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Forebrain GABAergic interneuron connectivity and BDNF signaling deficits in Engrailed-2 knockout (En2-/-) mice, a mouse model for autism spectrum disorder

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ABSTRACT

Autism spectrum disorders (ASD) comprise a genetically heterogeneous group of neurodevelopmental disabilities characterized by repetitive behaviors as well as deficits in communication and social/emotional interactions and behaviors.

Defects in GABA transmission have been hypothesized to underlie the symptoms of ASD (Ben-Ari, Khalilov, Kahle, & Cherubini, 2012). *Engrailed-2* (En2) is a homeodomain transcription factor involved in regionalization and patterning of the midbrain and hindbrain regions (Joyner, 1996; Gherbassi and Simon, 2006). *En2* is expressed in the developing and adult mouse midbrain/hindbrain region (Joyner, 1996), as well as the adult mouse forebrain (Tripathi et al., 2009). Genome wide association studies have shown that two intronic single-nucleotide polymorphisms in the human *En2* gene are significantly associated with ASD (Benayed et al., 2009). *En2-/-* mice lack the homeodomain of *En2* (En2hd/hd mice; Joyner et al., 1991, referred to as *En2-/-*) and display neuropathological and behavioral changes relevant to ASD. We first investigated the distribution of different interneuron subtypes in the cerebral cortex and hippocampus of En2-/- mice. Results showed a significant loss of parvalbumin, somatostatin and neuropetide Y positive interneurons, in both regions of En2-/- brains.

Brain-derived neurotrophic factor (BDNF) is a crucial factor for the postnatal development of forebrain GABAergic neurons, namely PV interneurons. We therefore investigated whether altered BDNF expression may be related to the altered development of GABAergic interneurons, described in *En2-/-* mice. Considering the important role of BDNF in forebrain GABAergic interneuron development, we decided to determine whether interneuron loss in *En2-/-* forebrain might be related to altered expression of BDNF and its signaling receptors. We evaluated the expression of different BDNF mRNA and protein isoforms in various brain areas from wild-type (WT) and *En2-/-* mice. Quantitative RT-PCR indicated an alteration of various splicing variants of BDNF mRNA in the cerebral cortex but not hippocampus and cerebellum of adult *En2-/-* mice, as compared to WT controls. Immunoblot analyses instead revealed increased levels of mature BDNF and reduced levels of truncated- and pro-BDNF isoforms in the hippocampus of *En2-/-* vs. WT mice. These data indicate a role of BDNF in the anatomical phenotype of *En2-/-* mice, suggesting that BDNF might be involved in the developmental defects of the GABAergic system in this mouse model of ASD. We next investigated the effect of BDNF on GABAergic and glutamatergic neuron connectivity using primary cultures of hippocampal neurons from WT and En2-/- mice. Results showed that BDNF is able to increase the number of GABAergic and glutamatergic synaptic contacts in En2-/- hippocampal neurons.

Taken together, our results suggest a beneficial role of BDNF in rescuing synaptic defects in *En2-/-* mice

LIST OF ABBREVIATIONS

AIS: Axon initial segment

ASD: Autism Spectrum Disorder

BDNF: Brain derived Neurotrophic Factor

CA1: Cornus ammoni 1

CA3: Cornus ammoni 3

CaMKII: Ca-calmoduline kinases II

cAMP: Adenosine 3'5' Cyclic Monophosphate

CB: Calbindin

CCK: cholecystokinin

ChC: Chandelier Cell

CNS: central nervous system

CNV: copy number variants

CR: Calretinin

CREB: cAMP-response-element binding protein

DAG: Diacyl glycerol

DG: Dentate gyrus

DIV: Days In Vitro

DSM-V: Diagnostic and Statistical Manual of Mental Disorders

EN: Engrailed

ER: Endosplamic Reticulum

FMRP: Fragile Mental Retardation protein

FXS: Fragile X Syndrome

GABA: *γ*-aminobutyric acid

$GABA^{A/B/C}$: γ-aminobutyric acid receptors type A/B/C

GAD: glutamic acid decarboxylase

GAT: GABA transporter

GFP: Green Fluorescence Protein

GWA: Genome-Wide Association

HRP: horse radish peroxidase

LGE: Lateral Ganglionic Eminence

MAP2: Microtubule associated protein-2

MGE: Medial Ganglionic Eminence

mRNA: messenger Ribonucleic Acid

MAPK: mitogen-activated kinase

MB: Multipolar Bursting

MECP2: Methyl CpG binding protein 2

mRNP: messenger ribonucleoparticles

NF1: Neurofibromatosis 1

NKCC: Na-K-Cl cotransporter

NLGN: Neuroligin

NPY: neuropeptide Y

NRXN: Neurexin

NT: Neurotrophin

NTR: Neurotrophin receptor

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PTEN: Phosphatase and Tensin homolog

p75NTR: p75 Neurotrophin Receptor

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PPD-NOS: Pervasive Developmental Disorder Otherwise Specified

