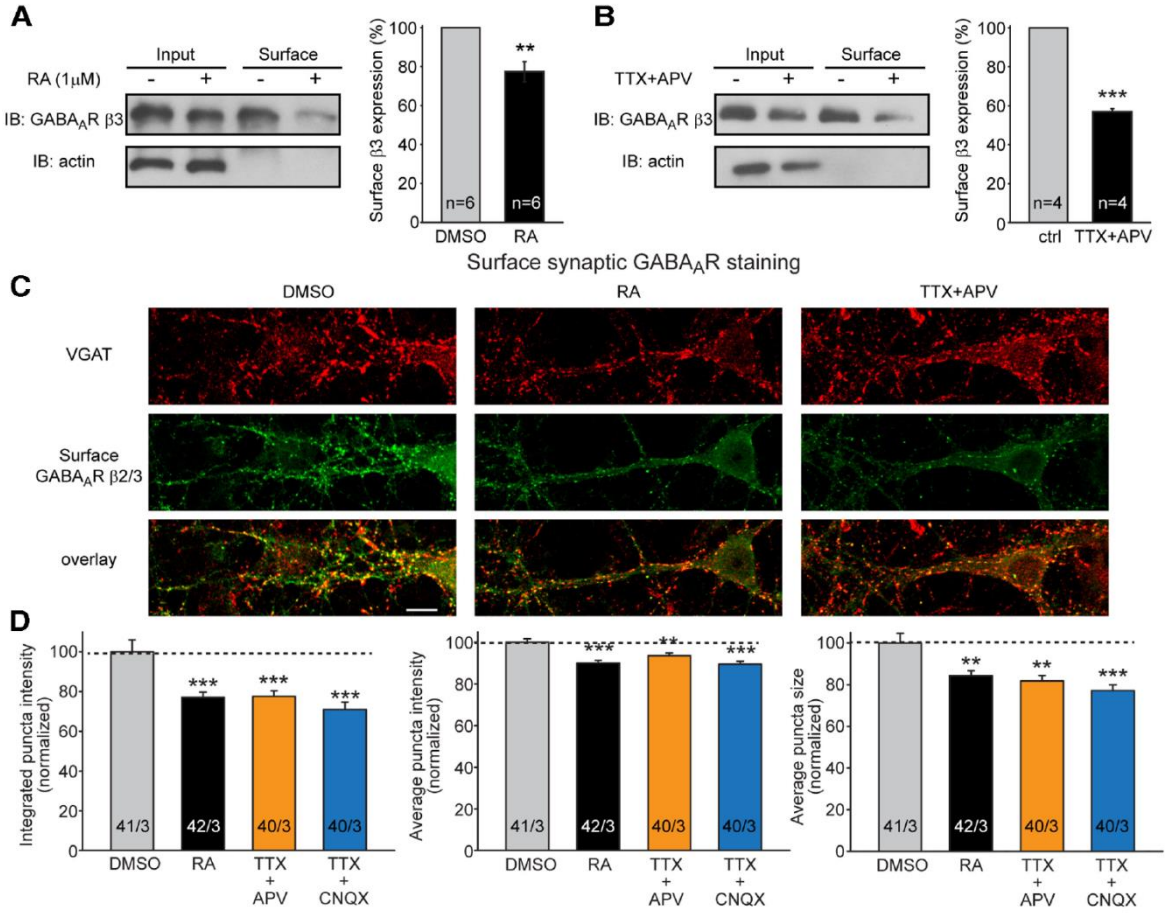

Figure 10: Activity blockade or RA treatment lead to a loss of total synaptic and surface $GABA_A$ receptors (**A**) Surface biotinylation assay for $GABA_AR$ β3 subunit in cultured hippocampal rat neurons treated with RA (**, p< 0.01). (**B**) Surface biotinylation assay for $GABA_AR$ β3 subunit in cultured neurons during activity blockade with TTX+APV (***, p<0.0001). (**C**) Immunolabelling of surface β2/3 subunit containing $GABA_ARs$ (green) and VGAT (red) from 14DIV rat hippocampal neurons treated with retinoic acid (1.5 hr), TTX+APV (24 hr) or TTX+CNQX (24 hr). Scale bar: 10 μm. (**D**) Quantification of synaptic $GABA_AR$β2/3 puncta (colocalized with VGAT) (**, P < 0.01; ***, p < 0.001).

