New insights into the molecular pathophysiology of fragile X syndrome and therapeutic perspectives from the animal model.

Arnau Busquets-Garcia[§], Rafael Maldonado, Andrés Ozaita*

Departament de Ciències Experimentals i de la Salut. Universitat Pompeu Fabra, 08003 Barcelona, Spain.

[§]Present address: INSERM U862 NeuroCentre Magendie, Bordeaux, France; Université de Bordeaux, Bordeaux, France.

*Corresponding author:

Andrés Ozaita, Laboratori de Neurofarmacologia, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, C/ Doctor Aiguader 88, 08003 Barcelona, Spain. Phone: +34-93-3160823; Fax: + 34-93-3160901; e-mail: andres.ozaita@upf.edu

Abstract

Fragile X syndrome is the most common monogenetic form of intellectual disability and is a leading cause of autism. This syndrome is produced by the reduced transcription of the fragile X mental retardation (*FMR1*) gene, and it is characterized by a range of symptoms heterogeneously expressed in patients such as cognitive impairment, seizure susceptibility, altered pain sensitivity and anxiety. The recent advances in the understanding of the pathophysiological mechanisms involved have opened novel potential therapeutic approaches identified in preclinical rodent models as a necessary preliminary step for the subsequent evaluation in patients. Among those possible therapeutic approaches, the modulation of the metabotropic glutamate receptor signaling or the GABA receptor signaling have focused most of the attention. New findings in the animal models open other possible therapeutic approaches such as the mammalian target of rapamycin signaling pathway or the endocannabinoid system. This review summarizes the emerging data recently obtained in preclinical models of fragile X syndrome supporting these new therapeutic perspectives.

Keywords: autism, fragile X syndrome, mGluR5, mammalian target of rapamycin (mTOR), endocannabinoid system, CB1 cannabinoid receptor, intellectual disability, anxiety, epilepsy, nociception.

Introduction

Fragile X syndrome (FXS) is the leading inherited neurological disorder causing intellectual disability and autism (Kooy et al., 2000). The causative mutation of almost all known cases of FXS is a trinucleotide CGG expansion in the promotor region of the fragile X mental retardation gene (*FMR1*) producing the loss of the fragile X mental retardation protein (FMRP) (Krueger and Bear, 2011; Penagarikano et al., 2007). FMRP is a RNA-binding protein that plays a major role in protein synthesis in neurons (Darnell et al., 2011).

The clinical symptoms of FXS are heterogeneous (Tranfaglia, 2011; Jacquemont et al., 2013). Although the most prominent neurological phenotype is intellectual disability (Fisch et al., 2002), patients with FXS often exhibit other neurodevelopmental problems, including attention deficit hyperactivity disorder and autistic-like behavior (Hagerman, 2006). Additional features of FXS include epilepsy, self-injurious behavior, sleep disorders and hypersensitivity to sensory stimulation (Chudley and Hagerman, 1987; Hagerman, 2006; Cornish et al., 2008).

Several cellular and molecular alterations have been demonstrated in FXS at the central nervous system level (Wang et al., 2012; Santoro et al., 2012). The most prominent is the alteration in the dendritic spine density and morphology (Hinton et al., 1991). This trait has also been observed in several autism spectrum disorders (Zoghbi, 2003; Spooren et al., 2012) and other neurological disorders (Penzes et al., 2011) leading to the idea that approaches that normalize this trait in FXS might also be worth tested in other brain disorders.

Animal models of the disease with preclinical implications

The most studied animal model for FXS was obtained by interrupting the murine

Fmr1 gene (Bakker, 1994) (Fmr1KO) that causes the loss of FMRP production. This animal model reproduces many aspects of the syndrome at the behavioral, cellular and molecular levels, allowing the evaluation of potential novel therapeutic approaches in the normalization of these pathological features.

Behavioral alterations observed in the animal model of FXS

The behavioral phenotype has been mainly characterized in the Fmr1KO mouse (Bakker, 1994). We will focus our attention to four of the behavioral traits better characterized: learning and memory impairment, seizure susceptibility, anxiety-like behavior and nociceptive desensitization, all sensitive to pharmacological or genetic intervention in the Fmr1KO.

