ROLE OF VOLTAGE SENSITIVE CALCIUM CHANNELS (VSCCs) IN THE MATURATION OF THE GABAERGIC SYSTEM IN THE FRAGILE X SYNDROME

by

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Abstract

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$GABA_A$ receptors are considered to be major inhibitory receptors in the brain. A significant down-regulation of the $GABA_A$ receptors in *Fmr1* knockout (KO) mice has been demonstrated in recent studies, which may underlie the mechanism for the anxiety and hyperactivity found in fragile X syndrome patients. However, during early developmental stages, activation of $GABA_A$ receptors exerts excitatory effects on neuronal networks. The excitatory effect of $GABA_A$ receptors early on sets the major tune in generating giant depolarizing potentials (GDPs), recurrent synchronized spontaneous network discharges which are features of neuronal activity of developing neurons. GDPs control extension and motility of neurites as well as synthesis and expression of the GABAergic phenotype through the activation of voltage sensitive calcium channels (VSCCs) during early developmental stages up to P14 in mice and rats. The developmental excitatory to inhibitory switch in the $GABA_A$ receptors function is mainly dependent upon the expression level of the cotransporters KCC2. In this study, we examined the expression level of $GABA_A$ receptors and VSCCs in the brain of FVB/NJ wild type (WT) and

Fmr1 knockout (KO) mice, and attempted to determine whether modulation of VSCC could affect the maturation of GABAergic system in the KO mice. First, we found reduced expression of $GABA_A$ receptors and VSCCs in KOs, and a shift in the time course of the excitatory to inhibitory functional switch of $GABA_A$ receptors in KOs. In addition, we modulated VSCCs activity in cerebellar granule cell culture with KCl and nifedipine to verify whether VSCCs can normalize reduced expression of $GABA_A$ receptors in KO mice. Large-scale nuclear translocation and exportation of the VSCC β3 subunit were detected, suggesting VSCC could play a regulatory role in gene expression. Finally, we pharmacologically manipulated VSCCs during embryogenesis by injecting pregnant mice with Bay K 8644 (a VSCC activator) and performed behavioral analyses on the offspring. Injection of VSCC activator rescued part of the KO behavior phenotype. In conclusion, the VSCCs in KO mice brain may be responsible for the activity-dependent, calcium-mediated gene expression resulting in the altered expression of $GABA_A$ receptors. Activating VSCCs during early development could partially normalize KO behavior.

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ABBREVIATIONS

ANOVA	analysis of variance between groups
BDNF	brain-drived neurotrophic factor
CCAT	calcium channel associated transcriptional regulator
CHCB2/HP1γ	chromobox protein 2/heterochromatin protein 1 γ
CREB	cAMP response element-binding protein
DIV	days *in vitro*
DRE	downstream regulatory element
DREAM	downstream regulatory element antagonist modulator
EPM	elevated plus maze
Fmr1	fragile X mental retardation 1
FMRP	fragile X mental retardation protein
FXS	fragile X syndrome
$GABA_A$ β	$GABA_A$ receptor β subunit
GABARAP	$GABA_A$ receptor associated protein
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GDPs	generating giant depolarizing potentials
HP1	heterochromatin protein 1
HVA	high voltage activated
IPSCs	inhibitory postsynaptic currents

KO	*Fmr1* knockout
KCC2	K^+-Cl^--coupled co-transporter
LVA	low voltage activated
mIPSCs	miniature inhibitory postsynaptic currents
NFDM	nonfat dry milk
NGS	normal goat serum
Nif	nifedipine
NLSs	nuclear localization signals
NMDA	*N*-Methyl-D-aspartate
OF	open field
PPI	pre-pulse inhibition
PBS	phosphate-buffered saline
RE	reticular thalamic nucleus
SSADH	succinate semialdehyde dehydrogenase
sIPSC	spontaneous inhibitory postsynaptic currents
VSCCs	voltage sensitive calcium channels

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INTRODUCTION

Fragile X syndrome and reduced GABAergic system in fragile X models

Fragile X syndrome (FXS) is the most common form of inherited mental retardation, with approximately 1 in 4,000 males affected. It is an X-linked disorder with remarkable inheritance pattern, in which certain alleles are passed from intellectually normal men to their affected grandsons through their unaffected daughters. The fragile X allele which is passed from generation to generation contains an over expansion of a CGG repeat in the 5'-untranslated region of *FMR1* gene (Loesch et al., 2007; Garber et al., 2008), the causative gene of fragile X syndrome, which encodes the fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein involved in translational regulation of target mRNAs. In FXS, *FMR1* gene is transcriptionally silenced, leading to the absence of FMRP.

Clinical diagnosis of FXS is usually based on the detection of the 200 or more copies of the CGG trinucleotide repeat. The physical hallmarks of FXS are relatively nonspecific because individuals with FXS may have almost anything from learning problems and a normal IQ to severe cognitive impairment. In addition, autistic-like features are common in individuals with FXS with prevalence between 18% and 33% and approximately 90% of male children with FXS show one or more features of autism (Belmonte et al., 2006; Brodkin, 2008). In general, anxiety, hyperactivity, depression and increased sensitivity to epileptic seizures are core features of individuals with FXS (Chonchaiya et al.).

The $GABA_A$ receptor is considered to be a major inhibitory receptor in the brain. Activation of $GABA_A$ receptors allow influx of Cl^- into GABAergic neurons, which hyperpolarize the

neuronal membrane and lead to inhibitory effects. The inhibitory effects of $GABA_A$ receptors may underlie the mechanism for the anxiety and hyperactivity common in FXS phenotypes.

The GABAergic system, particularly the $GABA_A$ receptor-related expression and function, is altered in FXS. In fragile X animal models, expression of $GABA_A$ receptors are reduced at the transcription and protein level. In *Fmr1* (*FMR1* gene in mouse) knockout (KO) mice, real-time PCR reveals significant decreased expression of $GABA_A$ receptor subunits including α_1, α_3, α_4 and α_5, β_1 and β_2, and γ_1 and γ_2, and δ (D'Hulst et al., 2006; Gantois et al., 2006; Curia et al., 2009). This seems to be an evolutionally conserved feature of fragile X syndrome. In fragile X deficient *Drosophila*, all three subunits that make up invertebrate $GABA_A$ receptors are under-expressed (D'Hulst et al., 2006). Under-expression of $GABA_A$ receptor subunits is further confirmed at protein level. Our lab has shown that expression of the β subunit is reduced in fragile X mice in cortex, hippocampus, diencephalon and brainstem (El Idrissi et al., 2005). Another study confirmed that α_5 and δ subunits are reduced in subiculum in KO mice (Curia et al., 2009).

Alteration of various additional elements of the GABA signaling system has been found in the brain of fragile X mice (D'Hulst et al., 2009). This includes the GABA transporter (GAT), succinate semialdehyde dehydrogenase (SSADH, an enzyme important in the degradation of GABA), and gephyrin (a protein involved in the clustering and targeting of $GABA_A$ receptors to the postsynaptic membrane). Under-expression of the GABAergic system seems to be site-specific. Decreased expression of several $GABA_A$ receptor subunits and gephyrin is observed only in the cortex (D'Hulst et al., 2006; Gantois et al., 2006), while mRNAs encoding proteins involved in transport, synthesis and degradation of GABA are under-expressed in both cortex and cerebellum (D'Hulst et al., 2009). The $GABA_A$ δ subunit is more than 2-fold under-

expression in hippocampus and neocortex but not cerebellum, caudate putamen, and thalamus of KO mice (Gantois et al., 2006).

Loss of subunit expression also leads to loss of functionality in the FXS. Electrophysiological recordings suggest a decreased GABAergic system efficiency in the hippocampus of the fragile X mouse (D'Antuono et al., 2003). This is consistent with down-regulation of $GABA_A$ expression (El Idrissi et al., 2005; D'Hulst et al., 2006; Gantois et al., 2006) and a decreased ratio between inhibitory and excitatory amino acids in fragile X mouse hippocampus. Tonic $GABA_A$ currents are down-regulated in KO subiculum, whereas no significant differences were observed in phasic $GABA_A$ currents (Curia et al., 2009). This may be accounted for by the under-expression of tonic $GABA_A$ receptor subunits $\alpha 5$ and δ in KO mouse subiculum (Curia et al., 2009). However, the extent of the reduction of these 2 subunits (~50% at protein level) cannot fully explain the degree of functional impairment (~91%) of the tonic $GABA_A$ receptor in subicular neurons in KO mice. This discrepancy between protein level and functional reductions could be due, at least in part, to alterations in the trafficking of the receptors to the membrane surface, as suggested for the glutamatergic receptors in fragile X model (Kooy 2003).

Contrary to the findings of reduced GABAergic function, enhanced GABAergic inhibition are found in striatum of the fragile X mice as revealed by increased frequency of the spontaneous inhibitory postsynaptic currents (sIPSC) and miniature inhibitory postsynaptic currents (mIPSCs) (Centonze et al., 2008). One possible explanation for the discrepancy between these results is that the down-regulation of $GABA_A$ receptors and of GABAergic synapses reflects an adaptive mechanism to counter the abnormal GABA release in fragile X mice. Together with evidence of site-specific under-expression of $GABA_A$ receptor subunits, these findings underscore the regional specificity effect of fragile X on the $GABA_A$ system.

Anchoring $GABA_A$ receptor to the postsynaptic membrane

The efficiency of GABAergic transmission is attributed to the release of the presynaptic neural transmitter GABA, the number of postsynaptic receptors and the mechanism for anchoring the receptors to correct locations on the plasma membrane.

GABARAP ($GABA_A$ receptor associated protein), a microtubule associated protein that bridges the microtubules with the large intracellular loops of the $GABA_A$ receptor subunits, is an important scaffolding element (Lüscher & Keller, 2004). Functional loss of GABARAP was reported to result in no $GABA_A$ receptor clusters in Japanese quail QT-6 fibroblasts (Chen et al. 2000). Gephyrin, a 93-kDa protein highly concentrated in the subsynaptic compartment of both glycinergic and GABAergic inhibitory synapses, is considered as the master scaffolding molecule of inhibitory synapses (Fritschy et al., 2008). Down-regulation of gephyrin expression by gene targeting or gene silencing leads to rapid disappearance of postsynaptic $GABA_A$ receptor clustering and loss of inhibitory postsynaptic currents (IPSCs) (Essrich et al., 1998; Kneussel et al., 1999; Kneussel et al., 2001; Jacob et al., 2005; Yu et al., 2007), indicating that gephyrin is required for normal postsynaptic clustering of $GABA_A$ receptors.

Postsynaptic clustering of gephyrin is disrupted in the absence of $GABA_A$ receptors. Targeted deletion of γ_2, α_1, or α_3 subunit genes resulted in a dramatic loss of postsynaptic gephyrin which parallels the loss of postsynaptic $GABA_A$ receptors (Essrich et al., 1998; Kralic et al., 2006; Studer et al., 2006). Gephyrin clusters are also lost when the γ_2 subunit gene is inactivated in mature neurons by conditional knockout (Schweizer et al., 2003). These observations suggest that gephyrin clustering is a regulated process, probably dependent on local synaptic activity. In this respect, it is interesting to note that $GABA_A$ receptors forms clusters in immature neurons

before being detectably colocalized with gephyrin. Gephyrin appears to contribute more to stabilize previously clustered $GABA_A$ receptors at inhibitory synapses (Lüscher & Keller, 2004).

It is not clear how the expression of the $GABA_A$ receptor is decreased in fragile X models. FMRP is an RNA-binding protein with particularly high expression in neurons and gonads. It plays an important role in transportation and translation of mRNA. A direct binding between FMRP and the mRNA of the δ subunit of the $GABA_A$ receptor has been demonstrated (Miyashiro et al., 2003). FMRP also controls mRNA stability (Zalfa et al., 2007). It could be postulated that in the absence of FMRP, RNAs normally bound to FMRP are misregulated and /or degraded, and $GABA_A$ receptor is directly under the control of the interaction of receptor subunit mRNAs with FMRP, or indirectly regulated through the alteration on the level of receptor-anchoring proteins, or the altered $GABA_A$ receptor or receptor anchoring proteins are regulated through alternative pathways beginning at early developmental stages.

Excitatory $GABA_A$ receptor-mediated effects during early developmental stages

The inhibitory effects of $GABA_A$ receptor activation are extensive in the brain. However during very early developmental stages, activation of $GABA_A$ receptors exerts excitatory effects on the neuronal networks. At very early developmental stages, the intracellular Cl^- concentration is higher than the extracellular Cl^- concentration in immature neurons, and activation of $GABA_A$ receptors leads to an outward Cl^- current which plays a depolarizing role on the neuronal membranes (Fig.1). The excitatory effects of $GABA_A$ receptors early on depolarize the membrane, activate voltage sensitive calcium channels (VSCCs) which is the only active route allowing calcium influx into the immature neurons at that stage, and set the major tune in generating giant depolarizing potentials (GDPs). GDPs are recurrent synchronized spontaneous

network discharges which is a feature of neuronal activity of developing neurons, and is critical in the establishment of neuronal networks (Ben-Ari et al., 2004). GDPs dominate ongoing activities at very early developmental stages, ensure large calcium oscillations through channels such as VSCCs, Early activation of VSCCs mediated by excitatory GABAergic-mediated effects induces increase of intracellular calcium concentration during early developmental stages up to P14, and subsequently activates a series of downstream calcium sensitive signaling cascades which are important for regulating DNA synthesis and therefore neuronal proliferation, migration and differentiation. Early activation of VSCCs triggers the formation of glutamatergic synapses during later developmental stages as well (Ben-Ari et al., 2001, 2002, 2004, 2007; Galanopoulou, 2008). GDPs also modulate the expression of the GABAergic phenotype through the activation VSCCs, as well as control extension and motility of neurites. GDPs disappear progressively and concomitantly with the excitatory to inhibitory switch of the function of $GABA_A$ receptors activation (Ben-Ari, 2001, 2002).

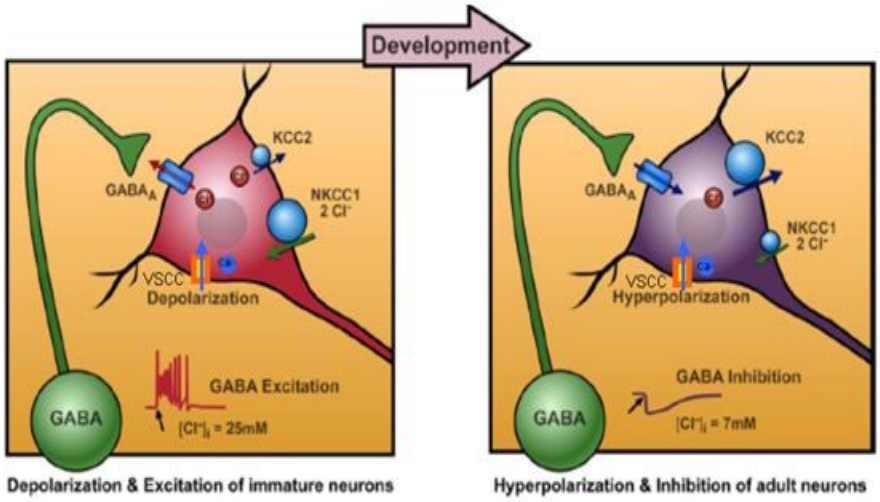


Figure 1. Excitatory to inhibitory switch of $GABA_A$ receptor-mediated effects during early developmental stages. The switch is mainly due to increased expression of K^+-Cl^--coupled co-transporter (KCC2) which extrudes Cl^- from GABAergic neurons (Adapted from Ben-Ari et al., 2007)

The excitatory to inhibitory switch of $GABA_A$ receptor-mediated responses results mainly from the drastically increased expression of K^+-Cl^--coupled co-transporter (KCC2) (Rivera et al., 1999). KCC2 extrudes Cl^- from GABAergic neurons, maintaining low intracellular chloride concentrations in relatively developed neurons, which contributes to GABAergic inhibitory effects by decreasing intracellular Cl^- concentrations and establishing an inward, not outward Cl^- current (Ben-Ari, 2007) (Fig. 1).

Voltage sensitive calcium channels

Calcium acts both as a charge carrier and a second messenger, and plays pervasive roles within neurons, directly or indirectly regulating almost all processes within the brain (Augustine et al., 2003). Many physiological processes, such as neurotransmitter release, neuronal excitability and plasticity and gene transcriptional regulation (Cao et al., 2006), are related to Ca^{2+} signaling events occurring at various cellular sites within neurons (Miller et al., 2001). Ca^{2+} influx through glutamate receptors as well as VSCCs provide the major sources of Ca^{2+} signals in neurons (Bloodgood et al., 2007).