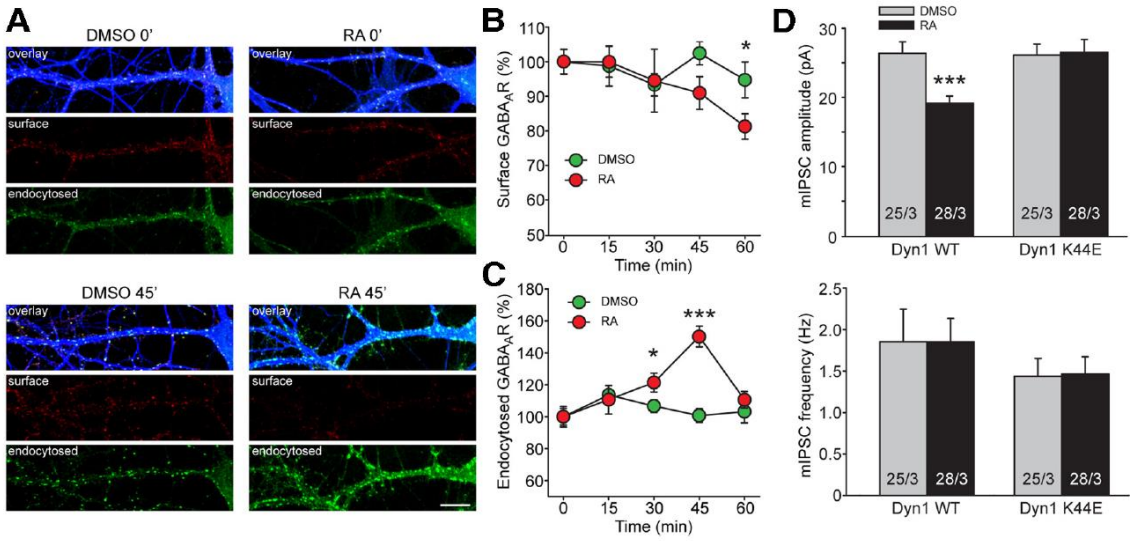

Figure 11: RA treatment enhances endocytosis of surface GABA$_A$Rs. (**A**) Representative images of endocytosis assay. Neurons were live-labeled with GABA$_A$R β2/3 antibody, treated with DMSO or RA, and then fixed at different time points and stained for endocytosed (green) and remaining surface (red) GABA$_A$Rs. Dendritic branches are labeled with MAP2 (blue). Scale bar: 10 µm. (**B**) Quantification of remaining surface GABA$_A$R (normalized to time 0) at different time points after DMSO or RA treatment (n/N = 25-34/3, *, $p < 0.05$). (**C**) Quantification of endocytosed GABA$_A$R (normalized to time 0) at different time points after DMSO or RA treatment (n/N = 25-34/3; *, $p < 0.05$; ***, $p < 0.001$). (**D**) mIPSC amplitude (top) and frequency (bottom) analysis from neurons overexpressing either wild-type Dynamin-1 or the dominant negative K44E, and treated with DMSO or RA (***, $p<0.0001$). All graphs show mean values ± SEM. n/N represents number of cells/number of independent experiments.

4.4 Non-genomic action of RA/RARα mediates the downscaling of synaptic inhibition

The effect of RA on excitatory synaptic transmission is mediated by a novel mechanism that does not require transcriptional regulation, but operates through translational de-repression of mRNAs that are bound to RARα (Aoto et al., 2008; Poon and Chen, 2008; Sarti et al., 2012b). Given these evidences, I asked whether the action of RA on inhibitory synaptic transmission uses a similar non-genomic mechanism. The transcription inhibitor Actinomycin D (Act D) had no effect on the RA-induced reduction of mIPSC amplitude, whereas inhibitors of protein synthesis such as cyclohexamide and anysomycin completely abolished RA's effect on the mIPSC amplitude (**Fig. 12A**).

The RA receptor RARα is required for RA's effect on synaptic excitation because both shRNA-mediated knockdown and cre-recombinase-mediated conditional knockout of RARα eliminated RA's action on excitatory synaptic transmission, and blocked homeostatic upregulation of synaptic excitation (Aoto et al., 2008; Sarti et al., 2012b). To investigate the requirement for RARα in downscaling of synaptic inhibition, I infected by injection CA1 pyramidal neurons from conditional RARα KO mice ($RAR\alpha^{fl/fl}$) with lentivirus expressing either an active or inactive GFP-tagged cre-recombinase (Cre or ΔCre) (Kaeser et al., 2011). I recorded evoked IPSCs (eIPSCs) simultaneously from an infected and an uninfected neuron adjacent to each other by stimulating the region of S. radiatum next to the two neurons (**Fig. 12B**). This allowed me to directly compare the size of eIPSCs of the infected and the uninfected neurons. Based on my observations above on the mIPSC amplitudes and synaptic $GABA_AR$ surface expression (**Fig. 8B and 10**), RA is expected to reduce eIPSCs as well. Therefore neurons with impaired downscaling of synaptic inhibition should exhibit bigger eIPSCs compared to wildtype neurons. Indeed, although knockout of RARα did not affect basal inhibitory synaptic transmission (**Fig. 12C**), RA treatment reduced the eIPSC amplitudes in uninfected neurons, rendering them significantly smaller than those from neighboring cre-recombinase expressing RARα KO neurons (**Fig. 12C**), indicating that the RA-induced down-regulation of eIPSC is significantly impaired in RARα KO neurons. This effect is not due to side effects of viral injection as infection with lentivirus expressing the inactive cre (ΔCre) did not cause such changes (**Fig. 12D**).

Structure-function analysis showed that different domains of RARα are associated with different functions. While the DNA-binding domain (DBD) mediates its nuclear function (Evans, 1988; Green and Chambon, 1988; Tasset et al., 1990), the C–terminal LBD/F domain is required for the non-genomic action of RARα through RA- and mRNA-binding (Poon and Chen, 2008; Sarti et al., 2012b). The deletion mutants of RARα thus provide useful tools to probe the involvement of different functions of RARα in downscaling of synaptic inhibition when expressed in RARα KO neurons. Co-expression of RARα LBD/F with Cre restored RA-induced homeostatic down-regulation of eIPSCs (**Fig. 12E**). By contrast, co-expression of RARα DBD failed to rescue downscaling of eIPSCs (**Fig. 12F**). These data indicate that the nuclear function of RA and RARα is not involved in the action of RA at inhibitory synapses.