Consistent with the cognitive impairment detected in FXS patients in attention-dependent tasks, the animal model shows clear deficits in the object recognition memory test (Ventura, 2004; Busquets-Garcia, 2013; Bhattacharya, 2012). Other tests demonstrate mild alterations such as those on spatial learning and reversal learning assessed in the Morris or plus-shaped water maze test (D'Hooge et al., 1997; Van Dam et al., 2000), or those on associative learning (Hayashi et al., 2007; Michalon et al., 2012; Paradee et al., 1999). These deficits have been also observed in the radial maze (Mineur et al., 2002). Interestingly, the deficits in spatial and associative learning seem to depend strongly on the strain where the Fmr1 mutation was studied (Paradee et al., 1999; Dobkin et al., 2000; Baker et al. 2010), an observation that reveals the critical role of the genetic background in certain behavioral outcomes.

Fmr1KO mice are also characterized by an anomalous reaction to sensory stimuli (Musumeci et al., 2000). FXS patients may also respond to olfactory, tactile, visual, and auditory stimuli with hyper-reactivity and convulsions (Miller et al., 1999; Berry-

Kravis et al., 2010), and the prevalence of epilepsy in FXS is larger than in the normal population (Berry-Kravis et al., 2010). In this regard, the abnormally high synchrony and hyperexcitability in Fmr1KO mice cortical networks may explain the hypersensitivity and the predisposition to seizures, since normal circuit function requires precise and efficient excitatory and inhibitory neurotransmission balance (Gonçalves et al., 2013). Indeed, hippocampal neuronal circuits in the Fmr1KO model are epileptogenic, and cortical network activity is enhanced due to enhanced group I metabotropic receptor activity in these mice, both as a consequence of the enhanced group I metabotropic glutamate receptor signaling (Chuang et al., 2005; Hays et al., 2011), and the reduced interneuron activity (Paluszkiewicz et al., 2011). The appearance of audiogenic seizures is a robust and reproducible phenotype of interest to evaluate the potential impact of therapies in FXS (Michalon et al., 2012; Busquets-Garcia et al., 2013; Dolan et al., 2013; Pacey et al., 2009). Notably, hyperexcitable networks have been observed in other autistic syndromes (Markram and Markram, 2010), and in this respect approaches that reduce circuit hyperexcitability may become common therapeutic strategies to different pathologies (Wondolowski and Dickman, 2013; Eichler and Meier, 2008).

Anxiety is another common symptom displayed by FXS patients (Bailey et al., 2008). Previous studies on anxiety-like behavior in Fmr1KO mice revealed mixed results that are not always consistent with the enhanced emotionality observed in patients. Reduced anxiety-like behaviors were reported in the Fmr1KO using different behavioral tests such as the open field, the light-dark box or the elevated plus-maze (Liu and Smith, 2009; Jung et al., 2012; Busquets-Garcia et al., 2013). Other studies did not report this alteration in anxiety measurements (Mineur et al., 2002; Thomas et al., 2011), a discrepancy that might be due to specific experimental settings of the

study, including differences in strain, age, phase of the circadian cycle or lighting conditions (Spencer et al., 2011).

Finally, self-injurious behavior is another important trait of FXS (Tranfaglia, 2011), a feature that could be related to a reduction in pain sensitivity (Symons et al., 2010). Accordingly, the Fmr1KO shows a decreased nociceptive sensitization in models of inflammatory pain (Price et al., 2007; Busquets-Garcia et al., 2013). In addition, FMRP is expressed by nociceptors and localized in pain-sensing neurons, as well as in regions implicated in nociceptive control (Price et al., 2007). However, these findings have not been directly associated to the self-injurious behavior observed in humans, since Fmr1KO mice do not demonstrate such a behavior. Nevertheless, it is reasonable that an elevated pain threshold or alterations in pain pathways could underlie the persistence of self-injurious behavior (Peebles et al., 2012). Although more studies are warrant, evidence showed that self-injurious behavior may be modulated by increasing pain sensitivity in neurodevelopmental disability patients (Symons et al., 2004), and this could be one of the therapeutic goals in FXS.