The 190-250 kDa pore-forming subunit of VSCCs α1 (Marcantoni et al., 2008; Halling et al., 2005) possesses the main channel machinery including an ion-conducting pore, ion selectivity, and voltage sensitivity (Fig. 2A). It constitutes four repeating domains, each with six hydrophobic transmembrane helical regions and one P loop which helps to form the narrowest region of the wall of the pore. The cytoplasmic linker regions of the α1 subunit contain phosphorylation and other regulatory sites and the intracellular carboxyl (C)- terminal region contains a binding site for calmodulin (CaM) which mediates various Ca^{2+}- triggered reactions (Benarroch et al., 2010) (Fig. 2B). Ten subtypes of α1 subunit (α1A, α1B, α1C, α1D, α1E, α1F,

α1G, α1H, α1I, α1S) are encoded individually by 10 genes in the CACNA1 gene family (Hayashi et al., 2007). The amino acid sequences of these various α1 subunits have been aligned for protein homology study, which grouped VSCCs into 3 categories: Ca_v1, Ca_v2 and Ca_v3. Ca_v3 family is less closely related to the other two families (Kisilevsky et al., 2008) and includes 3 different T-type channel isoforms ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$) that are activated in response to relatively small membrane depolarization. Ca_v1 and Ca_v2 families are HVA channels activated by a current which is much more positive than the resting membrane potential (Lipscombe et al., 2004; Vacher et al., 2008).

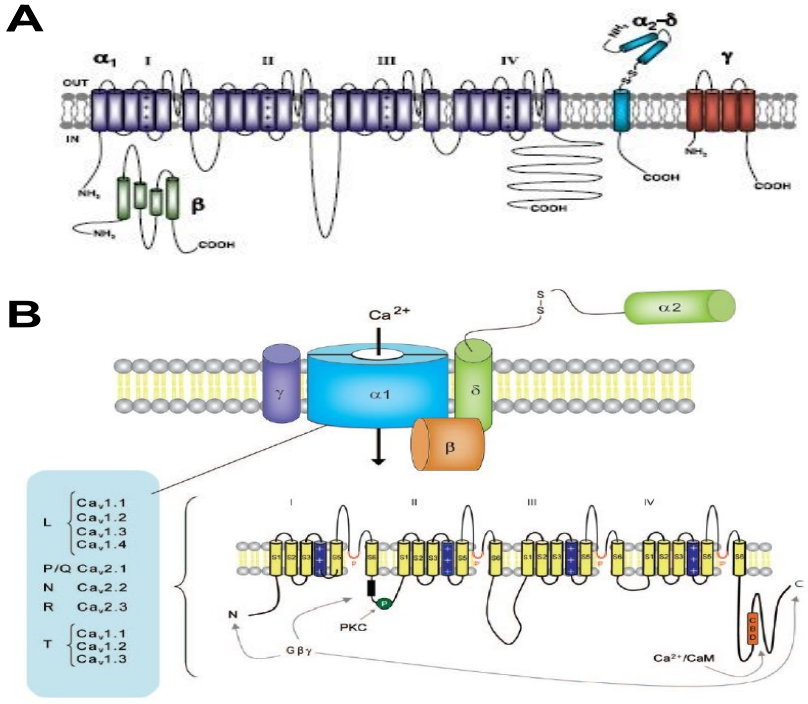


Figure 2. Subunit composition of VSCCs. (A) Schematic illustration of a typical HVA VSCC with the pore forming α1 subunit and the auxiliary β, $\alpha^2\delta$ and γ^2 subunits (From (Vacher et al., 2008). (B) Three dimensional demonstration of VSCC subunits (upper diagram) and detailed constitution of the α1 subunit with transmembrane domains (From Benarroch. 2010).

All four channel isoforms in the Ca_v1 family ($Ca_v1.1$~ $Ca_v1.4$) are L-type channels, while the Ca_v2 family consists of P/Q-type ($Ca_v2.1$), N-type ($Ca_v2.2$) and R-type ($Ca_v2.3$) channels. In addition to the pore hyphen forming subunitα1, HVA channels consist of 3 more auxiliary subunits: β, $\alpha^2\delta$ and γ^2 (Fig. 2). Each of the subunits is composed of several distinct isoforms that are produced through mechanisms such as alterative spicing. They provide various functions such as regulation of the rate of channel inactivation, the voltage-dependent gating of the channel and modulation of channel biophysical properties (Benarroch. 2010; Helton et al., 2002; Arikkath et al., 2003; Black et al., 2003; Dolphin et al., 2003). Each VSCC type has distinct physiological and pharmacological characteristics.

Distribution of VSCCs in neurons

Except for Ca_v1 .1 channels which are exclusively expressed in muscles, all the other VSCC isoforms are detected in the brain (Tab. 1). Ca_v1 channels (L-type VSCCs), especially the Ca_v1 .2 and Ca_v1 .3 are distributed predominantly in the soma and distal dendrites (Leitch et al., 2009).

Ca_v2 channels (P/Q-, N- and R- type VSCCs) are clustered at the presynaptic terminals, but also in soma and dendrites. Immunodetection of P/Q-, N- and R- type calcium channels demonstrated the distribution of these channels in both proximal and distal dendrites of rat globus pallidus (GP) neurons as well as in presynaptic terminals (Hanson et al., 2002). The $Ca_v2.2$ calcium channel (N-type VSCC) in the brain and spinal cord of mouse is prominently located in nuclei and it translocates during and after pilocarpine-induced status epilepticus (Xu et al., 2010).

Ca_v3 channels (T-type VSCCs) are located in the soma and dendrites. Different isoforms of Ca_v3 channels are present in a compartmentalized manner in the neurons. Immunocytochemistry and western blotting of Ca_v3 channels in rat brain showed that the $Ca_v3.1$ channels are present mainly in the soma or proximal dendritic region, $Ca_v3.2$ channels in the soma and proximal-mid dendrites, and $Ca_v3.3$ channels are in the soma and over-extended lengths of the dendritic arbor in CA1 pyramidal cells and in subicular pyramidal cells. (McKay et al., 2006) $Ca_v3.1$ channels in cat reticular thalamic nucleus (RE) bear a similar distribution pattern as in the rat brain, but Cav3.3 channels are on cell bodies.

Table 1. VSCCs in brain

	Channel	Subtypes in Brain	Neuronal Distribution	Physiological Function
HVA	Ca_v1 (L-type)	$Ca_v1.2$	soma and dendrites	gene expression, pacemaker activity, sensory transduction
	$Ca_v1.1$~ $Ca_v1.4$	$Ca_v1.3$		
		$Ca_v1.4$		
	Ca_v2	$Ca_v2.1$ (P/Q type)	presynaptic terminals	transmitter release, neuronal excitability, gene expression
	$Ca_v2.1$~ $Ca_v2.3$	$Ca_v2.2$ (N type)		
		$Ca_v2.3$ (R type)		
LVA	Ca_v3 (T-type)	$Ca_v3.1$	soma and dendrites	pacemaker activity, rhythmic burst firing, oscillatory behavior, hormone secretion, smooth muscle contraction
	$Ca_v3.1$~ $Ca_v3.3$	$Ca_v3.2$		
		$Ca_v3.3$		

Physiological effects and gene expression regulation by Ca^{2+} signaling through VSCCs in neurons

Local Ca^{2+} signaling starts from the passive entry of Ca^{2+} through Ca^{2+} permeable membrane channels, and associates with the subsequent diffusion of Ca^{2+} into the cytoplasm which then targets Ca^{2+} signals to their intracellular Ca^{2+} sensors (Ca^{2+} binding proteins), and in turn activates Ca^{2+} effectors (Ca^{2+} activated enzymes) resulting in various physiological processes. Because of the spatial proximity of Ca^{2+} signals and Ca^{2+} sensors, Ca^{2+} signals can be compartmentalized to segments of the neurons. To regulate different neuronal activities, the speed and magnitude of Ca^{2+} signaling is inversely related to the distance between Ca^{2+} signal and Ca^{2+} sensor (Augustine et al., 2003).

Numerous physiological effects are triggered by Ca^{2+} signaling through VSCCs. Knockout and natural mutant studies suggest many VSCCs act as pharmacologic targets for absence epilepsy, cerebellar ataxia and naturopathic pain treatments (Benarroch et al., 2010). Ca_v1 channels are associated with Parkinson disease, Alzheimer's disease and other neurological disorders (Willis et al., 2010). Channel activity can be blocked by dihydropyridine calcium channel blockers such as efonidipine and nilvadipine and by non-selective mibefradil (Mishra et al., 1994; Bezprozvanny and Tsien, 1995; Masumiya et al., 1998; Ishibashi et al., 1998; Furukawa et al., 2005).

Studies on effects of VSCCs and the underlying mechanisms of VSCC-related neurological disorders indicate that VSCCs are critical in mediating rhythmic burst firing and pacemaker activity (Benarroch. 2010), regulating neurotransmitter release, cell differentiation, synaptic plasticity, modulating excitatory activity-dependent gene expression and other aspects of nervous system physiology(Table 1).

Ca_v3 channels are recognized as primary pacemaker activity candidates that contribute to neuron excitability regulation. Recording of the membrane current of an NG108-15 cell line infected with pBK-CMV plasmid constructs encoding for the $Ca_v3.3$ α subunit showed $Ca_v3.3$-expressing cells trigger repetitive action potentials that generate spontaneous membrane potential oscillations (MPOs) (Chevalier et al., 2006).

Ca_v2 channels mediate transient action potential-evoked transmitter release. N-type channels are more frequently linked to inhibitory transmission while P/Q type channels contribute more to excitatory transmission (Potier et al., 1993). Ca_v1 channels (L-type VSCCs) are effective in regulating synaptic plasticity through Ca^{2+} signaling pathways to affect learning and memory. A fundamental alteration of the mechanism of Hebbian plasticity through the synapse homeostatic change is due to the genetic loss of $Ca_v1.2$, which plays important roles in mouse fear memory acquisition (Langwieser et al., 2010).

DNA is the repository for genetic information and gene transcription and expression, which are the basis for all physiological alterations. The activity-dependent changes in gene expression are usually triggered by activity-induced increase of cytoplasmic Ca^{2+} concentration. High throughput gene chip analysis reveals a list of 248 genes that are potentially affected by Ca^{2+} signals. Among 27,000 rat gene transcripts, expression of 117 genes were blocked in response to the L-type channel antagonist nifidipine, indicating a critical role of Ca^{2+} influx through VSCCs in gene transcription (Xiang et al., 2007). L-type channels are considered to play a major role in triggering changes in gene expression (Gallin and Greenberg, 1995). Ca^{2+} influx through L-type channels induces a rapid and sustained increase in BDNF mRNA, which plays an important role on neuronal survival (Ghosh et al., 1994). L-type channels influence gene transcription through

various ways, including associating the channel-mediated Ca^{2+} entry with local Ca^{+} sensor proteins, which initiate activation or inactivation of distinct target transcription factors, or by associating with the transcription factor itself, or by acting as a transcription factor. The L-type channe induces significant Ca^{+} passage and is able to activate CaM (Ca^{+}/Calmodulin) and CaMK (Ca^{+}/Calmodulin-dependent protein kinase) within seconds, which reliably phosphorylate Ser133 or Ser142 of the transcription factor CREB (a transcription factor regulating a wide range of gene transcription) (Jensen et al., 1991; Srinivasan et al., 1994). The Ca^{+} binding form of DREAM (downstream regulatory element antagonist modulator) protein was found to dissociate from the downstream regulatory element (DRE), which activates DRE-regulated genes such as the prodynorphin gene by relieving the trascriptional repression of DREAM to DRE-containing promoters (Lusin et al., 2008). Even the fragments of L-type channels possess the ability to affect gene expression by acting directly as transcription factors. A domain of the C-terminus of the α_1 subunit of L-type channels (calcium channel associated transcriptional regulator, CCAT) was shown to up- or down-regulate several genes including a gap junction protein coding gene essential for neuron extension (Gomez-Ospina et al., 2006). The β_4 subunits of VSCCs dissociated from the channel also translocate to the nucleus, which then induces hypen or down-regulation of additional genes (Escayg et al., 2000). Non-L-type channels are also involved in excitation-transcription coupling. Tyrosine hydroxylase mRNA expression is induced by specific activation of N-type channels in primary sensory neurons (Brosenitsch and Katz, 2001). Expression of syntaxin 1A mRNA which is crucial in neurotransmitter release and is reduced when P/Q type channels are blocked in cerebellar granule cells (Sutton et al., 1999).

Association of VSCCs with GABAergic system

Elevation of intracellular Ca^{2+} or activation of Ca^{2+}/Calmodulin-dependent protein kinase or phosphatases has been shown to influence $GABA_A$ receptor mediated Cl^- currents (De Koninck & Mody, 1996; Smart, 1997; Poisbeau et al., 1999). Blockade of L-type VSCCs is related to the regulation of the tolerance of $GABA_A$ receptor synaptic function to chronic benzodiazepine treatment in rat hippocampal slice preparations (Xiang & Tietz, 2008). Attenuation of Ca^{2+}–induced GABA synaptic potentiation followed by inhibition of tubulin polymerization indicates that GABA synaptic potentiation might be affected by Ca^{2+} signaling cascades through the contribution of microtubule-mediated processes (Wei et al., 2004). Traced by two photon excitation fluorescence microscopy, there is no significant changes in GABA-elicited Cl^- currents following VSCC activation and Ca^{2+} influx driven through VSCCs is short-lived (Cupello et al., 2005). Calcium is broadly regarded as a second messenger. Even though the signal itself does not last long, its effect could be profound through a series of downstream pathways. The interaction of VSCCs with $GABA_A$ receptor could be mediated by calcium-dependent processes.

Hypothalamic neurons from postnatal day 0-14 (p0-14) rats and HEK293 cells expressing cloned $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ $GABA_A$ receptor were tested for GABA-activated Cl^- currents by patch clamp. It was found that nitrendipine (an L-type channel blocker) inhibited GABA-gated currents. The inhibitory actions were fully reversed after removal of the drug. Based on the hypothesis that Ca^{2+} channel blockers act directly on the $GABA_A$ receptor by associating with it, several pharmacological binding sites on $GABA_A$ receptor, such as benzodiazepine site, picrotoxin site and Zn^{2+} site were examined for their interaction with nitrendipine (Das et al., 2004). However, none of the selected sites was shown to be the action site of nitrendipine, which

provides the possibility that Ca^{2+} influx through calcium channel acts on GABAergic activity through the function of VSCCs instead of interacting directly with $GABA_A$ receptors.

In addition to disrupted $GABA_A$ receptor-mediated function in response to the blockage of VSCC activities, the expression level of $GABA_A$ receptors are altered as well. Application of the VSCC blocker nifedipine or nimodipine to rat hippocampal neuron cultures, causes a 40% reduction in the expression of cell surface $GABA_A$ receptor. Nifedipine specifically down regulates the number of $GABA_A$ β_3 subunit. The insertion of $GABA_A$ receptor into the membrane was significantly reduced by application of nifedipine and recovery of the $GABA_A$ receptor trafficking largely occurred with the addition of the proteosome inhibitor MG132, indicating that the anchoring of $GABA_A$ receptor into the plasma membrane might be affected by the function of VSCCs, especially L-type VSCCs (Saliba et al., 2009).

All together, the function, expression and trafficking of $GABA_A$ receptors are altered in response to altered VSCC activites. Based on the fact that there is no active VSCC blocker binding site on $GABA_A$ receptors and VSCCs play critical role in gene expression regulating the establishment of neuronal circuits during early developmental stages, we think VSCCs may be responsible to regulate the GABAergic system through gene expression, expecially through the regulation on scaffolding proteins which anchor $GABA_A$ receptors on the membrane.

RESULTS

Expression of VSCCs and $GABA_A$ receptor in KO mice

The fragile X mental retardation syndrome results from the transcriptional silencing of the fragile X gene, *Fmr1*, and to the ensuing loss of the *Fmr1* gene product, FMRP. Our lab has found that expression of the $GABA_A$ receptor β subunit ($GABA_A$ β), which is required for receptor function, is reduced in the cortex, hippocampus, diencephalon and brainstem in adult male fragile X mice (El Idrissi et al., 2005). Concomitantly, expression of glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis, was increased in the same regions that showed $GABA_A$ β reduction, indicating a compensation for the reduction of $GABA_A$ β.

Here we checked whether there is any alteration in the expression level of $GABA_A$ receptor in the brains of our 2-month-old FVB/NJ age matched WT and KO lab mice. In an attempt to check whether there is any interaction between $GABA_A$ receptor and VSCCs, we also checked the expression level of VSCCs in the brains of all tested mice.