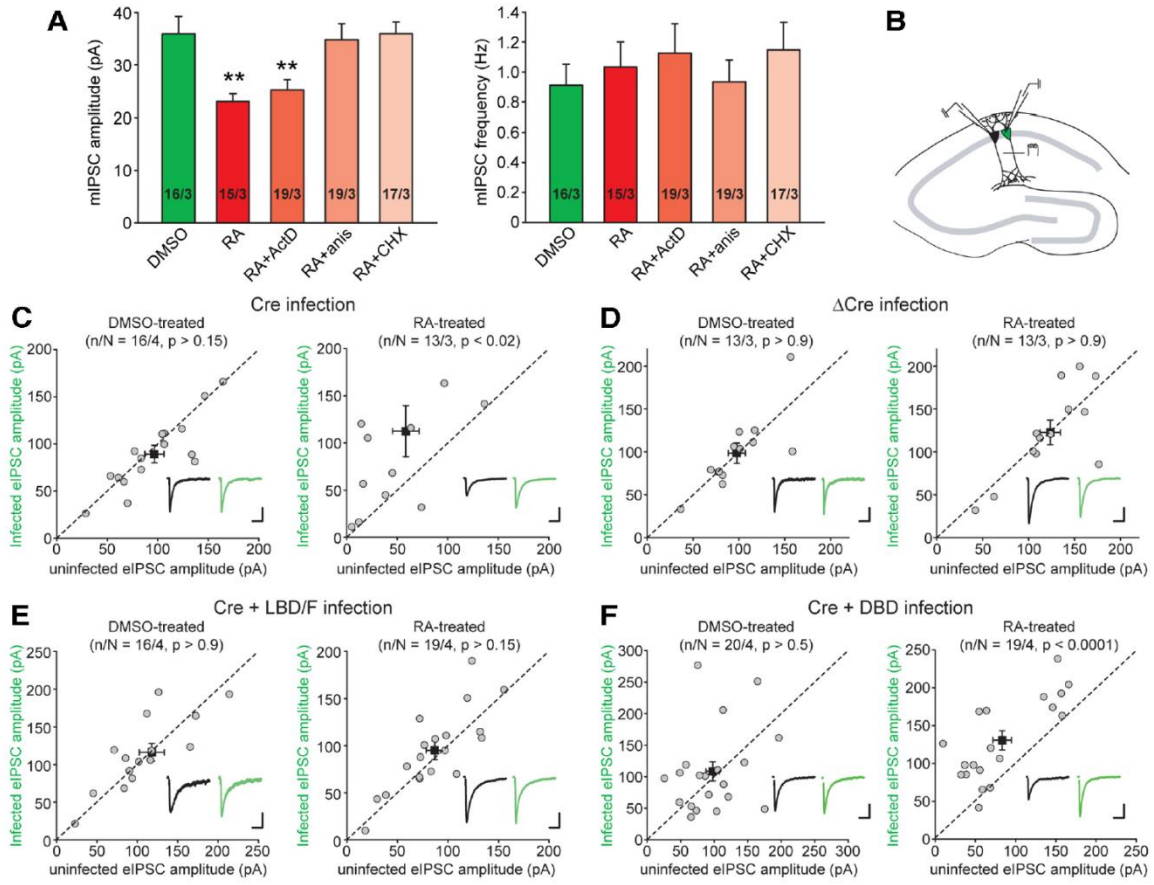

Figure 12: New protein synthesis and non-nuclear functions of RARαare required for downscaling of inhibitory synaptic transmission (**A**) Amplitude and frequency analysis of mIPSCs obtained from rat hippocampal neurons co-treated with transcription (Actinomycin) or translation (Anisomycin, Cyclohexamide) inhibitors and RA (**, $p < 0.01$). (**B**) Recording configuration for paired-recordings of eIPSCs. Cultured hippocampal slices from RARα floxed mouse were infected with Cre-expressing lentivirus. Paired recordings of eIPSCs were obtained simultaneously from two neighboring CA1 pyramidal neurons, one infected (green), one uninfected (black). The stimulating electrodes were positioned in S. radiatum as shown. (**C-F**) Scatter plots of eIPSCs from individual pairs (gray circles) and group mean ± SEM (black squares) of simultaneously recorded neurons. Insets are representative traces for infected neurons (green) and RARα *floxed* neurons (black) recorded after treatment with RA or DMSO. Neurons were infected with a lentiviral vector expressing (**C**) Cre recombinase (to generate RARα KO neurons); (**D**) inactive recombinase ΔCre; (**E**) Cre and RARα LBD/F; (**F**) Cre and RARα DBD. Scale bars: 40 pA, 100ms. n/N represents number of cells/number of independent experiments.

4.5 RA alters the synaptic excitation/inhibition balance in acute hippocampal slices

Given the rapid action of RA on synapses, we wondered if we could reproduce the effect of RA observed in cultured neurons and cultured slices also in acute slices, where local circuits are better preserved than in culture preparations. Zhenjie Zhang, a postdoc working in Lu Chen's group, incubated acute hippocampal slices from young mice (P10) with RA (2 μM) and noticed an induced significant downscaling of mIPSCs recorded from CA1 pyramidal neurons (**Fig. 13A**). The mIPSC frequency was not affected (**Fig. 13B**).

These data suggest that RA, by acting both on synaptic excitation and inhibition, can rapidly and robustly shift the E/I balance of synaptic inputs to a neuron. EPSCs and IPSCs exhibit different rise and decay kinetics. Moreover, inhibitory and excitatory synapses often display different release probabilities and distinct forms of short-term plasticity (depressing versus facilitating synapses) (Zucker, 1989). Therefore, the E/I ratio of a given set of inputs can exhibit rapid dynamics within a pulse (due to difference in EPSC and IPSC kinetics) and from pulse to pulse (due to difference in short-term plasticity) in a high frequency burst of action potentials. Given that RA reduces both mIPSCs and eIPSCs, we set out to explore its effect on synaptic E/I balance with evoked responses. Zhenjie stimulated the S. radiatum with a 5-pulse 25 Hz stimulus train and recorded eEPSCs and eIPSCs in the same neurons (**Fig. 13C**) and found that the synaptic excitatory/inhibitory conductance ratio (Ge/Gi) changed significantly over time (**Fig. 13D**). For each pulse, due to the more rapid onset and faster rise times of EPSCs than those of IPSCs, the synaptic Ge/Gi ratio was high at the beginning of each response. This was even more evident for the first pulse as Gi was near zero when Ge ramped up rapidly. But the Ge/Gi ratio decreased rapidly within 10 ms of stimulus onset as soon as inhibition increased (**Fig. 13D**). Additionally, the peak Ge/Gi for each pulse exhibited an overall decreasing trend due to the slow decay kinetics of synaptic inhibition (**Fig. 13D**). RA treatment significantly increased the peak Ge/Gi (**Fig. 13D**, red traces).

4.6 Downscaling of inhibitory synaptic transmission is absent in Fmr1 KO neurons

Activity blockade induces RA synthesis in neurons from Fragile-X model mice (*Fmr1* KO), but both synaptic activity blockade- and RA-induced upscaling of excitatory synaptic transmission are absent (Soden and Chen, 2010b). These data demonstrated a critical role of FMRP, the protein encoded by *fmr1*, in synaptic RA signaling and homeostatic synaptic plasticity. I thus asked whether FMRP is specifically required only for homeostatic regulation of excitatory synaptic strength, or is universally involved in RA-mediated regulation of both synaptic excitation and synaptic inhibition.