New insights into the cellular and molecular alterations

Fragile X Mental Retardation Protein acts as an inhibitor of local translation for specific mRNAs in neuronal dendritic spines (Darnell et al., 2011). The most frequent cellular phenotype associated with the loss of FMRP in rodents is an aberrant increase in the immature dendritic protrusions or filopodia (Hinton et al., 1991; Grossman et al., 2006). In agreement, alterations in neuronal spine density and morphology are *post-mortem* features of neurons in different brain areas of FXS patients (He and Portera-Cailliau, 2013). The altered dendritic spine number and morphology/functionality may underlie the hyperexcitability of Fmr1KO neuronal

circuits, which may play a relevant role in the pathophysiology of the disease (Gibson et al., 2008). Similarly, other neurological disorders, including epilepsy, tuberous sclerosis complex or Rett syndrome, show hyperexcitable neuronal circuits resulting from the unbalance between excitatory and inhibitory inputs (Eichler and Meier, 2008; Bateup et al., 2013). The structural abnormalities of synapses in Fmr1KO mice are associated to changes in functional synaptic connectivity (Pfeiffer and Huber, 2009), revealing the significance of FMRP in regulating neuronal and spine development, and the possibility of targeting this structural dysfunction pharmacologically (Dolan et al., 2013).

Synaptic transmission has been found altered in Fmr1KO mice. Long-term potentiation (LTP) and long-term depression (LTD) are phenomena widely accepted to underlie synaptic changes during learning and memory (Lynch, 2004; Massey and Bashir, 2007). Exaggerated hippocampal LTD, and specifically that dependent on metabotropic glutamate receptor (mGluR) activation (mGluR-LTD) has been reproducibly reported in Fmr1KO mice (Huber et al., 2002; Zhang et al., 2009). In this case, the lack of mRNA repression by FMRP in the Fmr1KO, increases the translation of different mRNAs necessary for LTD and enhances the internalization of AMPA receptors (Waung et al., 2008). The supposition that particular FXS symptoms could be explained by excessive protein synthesis downstream of over-active mGluR signaling led to the proposal entitled “mGluR theory of fragile X” (Bear and Huber, 2004). This was followed by the design of pharmacological approaches targeting mGluR5 (Krueger and Bear, 2011). Interestingly, the antagonists of mGluR5 CTEP was effective in the normalization of the Fmr1KO phenotype (Michalon et al., 2012; Michalon et al., 2014). Indeed, chronic CTEP administration normalized ERK and mTOR activity in Fmr1KO mice, and corrected the altered hippocampal GluR-LTD,

the seizure susceptibility, the deficits in memory and the in FXS, modifying to some extend the activity of brain regions such as the amygdala, the hypothalamus and the hippocampus (Michalon et al., 2012; Michalon et al., 2014). Interestingly, several mGlu5 inhibitors are under clinical examination in FXS: RO4917523 (Hoffmann-La Roche), STX107 (Seaside Therapeutics) and mavoglurant/AFQ056 (Novartis), although this last one has been recently called off (FRAXA, personal communication). It will be of great interest to analyze the results of these clinical trials to better design clinical tests for this and other therapeutic strategies (Gomez-Mancilla et al., 2014).

The studies in hippocampal LTP found either reduced (Hu et al, 2008; Lauterborn et al, 2007; Lee et al, 2011; Shang et al, 2009), or no major alterations in this parameter (Auerbach and Bear, 2010; Bear et al, 2004; Godfraind et al, 1996; Paradee et al, 1999), maybe due to the use of different induction protocols. The deficient LTP, specifically NMDA-dependent LTP, was related to a selective impairment of the signal transduction between the small GTP-ase Ras and the phosphoinositide 3-kinase (PI3K), that affects AMPA-type ionotropic glutamate receptor trafficking to the synapse (Hu et al., 2008; Stornetta and Zhu, 2011; Lim et al., 2014). In this regard, the modulation of the Ras/PI3K signaling, observed after combined treatment with activating compounds for serotonin 5HT2B and dopamine D1 receptors enhanced the Ras/PI3K and recovered AMPA receptor-dependent plasticity, correlating with the restoration of associative learning (Lim al., 2014). The fact that this approach used FDA-approved psychoactive drugs, and that these drugs are commonly used to treat a number of mental and psychiatric disorders (Roth et al. 2004; Beaulieu, 2012), may accelerate its testing in FXS patients.