With immunofluorescence labeling of the $GABA_A$ receptor β subunit, we confirmed our previous finding of the reduction in $GABA_A$ receptor expression in the hippocampus of the KO mice. As shown in Fig. 3, expression of the $GABA_A$ receptor is reduced in the CA3 region in KOs. Based on the fluorescence intensity of the labeling, $GABA_A$ receptor expression was significantly reduced in KOs. We also compared the expression level of VSCCs in brains of WTs and KOs using an antibody to the VSCC β subunit. A significant reduction of VSCC expression was found in the same region of the hippocampus of KO mice compared to WTs (Fig. 3B). This is similar to our previous finding of reduced VSCC expression in the pancreas of the KOs (El-

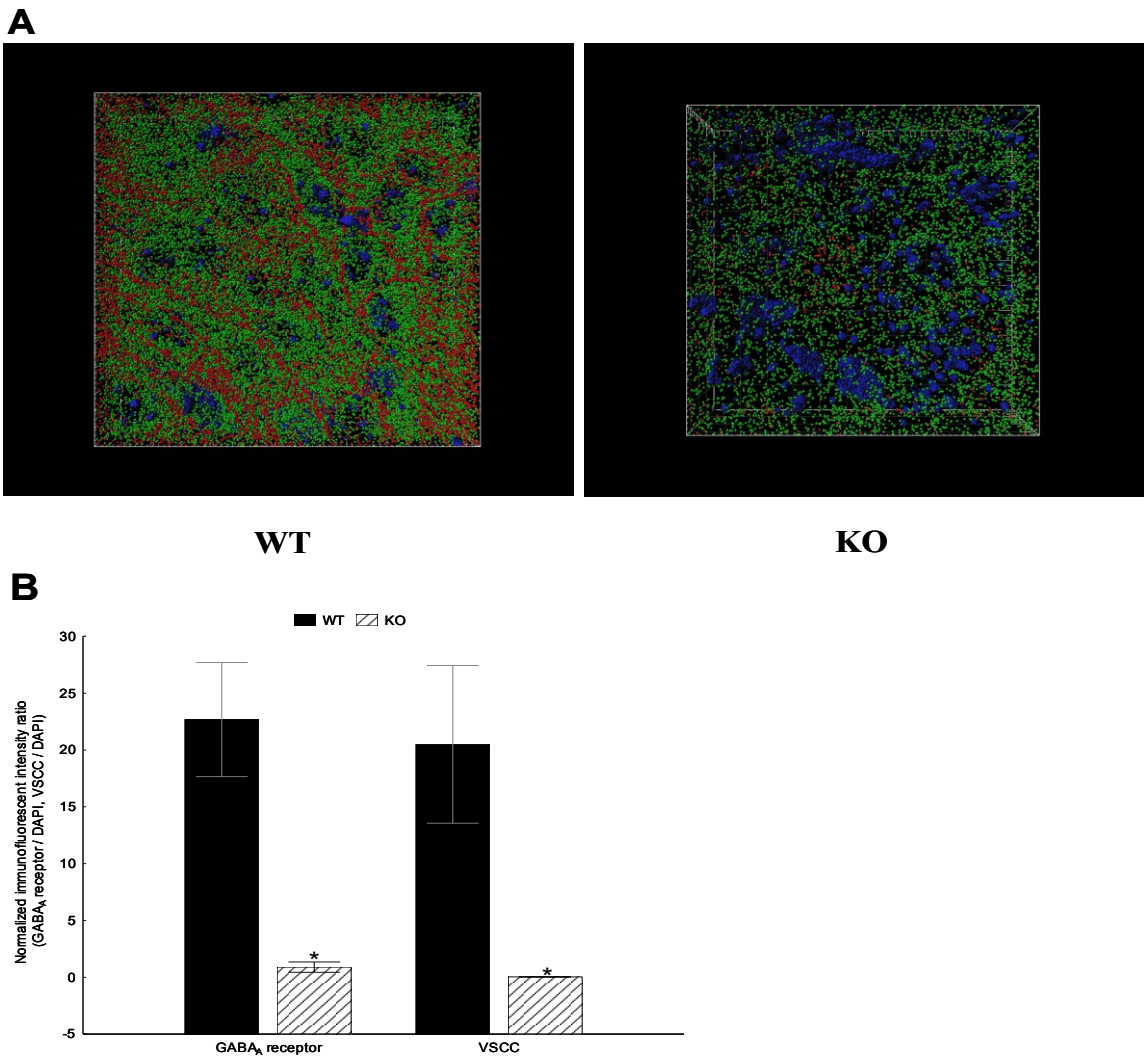


Figure 3. Reduced expression of $GABA_A$ receptor and VSCCs in the CA3 region of KO mice. The tissue was labeled with three colors: Red: VSCC, Green: $GABA_A$ receptor, Blue: DAPI, marking the nuclei. Z-stacks of 19-22 µm optical sections were captured from a mouse brain tissue slice using a Leica SP2 AOBS confocal microscope with a Leica Plan Apochromat 63x/1.4-0.60 oil immersion objective (0.5-µm optical slice thickness, 37-44 z-sections collected). Image stacks were then reconstructed in 3D (A) and immunointesity of $GABA_A$ receptor and VSCCs were quantified (B) using Imaris software (Bitplane). *P <0.05 (KO vs. WT mice), Fisher test for significant one-way ANOVA data.

Idrissi et al., 2010). The expression of $GABA_A$ receptors is activity-dependent and requires calcium for gene activation. Amongst these genes are the ones that code for the various subunits

of the $GABA_A$ receptors. Reduced expression of the VSCCs in KO mice brain may be responsible for the reduced activity-dependent, calcium-mediated gene expression resulting in the reduced expression of to the $GABA_A$ receptor.

Time-course of excitatory to inhibitory switch of $GABA_A$ receptor-mediated effects during development is shifted in the KOs.

We confirmed reduced $GABA_A$ receptor expression in adult KO mice brains in the previous section, and which triggers our interest to look at GABAergic system at earlier developmental stages. As mentioned above, at early stages of development, the $GABA_A$ receptors-mediated effect is excitatory, which causes GDPs and activation of VSCCs. GDPs in turn regulate expression of the GABAergic phenotype through the activation of VSCCs (Ben-Ari et al., 2007). The $GABA_A$ receptors and VSCCs act as synergistic partners in establishing functional neuronal networks at early developmental stages. The excitatory to inhibitory switch of $GABA_A$ receptors-mediated effect is a critical feature of the GABAergic system during development. As a result, we attempted to map the time course of the excitatory to inhibitory switch in both WTs and KOs, so that the VSCCs could be controlled while $GABA_A$ receptors still mediate excitatory responses. KCC2 plays a major role in the excitatory to inhibitory switch of the function of the $GABA_A$ receptor by extruding Cl^- from GABAergic neurons and maintaining low intracellular chloride concentrations. An elevated expression of KCC2 during development suggests the excitatory to inhibitory switch of the GABAergic effect. Here, we examined the expression level of KCC2 with western blotting in brain samples from P1, P3, P5, P7 and P10 WTs and KOs, to determine the change of KCC2 expression in various brain regions during these 5 time points (Fig. 4).

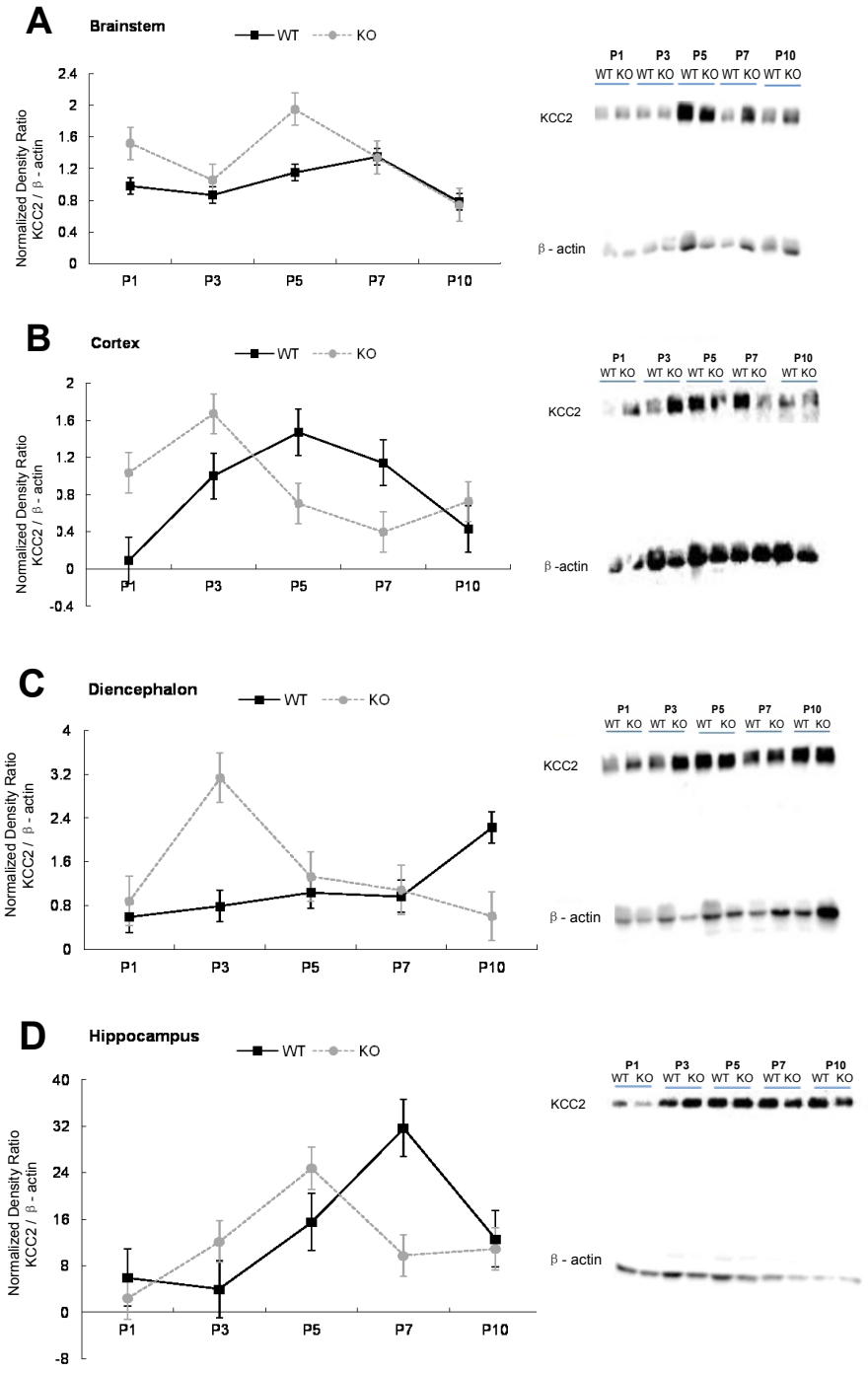


Figure 4. Shift of peak expression of KCC2 in WTs and KOs during development. Shifted peak expression of KCC2 based on western blotting in brainstem(A), cortex (B), diencephalon (C) and hippocampus (D). Western blots showing the expression of KCC2 during development are illustrated on right pannel. Densitometric analysis of the western bots is shown in the left panel. The values on the figures represent relative density of the bands normalized to β-actin using ImageJ software.

Western analysis indicated that expression of KCC2 peaked at different times in different regions of the brain. Surprisingly, a shift to earlier developmental stages was observed in cortex, hippocampus, diencephalon and brain stem of KOs (Fig. 4). In the brainstem, the expression of KCC2 in the WTs peaked at P7 but at P5 in the KOs (Fig. 4A). Similarly, the peak expression shifted from P5 in WTs to P3 in KOs in the cortex (Fig. 4B), and from P7 in WTs to P5 in KOs in the hippocampus (Fig. 4C). The shift is most dramatic in the diencephalon, where the peak of KCC2 expression was reached at P3 in KOs, about seven days earlier than WTs (Fig. 4D). The shift in peak expression of KCC2 in KOs suggests functional disturbances in GABAergic system development of KOs.

Pharmacological manipulation of VSCCs and $GABA_A$ receptors

In the previous section we demonstrated a switch of peak expression of KCC2 in KOs in comparison to WTs during early post natal developmental stages (P3 – P5), which indicates an abrrent depolarizing-to-hyperpolarizing switch of GABAergic responses in KOs. We sought to pharmacologically activate the VSCCs and the $GABA_A$ receptors in an attempt to normalize the timing of the switch. Studies on neuronal cell cultures *in vitro* showed that excitatory GABAergic responses last up to 2 weeks after plating (Connor et al., 1987; Ganguly et al., 2001). We administered 15mM KCl to depolarize the membrane for activating VSCCs, VSCC blocker and $GABA_A$ receptor agonist to 1 DIV (days *in vitro*) primary cerebellar cultures for 48hrs. We investigated the morphology of neurons and the expression pattern of VSCCs and $GABA_A$ receptors in the cultures. Application of 15mM KCl induced prominent outgrowth of neurites was shown in WTs following KCl administration (Fig. 5B).

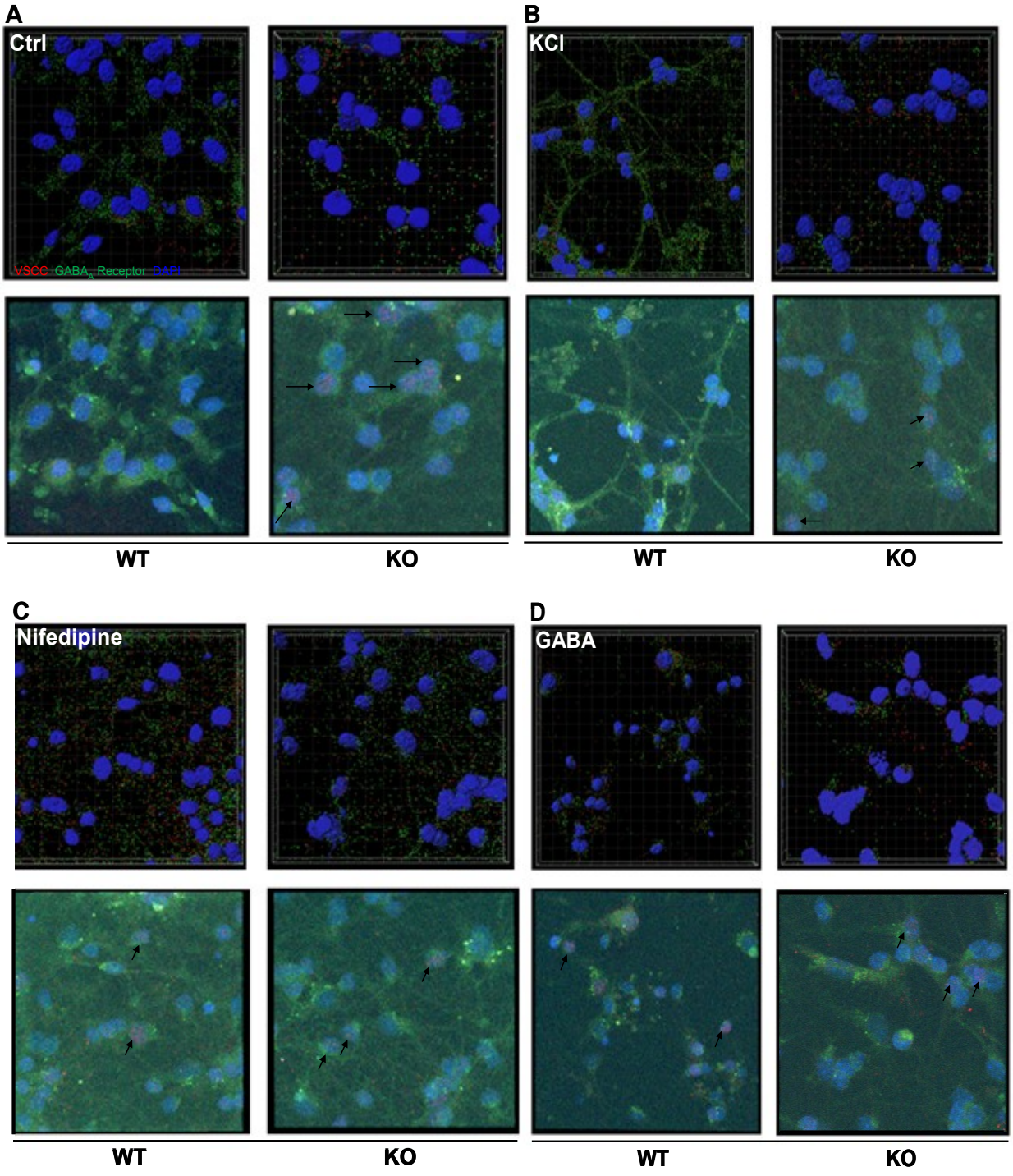

A
Ctrl
VSCC GABA$_A$ Receptor DAPI
B
KCl
WT
KO
WT
KO
C
Nifedipine
D
GABA
WT
KO
WT
KO

Figure5.

Figure 5. continued

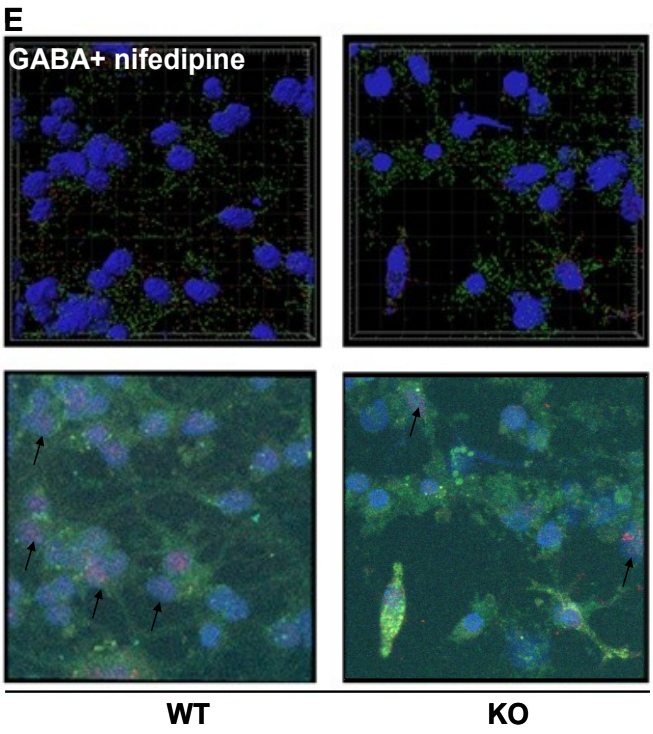


Figure 5. Caption The expression pattern of both $GABA_A$ receptor and VSCC in primary cerebellar cell cultures reflected by immunohistochemical signals under various treatment conditions. Red sphere: VSCC, Green sphere: $GABA_A$ receptor. Upper panels in each 4-image diagram: 3D-Imaris software reconstructions from z-stacks taken from Leica SP2 AOBS confocal microscope, Lower panels in each 4-image diagram: maximum projections from Leica confocal software of z-stacks taken from Leica SP2 AOBS confocal microscope. Different treatments are illustrated on the upper left corner of the diagram with whitish labeling. Nuclear localization of VSCCs are illustrated with black arrows. (A) WT and KO control group (Ctrl) received no treatment. (B) WT and KO group received 15mM KCl treatment. (C) WT and KO group received 1μM nifedipine treatment. (D) WT and KO group received 10μM GABA treatment. (E) WT and KO group received co-treatment of 10μM GABA+1μM nifedipine.