PV: Parvalbumin

RELN: Reelin

RT: Room Temperature

RTT: Rett Syndrome

SST: Somatostatin

TGN: Trans-golgi network

Trk: Tropomiosin receptor kinase

vGAT: vescicular GABA Transporter

vGlut: vescicular Glutamate Transporter

VIAAT: Vesicular Inhibitory amino acid transporter

VIP: vasoactive intestinal peptide

WT: Wild Type

1. INTRODUCTION

1.1 Autism spectrum disorders

Autism spectrum disorders (ASD) comprise a highly heterogeneous set of related neurodevelopmental disorders classified as part of Pervasive Developmental Disorders (PDDs) in the *Diagnostic and Statistical Manual of Mental Disorders*, fifth edition, published in 2013. In addition to autism, in DSM-V, have been included disorders that were previously diagnosed separately, such as autistic disorder, Asperger's disorder, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified.

ASD are characterized by some common aspects, such as impairment in reciprocal social interaction, marked deficits in verbal and nonverbal communication, restricted, repetitive and stereotyped interests and behaviors (DiCicco-Bloom et al., 2006). Autistic symptoms are typically noticed in the first or second year of life: clinical diagnosis is made at about two years of age, because of the necessity to assess motor and communication skills of the children. By the age of three, deficits characterizing ASD are generally recognizable (Miles, 2011). Manifestations of these disorders consist in delay or abnormality in language and play, repetitive behaviors, unusual interest.

Recent studies demonstrated that ASD are much more common than previously believed (Payakachat et al., 2012). Before 1982, the estimated prevalence of autism was 4.5 per 10.000 according to Kanner's criteria (Lotter, 1966) (Fombonne, 1999); subsequently, DSM-IV studies found proportions of autistic disorder at 15 to 40 per 10.000 (Wing & Potter, 2002). Other studies revealed a prevalence of 70 to 113 per 10.000, or about 1% of the population (Fombonne et al., 2009). In 2013, Blumberg and colleagues showed parent-reported rates of 2% of children (Blumberg et al., 2013). The mode of inheritance seems to be complex and boys being affected more frequently than girls (Crawley et al., 2004)(Llaneza et al., 2010). ASD appear to be a syndrome of complex genetic traits and during pregnancy different exposures to allergenic environmental factors, autoimmune problems, viral infections, maternal anticonvulsants (valproic acid) may be implicated in the etiology of ASD (London, 2000) (Trottier at al.,1999)(Arndt, et al., 2004). Even premature birth has been implicated as a cause of ASD, therefore, it has been demonstrated that babies from gestations of less than 28 weeks have a high risk of neurological problems (Agnes Cristina Fett-Conteal et al., 2013). Etiology is not well known and in a minority of cases (less than 10%) autism is part of another condition: cases include tuberous sclerosis, Fragile X syndrome (FXS), phenylketonuria and congenital infections secondary to rubella and cytomegalovirus (Faras, et al., 2010).

ASD is diagnosed when the characteristics deficits of social communications are complemented to repetitive behavior and restricted interests: it has been well-defined that genetic conditions could give rise to distinct patterns of autistic disease (Bruining et al., 2014). Patterns of autistic symptomatology can be associated with specific genetic disorders: different studies have indicated that a specific disorder referred to specific behavioral phenotype (Hall et al.,2010)(Pride et al.,2012) (Bruining et al., 2014). Several well-described single-gene disorders have been reported in ASD, as a part of the expanded phenotype associated with changes in that gene (Agnes Cristina Fett-Conte, 2013).