Another well-accepted theory to understand the pathophysiology of FXS involves an

alteration of the inhibitory GABAergic signaling (D'Hulst and Kooy, 2007). Different alterations in brain regions of the Fmr1KO involving the GABAergic system include, a decreased GABA receptor signaling efficacy, a down-regulation of tonic GABA receptor-mediated inhibition and morphological defects of GABA releasing interneurons (**Figure 1**) (Levenga et al., 2010; Paluszkiewicz et al., 2011). Interestingly, a $GABA_B$ agonist, baclofen, was found effective in preventing the epileptic phenotype in Fmr1KO mice (Pacey et al., 2009), and arbaclofen, (R-baclofen) corrected synaptic abnormalities and reduced the elevated AMPA receptor internalization in Fmr1KO to control values (Henderson et al., 2012).

Overall, the glutamatergic and the GABAergic theories propose an exaggerated excitatory mGluR signaling and a decreased GABA signaling, suggesting an excitatory/inhibitory unbalance that could underlie most of FXS central traits, and motivate the most advanced therapies under clinical studies. However, in this review we want to highlight interesting alternative theories that have appeared recently in the fragile X syndrome field.

Mammalian target of rapamycin pathway as an intracellular therapeutic strategy

FXS has also been related to alterations in the intracellular signaling of the mammalian target of rapamycin (mTOR) pathway (Sharma et al., 2010). The deregulation of mTOR has also been associated with other neurological disorders characterized by presenting cognitive deficiencies (Troca-Marin et al., 2012). mTOR pathway components are present at synapses and influence synaptic plasticity through the control of local protein synthesis (Tang and Schuman, 2002). mTOR is activated in dendrites by stimulation of group I mGluRs and is required for mGluR-LTD at CA1 hippocampal synapses (Hou and Klann, 2004). Several groups have recently analyzed the protein kinase B (Akt)/mTOR pathway in Fmr1KO mice (Gross et al.,

2010; Sharma et al., 2010; Busquets-Garcia et al., 2013). Thus, increased activities of phosphoinositide 3-kinase (PI3K), Akt, and mTOR (**Figure 1**) have been detected in cortical synaptoneurosomes and hippocampal lysates from Fmr1KO mice (Sharma et al., 2010; Gross et al., 2010) and post-mortem tissues from FXS patients (Hoeffer et al., 2012). Additionally, PI3K inhibition rescues the excess translation and subsequent AMPA receptor endocytosis revealed in the Fmr1KO mice (Gross et al., 2010). In contrast, when Ras/PI3K signaling was tested during active learning, the maximal PI3K signaling was reduced in Fmr1KO mice (Hu et al., 2008; Lim et al., 2014). Indeed, as the signaling dynamic range of PI3K could be more important than the absolute signaling level for the capacity of synaptic plasticity and learning it can not be discarded that drugs stimulating the PI3K-Ras cascade could have beneficial effects in FXS (Lim et al., 2014). Although more studies are needed, the overall deregulation of PI3K/Ras signaling and aberrant mTOR-dependent protein translation seem to crucially contribute to the symptomatic manifestations in FXS.

Interestingly, genetic reduction of an mTOR pathway component, p70 ribosomal S6 kinase 1 (S6K1) (**Figure 1**), prevents the molecular, cellular and behavioral phenotypes in Fmr1KO mice (Bhattacharya et al., 2012). At the pharmacological level, the mTOR inhibitor temsirolimus (Guertin and Sabatini, 2009) also normalized in Fmr1KO mice the cognitive deficit in the object recognition test and the susceptibility to audiogenic seizures (Busquets-Garcia et al., 2013). In summary, the genetic reduction of mTOR pathway components, the direct pharmacological blockade of the mTOR pathway or the modulation of PI3K/Ras cascade can prevent a broad range of phenotypes and this could lead to find new therapeutic options for FXS.