VSCC β3 subunits were mainly distributed in the cytoplasm of WT control cells (Fig. 5A), redistribution to the nucleus occurred following nifedipine (Fig. 5C), GABA (Fig. 5D) and GABA and nifedipine co-treatment (Fig. 5E). In contrast to that of WT control, VSCC β3 subunits were localized mostly in the nucleus of KO control cells (Fig. 5A). Extensive translocation of the VSCC β3 subunits from the nucleus to the cytoplasm occurred only following KCl treatment (Fig. 5B). In general, WT cells were more sensitive to the stimulation

mediated by nifedipine and GABA, KO cells, on the other hand, were more sensitive to the chronic deploarization induced by KCl. Large scale translocation of VSCCs β3 subunits between the nuclei and the cytosol was shown following various treatments, suggesting a potential role of VSCCs β3 subunit in the regulation of gene expression.

Administration of VSCC activator and $GABA_A$ receptor allosteric modulator restore behavioral performance in KOs

We found that the induction of neurite outgrowth was increased following VSCC activation. Furthermore, we showed a significant shuttling of the β3 subunit of the VSCC between the nucleus and the cytoplasm. This indicated that active regulation of gene expression took place during VSCC activation. In addition, higher sensitivity of KOs to the calcium channel activator lead us to further investigate the functional significance of this increased nuclear translocation of the β3 subunit of the VSCC and the resulting alteration in gene expression. In an attempt to check whether early activation of VSCC could normalize aberrant KO behavior through gene expression, we performed behavioral tests on offspring of pregnant mice that were injected with VSCC activator, $GABA_A$ receptor allosteric modulator, or combined injection of both drugs. In the previous section we demonstrated that the expression of KCC2 peaked in WTs and KOs took place during early developmental stages (P3 - P10) (Fig. 4), which indicates that the depolarizing-to-hyperpolarizing switch of GABAergic responses occurs through P3 to P10. To study the impact of VSCCs on the expression of $GABA_A$ receptor and $GABA_A$ receptors-related activities, pharmacological manipulation of VSCCs should be applied prior to P3. We injected pregnant mice on E16 and E19 (embryonic day 16 and 19). Information of mice used for

behavior tests are summarized in Tab. 2. The following behavioral tests were carried out sequentially based on increased aversiveness of the test: the open field (OF), the elevated plus maze (EPM), social interaction, acoustic startle test and contextual and cued fear conditioning.

Table 2. Mice used for behavior tests

		Genotype	Treatment	Drug fact
Pregnant mice	**A**	*WT*	none	none
	B	*KO*	none	none
	C	*KO*	10 mg/kg Ganaxolone	positive $GABA_A$ receptor allosteric modulator
	D	*KO*	0.5 mg/kg Bay K 8644	VSCC activator
	E	*KO*	0.5 mg/kg Bay K 8644 + 10 mg/kg Ganaxolone	positive $GABA_A$ receptor allosteric modulator + VSCC activator

			Male number	Female number
Offspring mice	**WT**	*from pregnant A*	4	10
	KO	*from pregnant B*	10	5
	KO GABA+	*from pregnant C*	5	9
	KO Ca+	*from pregnant D*	5	2
	KO GC+	*from pregnant E*	5	13

The open field (OF)

The open field (OF) is an exploration-based test for motor function, as well as anxiety-like behavior, which is built on the premise that the innate tendency to explore a novel place is inhibited by the aversiveness of the environment (Treit and Fundytus, 1988). A high level of exploration in a novel environment is an indication of low anxiety. In the open field test, anxiety-like behavior is manifested as the avoidance of the strong-illuminated central, open area as the natural tendency of a mouse is to stay near the perimeters of a novel environment. On the other hand, longer immobility in the center area indicates less anxiety. As shown in Fig. 6B there were

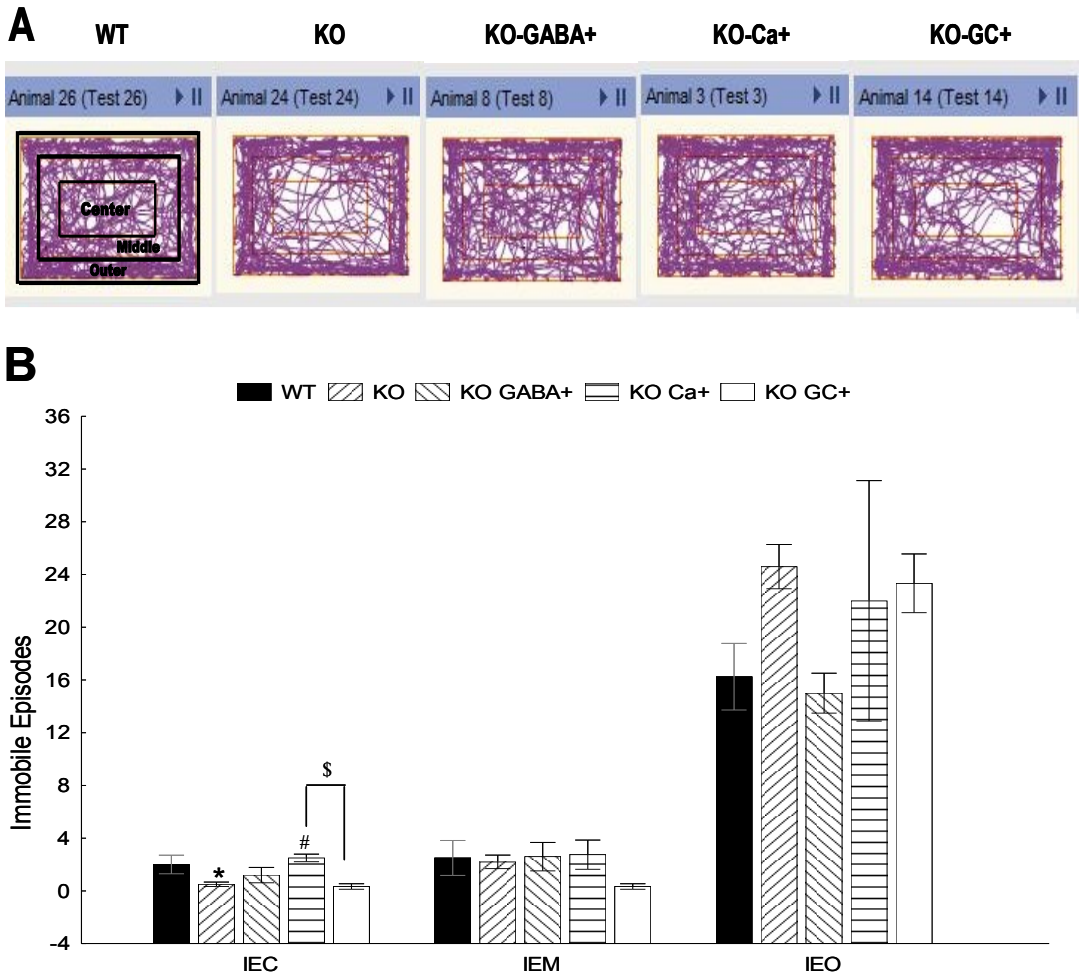


Figure 6. Behavioral performance of male offspring of wild type control mice (WT), *Fmr1* knockout mice (KO), *Fmr1* knockout mice injected with 10 mg/kg Ganaxolone (KO GABA+), *Fmr1* knockout mice injected with 0.5 mg/kg Bay K 8644 (KO Ca+) and *Fmr1* knockout mice injected with both 10 mg/kg Ganaxolone and 0.5 mg/kg Bay K 8644 (KO GC+) in the open field test. * P <0.05 (vs. WT mice), $^{\#}$P <0.05 (vs. KO mice), $^{\$}$P <0.05 (vs. each other), Fisher test, Scheffé test and Tukey HSD for unequal N for one-way significant analysis of variance between groups (ANOVA) data. (A) Track plots of the 10-min open field test of a representative animal from each group. Different zones within the open field arena (center zone, middle zone and outer zone) are illustrated in the left diagram. (B) Immobile Episodes of mice from respective groups in each zone. IEC: Immobile episodes in center zone, IEM: Immobile episodes in middle zone, IEO: Immobile episodes in outer zone.

significant differences in the immobile episodes (number of stops) in the center zone between male WT and KO (P <0.05), male KO Ca+ and KO GC+ (p<0.05), as well as between male KO

Ca+ and KO ($p<0.05$). KO males showed significantly less immobile episodes in the center zone when compared to WTs indicating a high anxiety level in KOs. KO Ca+ males exhibited significantly more immobile episodes in the center zone when compared with KO and KO GC+ males during the same amount of testing time (10 min) ($p<0.05$), which suggests that VSCC activation reduced anxiety level in KOs. There were no significant differences in immobile episodes in the center zone compared among all other groups of males. Aditionally there were no differences between exploration of the middle or outer zones when comparing all groups of males. Female offspring were also tested in the open field, but revealed no phenotype in any of the groups.

The Elevated Plus Maze (EPM)

The EPM is also an exploration-based test for anxiety-like behavior. It is based on the observation of rat's innate preference of elevated closed alley over elevated open valley. The test apparatus consists of two open arms perpendicular to two enclosed arms in a plus configuration with a central connecting platform elevated 40-70 cm above floor. The test produces a conflict between the instinct of mice to explore a novel environment and the natural tendency of mice to avoid the aversive properties of an elevated open area (Kalueff et al., 2007). It is a stronger test for anxiety than the open field test. Anxiety-like behavior is exhibited as avoidance of the open arms which is measured by the proportion of time spent in the open arms and proportions of entries into the open arms. Mice performance scored by EPM is shown in Fig. 7. In general, all groups tested explored the open arms more frequently than closed arms. Male KO mice displayed a significant reduction in the exploration in the open arms compared with WT males

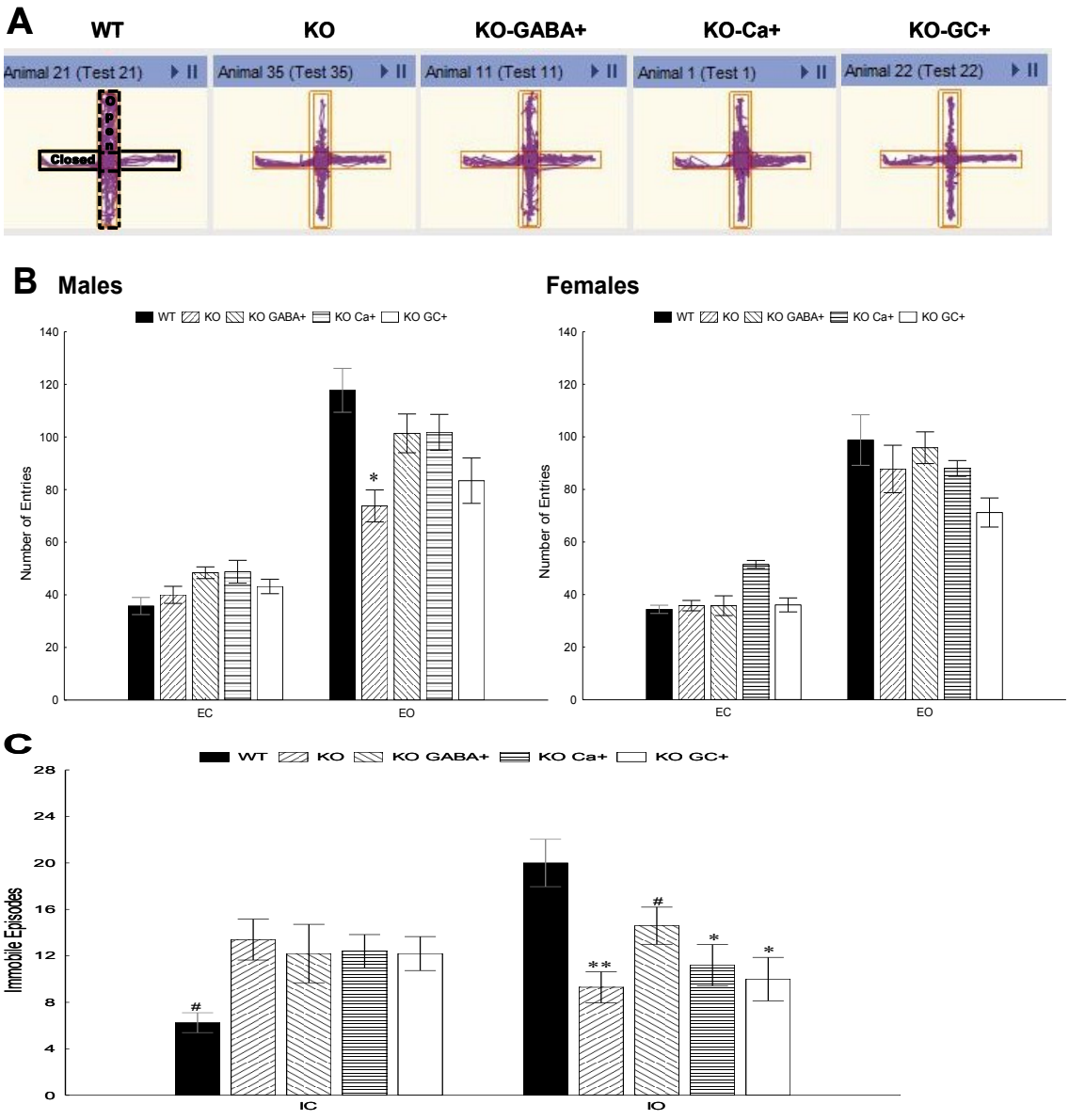


Figure 7. Behavioral performance of offspring of wild type control mice (WT), *Fmr1* knockout mice (KO), *Fmr1* knockout mice injected with 10 mg/kg Ganaxolone (KO GABA+), *Fmr1* knockout mice injected with 0.5 mg/kg Bay K 8644 (KO Ca+) and *Fmr1* knockout mice injected with both 10 mg/kg Ganaxolone and 0.5 mg/kg Bay K 8644 (KO GC+) in the EPM test. $^{*}P < 0.05$ (vs. WT mice), $^{**}P < 0.01$ (vs. WT mice), $^{\#}P < 0.05$ (vs. KO mice), Fisher test, Scheffé test and Tukey HSD for unequal N for significant ANOVA data. (A) Track plots of the 10-min EPM test of a representative animal from each group. Different zones within the elevated plus maze (open arm and closed arm) are illustrated in the left diagram. (B) Number of entries to each arm. EC: Number of entries to the closed arm, EO: Number of entries to the open arm. Right panel: male mice, Left panel: female mice. (C) Immobile Episodes of male mice from respective groups in each arm. IC: Immobile episodes in the closed arm, IO: Immobile episodes in the open arm.

(Fig. 7B, P <0.05) indicating an elevated anxiety level. A significant reduction of immobile episodes in the open arms was also detected (Fig. 7C, P <0.01) in the male KO group. Male KO GABA+, KO Ca+ and KO GC+ groups exhibited more active exploration than the male KO group (Fig. 7B) which suggests reduced anxiety level compared to KO males. The immobile episodes in the open arm displayed by male KO GABA+ were shown to be significantly more (Fig. 7C, $p_{KO\ GABA+} < 0.05$) than those by KO males. Female offspring tested in the EPM Showed the same but less pronounced profile of number of entries to each zone as male groups (Fig. 7B). All females explored in the open arm more often than in the closed arm. KO females exhibited fewer entries to the open arms than those of WT, KO GABA+ and KO Ca+. The results described so far indicate a significantly higher anxiety level in male KOs compared with other groups. The anxiety level in VSCC activator and GABAA receptor positive allosteric modulator treated KO mice is normalized with no difference from WTs.

Social Interaction

We subjected all groups to the social interaction test. Social interactions in rodents include sniffing, following, crawling over and grooming each other (File, 1985). The social interaction test, first designed for rats (File and Hyde, 1978), then modified and successfully applied to mice (Egashira et al., 2007), can be used to measure aspects of social approach behaviors, reciprocal social interactions, social recognition and social preference. Social behaviors are often tested in mice using the classical three-chamber test, where the subject mice were placed in a three-chambered open field and were provided with choices of either unfamiliar conspecific or non-moveable toys (Bolivar et al., 2000; Ferguson et al., 2000; Moy et al., 2004; Nadler et al., 2004).