Different genetic diseases are associated with ASD, such as Rett syndrome, Asperger Syndrome, tuberous sclerosis and Fragile X syndrome. Sakai and colleagues evaluated the association of identified genes with ASD and recognized the molecular clusters associated with this syndrome (Neale et al., 2012). They discovered that two autism associated protein, SHANK and NLG3, may interact with protein associated to ASD (Sakai et al., 2011)(Baudouin, 2014). ASD is a constellation of monogenic disorders that converge to a narrower set of behavioral symptoms (Baudouin, 2014). The regulation of clusters of genes involved in the cortical development has been studied by Voineagu and colleagues, which discovered a difference in the gene expression in ASD patients (Voineagu et al., 2011). They identified a group of genes correlated with synaptic function and neuronal signaling involved in development (Baudouin, 2014). About 500 genes have been associated with diverse forms of ASD, each of them have been reported in a small fraction of the cases of ASD (Baudouin, 2014)(Gilman et al., 2011).

1.1.1 Genetics of autism

Autism is a common and heritable neuropsychiatric disorder that can be divided into two types of pathology: syndromic autism (associated with a known genetic syndrome) and non-syndromic autism (Miles et al., 2011) (Singh & Eroglu, 2013), which comprises a vast majority of autism cases (Geschwind et al., 2011). About 10%-20% of individuals with an ASD have an identified genetic etiology. Chromosomal alterations have been reported in ~5% of cases copy number variants (CNVs) and single-gene disorders (~5%)(Gilman et al., 2011)(Muhle et al., 2004)(Neale et al., 2012). Syndromic ASD genes are defined as those genes predisposing to autism in the context of a syndromic disorder. The distinction between syndromic and non-syndromic terminology is not precise, and several genes, initially identified in syndromic conditions, were later reported in subjects with non-syndromic forms (Betancurlet et al., 2011) (Betancur et al., 2009). Monogenic disorders

described in ASD include Neurofibromatosis *(NF1),* Angelman syndrome *(UBE3A), SHANK3* alteration*,* Rett Syndrome *(MECP2)* and PTEN mutations in patients with macrocephaly and autism (Berkel et al., 2010)(Durand et al., 2007) (Geschwind & Levitt, 2007) (Hatton et al., 2006) (Veenstra-Vanderweele et al., 2004). In addition, genome wide association studies revealed mutations in DLX, Reelin and Engrailed genes, resulting in autism phenotypes and neuropathology (Gadad et al., 2013). Disorders known for their high comorbidity with ASD include Fragile X syndrome (*FMR1*), the most common single gene mutation account to autism, present in ~ 2% of cases (Betancur et al., 2011).
Rare mutations have been identified in synaptic genes, including *NLGN3* and *NLGN4X* (Jamain et al., 2003): Neurexins and Neuroligins are synaptic cell-adhesion molecules that connect pre- and postsynaptic neurons at synapses (Laumonnier et al., 2004). Four Neuroligin isoforms have been identified in mice and five in humans (Südhof, 2008). Series of mutations have been found in *NLGN1, 3* and *4* genes in patients with familial ASD, which primarily lead to a decrease or an absence of protein expression (Jamain et al., 2003) (Südhof et al., 2008). Non-syndromic disease is not correlated to other neurological syndromes and is heritable: loss-of-function and gain-of-function mutations in *NLGN3* and *NLGN4* genes are linked to class of autism (Betancur, 2011) (Singh et al., 2013). In line with this, only NLGN3 and NLGN4 mouse models present autism-related behavioral phenotypes (Tabuchi et al., 2007).

Another important disorder recognized to have an increased risk in autistic population is neurofibromatosis (Gillberg & Forsell, 1984)(Caglayan, 2010): 5%–10% of patients with neurofibromatosis 1 (NF1) have a 17q11 deletion involving *NF1* and other genes (Betancur, 2011) (Tonsgard et al.,1997). The *NF1* gene encodes for a protein called neurofibromin, a GTPase activator involved in the regulation of the Ras/ERK signaling (Martin et al., 1990). The NF1 microdeletion syndrome is characterized by a more severe phenotype than that observed in patients with intragenic *NF1* mutations (Caglayan et al., 2010).

Autism is considered part of the behavioral phenotype found in Angelman syndrome (AS) (Bonati et al., 2007). This syndrome presents genetic alterations, in detail: deletion on the maternal chromosome 15 (70%), paternal chromosome 15 uniparental disomy (UPD; 2%) point mutations of the *UBE3A* gene (10%) and epimutations. No identifiable molecular abnormality is found in the remaining AS patients (10–13%) (Bonati et al., 2007). The most common chromosomal rearrangement is the maternal duplication of 15q11-q13, which accounts for approximately 1-2% of ASD cases (Bonati et al., 2007) (Kishino et al., 1997).