The endocannabinoid system: a new possible therapeutic target for FXS

Several of the behavioral and biochemical responses that are modified in FXS are modulated by the endocannabinoid system (Kano et al., 2009; Fride, 2005), an important neuromodulatory system regulating synaptic plasticity (Castillo et al., 2012). Indeed, the activation of CB1 receptors, the main cannabinoid receptor in the brain, produces dose-dependent deficits in cognitive performance, analgesic effects, modifies anxiety-like behavior and affect neuronal network excitability (Fride, 2005; Kano et al., 2009). Moreover, some of the pharmacological effects of cannabinoid agonists are mediated by the activation of the Akt/mTOR signaling pathway in the brain (Puighermanal et al., 2012). CB1 receptors are abundantly expressed at presynaptic GABAergic contacts and, to a lesser extent, on glutamatergic terminals (Marsicano and Lutz, 1999), where they modulate neurotransmitter release. Cannabinoid receptors, upon activation by their endogenous ligands (endocannabinoids) that are produced "on demand" after depolarization, may reduce presynaptic neurotransmitter release through feedback inhibition (Wilson and Nicoll, 2002). Interestingly, postsynaptic activation of mGluR5 is a key physiological mechanism that promotes the synthesis of endocannabinoids in response to synaptic activity (Varma et al., 2001).

The responses mediated by endocannabinoids on GABAergic synapses in the hippocampus and dorsal striatum after mGluR5 activation are enhanced in Fmr1KO mice (Zhang and Alger, 2010; Maccarrone et al., 2010). However, other responses to endocannabinoid, such as the LTD in excitatory terminals at ventral striatum and prefrontal cortex are abolished in Fmr1KO mice (Jung et al., 2012). These findings point to the possibility that defective endocannabinoid modulation of synaptic function in different brain areas may contribute to the symptoms of FXS.

Two therapeutic strategies apparently opposed and targeting the endocannabinoid system have been tested in the Fmr1KO model. A first strategy consisted in the enhancement of the main endocannabinoid in the brain, 2-arachidonoylglycerol (2-AG) (Jung et al., 2012) using JZL184, a specific inhibitor of the 2-AG degrading enzyme monoacylglycerol lipase (Long et al., 2009). This treatment increased brain 2-AG content and normalized both the anomalous synaptic plasticity in the prefrontal cortex and ventral striatum, and the hyperlocomotion and reduced anxiety-like behavior of Fmr1KO mice (Jung et al., 2012). A second strategy, based in the blockade of CB1 receptors (either pharmacologically or genetically), normalized the cognitive deficit, the enhanced seizure susceptibility, the decreased pain sensitivity and the abnormal mTOR signaling and dendritic spine morphology in the hippocampus of Fmr1KO mice (Busquets-Garcia et al., 2013). Both studies demonstrate the involvement of the endocannabinoid system in the pathophysiology of FXS, but further investigation is required to clarify the most beneficial therapeutic venue.

Other experimental approaches to treat FXS

Additional therapeutical approaches have been tested in FXS based on the molecular pathophysiology of the disease (Hagerman et al., 2012). Among those, the use of the mood stabilizer lithium was found to improve seizure susceptibility in Fmr1KO mice (Min et al., 2009), probably through its inhibitory action on glycogen synthase kinase 3 (GSK3), found constitutively overactive in the Fmr1KO (Min et al., 2009; Franklin et al., 2014). Although its beneficial effects reversing some FXS traits, lithium exerts side effects that could prevent its therapeutic utilization. Minocycline, a tetracycline antibiotic with neuroprotective effects in neurodegenerative conditions (Domercq and