The test apparatus we developed was adapted from the three-chambered social approach task (Yang et al., 2011). It consisted of three separable chambers (Fig. 8A): dark, target, and test chamber placed in the middle. The dark chamber was painted dark, while the test and target chambers are exposed to room light which was illuminated by 6 Lux lights facing upwards towards the ceiling. The dark and social chambers had matching openings and the subject mouse could freely move between the two chambers. The target and test chambers have matching openings (social zones) as well, although much smaller to only allow minimal direct physical sniffing between the subject and stranger mice yet allow air exchange between the two chambers. A video camera was mounted on the side at a distance that provided complete coverage of the arena. At the beginning of a test, the subject mouse was placed in the test chamber and allowed to acclimate to the environment for 10 minutes (set 1). Then a stranger mouse (untreated WT of opposite sex) was introduced into the target chamber for a 10-minute test session (set 2). To quantify the social tendencies of the subject mouse, the following parameters were measured: a) the time spent in the dark chamber; b) the time spent in the social zone of the test chamber; c) the time and frequency of contact with the stranger mouse.

In accordance with the natural preference for darkness, all mice tested spent considerably more time in the dark chamber than in the social zone of the test chamber during the first 10-min (set1) test (Fig. 8B and C). Time spent in the social zone of the test chamber was significantly increased after the stranger mouse was placed in the target chamber during the second 10-min (set2) test (Fig. 8B and C). It seemed that male KO mice displayed longer stays in the dark chamber and a decreased duration in the social zone of the test chamber compared to the WT males within the 10-min test (set 2) (Fig. 8B and C). Male KO Ca+ and KO GABA+ mice showed spent similar time in the dark chamber and the social zone of the test chamber as male

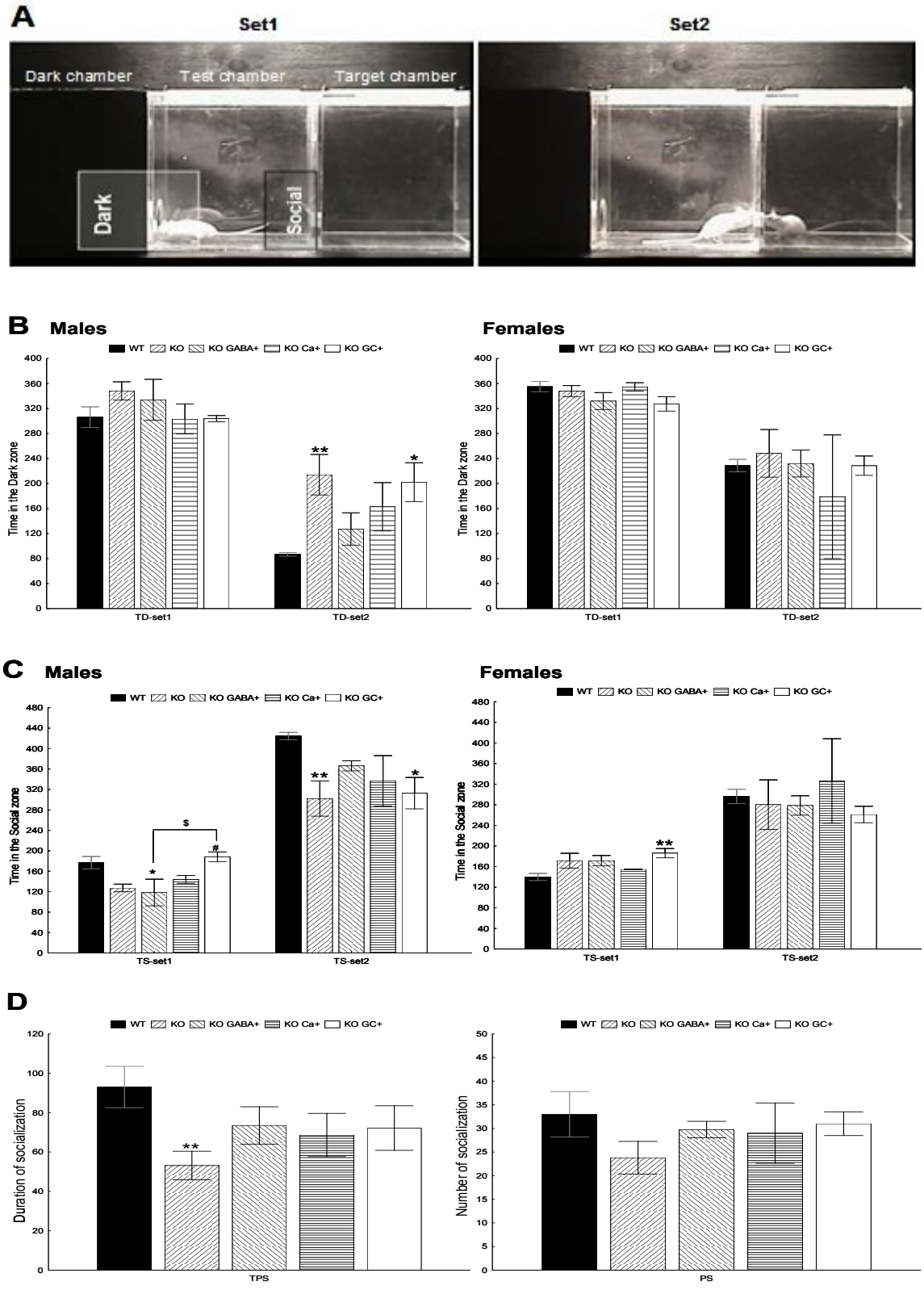

A
Set1
Set2
Dark chamber
Test chamber
Target chamber
Dark
Social
B
Males
Females
WT
KO
KO GABA+
KO Ca+
KO GC+
Time in the Dark zone
TD-set1
TD-set2
C
Males
Females
Time in the Social zone
TS-set1
TS-set2
D
Duration of socialization
TPS
Number of socialization
PS

Figure 8.

Figure 8. Caption. Behavioral performance of offspring of wild type control mice (WT), *Fmr1* knockout mice (KO), *Fmr1* knockout mice injected with 10 mg/kg Ganaxolone (KO GABA+), *Fmr1* knockout mice injected with 0.5 mg/kg Bay K 8644 (KO Ca+) and *Fmr1* knockout mice injected with both 10 mg/kg Ganaxolone and 0.5 mg/kg Bay K 8644 (KO GC+) in the Social Interaction test. $^{*}P < 0.05$ (vs. WT mice), $^{**}P < 0.01$ (vs. WT mice), $^{\#}P < 0.05$ (vs. KO mice), $^{\$}P < 0.05$ (vs. each other), Fisher test, Scheffé test and Tukey HSD for unequal N for significant ANOVA data. (A) Exemplary 10-min-set1 and 10-min-set2 social interaction tests of a representative animal from each group. Different zones within the social chamber (dark zone and social zone) are illustrated in the left diagram. (B) Time in the dark zone. TD-set1: Time in the dark zone during the first set of the test, TD-set2: Time in the dark zone during the second set of the test after a stranger mouse was placed in the neutral chamber. Right panel: male mice, Left panel: female mice. (C) Time in the social zone. TS-set1: Time in the social zone during the first set of the test, TS-set2: Time in the social zone during the second set of the test. Right panel: female mice, Left panel: male mice. (D) Socializations of male mice from respective group in the social zone. TPS: Duration of contact measured as the time the test subject spent interacting with the stranger mouse, PS: number of contacts between the test subject and the stranger mouse.

WT mice after strager mice were placed in the target chamber (Fig. 8B and C set2). Total duration of contacts (TPS) was significantly reduced in the male KOs compared with WT males ($P < 0.05$) (Fig. 8D). None of other three groups tested (KO GABA+, KO Ca+, KO GA+) showed any difference versus WT counterparts. All together, male KO mice displayed a deficit of social preference in our test and KO groups with treatments showed a restoration of social ability in comparison to KOs. Female offspring showed no difference between groups (Fig. 8B and C).

Acoustic Startle test

The startle response is a stereotyped motor reaction to sudden startling stimuli, usually elicited by acoustic or tactile stimuli. The startle response in rodents is characterized by contractions of major muscles that generally lead to the flinch of the whole body (Koch and Schnitzler, 1997). The acoustic startle response in rodents is mapped to a simple neural pathway that involves the cochlear nucleus and reticular pontine nucleus (Davis et al., 1982, Koch and Schnitzler, 1997),

but is modulated by higher brain structures (Braff et al., 2001). One example of the modulation is the prepulse inhibition (PPI) of the startle response, which is mediated by the forebrain neural circuits (Swerdlow et al., 2001, Swerdlow et al., 2006). PPI is the attenuation of a startle response by a preceding non-startling sensory stimulus (Braff et al., 2001). It is considered an operational measure of sensorimotor gating, an endogenous brain mechanism that filters out a multitude of irrelevant sensory stimuli from those of importance (Braff et al., 1992). Both patients with fragile X syndrome and *Fmr1* knockout mice display sensorimotor gating abnormalities (Frankland et al., 2004).

We examined PPI of acoustic startle response in all groups of mice. As described in Materials and Methods, the test consisted of three blocks. In Block 1, the subject mouse was presented with four successive trials of 40-msec noise bursts at 115 dB. This was to establish a stable response baseline, as there was a rapid habituation to startle responses in the first few trials. In Block 2, the animal was subjected to 16 trials of pre-pulse followed by pulse, with pre-pulse set at four sound levels of 75dB, 85dB, 95 dB and 105 dB of 20ms duration and pulse at 115 dB for 40 msec. in duration, followed by a startle stimulus 115dB. The trials were presented in random order and with varying inter-trial intervals to rule out effects of attention. Block 3 was the exact repeat of Block 1.

Compared with WTs, male KO mice demonstrated significantly decreased acoustic startle response to the 115 dB stimulus in Block 1 and Block 3 ($P < 0.01$) (Fig. 9A and C). This was consistent with previous reports of reduced startle reactivity in *Fmr1* knockout mice (Nelsen et al., 2002, Errijgers et al., 2008, Pietropaolo et al., 2011). KO GABA+ and KO Ca+ were close to the WT level of startle response for the 115 dB stimulus in Block 1 and Block 3, indicating normalized startle responses following VSCC activator and positive $GABA_A$ receptor allosteric

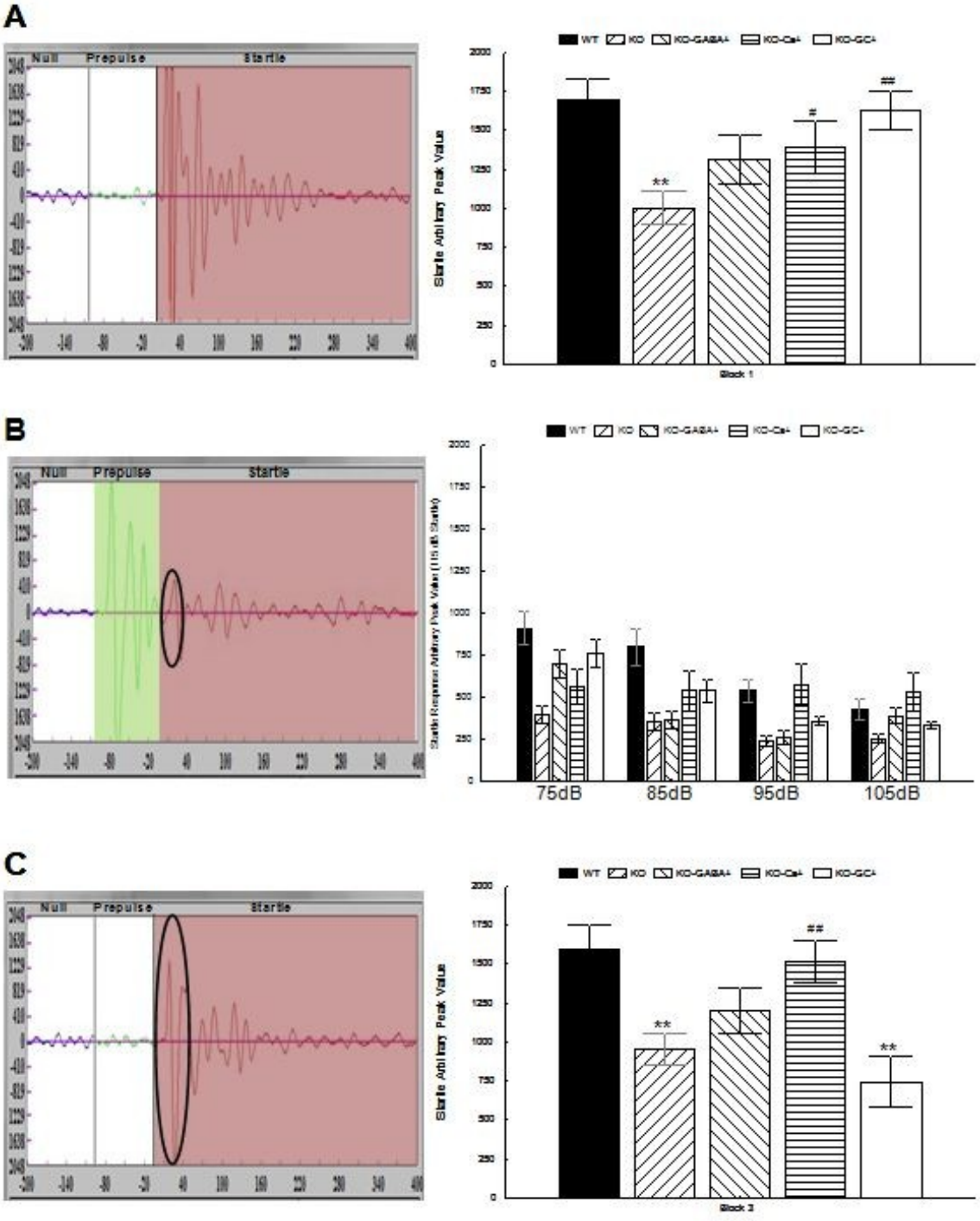

A
Null
Prepulse
Startle
WT
KO
KO-GABAA
KO-Ca
KO-GCA
Startle Arbitrary Peak Value
Block 1
B
Null
Prepulse
Startle
75dB
85dB
95dB
105dB
C
Null
Prepulse
Startle
Startle Arbitrary Peak Value
Block 2

Figure 9.