I found that TTX+CNQX treatment produced no reduction in the mIPSC amplitude of neurons in organotypic cultured hippocampal slices from *Fmr1* KO mice, but caused a robust reduction of the mIPSC amplitude in wildtype slices (**Fig. 14A**). Moreover, RA significantly decreased the mIPSC amplitude in WT but not *Fmr1* KO neurons (**Fig. 14B**). The frequency of mIPSCs was not altered by RA in either WT or Fmr1 KO slices (**Fig. 14B**). Therefore, FMRP is required for homeostatic regulation of both synaptic inhibition and excitation. Next, Lu Chen and Zhenjie Zhang examined the effect of RA on the synaptic E/I balance in acute slices from the *Fmr1* KO mice. Although the basal Ge/Gi ratio was similar to that of the wildtype neurons, RA failed to shift the Ge/Gi balance (**Fig. 15C and 15D**), confirming my observations in cultured slices that RA-dependent regulation of synaptic strength is missing in the absence of FMRP expression.

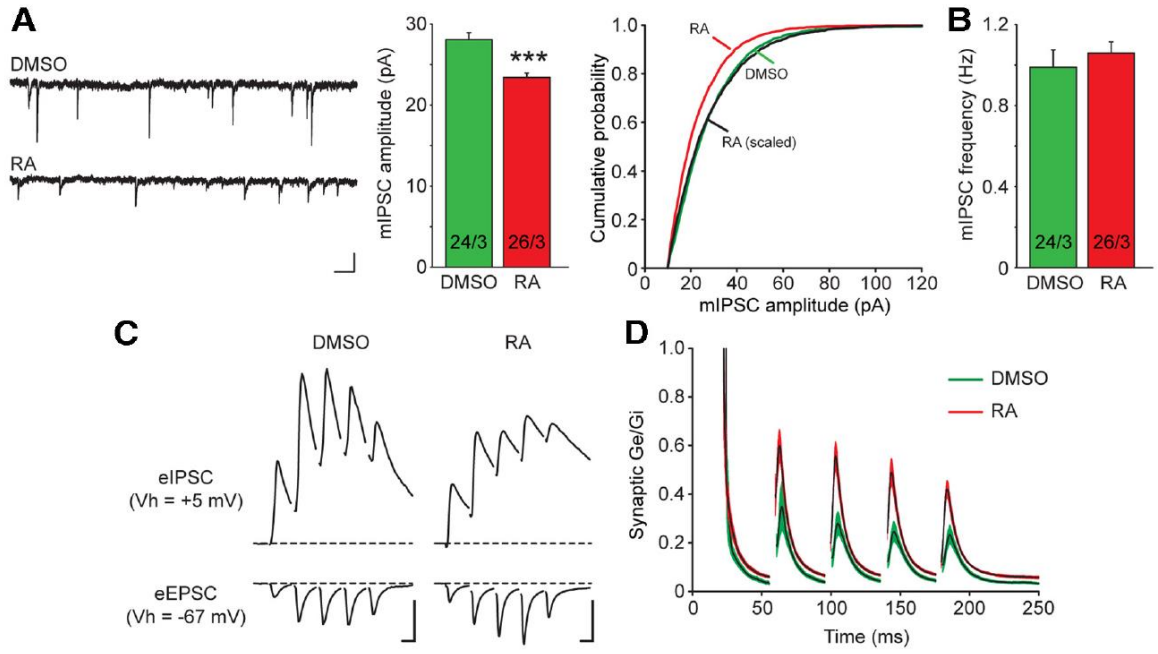

Figure 13: RA induces downscaling of synaptic inhibition and increases excitation/inhibition ratio in acute hippocampal slices. (**A**) Representative traces and amplitude analysis of mIPSC recordings obtained from CA1 pyramidal neurons of acute hippocampal slices incubated with DMSO or RA (***, $p < 0.001$). Scale bar: 20 pA, 0.5 s. (**B**) Frequency analysis of mIPSCs recorded in (A). (**C**) Example traces of 25 Hz five-pulse train evoked IPSCs and EPSCs recorded in the same wildtype CA1 pyramidal neurons treated with DMSO or RA. Scale bars: 200 pA, 20 ms. (**D**) Synaptic excitatory/inhibitory conductance ratio Ge/Gi (black line: mean; red or green shades: ± s.e.m.) of the synaptic responses to train stimulation in wildtype neurons (n/N = 8/3). Note the peak of first pulse is cut off at 1 for display purpose.