Matute, 2004) has been found to enhance the maturation of hippocampal dendritic spines and to improve anxiety-like and exploratory behaviors in Fmr1KO mice (Bilousova et al., 2009). Memantine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist widely prescribed for treatment of Alzheimer's disease (Rogawski and Wenk, 2003) that inhibits the Ca^{2+} influx. Experimental evidence showed that memantine could restore dendritic spines to normal values in the Fmr1KO neurons (Wei et al., 2012). Another promising compound is acamprosate, a mixed agonist/antagonist at NMDA receptors and activator of GABA-A receptors with possible inhibitory effects at group I mGluRs (Mann et al., 2008). In humans, acamprosate was well tolerated and improved social behavior, reducing the inattention/hyperactivity in FXS patients (Erickson et al., 2013).

3- Conclusion

At the present time, the treatment of patients with FXS is only symptomatic. These symptomatic treatments include psychostimulants that ameliorate attention deficit and hyperactivity, and selective serotonin reuptake inhibitors that reduce aggression and anxiety-like responses. New strategies for therapeutic intervention have been recently proposed based on the basic research presented above. These new findings reinforce the potential therapeutic value of drugs that modulate mGluR5, $GABA_B$, mTOR or CB1 receptor function. The ongoing clinical trials targeting mGluR5 and $GABA_B$ receptors, and the possibility to initiate novel trials with mTOR or CB1 receptor modulators will soon validate the translational power of the animal models of FXS to predict the most suitable treatments in humans. Importantly, all these promising approaches are able normalize cognitive deficits together with other phenotypes of

FXS in the preclinical models and, if confirmed in humans, they could improve enormously the quality of life of FXS patients compared to the actual symptomatic treatments.

Figure legend

Figure 1. Schematic diagram showing the main molecular alterations described in FXS and the possible therapeutic site of action of the principal therapeutic approaches currently under study.

Right panel: The physiological situation in control conditions is represented. FMRP regulates the translation of several transcripts important for synaptic plasticity (Darnell et al., 2011) among those MMP-9 (Janusz et al., 2013), contributing to the normal behavioral output. The activity of the mTOR pathway is modulated by the balance between the excitatory and inhibitory drives in principal excitatory neurons. Presynaptic $GABA_B$ receptors and CB1 receptors modulate neurotransmitter release. CB1 receptors are more abundant in GABAergic terminals than in glutamatergic terminals.

Left panel: In the fragile X syndrome condition, the uncontrolled over-activity of mGluR5 (Bear et al., 2004) and the reduced GABAergic transmission (Levenga et al., 2010) may shift the excitatory/inhibitory input in principal neurons leading to the uncontrolled over-activation of the mTOR pathway and the phosphorylation of different upstream or downstream targets such as phosho-Akt or phosho-p70S6K, respectively (Sharma et al., 2010; Busquets-Garcia et al., 2013). The Ras/PI3K/Akt has been found impaired during active learning in Fmr1KO (Lim et al., 2014). Serotonin and dopamine receptor-acting compounds manage to re-establish the Ras/PI3K signaling recovering normal synaptic plasticity and memory performance (Lim et al., 2014). Under FXS conditions, the activation of protein translation machinery is favored, leading to aberrant enhanced protein synthesis and synaptic plasticity, enhanced internalization of AMPA receptors, changes in the structure of

spines to immature appearance, decreased phosphorylation of GSK3 to enhance its kinase activity, and enhanced activity of MMP-9. Treatments acting on mGluR5 or the $GABA_B$ receptor agonist, CTEP and arbaclofen, respectively (Michalon et al., 2012; Henderson et al., 2012) were effective in normalizing specific Fmr1KO phenotypes. The pharmacological reduction of actin polymerization by FRAX486, a p21-activated kinase inhibitor, was also valuable (Dolan et al., 2013). The genetic or pharmacological attenuation of CB1 receptor signaling, and the inhibition of mTOR activity also improved the cognitive performance (Busquets-Garcia et al., 2013). Lithium and other GSK3 inhibitors were found effective in reducing GSK3 activity (Min et al., 2009; Franklin et al., 2014), while minocycline may decrease MMP-9 activity in the FXS condition (Bilousova et al., 2009)