Figure 9. continued

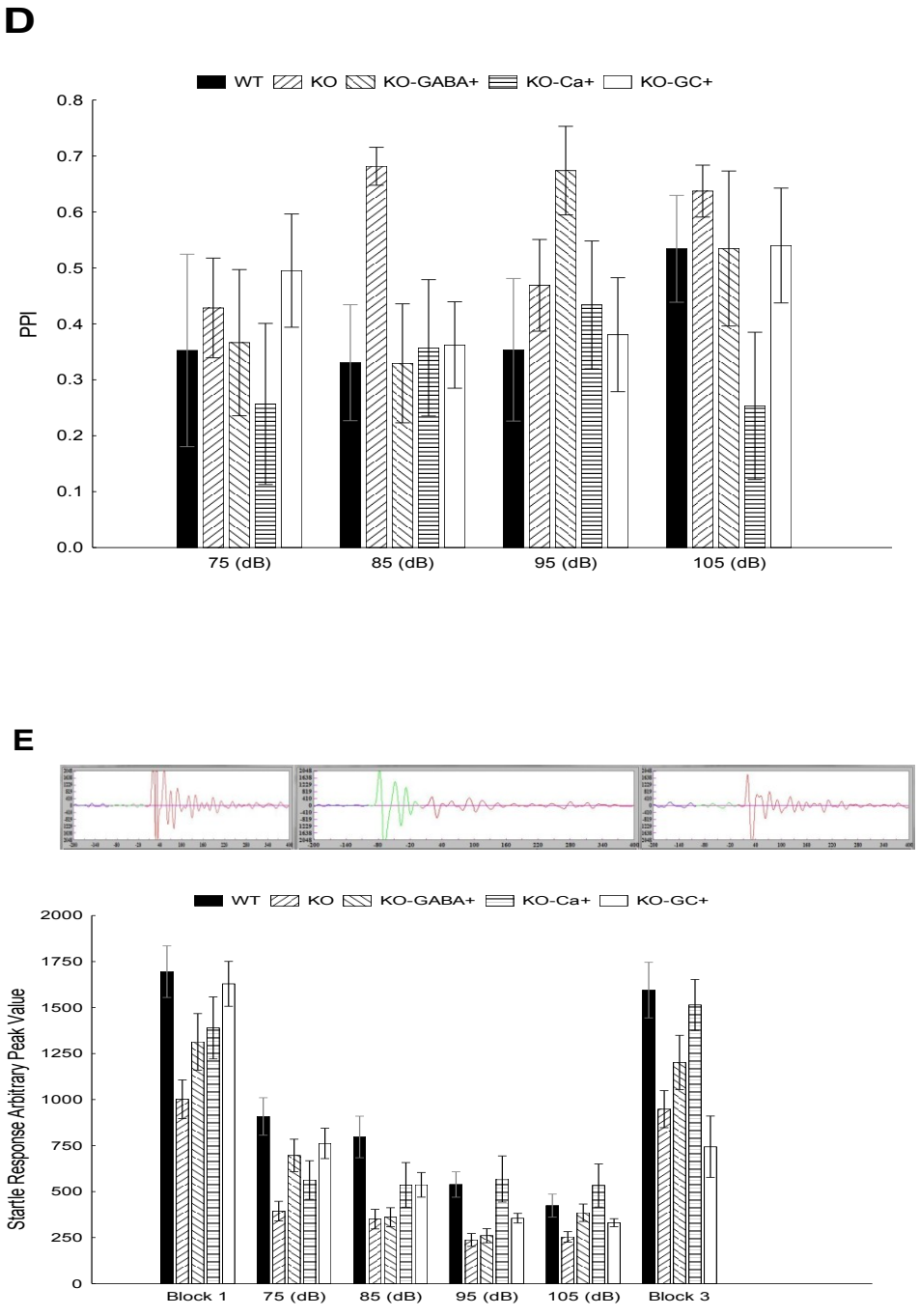


Figure 9. continued

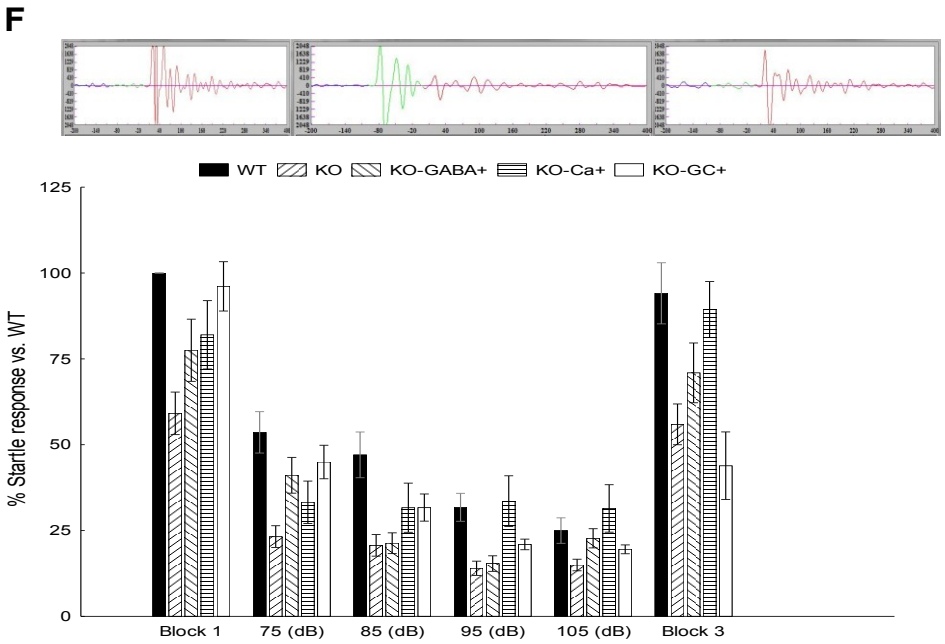


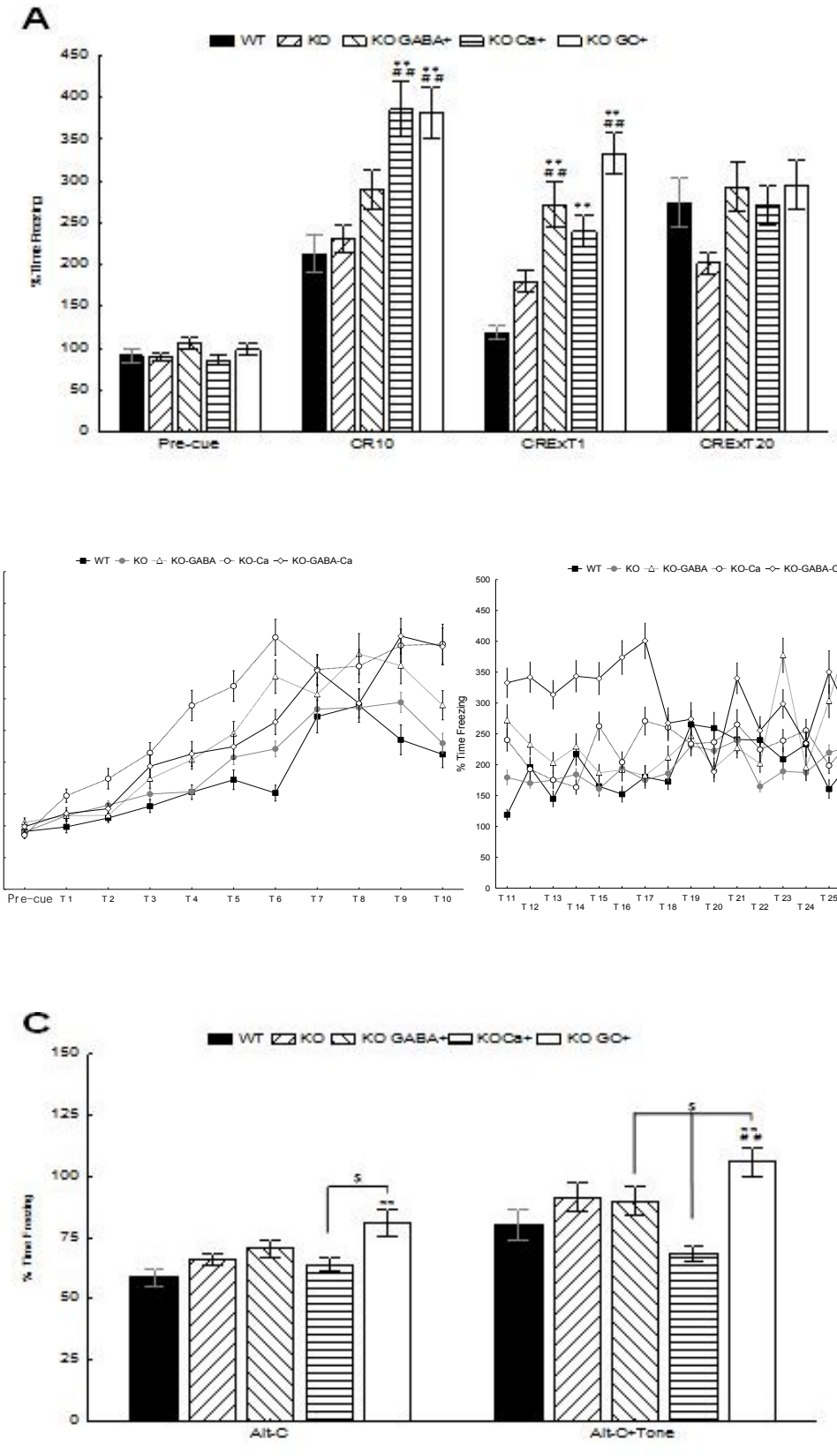
Figure 9. Behavioral performance of male offspring of wild type control mice (WT), *Fmr*1 knockout mice (KO), *Fmr*1 knockout mice injected with 10 mg/kg Ganaxolone (KO GABA+), *Fmr*1 knockout mice injected with 0.5 mg/kg Bay K 8644 (KO Ca+) and *Fmr*1 knockout mice injected with both 10 mg/kg Ganaxolone and 0.5 mg/kg Bay K 8644 (KO GC+) in the Acoustic Startle test. $^{*}P < 0.05$ (vs. WT mice), $^{**}P < 0.01$ (vs. WT mice), $^{\#}P < 0.05$ (vs. KO mice), $^{\#\#}P < 0.01$ (vs. KO mice), Fisher test, Scheffé test and Tukey HSD for unequal N for significant ANOVA data. (A) Acoustic startle response of male mice to the 115dB startle stimulus in Block 1. The shadowed zone represents when the subsequent startle stimulus (115dB) was delivered. The circle represents the peak value of the startle response. (B) Prepulse inhibition (PPI) to the 75, 85, 95and 105 dB auditory prepulse followed by a 115 dB startle stimulus in male mice (Block 2) after Block 1. The first shadowed zone represent the time when the prepulse (75dB, 85dB, 95dB and 105dB) was presented. The second shadowed zone represents when the subsequent startle stimulus (115dB) was delivered. The circle represents the peak value of the startle response. (C) Acoustic startle response of male mice to the 115dB startle stimulus in Block 3 administered after Block 2. The shadowed zone represents when the subsequent startle stimulus (115dB) was delivered. The circle represents the peak value of the startle response. (D) Percent PPI in Block 2 for the four levels of prepulse administered. (E) Representative data for one set of acoustic startle test started with Block 1, followed by Block 2 and finished by Block 3. (F) Representative data for one set of acoustic startle test with Block 1, followed by Block 2 and finished by Block 3. Data are shown as percentage of the startle response of WTs.

modulator treatments. While the response level in Block 1 is comparable to WT for KO GC+, it became much reduced when tested in Block 3 (Fig. 9A and C). PPI increased with prepulse intensity as evidenced by the decreasing startle response in the WT (Fig. 9B, D and E). This is in agreement with published reports (Csomor et al., 2005, Yee et al., 2005, Pietropaolo et al., 2008, Pietropaolo et al., 2011). There was no statistically significant difference in PPI between WT and KO and among the treatment groups (Fig. 9B, D, E and F). However a strong trend of enhanced PPI in KOs compared with other counteparts was shown in Fig. 9D. It has been shown that the *Fmr1* knockout in FVB background, which is the strain we used in the current study, has similar PPI as WT with the same genetic background, while the *Fmr1* knockout in C57B6/J background exhibits enhanced PPI versus its WT counterpart (Frankland et al., 2004, Pietropaolo et al., 2011). Except for KO GC+, all other groups showed similar startle response in Block 3 as they did in Block 1. This demonstrates that the PPI observed in Block 2 is a result of prepulse rather than habituation or learning by attention. The results shown so far suggest that KO GABA+ and KO Ca+ VSCC activator and positive $GABA_A$ receptor allosteric modulator treatments partially normalized startle responses in KOs.

Contextual and cued fear conditioning

We assessed the cognitive function of all five groups of mice using the contextual/cued fear conditioning test, a Pavlovian associative learning and memory paradigm. In this test, mice were trained to associate contextual cue and auditory cues [conditioned stimulus (CS)] with an aversive unconditioned stimulus (US), a mild foot shock. The dependent measure of the test is a freezing response that took place following pairing of the US with CS.

All male mice showed similar amounts of freezing behavior in the contextual chamber during the first 10 sec exploratory time, before they were subjected to 10 trials of paired auditory cues followed by a 0.5mA foot shock. As expected, by the last trial on day1, all groups displayed increased freezing time compared with the pre-cue. The freezing time of KO GABA+ and KO GC+ was significantly longer than that of WT and KO (Fig. 10A). Responses to the auditory cue without the foot shock in the identical contextual chamber were reduced to different degrees in all groups but KO GABA+ in Trial 11 (the first trial of the 20-trial Day2 test) as compared to Trial 10 (the last trial of the 10-trial Day1 test). Overall, a higher level of freezing was shown in KO GABA+, KO Ca+ and KO GC+ in both Day 1 and Day 2 tests (Fig. 10B), indicating higher contextual learning. On day 3 the sensory cues had been changed as much as possible, so that the mouse perceived the chamber as a novel context unrelated to the first-two-day environment. All groups displayed less freezing time in the altered contextual environment compared to the freezing time in the conditioning chamber. It seemed that cued learning was not as robust as contextual learning when results from Day 2 and Day 3 were compared (Fig. 10B and C). It may partly due to the experimental design that the CS also included light but in cued test only the tone was presented. No difference was detected between WT and KO in the contextual/cued learning. This corroborates with published results (Dobkin et al., 2000, Van Dam et al., 2000). Although some reported deficit in fear conditioning test for *Fmr*1 knockout mice (Paradee et al., 1999), it turns out to be dependent on genetic background (Dobkin et al., 2000, Van Dam et al., 2000). And for the particular strain of *Fmr*1 knockout mice, it was reported that no difference is revealed in fear conditioning between knockout and normal mice (Dobkin et al., 2000).


A
WT
KO
KO GABA+
KO Ca+
KO GO+
% Time Freezing
Pre-cue
CR10
CRExT1
CRExT20
B
WT
KO
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KO-Ca
KO-GABA-Ca
% Time Freezing
Pre-cue
C
WT
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KO GABA+
KOCa+
KO GO+
% Time Freezing
Alt-C
Alt-O+Tone

Figure10.

Figure 10. Caption. Behavioral performance of male offspring of wild type control mice (WT), *Fmr1* knockout mice (KO), *Fmr1* knockout mice injected with 10 mg/kg Ganaxolone (KO GABA+), *Fmr1* knockout mice injected with 0.5 mg/kg Bay K 8644 (KO Ca+) and *Fmr1* knockout mice injected with both 10 mg/kg Ganaxolone and 0.5 mg/kg Bay K 8644 (KO GC+) in the contextual and cued fear conditioning test. $^{**}P < 0.01$ (vs. WT mice), $^{\#\#}P < 0.01$ (vs. KO mice), $^{\$}P < 0.05$ (vs. each other), Scheffé test and Tukey HSD for unequal N for significant ANOVA data. (A) Proportion freezing time of male mice in the contextual chamber on Day 1 and Day 2 tests. Pre-cue: Unconditioned response of mice during the first 10-sec exploration time before any stimulus in the contextual chamber on Day1, CR10: Conditioned response of mice from the last trial (trial 10) on Day 1, CRExT1: Conditioned response of mice from the first trial (Trial 1) on Day 2, CRExT20: Conditioned response of mice from the last trial (Trial 20) on Day 2. (B) Proportion freezing time of male mice in the contextual chamber during acquisition and extinction on Day 1 and Day 2 tests. Pre-cue: Unconditioned response of mice during the first 10-sec exploration time before any stimulus in the contextual chamber on Day 1, T1-T10: Conditioned response of mice during Trial 1 to Trial 10 on Day 1, T11-T30: Conditioned response of mice during Trial 11 to Trial 30 on Day 2. (C) Proportion freezing time of male mice in altered contextual environment on Day 3 tests. Alt-C: Response of mice during the first 3-min exploration time before the high tone stimulus in altered contextual chamber on Day 3, Alt-C + tone: Response of mice during the last 3-min testing time with the high tone stimulus in altered contextual chamber on Day 3.

DISCUSSION

The findings of this study can be summarized as follow:

- Expression of VSCCs and $GABA_A$ receptor is reduced in *FMR1* knock out (KO) mice.
- The time course of excitatory to inhibitory switch of the function of $GABA_A$ receptor in WTs and KOs is shifted during development.
- Peak of KCC2 expression was reached earlier in KOs than in WTs.
- VSCCs β3 subunits translocated between the nuclei and the cytosol.
- VSCC activator normalized part of the KO behavior.

Our lab has previously demonstrated that expression of the β subunit $GABA_A$ receptor is reduced in the brain of the fragile X mouse (El Idrissi et al., 2005), which might act as a mechanism underlying the hyperactivities observed in fragile X patients. This reduction was confirmed in the present study (Fig. 3).

The current results are in agreement with previous finding of reduced VSCC expression in the islets of the Langerhans in the pancreas of KOs (Fig. 11) (El Idrissi et al., 2010).The islets of Langerhans in the moose pancreas contain the 65-kD isoform of GAD (GAD65), a subset of $GABA_A$ receptors, and high concentrations of GABA(Sorenson et al., 1991; Thomas-Reetz and De Camilli, 1994; Yang et al., 1994; Chessler et al., 2002). The most prominent function of the pancreas lies in the release of insulin and glucagon. Insulin and GABA are co-released from β cells (Braun et al., 2009). While insulin lowers plasma glucose concentration, GABA acts via $GABA_A$ receptors inhibiting the constitutive secretion of glucagon from the α cells (which raises

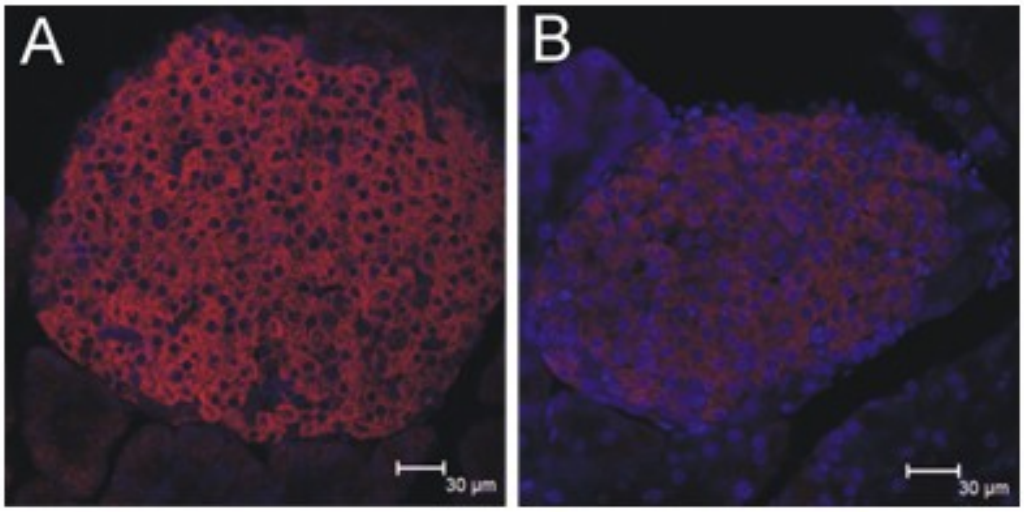

Figure 11. Reduced expressions of VSCCs in the islet of Langerhans in the pancreas of KO mice. Representative confocal images of the islet of Langerhans of two-month-old FVB/NJ WT mice (A) and KO mice (B). Red: VSCC; Blue: DAPI. (Modified from El Idrissi et al. 2010).

very low blood sugar level). The co-release of GABA and insulin is dependent on the activation of the VSCCs. These channels are widely expressed by all pancreatic islet cells and are required for calcium-dependent exocytosis of hormones and neurotransmitters. During early development, VSCCs play an important role in calcium-dependent gene expression, and in calcium-mediated vesicular release of neurotransmitters and pancreatic hormones. The perturbation of pancreatic function in KOs could be associated with reduced expression of the VSCCs.