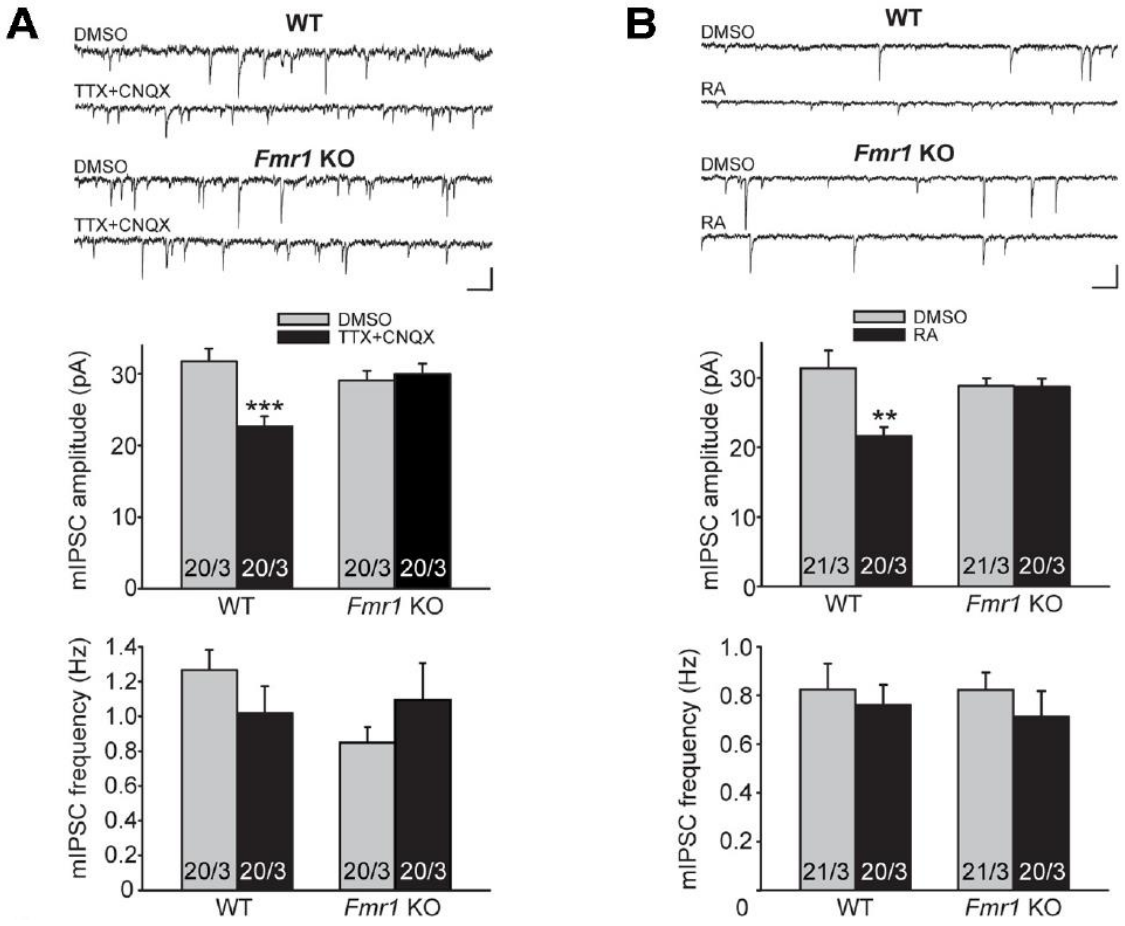

Figure 14: RA-mediated downscaling of synaptic inhibition is absent in *Fmr1* KO neurons (**A**) Representative traces (top) and quantification (bottom) of mIPSCs recorded from WT or *Fmr1* KO CA1 pyramidal neurons in cultured hippocampal slices treated with DMSO or TTX+CNQX (***, $p < 0.001$). (**B**) Representative traces (top) and quantification (bottom) of mIPSCs recorded from WT or *Fmr1* KO CA1 pyramidal neurons in cultured hippocampal slices treated with DMSO or RA (**, $p < 0.01$). All scale bars are 50 pA and 0.5 s.

4.7 RA alters neuronal excitability through modulation of the synaptic E/I balance

What could be the functional impact of such an RA-dependent shift in synaptic Ge/Gi? Although the synaptic Ge/Gi ratio resides largely well below 1, the actual synaptic E/I balance is the result of the interaction between the driving forces of ion fluxes (dictated by the membrane potential and the reversal potentials of EPSCs and IPSCs) and the synaptic conductance. Prof. Lu Chen therefore simulated synaptic EPSC/IPSC ratios in a 25 Hz 5-pulse train in two scenarios that neurons commonly experience, a resting condition (Vm = -60 mV) and a partly depolarized condition (Vm = -50 mV, just below the action potential firing threshold), using the Ge/Gi ratio calculated from our recordings (Fig. 6C and 6D). Here, she adopted well-accepted reversal potentials of EPSCs and IPSCs (E_{EPSC} = 0 mV, E_{IPSC} = -65 mV). At -60 mV, synaptic excitation dominates inhibition at the onset of each stimulus even in DMSO treated condition because of the vast difference between driving forces (60 mV for EPSC and 5 mV for IPSC), and RA treatment significantly exaggerated the dominance of synaptic excitation (**Fig. 15A**). By contrast, at Vm = -50 mV, the synaptic excitation was largely shunted by inhibition because of the shift in driving force after the first pulse (**Fig. 15B**). RA treatment significantly increased the synaptic EPSC/IPSC ratio and restored the dominance by excitation (**Fig. 15B**). Thus, for a neuron in a slightly depolarized state, RA robustly enhances the firing probability induced by a bursting input through reversing the E/I balance. In the *Fmr1* KO mouse, due to the deficiency in synaptic RA signaling and homeostatic synaptic plasticity, such modification of the E/I balance is lost (**Fig. 15C and 15D**).