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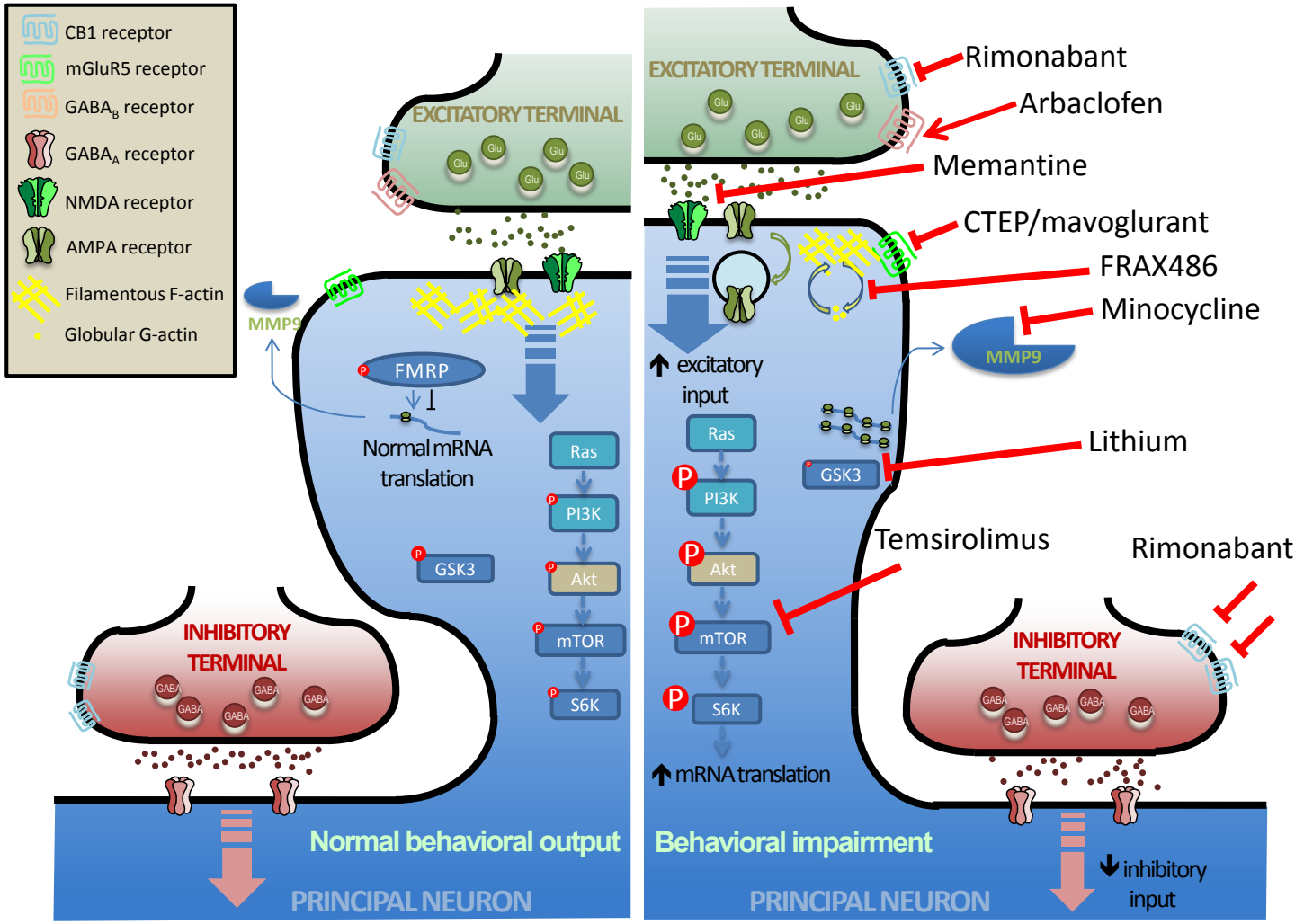

BC-D-14-00121R1- Busquets-Garcia et al., Figure 1