ASD or autistic features are associated with a wide mutation spectrum, that includes missense, frameshift, nonsense, and intragenic deletions (Moretti et al., 2006). For example, *MECP2* mutations or deletions cause Rett syndrome in females, and congenital encephalopathy (Herman et al., 2007). The *MECP2* gene is located in Xq28 and is subject to X-chromosome inactivation (Young et al., 2004). It is a member of a family of proteins that bind regions of DNA enriched with methylated CpG regions; human *MECP2* gene has four exons which generate by alternative splicing two protein isoforms, differing in their N-termini (Kriaucionis et al., 2004). Duplication of *MECP2* lead to the Xq28 duplication syndrome, that is mostly reported in males (females are protected by X inactivation) and is often associated with ASD or autistic features (Ramocki et al., 2009) (Betancur et al., 2011).

A particularly intriguing form of autism is a *PTEN* (phosphatase and tensin homolog) macrocephaly syndrome (Butler et al., 2005) (Miles et al., 2011). The frequency of *PTEN* mutations in children with ASD and macrocephaly is unknown; in one study, 15% (4/26) of children with *PTEN* mutations had ASD (Betancur et al., 2011)(McBride et al., 2010). More recently, heterozygous *PTEN* gene mutations have been identified in a subset of individuals with autism and macrocephaly (Butler et al., 2005). PTEN is a major lipid 3-phosphatase, which signals down the PI3 kinase/AKT pro-apoptotic pathway PTEN acts dephosphorylating both serine and threonine residues and regulating various cell-survival pathways, such as the mitogen-activated kinase (MAPK) pathway (Waite et al., 2002). The protein-phosphatase/MAPK pathway is important in the mediation of growth arrest and other crucial cellular functions.

Phelan-McDermid syndrome (PMS) is associated to ASD and schizophrenia and is characterized by severe intellectual disability (ID) and poor language (Guilmatre et al., 2014). In this syndrome a deletion of *SHANK3 (*22q13 deletion) has been described (Berkel et al., 2010)(Durand et al., 2007) (Miles, 2011). Studies demonstrated that SHANK3 mutations and the small cytogenetic rearrangements have been implicated with an ASD phenotype (Durand et al., 2007).

The most common single gene mutation in ASD is FMR1 associated with FXS, present in ~2% of cases. FMRP gene, located on chromosome Xq27-3, is expressed in brain tissue, with the highest concentrations in the granular layers of the hippocampus and the cerebellum (Abrams et al., 1995). FXS is caused by the expansion of the CGG trinucleotide repeat in the 5' untranslated region of the *FMR1* gene, which activate the hypermethylation inducing partial or complete gene silencing. This alteration leads to

partial or complete lack of the gene which product fragile X mental retardation protein (FMRP) (Agnes Cristina Fett-Conte, 2013). The *FMR1* gene encodes for FMRP (Bassell et al., 2008) (De Rubeis et al., 2012), an RNA-binding protein that contributes to the post-transcriptional control of gene expression. In neurons, FMRP is part of messenger ribonucleoparticles (mRNPs) and its functions are multiple, such as, role in the RNA metabolism, dendritic targeting of mRNAs, protein synthesis and in particular, regulates dendritic transport of associated mRNAs, their stability and local translation (Darnell & Richter, 2012)(De Rubeis et al., 2012).

Examples of genes with suggestive evidence of ASD association are *RELN and EN2*. The *RELN* gene codes for the extracellular matrix glycoprotein Reelin, which is involved in neuronal migration and lamination of the cerebral cortex during embryogenesis. *RELN* maps to 7q22 human chromosome, a region linked to ASD. Genetic and molecular studies showed that Reelin messenger-RNA and its protein are downregulated in cortical, hippocampal, and cerebellar neurons of patients affecting by schizophrenia and autism (Guidotti et al., 2000)(Fatemi et al., 2001). EN2 codes for the homeobox-containing transcription factor Engrailed-2, a key regulator of posterior brain embryonic development. The human *En2* gene maps to a region of chromosome 7 implicated in ASD susceptibility and genome-wide association (GWA) indicated *En2* as a candidate gene for ASD (Joyner et al., 1991) (Gharani et al., 2004).