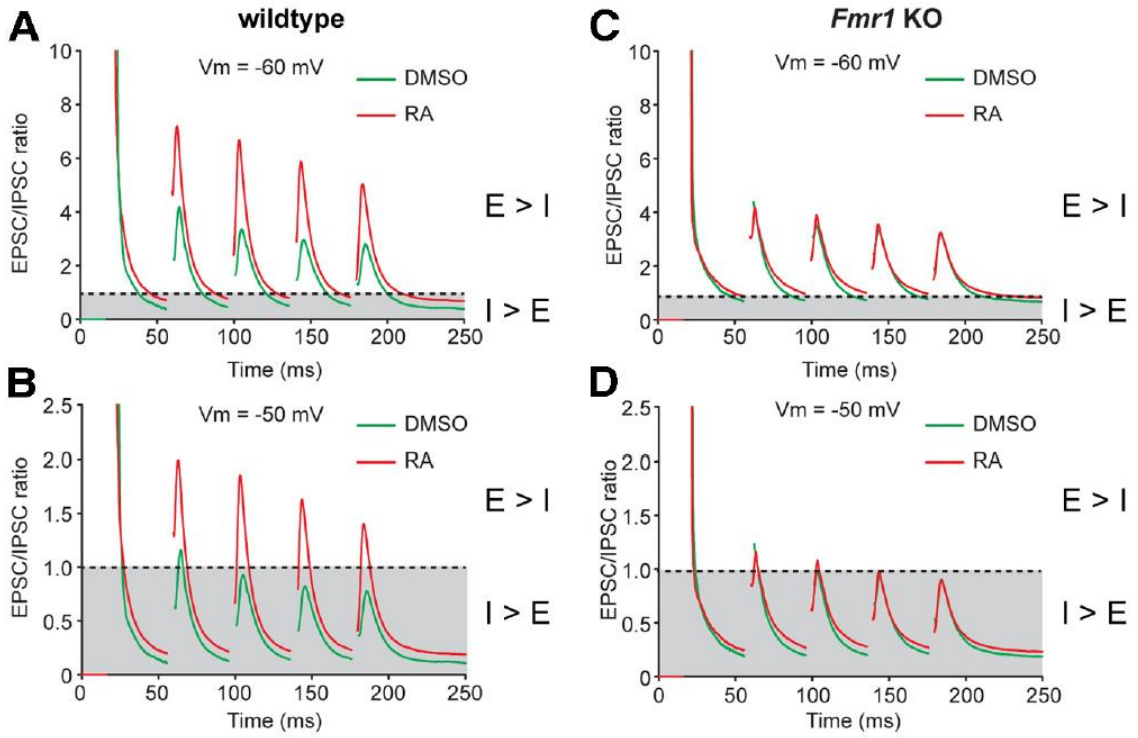

Figure 15: RA alters neuronal excitability (**A**) Simulated EPSC/IPSC ratio in DMSO- or RA-treated wildtype neurons at resting membrane potential (-60 mV). EPSC/IPSC = $(V_m-E_{EPSC})/(V_m-E_{IPSC})*(Ge/Gi)$, using Ge/Gi values obtained in Figure 6D. Gray area depicts inhibition-dominating zone. Note that for both treatment, synaptic excitation dominates at the beginning of each pulse due to large excitatory driving force (0 mV) and small inhibitory driving force (65 mV). (**B**) Same simulation in wildtype neurons at membrane voltage just below action potential firing threshold (-50 mV). Due to a shift in excitatory and inhibitory driving force (50 mV and 15 mV, respectively), only synaptic responses in RA-treated neurons are consistently excitation-dominant. (**C**) and (**D**) Simulation of EPSC/IPSC ratio in Fmr1 KO neurons at -60 mV and -50 mV. The effect of RA on boosting synaptic excitation is absent.

DISCUSSION

In this study, I show that RA mediates homeostatic downscaling of synaptic inhibition, and that similar to RA-dependent upscaling of synaptic excitation, RA-dependent downscaling of synaptic inhibition requires FMRP (Soden and Chen, 2010b). Blocking RA synthesis completely prevented the activity blockade-induced down-scaling of synaptic inhibition. Direct application of RA rapidly and robustly suppressed synaptic inhibition by triggering the removal of $GABA_A$Rs from synapses. The effect of RA on synaptic inhibition was occluded by prolonged synaptic activity blockade. Similar to RA-induced increase in synaptic excitation, the RA-induced decrease in synaptic inhibition required protein synthesis but not gene transcription, and involved a non-transcriptional function of RARα. However, the upscaling of synaptic excitation and downscaling of synaptic inhibition by RA were not dependent on each other since blocking excitatory upscaling did not affect inhibitory downscaling, suggesting that RA acts as a master organizer of neuronal activity by independently regulating excitatory and inhibitory synapses. In organizing the activity of neurons, RA appears to activate parallel cellular pathways to modulate synaptic excitation and inhibition in a coordinated fashion. The result of such orchestrated modulation in response to reduced synaptic excitation is a rapid increase in the synaptic E/I ratio, which may be responsible for subsequent changes in Hebbian plasticity at the affected synapses (Chen et al., 2013).

$GABA_A$Rs mediate most fast synaptic inhibition in the CNS. Modulation of $GABA_A$R trafficking to and out of synapses underlies many neuronal excitability changes under both physiological and pathological conditions (reviewed by (Luscher et al., 2011). Endocytosis of $GABA_A$Rs occurs primarily through clathrin- and dynamin-dependent mechanisms, and requires interactions of $GABA_A$R β and γ subunits with the clathrin adaptor protein AP2 (McDonald et al., 1998; Brandon et al., 2000; Kittler et al., 2000; Brandon et al., 2002; Brandon et al., 2003; Kittler et al., 2005; Kittler et al., 2008; Smith et al., 2008). Here, I found that a dominant-negative dynamin mutant blocked the RA-mediated reduction of synaptic inhibition (although somewhat surprisingly it did not affect basal inhibitory transmission), suggesting that inhibitory down-scaling is mediated by enhanced endocytosis of $GABA_A$Rs. Interestingly, the effect of RA on inhibitiory synapses also required *de novo* protein synthesis, suggesting that a newly synthesized protein activates $GABA_A$R endocytosis. No such protein has yet been described – indeed, the very existence of a protein synthesis-dependent endocytosis pathway is novel.

While RA's action at excitatory and inhibitory synapses both require protein synthesis and expression of FMRP, the end result of the two processes is diametrically opposite: upscaling of synaptic excitation involves new synthesis of AMPARs that are then inserted into synapses, whereas downscaling of synaptic inhibition involves new protein synthesis that causes the removal of $GABA_A$Rs. This raises the question whether our previous view that upscaling of synaptic excitation is simply due to new AMPAR synthesis may have been too limited, and whether upscaling may also involve new synthesis of a regulatory protein that controls synaptic AMPAR trafficking, in addition to stimulating new AMPAR synthesis itself. Thus, we would like to posit that the protein-synthesis dependent actions of RA may be more regulatory than executive for both up- and downscaling, and involve the synthesis of one or several short-lived protein core factors that then promote both AMPAR insertion and GABAR endocytosis. This hypothesis provides the simplest explanation for all available data, but alternative, more complicated

scenarios – such as multiple independent regulatory and executive pathways – cannot be ruled out.

RA is the first molecule identified that mediates homeostatic heterosynaptic regulation of inhibitory synapses. This role of RA is similar to that of endocannabinoids, BDNF, and nitric oxide in mediating heterosynaptic forms of inhibitory synaptic plasticity, for example i-LTD or i-LTP (Castillo et al., 2011). Moreover, endocannabinoids, BDNF, and nitric oxide also act on excitatory synapses like RA. Likewise, TNFα has been shown to induce a rapid insertion of AMPA receptors and endocytosis of GABAA receptors (Stellwagen et al., 2005). However, RA-signaling exhibits one fundamental distinction: i-LTP and i-LTD are induced by activation of excitatory synapses and an increase in dendritic calcium levels, whereas RA-mediated regulation of synaptic inhibition and excitation is triggered by silencing excitatory synapses and a decrease in dendritic calcium levels. Thus, RA acts as a critical component of the feedback loop linking reduced synaptic excitation to shifts in synaptic E/I balance through homeostatic up-regulation of excitatory synaptic strength and down-regulation of inhibitory synaptic strength.