The GABAergic system in pancreas and brain shares considerable similarities. Proteins such as $GABA_A$ receptors, somatostatin, GAD and taurine transporter which are widely expressed in the pancreas are also detected in the brain. VSCCs provide one of the main routes of calcium entry into cells and are also responsible for vesicular exocytosis. The function of these channels is critically important especially during the early developmental stages of the neuronal network. VSCCs, as one of the major sources of Ca^{2+} signals and initiate many physiological events

including gene expression (Flavell and Greenberg, 2008; Catterall, 2011). Particularly, VSCCs plays a critical role in mediating Ca^{2+} influx during early development and therefore, could have a great effect on regulation of gene expression in early developmental stages (Ben-Ari et al., 1994; Flavell and Greenberg, 2008). Blocking Ca^{2+} influx through L-type VSCCs reduces the expression levels of $GABA_A$ receptors (Saliba et al., 2009). Our findings of the concomitant reduction of $GABA_A$ receptors and VSCCs in both the brain and pancreas in *Fmr1* KOs may indicate a causal effect of VSCCs on $GABA_A$ receptor expression level.

To check whether VSCCs and $GABA_A$ receptor expression are activity-dependent, we mimicked the tonic electrical activity of activating VSCCs by treating cultured cerebellar granule cells with 15mM KCl, and blocking VSCCs functions via nifedipine treatment. Interestingly, we observed nuclear localization of the β3 subunit of VSCC in cerebellar granule cell culture (Fig. 4), which suggested the role of β3 subunit of VSCC in gene transcription. This is not the only case that subunits of VSCCs are found in the nucleus. Two domains of VSCC were reported to translocate between the nucleus and the cytoplasm and have been shown to be involved in transcriptional regulation (Gomez-Ospina et al., 2006; Hibino et al., 2003). A C-terminal fragment of L-type VSCC, a calcium channel associated transcription factor (CCAT), is localized in the nucleus of many neurons, especially GAD65-expressing GABAergic inhibitory interneuron (Gomez-Ospina et al., 2006). This L-type VSCC fragment is categorized as a member of the family of transcription factors, which significantly upregulates 16 mRNAs and downregulates 31 mRNAs expressed in Neuro2A cells. The fragment is exported from the nucleus in response to lower intracellular calcium concentration caused by depolarization or alternative activities (Gomez-Ospina et al., 2006). The second nuclear localized VSCC fragment is the β4c subunit, a short particular splice variant of β4. The β4c subunit was detected in the

chicken cochlea, eye, heart, lung, as well as in the brain. Interaction of the β4c subunit with the chromobox protein 2/heterochromatin protein 1 γ (CHCB2/HP1γ) or other types of heterochromatin protein 1 (HP1), nuclear proteins involved in gene repression and transcriptional regulation, redistribute it from the cytoplasm into the nuclei. Coexpression of β4c and CHCB2 in Cos-1 cells in the GAL4-CAT reporter assay dramatically attenuats the gene-silencing effect of CHCB2 on CAT activity (Hibino et al., 2003). However, the β4c subunit alone does not influence gene transcription, which indicates it may function as a transcriptional regulator reducing CHCBs/HP1s-mediated gene silencing (Hibino et al., 2003).

We have not determined through what kind of mechanism the activity-dependent translocation of β3 subunit of VSCC was triggered and what the effect of this translocation is, or whether the increased neurite outgrowth is directly induced by the translocation. It is possible that VSCC β3 subunit may be involved in transcriptional regulation as the CCAT and the β4c subunit. Translocation of transcription factors between the nucleus and the cytoplasm has been demonstrated in various cell types (Okamura et al., 2000; Meffert et al., 2003). The function, localization, or expression of transcription factors in the nucleus can be altered through activity-regulated signaling pathways (West et al., 2002). Classically, the nuclear localization signals (NLSs) contained in transcription factor sequences recognize and bind to certain subunits of nuclear transport proteins, the transcription factor- nuclear transport protein complex will be translocated subsequently to the nucleus through nuclear pores (Otis et al., 2006). The VSCC β3 subunit may also contain domains with nuclear transport proteins. Changes in the level of intracellular calcium may provide enough signals to pass the threshold for activation of nuclear transporting complex formation and nuclear redistribution that could be induced which temporarily causes a burst of nuclear proteins that activate or suppress downstream processes.

Immunoprecipitation or alternative methods can be used to screen binding partners of β3 subunit. Mutants of the β3 subunit can then be constructed for further identification of the binding domain of the β3 subunit with the interacting protein.

We observed prominent neurite outgrowth following KCl treatment (Fig. 4B), indicating active regulation of gene expression taking place during activation of VSCCs. Activation of VSCCs allows large transient calcium influx into neurons a process that has been shown to be important for synaptic growth and neuronal survival. It also has been shown that L-type VSCC-associated CREB activation transcriptionally regulates genes that are important for dendritic growth in cortical cultures (Redmond and Ghosh, 2005). Active transcriptional regulation of gene expression leads to alteration of the expression levels of proteins. The translocation of the VSCC β3 subunit mediated gene transcription possibly induces gene expression of the $GABA_A$ receptor scaffolding proteins.

GABARAP and gephyrin are two important $GABA_A$ receptor scaffolding proteins. GABARAP, a 17-kDa protein, is important for the membrane trafficking of the $GABA_A$ receptor. As a member of a family of homologous small microtubule binding proteins, GABARAP is regarded as a key factor regulating $GABA_A$ receptor function (Arancibia-Cárcamo and Kittler, 2009). The colocalization of GABARAP and $GABA_A$ receptor clusters has been shown in cultured neurons (Wang et al., 1999). Functional loss of GABARAP reportedly results in no $GABA_A$ receptor clusters in Japanese quail QT-6 fibroblasts (Chen et al. 2000). Gephyrin, a 93-kDa protein, considered as the master scaffolding molecule of inhibitory synapses, serves as a marker of GABAergic and glycinergic postsynaptic sites in the central nervous system (Fritschy et al., 2008). It is highly concentrated in the postsynaptic compartments of GABAergic inhibitory synapses and its clusters are usually found to be colocalized with $GABA_A$ receptors (Studer et al.,

2006; Peden et al., 2008). Down-regulation of gephyrin expression by gene targeting or gene silencing leads to rapid disappearance of postsynaptic $GABA_A$ receptor clustering and loss of IPSCs (Jacob et al., 2005; Yu et al., 2007), indicating that gephyrin is required for normal postsynaptic clustering of $GABA_A$ receptors, and it plays a specific role in limiting the mobility of $GABA_A$ receptor clusters, thereby enhancing cluster stability. We examined the expression level of both GABARAP and gephyrin in the pancreas of two-month-old wild type (WT) and *Fmr1* knockout (KO) FVB/NJ mice. GABARAP expression in the pancreas was significantly reduced in the KO (Fig. 12). Similar decreased expression pattern was observed for gephyrin in KO mice (Fig. 13). Together with the observation that VSCCs levels were reduced as well in the same pancreatic samples (Fig. 11) (El Idrissi et al. 2010), and together with the results of current study, we conclude VSCCs might affect the expression of GABARAP and gephyrin through gene expression, and subsequently regulate the expression level of $GABA_A$ receptors.

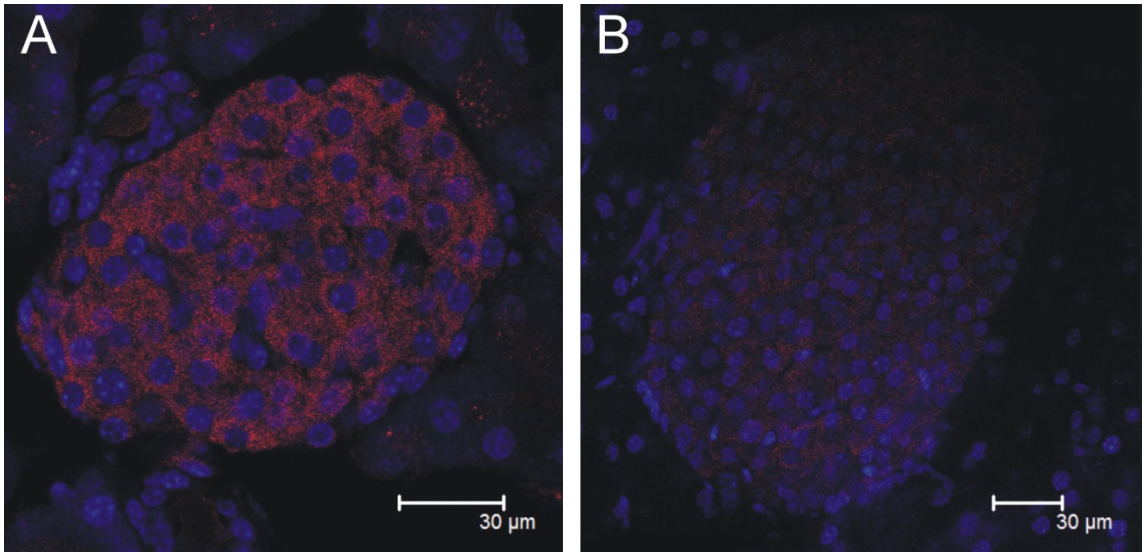

Figure 12. Reduced expression of GABARAP in the islet of Langerhans in the pancreas of KO mice. Representative confocal images of the islet of Langerhans of two-month-old FVB/NJ WT mice (A) and KO mice (B). Red: GABARAP. Blue: DAPI.

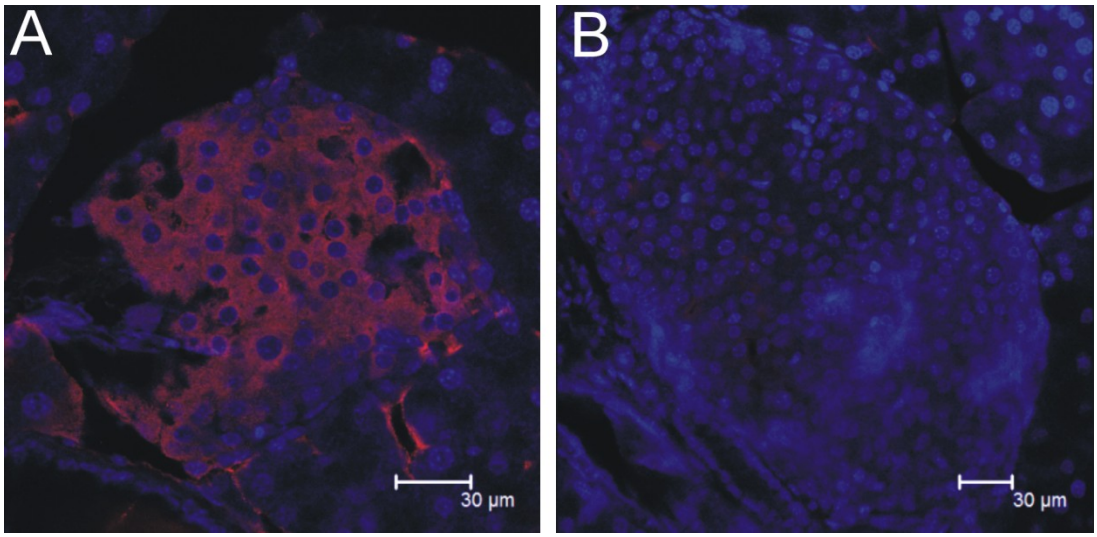

Figure 13. Reduced expression of gephyrin in the islet of Langerhans in the pancreas of KO mice. Representative confocal images of the islet of Langerhans of two-month-old FVB/NJ WT mice (A) and KO mice (B). Red: gephyrin. Blue: DAPI.

Further work needs to be performed to study the effect of VSCCs regulation of expression of $GABA_A$ receptors anchoring proteins in the brain.

Our finding of the distinct temporal KCC2 expression patterns in the four regions of mice brain agrees with published studies that have shown that KCC2 expression follows different time courses in different cell types as well as different regions of the brain (Rivera et al., 2005; Zhang et al., 2006; Belenky et al., 2008). In the embryonic CNS, KCC2 expression increases differentially in different regions of the brain with time that corresponds to the neuronal maturation pattern in the brain (Rivera et al., 1999; Wang et al., 2002; Ikeda et al., 2003; Stein et al., 2004). As KCC2 is the key player in the developmental switch from GABA-mediated excitation to inhibition, it is not surprising that the timing of the developmental shift of GABA receptor mediated responses exhibits significant regional differences (Ben-Ari et al., 2007). In the developing rat hippocampus, the increase in the efficacy of Cl^- extrusion and KCC2

expression take place by the second postnatal week (Rivera et al., 1999). One study in mice has shown that the adult inhibitory response to GABA is established earlier in the spinal cord than in the hippocampus (Stein et al., 2004). Caution has to be taken when comparing time courses across species since time of birth provides no solid point of reference for brain or pancreas development.

During early neuronal development, $GABA_A$-receptor-mediated responses are a key factor in the control of several Ca^{2+}-dependent developmental phenomena, including neuronal proliferation, migration and targeting. These events occur prior to synapse formation and are important for the construction of the nervous system. As a molecular switch turns $GABA_A$ receptor-mediated response from excitatory to inhibitory, KCC2 is regarded as a marker for neuronal maturation (Rivera et al., 1999; Stein et al., 2004; Rivera et al., 2005). It is important for $GABA_A$-receptor and KCC2 to be expressed both at the right time and at the right place. The shift of KCC2 temporal expression that have detected in the KOs (Fig. 4) may result in immature GABA physiology and potential disturbance of brain development.

As outlined above, reduced VSCC found in KOs could mediate altered gene expression and a shift of KCC2 in KOs may be responsible for disrupted brain development, both of which may result in an aberrant behavioral phenotype in KOs. *Fmr1* KO mice exhibit several physical and behavioral characteristics of the human FXS syndrome such as macroorchidism, hyperactivity (Bakker et al., 1994). Social anxiety is a prominent feature of FXS. *Fmr1* KO mice display deficits in social interaction manifested as abnormal anxiety-related responses and sensorimotor gating (Mineur et al., 2002, Frankland et al., 2004, Mineur et al., 2006, Errijgers et al., 2008, McNaughton et al., 2008, Pietropaolo et al., 2011). Some mild learning deficits have been observed using paradigms including contextual fear conditioning and Morris water maze (Bakker

et al., 1994, Paradee et al., 1999, Dobkin et al., 2000, Van Dam et al., 2000, Mineur et al., 2002), although they are largely dependent upon genetic background (Dobkin et al., 2000, Van Dam et al., 2000). Disrupted VSCCs function is associated with disrupted behavior in various mouse strains. McKinney and Murphy showed that L-type VSCC Cav1.3 was involved in consolidation of contextually conditioned fear in C57BL/6 mice using Cav1.3 knockout mice (McKinney and Murphy, 2006). Bader et al., 2011 reported that mutant L-type VSCCs with one single mutation on the coding region Cav1.2 (the causal factor of Timothy syndrome, a rare disorder with multi-organ dysfunction) in mouse, when expressed at a fairly low level in mice, results in autistic-like behaviors such as repetitive and perseverative behavior, and reduced socialization (Bader et al.).

To examine possible roles of VSCCs in KO mice, we performed a battery of behavioral tests treated during embryogenesis with a VSCCs agonist, with a positive allosteric modulator of the $GABA_A$ receptor or both, by injection of the drugs into their pregnant mothers. Overall, KO mice displayed behaviors more or less consistent with results from published studies. They were less active in exploring open areas in the EPM test (Fig. 7) and spent less time socializing with a stranger mouse (Fig. 8). KO mice also had reduced startle response to acoustic stimuli (Fig. 9). Although they failed to show any difference from WT in PPI and contextual/cued fear learning, it was in line with reports of the specific *Fmr1* KO strain we used (Dobkin et al., 2000, Frankland et al., 2004, Pietropaolo et al., 2011).

Effects of the treatments seemed to normalize part of the disrupted KO behaviors. Administering VSCCs agonist and $GABA_A$ receptor positive modulator seem to improve the exploring activity in the open arms of the EPM test (Fig. 7B) and social interaction with stranger mice (Fig. 8). One notable observation is that both treatments improved learning in the fear conditioning (Fig. 10). Interestingly, treatment with both did not necessarily bring about a strong

effect, sometimes even the opposite (Fig. 7). These data suggest that the VSCCs and $GABA_A$ receptors may interact synergistically during early development. Pharmacological manipulation of the channels could bring about improvement of the KO behaviors. However further investigation is necessary to determine the optimal dosage and timing for administration of these drugs.