Throughout the development of sensory systems, the E/I balance is thought to be generally well preserved. Balanced synaptic excitation and inhibition (e.g. feed-forward excitation versus feed-forward inhibition) is critical for network stability and information processing (Liu et al., 2007; House et al., 2011; Zhang et al., 2011). Failure to maintain a proper E/I balance has been linked to neurological disorders including epilepsy, schizophrenia and autism spectrum disorders (Rubenstein and Merzenich, 2003; Lewis et al., 2005; Ramocki and Zoghbi, 2008; Sudhof, 2008; Chao et al., 2010). In the mouse model of FXS (the *Fmr1* KO), an imbalance of excitation and inhibition and network hyper-excitability was reported in layer 4 of the barrel cortex (Gibson et al., 2008). Additionally, we observed a complete absence of RA-mediated homeostatic regulation of both synaptic excitation (Soden and Chen, 2010b) and inhibition (**Fig. 8**). Moreover, although the E/I ratio in the hippocampal CA1 region appears to be similar between WT and *Fmr1*KO neurons (likely through compensation at the circuit level), the ability of RA to alter the E/I ratio in a stimulus-dependent fashion is lost in *Fmr1*KO neurons (Fig. 7).

As the mechanistic links between E/I imbalance and altered social behavior are just beginning to be uncovered (Yizhar et al., 2011), future investigations are needed to provide molecular, cellular, and circuitry explanations for the behavioral abnormalities in psychiatric diseases. The ability of RA to activate two independent and parallel pathways that mediate concomitant upscaling of synaptic excitation and downscaling of synaptic inhibition allows a greater dynamic range for synaptic homeostasis organized by one single molecule. Thus, RA is a unique synaptic signaling molecule that may play an instructive role in sculpting synaptic inputs based on their activity history to enable efficient encoding, processing and storing of stimulus-specific information.

Chapter 5

CONCLUSIONS, SIGNIFICANCE AND REMAINING QUESTIONS

Until recently, RA was primarily known as a regulator of nuclear transcription in development. The newly discovered non-transcriptional function of RA in mature neurons represents a fundamental shift in the understanding of the mechanisms underlying RA's role in the adult brain, and allows us to explore the biological significance of this important molecule under a different light. The established role of RA in synaptic scaling through synaptic activity-dependent modulation of local protein synthesis in neuronal dendrites indicates that RA participates actively in cellular processes beyond development, and may potentially influence the cognitive functions of an organism throughout life. The mere fact that a nuclear signaling molecule and its receptor are also dendritic regulators of synaptic strength is amazing, although it accounts for previous observations that RA synthesis and receptor expression persist far beyond development into adulthood.

5.1 RA and other types of synaptic plasticity

Although the synaptic action of RA has been tightly linked to HSP, these findings do not mean that the impact of RA on synapses is limited to HSP. In addition to evidence showing that vitamin A deficiency leads to impaired hippocampal Hebbian plasticity and learning, a recent study using a dominant negative form of RARα expressed in the adult forebrain demonstrated impairments in AMPA receptor-mediated synaptic transmission, hippocampal LTP, hippocampal-dependent social recognition, and spatial memory (Nomoto et al., 2012). These findings suggest that the functional impact of RA may go beyond HSP.

The history of a neuron's activity determines its current biochemical state and its ability to undergo synaptic plasticity, a phenomenon referred to as meta-plasticity (Abraham and Bear, 1996). A number of factors have been proposed to contribute to meta-plasticity through influencing the state of the neuron/synapse. For example, the postsynaptic NMDA receptor composition (GluN2A- versus GluN2B-containing (Yashiro and Philpot, 2008)), the phosphorylation state of AMPA receptors (Lee et al., 2000), the ratio of CaMKIIα to CaMKIIβ (Thiagarajan et al., 2007), presynaptic endocannabinoid receptors (Chevaleyre and Castillo, 2004), as well as signaling by various neuromodulators (Scheiderer et al., 2004; Seol et al., 2007; Lee et al., 2010; Huang et al., 2012) have all been shown to act as mechanisms for meta-plasticity. Aside from its ability to rapidly enhance excitatory synaptic transmission (Aoto et al., 2008), RA also alters inhibitory synaptic transmission (unpublished observation). Therefore, RA likely is a candidate 'metaplasticity molecule' that may change the state of a neuron (e.g., its excitatory/inhibitory balance) and thus influence Hebbian plasticity, through a mechanism potentially distinct from that mentioned above. Thus, although the primary functional significance of HSP has always been thought to maintain neural network stability by dynamically regulating neuronal excitability, the biochemical events involved in the homeostatic adjustment of synaptic activity may well directly influence the ability of the neuron to undergo Hebbian-type plasticity.

5.2 RA signaling-global versus local regulation

HSP was initially thought to operate at a global level (e.g. changes occur to all synapses of a neuron, Fig. 2A) (Turrigiano, 2008). A number of recent studies provided evidence for additional "local" processes that can act on individual synapses or a small subset of synapses (Ju et al., 2004; Sutton and Schuman, 2006; Branco et al., 2008; Hou et al., 2008; Yu and Goda, 2009). In this sense, 'local HSP' would define HSP as a process that does not maintain a constant activity level in an entire neuron, but in a neuronal subcompartment, such as a dendritic segment. In this context, many properties of synaptic RA make it an attractive molecule for mediating both global (not input-specific) and local (input-specific) HSP. The RA synthesis enzyme RALDH is expressed throughout the soma and dendrites of neurons (Aoto et al., 2008). It has been shown that RA is synthesized within a neuron experiencing reduced synaptic activity, and that the RA synthesis machinery is sensitive to changes in synaptic/dendritic calcium influx but not somatic calcium influx (Wang et al., 2011b). Together, these observations suggest that RA synthesis can occur in a subset of synapses or in discrete regions of dendrites experiencing decreased synaptic activity.