Material and Methods

Mouse husbandry

All mice were housed in opaque cages on hardwood chips and supplied with food and water *ad libitum*. The animal room was maintained under controlled temperature (24 ± 1°C) and humidity (55 ± 5%) with a 12-hr light: 12-hr dark cycle. The *Fmr1* Knockout mutation was originally engineered in E14 embryonic stem cells derived from the 129/Ola strain (Bakker et al., 1994). FVB/NJ-129 hybrid mice carrying this mutation were obtained (a generous gift, B. Oostra & R. Willemsen) and repeatedly backcrossed to FVB/NJ at the CSI animal facility. KO and control littermates used in the experiments were the product of >20 generations of this process. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines.

Mouse strains and genetics for protein tests

Two-month-old FVB/NJ males and *Fmr1* knockout FVB/NJ males were used in identification of $GABA_A$ receptor and VSCC expression in CA3 region in the brain. The number of mice used in these studies was sufficient to provide statistically reliable results were used in these studies.

FVB/NJ and *Fmr1* knockout FVB/NJ mice were used at 5 time points were used to study KCC2 expression level in the brain to map the $GABA_A$ receptor-mediated effect switch. Wild

type control (WT) and *Fmr1* knockout mice (KO) on P1, P3, P5, P7 and P10 were used (P: postnatal day).

Mouse strains and treatments for behavior tests

FVB/NJ and *Fmr1* Knockout FVB/NJ mice were used (Table 2). Mice were tested from the following groups: offspring of wild type control mice (WT, male: N=4, female: N=10), offspring of *Fmr1* knockout mice (KO, male: N=9, female: N=5), offspring of *Fmr1* knockout mice injected with 10 mg/kg Ganaxolone (KO GABA+, male: N=5, female: N=9), offspring of *Fmr1* knockout mice injected with 0.5 mg/kg Bay K 8644 (KO Ca+, male: N=5, female: N=2) and offspring of *Fmr1* knockout mice injected with both 10 mg/kg Ganaxolone and 0.5 mg/kg Bay K 8644 (KO GC+, male: N=5, female: N=13). Timed breeding was performed on KOs. Titration of Bay K 8644 was performed prior to the test. 1mg/kg and 0.8mg/kg Bay K 8644 induced severe seizures in the injected pregnant KO mice. 0.5mg/kg Bay K 8644 did not cause any seizure and were subsequently applied to the test animals. Pregnant KO mice were treated with 0.5 mg/kg Bay K 8644 at E15, 5 mg/kg Ganaxolone at E15 and E19, or 0.5 mg/kg Bay K 8644 with 5 mg/kg Ganaxolone at E15, followed by 5 mg/kg Ganaxolone at E19 respectively. Offspring mice were caged separately by sex, and genotyping was determined by PCR (Bakker et al., 1994) following weaning at postnatal day 21. Mice were behaviorally tested when they were 8 weeks old.

Immunohistochemistry

The cryosectioned brain slides were washed with phosphate-buffered saline (PBS; 0.01M, ph7.4) 3 times (10min each), permeabilized and blocked with 2% nonfat dry milk (NFDM) in PBS, containing 0.02% TritonX-100 and 10% Normal goat serum (NGS) for 1hr, and incubated overnight (at 4 ^{o}C) with the primary antibody after 3 washes with PBS(D'Ascenzo M et al., 2006). The *in vitro* expression of VGCCs and various scaffolding proteins was analyzed with the following antibodies (diluted in NFDM-PBS with 2%NGS): rabbit anti- VSCC β3 (1:500; Millipore Corp. MA, USA) and mouse anti-$GABA_A$ receptor β chain (1:500; Chemicon International Inc., CA, USA). The following day, the samples were blocked for 1hr with 10% NGS in NFDM-PBS, and then washed and incubated for 1 hr at room temperature with secondary antibodies diluted in NFDM-PBS with 2%NGS: fluorescein goat F(ab')2 anti-mouse IgG-FITC (1:250; Santa Cruz Biotechnology, Inc., CA, USA), Cy5-conjugated affinipure goat anti rabbit IgG (H+L) (1:250; Jackson ImmunoResearch laboratories, Inc., PA, USA). The nuclei were counterstained and finally cover-slipped with Prolong Gold antifade reagent with DAPI (Invitrogen, Eugene, OR, USA). All experiments were repeated independently at least three times.

Western blotting

The concentration of different protein extracts were measured by a BCA assay kit (SigmaeAldrich) using a microplate spectrophotometer (Spectra MAX 340PC; Molecular devices, Inc., CA, USA) and the isolated proteins from WT and KO brains are then subjected to SDS-PAGE. Western blotting was performed as described previously (Adusei DC et al., 2010). The following antibodies were used in this study: mouse anti- KCC2 (1:500; UCDavis, CA,

USA), mouse anti-β actin (1:10,000, Sigma, MO, USA), goat anti-mouse IgG, (H+L), peroxidase conjugated (1:5000; Thermo Scientific, IL, USA), goat anti-rabbit IgG, (H+L), peroxidase conjugated (1:5000; Thermo Scientific, IL, USA). All experiments were repeated independently at least three times.

Microwell cell culture of mouse cerebellum

Cerebellums from P7 WT and KO mice were cleanly isolate and were then transferred to sterile centrifuge tube containing 2ml calcium and magnesium free- physiological buffered solution (CMF-PBS). 5-minute incubation of cerebellums in 1ml 1% Trypsin-DNase with gentle rotation at room temperature was performed following the removal of the CMF-PBS. Cerebellar tissues were then thoroughly triturated in 1ml EG-0.05%DNase to remove excess DNA. Clumps of tissue were allowed to settle by sitting on ice for 2 minutes. The supernatant were centrifuged at 1000rpm for 5 minutes in the IEC Centra GP8R centrifuge (International Equipment Company, MA, USA). Cell pellets were throroughly dissolved in medium, and were plated at a density of $5x10^6$ cells/ml in the minimum essential medium (MEM) eagles serum. The primary cerebellar cultures were incubated in the napco 6300 CO_2 incubator (Thermo Electron Corp., MA, USA) at 37.5^oC, and with 5% CO_2 in the atomasphere. The following day N_2 medium were added to the culture medium to promote the growth of neurons instead of glial cells.

Cell culture treatments

Primary cerebellar cell cultures were obtained from P7 (postnatal day 7) WT mice and P5 KO mice. Both groups were cultured in N_2 supplement media to facilitate neuronal growth instead of glial cell growth, and were treated with 15mM KCl, 1μM nifedipine (Nif) prepared in DMSO (with final DMSO concentration in media lower than 0.1%), 10μM GABA prepared in PBS and both 10μM GABA and 1μM Nif respectively at 1 DIV (days *in vitro*) for 48 hrs (Tab. 3).

Table 3. Treatment for primary cerebellar granule cultures

Treatment group	**Chemicals administrated (48 hours)**	**Concentration**
Control (Ctrl)	/	/
Activation of the VSCCs	KCl	15mM
Blockade of the VSCCs	Nifedipine (Nif)	1μM
Activation of the $GABA_A$ receptors	GABA	10μM
Activation of the $GABA_A$ receptors & Blockade of the VSCCs	GABA + Nif	10μM+1μM

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT; 21-23 ^{o}C), washed with phosphate-buffered saline (PBS; 0.01M, ph7.4) 3 times (10min each), permeabilized and blocked with 2% nonfat dry milk (NFDM) in PBS, containing 0.02% TritonX-100 and 10% Normal goat serum (NGS) for 1hr, and incubated overnight (at 4 ^{o}C) with the primary antibody after 3 times washes with PBS(D'Ascenzo M et al., 2006). The in vitro

expression of VGCCs and various scaffolding proteins were analyzed with the following antibodies (diluted in NFDM-PBS with 2%NGS): rabbit anti-VSCC β3 (1:500; Chemicon International Inc., Temecula, CA, USA) and mouse anti-$GABA_A$ receptor β chain (1:500; Chemicon International Inc., Temecula, CA, USA). The following day, the samples were blocked for 1hr with 10% NGS in NFDM-PBS, and then washed and incubated for 1 hr at room temperature with secondary antibodies diluted in NFDM-PBS with 2%NGS: fluorescein goat F(ab')2 anti-mouse IgG-FITC (1:250; Santa Cruz Biotechnology, Inc., CA, USA), Cy5-conjugated affinipure goat anti rabbit IgG (H+L) (1:250; Jackson ImmunoResearch laboratories, Inc., PA, USA) . The nuclei were counterstained and finally cover-slipped with Prolong Gold antifade reagent with DAPI (Invitrogen, Eugene, OR, USA). All experiments are required to be repeated independently at least three times.

Behavior test:

Open field activity

At 8 weeks of age, mice were subjected to an open-field test. The mice were allowed to acclimate in a procedure room dimly lit by red lighting (100 Watts/30 Lux) for at least 20 min prior to the test. Each individual mouse was then transferred to the testing room and placed into the open field apparatus from the center of the arena. The open-field apparatus was a rectangular field (56 cm W × 39 cm L with 22.86 cm H) (Fig. 6A); none of the mice were familiar with the apparatus. A video camera was mounted above the box to record activity. Open-field behavior of each mouse was recorded for 10 min by the video camera positioned above the apparatus.

Lighting during testing was provided by two 15W fluorescent lights. The animals were tested during the dark phase.

Elevated plus-maze test

The mice were tested in the elevated plus-maze (EPM) after the open field test. The EPM used in this study comprised two opposing open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm), which joined at a square central area (5 × 5 cm) to form a plus sign (Fig. 7A). The maze floor was constructed of wood with a gray rubber covering and the side/end walls (15 cm height) of the enclosed arms of black painted wood. To reduce the likelihood of falling-over, a slightly raised edge (0.25 cm) around the perimeter of the open arms provided additional traction for the animals. The entire apparatus was elevated to a height of 45 cm above the floor by a single central support and four 25-W white fluorescent lights arranged as a cross at 100 cm above the maze were used as the source of illumination (Chen et al., 2001). Experimental procedures were the same as described above. During each test, an individual mouse was placed on the central platform of the maze facing an open arm. Its EPM exploratory behavior on the plus-maze was recorded for 10 min by a vertically mounted video camera linked to a monitor and video recorder in an adjacent computer. After each trial, the mouse was returned to its home cage and the maze was cleaned with 70% ETOH to remove any trace of olfactory cues.

Social interaction test

The mice were then tested for the social interaction task. The social interaction apparatus consisted of one black box and two clear boxes lined up on the counter top with holes on the sides and lids on the tops (Fig. 8A). The large holes between the black box and the middle clear box were exactly aligned together, allowing mice to enter each box freely. The smaller holes

between the two clear boxes were shifted to prevent mice crossing over. A black board was placed behind the boxes and against the wall to increase contrast. Any objects that can be perceived as visual distracters were moved off the counter top surrounding the clear boxes. The room was dimly lit with 6 Lux lights facing upwards towards the ceiling. A camera was set up on tripod across from the counter top, to provide a video recording as a side tri-pod view. Mice were tested individually. Experimental procedures were the same as described above. Each mouse was subjected to 2 sets of tests. **Set 1:** the test mouse was placed in the middle clear box and allowed recorded for 10 min. **Set 2:** a stranger mouse was placed into the side clear box, while the test mouse remained in the middle clear box, and was recorded for 10 min.

Acoustic startle response test

Mice were tested with equipment from MED Associates, Inc. (St. Albans, VT) for the acoustic startle response test. The foam-lined sound-attenuated chamber (ENV-022S) was equipped with a Pro-Series Startle Platform (a movement-sensitive loading cell platform), a transducer and amplifier (PHM-250B). All audio stimuli and background noise were white noise. Decibel levels were verified by a Quest Technologies Impulse Sound Level Meter Model 2700 (Oconomowoc, WI). A fan and a red light were provided inside the chamber for the comfort of the animal.

On the day of testing, a 3.2cm round acrylic restrainer ENV-263 A (Med Associates, Inc. St. Albans, VT) was secured on top of the loading cell platform within the sound attenuated test chamber allowing adequate space and sound diffusion for the animals. Experimental procedures were the same as described above. All mice were tested individually. Mice remained in the test chamber for the duration of the test. Each mouse received a 5-min acclimation in the acrylic restrainer prior to the testing with the box fan and red light turned on, where no acoustic startle

stimuli were presented. Background noise was maintained at 65dB throughout testing. Mice were subjected to 3 Blocks after the 5-min acclimation in each test. **Block 1:** Null period 100ms followed by a startle stimulus 115dB for 4 trials with 40ms duration, a 1ms rise/fall time, and a 5-25sec inter-trial-interval. Variable interstimulus intervals may aid in the prevention of habituation to a particular stimulus. **Block 2:** this block consisted of 16 trials with a 5-25sec inter-trial-interval. Null period 100ms followed by a pre-pulse of 75 dB, 85dB, 95 dB or 105 dB 20ms in duration, followed by a startle stimulus 115dB with 40ms duration, a 1ms rise/fall time. The various trial types were presented 4 times each and constituted a 4 (75 dB, 85dB, 95 dB or 105 dB prepulse) X 4 (115dB startle stimulus) random array. **Block 3:** exact replication of block 1.

Contextual and cued fear conditioning test

The mice were subjected to the contextual and cued fear conditioning test at the end of all the behavior tests to test their learning and memory levels. The context fear chamber apparatus (FRZ 123485 Freeze Monitor, San Diego Instruments, Inc., CA, USA) has 3 infrared sensors on each side that permit freezing records by measuring the latency to break the infrared beams every 10s. The data were transduced on line during testing using Freeze Monitoring Software MED-PC-IV®. At 8 weeks of age, mice were subjected to the test. Mice were allowed to acclimate in a procedureroom dimly lit by red lighting (100 Watts/30 Lux) for at least 20 min prior to the test. The contextual and cued fear conditioned stimuli in the testing paradigm were adapted from Wehner and Radcliffe (2001) (Wehner and Radcliffe, 2001) and are as follows: (a) **Day 1 Acquisition Phase:** 120s acclimation, followed by a tone emitted for 30s duration, after 10s of the tone onset the mice were presented with a light that was illuminated for 10s then shut off and finally during the last 2s of the tone presentation a 0.5mA floor grid shock was given for 5s in

duration as the unconditioned aversive stimulus. Prior to and following the delivery of the shock, mice latencies to break 3 infrared beams were measured every 10s for 120s followed by a 70s inter-trial-interval. 10 trials were presented during day 1 which was considered the learning acquisition phase. (b) **Day 2 Retention Phase:** Identical testing procedures were administered as in day 1 except that all trials were presented without shock and 20 trials were presented during day 2. (c) **Day 3 Altered Context Phase:** In order to assess the cued specific learning in separation of the contextual environment in which training occurred, alterations were experimentally manipulated to evaluate fear generalization between contextual and altered (i.e. novel) environments without tone and then with the conditioned tone presentation. The floor grids were covered with a smooth black rubber matt and a black plexi-glass diagonal divider was inserted into the chamber to separate the chamber into two equal triangular compartments. Opposite of the side in which the mice were tested an inaccessible petri dish was placed containing vanilla extract to increase exploratory locomotor behavior in the mice as an internal positive test control in the altered environment.

Behavior test Data analysis

The open field, EPM and social interaction data were recorded by a video camera as digital video clips using an analog-digital converter. The movies were analyzed using AnyMaze® software. Tracking of the animal was based on contrast relative to background. Different zones were labeled and indicated on the monitor. Two tracking points were specified: one the head and the other the center of gravity of the animal. An excel spreadsheet was generated containing all the parameters specified.

The startle response data was analyzed with the SOF-825 Startle Reflex Software (Med Associates, Inc. St. Albans, VT). Programming was set for pre-pulse inhibition (PPI) analyses. Data detection was set for startle response only at a minimum latency of 5ms, first peak minimum value of 10 arbitrary units (both PP and startle period), and peak data points with a minimum time of 10ms. An excel spreadsheet was generated containing all the parameters specified.

The contextual and cued fear conditioning data were transduced during testing with Freeze Monitoring Software MED-PC-IV®. An excel spreadsheet was generated containing all the parameters specified. The mean latency to freeze value of the control group during the first 10s of experimentation was used as a baseline measure. Data from all treatment groups, within and between, were normalized against the baseline value using the following formula: (animal latency to freeze raw data following exposure of auditory cue with foot shock * 100 / control mean value of first 10s exposure to context = % time freezing difference from baseline). This formula was computed for all comparisons of baseline versus all experimental manipulations across the 3 day testing paradigm for relative comparisons of auditory cued fear conditioning. Day 2 retention data were compared against the trial 4 data from day 1. Retention data comparisons were made by normalizing day 1 trial 4 data against day 2 retention data using the following formula: (animal latency to freeze raw data from day 2 trial 1 following exposure of auditory cue without foot shock * 100 / control mean value of first 10s following exposure of auditory cue with foot shock from day 1 trial 4 = % time freezing difference of auditory cued conditioned retention from acquisition). Day 3 altered context data were compared against itself in the presence or absence of a fear conditioned auditory cue. Altered context data comparisons

were made by normalizing day 3 altered context data against day 3 altered context with fear conditioned auditory cue data using the following formula: (animal latency to freeze raw data from day 3 trial 1 following exposure of an auditory cue in altered context * 100 / control mean value of first 10s exposure to altered context = % time freezing difference from baseline).

Statistical analysis

All data was post analyzed offline and statistics were computed in Statistica V 6.1 (Statsoft, Inc. Tulsa, OK). Data were analyzed with one-way ANOVA. Post-hoc comparisons were made Fisher test, Scheffé test and Tukey HSD for unequal N. Significance was set at a confidence level of 95%. Data are presented as mean ± SEM.

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