A fascinating property of RA is that it is a small lipophilic molecule that can potentially freely diffuse inside a neuron and through cell membranes, but such diffusion is restricted due to the presence of cellular RA-binding proteins and abundant expression of CYP26, an RA degradation enzyme (Ray et al., 1997). This means that RA's action may be local, in the vicinity of the synapses experiencing reduced synaptic activity and calcium influx, but at the same time its action may extend beyond those synapses. One intriguing observation is that decreased excitatory synaptic activity during blockade of AMPA receptors not only induces HSP at excitatory synapses, but also decreases the strength of inhibitory synapses.

The changes at excitatory and inhibitory synapses both require RA synthesis. This is one example that RA's action is likely not restricted to synapses next to which RA synthesis is triggered, but can act as a communicator between excitatory and inhibitory synapses (or other neighboring excitatory synapses). Additionally, changes in presynaptic function (manifested as an increase in mEPSC frequency) have been observed when synaptic scaling was induced in more mature neurons (Thiagarajan et al., 2005, Jakawich et al, 2010, Wang et al., 2011). These changes may be dependent on postsynaptic BDNF as a secreted signal (Jakawich et al., 2010), but also require RA synthesis (Wang et al., 2011). It is possible that RA stimulates *de novo* synthesis of trans-synaptic signaling molecules such as BDNF, which then communicates the initially cell-autonomous postsynaptic action of RA to presynaptic partners that send their inputs to the RA-synthesizing neuron. When multiple excitatory synapses on the same dendritic segment experience reduced activity, RA synthesized at these locations could even act synergistically to influence all synapses on this segment, switching RA's action from a more local to a more global mode. Thus, depending on the location and scale of RA synthesis, RA can potentially alter the strength of a single synapse, a subset of neighboring synapses, or all of the synapses in a dendritic segment. A number of recent studies in the hippocampus and cortex show that neighboring synaptic inputs on the same dendrites tend to have synchronized activity (Kleindienst et al., 2011; Takahashi et al., 2012), and that behaviorally induced synaptic plasticity and spine dynamics exhibit highly structured spatial patterns (Makino and Malinow, 2011; Fu et al., 2012; Lai et al., 2012). Although much more work is required, it will be fascinating to

investigate whether RA and possibly other small molecules play an instructive role in sculpting the strength of synaptic inputs that is critical for encoding, processing and storing stimulus-specific information.

5.3 The interplay between RA and FMRP in the regulation of synaptic strength

A big question arising from the evidences shown in this thesis work is however regarding protein and activity regulation operated by RA in the context of FMRP. How do FMRP and RA/RARα work together to regulate RA-mediated translation during homeostatic plasticity of excitatory and inhibitory currents? One possibility is that in the absence of FMRP and inhibition of protein synthesis of FMRP-bound mRNAs is removed. Elevated levels of basal mRNA translation may produce a "ceiling effect" that prevents further increases in protein synthesis by RA. However, reintroduction into *Fmr1* knockout neurons of a mutant form of FMRP (I304N) (Soden & Chen, 2010) that suppresses basal AMPA receptor translation, failed to restore RA-dependent HSP, suggesting that simply reducing AMPA receptor protein levels through FMRP binding to mRNA is not sufficient to rescue plasticity.

Understanding other cellular processes altered in the *Fmr1* knockout mouse may also shed some light on the specific involvement of FMRP in RA signaling. For example, in addition to removal of translational inhibition through the uncoupling of mRNA from FMRP, the activities of MAPK/Erk1/2 and PI3K/AKT/mTOR pathways in *Fmr1* knockout mice are elevated (Osterweil et al., 2010; Sharma et al., 2010; Bhakar et al., 2012; Ronesi et al., 2012). Coincidently, RA has been reported to activate MAPK/Erk as well as PI3K signaling pathways in various cell lines (Uruno et al., 2005; Ko et al., 2007; Masia et al., 2007), making these pathways likely candidate for linking FMRP and RA functions. Although no direct binding of FMRP to RARα has been observed (Soden and Chen, 2010a), it is possible that they interact by binding to the same RNA molecules.

5.4 Lack of RA mediated HSP in the Fragile-X mouse model and its implications

The blockage of excitatory and inhibitory HSP in *Fmr1* knockout mice and the requirement for FMRP in synaptic RA signaling raise the intriguing possibility that impaired HSP may underlie some symptoms associated with intellectual disability and cognitive dysfunction. Hebbian-type synaptic plasticity is considered the cellular mechanism for learning and memory. *Fmr1* knockout mice, as an animal model for Fragile-X mental retardation, have been studied extensively for defects in neuronal function and learning and memory. Indeed, impaired Hebbian-type synaptic plasticity in *Fmr1* knockout mice (Huber et al., 2002; Larson et al., 2005) may contribute to their learning deficits (Mineur et al., 2002; Yan et al., 2004; Koekkoek et al., 2005). The newly discovered link between FMRP, HSP and RA-mediated translational regulation of synaptic proteins suggest that FMRP and its regulation of protein synthesis participate in multiple forms of activity-dependent synaptic plasticity, though seemingly through distinct mechanisms. These findings provide a new perspective on the phenotype in *Fmr1* knockout mice and on the symptoms of human Fragile-X patients. Lack of excitation/inhibition balance regulation may explain, for example, the global alterations of neural activity that have been observed in *Fmr1* knockout mice and FXS patients (Berry-Kravis, 2002; Yan et al., 2004). Moreover, as we discussed above, although HSP may not participate directly in the cellular

processes for memory encoding (e.g. input-specific neural circuitry modification), its contribution to network stability and its influence to neuronal coding capacity through meta-plasticity nonetheless could significantly alter the cognitive function of an organism. Understanding the interplay between these different processes will provide significant further insight into the circuitry underpinning of memory formation as well as synaptic dysfunction in neurological diseases.

5.6 Conclusion

Future work is needed to understand how the RA-dependent control of synaptic strength relates to the overall properties of neural circuits and to the behavior of a whole animal, as well as how RARα controls synaptic strength as a function of RA and whether this control is important for overall nervous system function. The discovery that RA is more than a developmental molecule, but also a diffusible mediator of synaptic signaling, opens up new avenues to our understanding of synaptic communication in the brain, and fits well into the emerging concept that developmental signaling mechanisms are reused in adult organisms in multifarious ways for a variety of functions.

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