1.1.2 Neuroanatomical abnormalities in ASD

Neuropathological changes associated to the development of ASD symptoms have been shown in several brain areas, however a clear consensus on the exact areas has not been reached and several aspects remain open to investigation. Several studies in post mortem brain patients, showed abnormal brain growth and white matter abnormalities, which led to consider functional connectivity in areas involved in the development of social, communication and motor abilities (Baribeau et al., 2013) (Mostofsky et al., 2009). In details, neuroimaging studies have shown altered pattern of brain overgrowth in areas of the frontal lobe, cerebellum and limbic structures and a volume loss in the corpus callosum and cingulum (Baribeau et al., 2013). Investigations have shown also a decreases in both cerebellar gray and white matters regions (Baribeau et al., 2013) (Gadad et al., 2013).
Kemper and Bauman have described three different types of pathological abnormalities in autism: alteration in the development of neurons in the forebrain limbic system, a decrease in the cerebellar Purkinje cell population and age-related changes in neuronal size and number in the cerebellar nuclei and the inferior olive (Kemper et al., 1998) (Romero-

Munguía, 2011). Several studies reported a significant data on the anatomy alteration in ASD. These variations regard the increase of volumes of the caudate, frontal lobe, temporal lobe and cerebellum. Purkinje cells loss and atrophy in fetal period have been shown predominantly in the posterolateral neocerebellar cortex (Schmahmann et al., 2004). Loss of Purkinje neurons is correlated with dysfunctions in GABA neurotransmission (the main inhibitory neurotransmitter in the brain): Purkinje cells release GABA and alterations of these cells could be related to the increase in GAD67 mRNA levels in cerebellar interneurons ASD patients (Yip et al.,2008). All these abnormalities are part of some important aspect during development, such as, neuron migration, axodendritic outgrowth, synaptogenesis, and pruning (DiCicco-Bloom et al., 2006). These observations suggest that delays in neuronal maturation is important in the pathogenesis of autism (Pardo et al., 2007)(Minshew et al., 2007).

Anatomical studies have also shown enlargement of the amygdala in autistic children, a phenotype associated with the severity of the social communication deficits and elevated anxiety (Haznedar et al., 2000). Neuropathological analysis of the limbic system in autism have found decreased neuronal size, increased neuronal packing density, and decreased complexity of dendritic arbors in the hippocampus, amygdala, and other limbic structures (Schumann et al., 2009)(Gadad et al., 2013). Overall, consistent evidence confirms that anatomical abnormalities in amygdala and cortical structure may lead to deficits in dendritic spine reorganization and consolidation (Gadad et al., 2013). Abnormal spine generation or deficits in spine reorganization, elimination, and pruning are anatomical traits involved in the pathology of autism (Hutsler et al., 2010).

1.2 GABAergic system

The major inhibitory neurotransmitter in the adult central nervous system (CNS) of vertebrates is *γ*-aminobutyric acid (GABA). GABA plays a central role in controlling several aspects of neuronal development and communications. GABA does not penetrate the blood brain barrier (BBB) and it is synthesized directly in the brain from glutamate by the glutamic acid decarboxylase enzyme (GAD). In the cerebral cortex, roughly 80% of the neurons are excitatory glutamatergic neurons and 20% are inhibitory GABAergic neurons. GABAergic neurons contribute to local circuit properties, whereas the majority has long-range inhibitory projections to other subcortical nuclei or cortical areas (Markram et al., 2004).

There are two main enzyme isoforms, GAD65 and GAD67 (Briggs et al., 2011): GAD65 is involved in synaptic transmission and it is the major GABA synthetic enzyme in mouse

brains. GAD67 is involved in the synthesis of GABA for general metabolic activity (Gogolla et al., 2009)(Chattopadhyaya et al., 2007). Levels of GAD expression can be modulated post-transcriptionally by GABA: moreover, glutamate can stimulate carrier mediated non-vesicular GABA release via upregulation of GAD67 (Soghomonian et al., 1998).

In the central nervous system, GABA is produced and released by inhibitory interneurons: these interneurons are defined on the basis of their morphology, electrical activity and connectivity (Ascoli et al., 2008). The different interneurons subpopulations are classified in: neurons expressing calcium binding proteins such as parvalbumin (PV), calbindin (CB), or calretinin (CR) and/or neuropeptides such as somatostatin (SST), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), or cholecystokinin (CCK) (Kelsom et al., 2013)(Chattopadhyaya et al., 2012a) with distinct electrophysiological and functional properties (De Marco García et al., 2011). Purkinje cells are a distinct set of GABAergic cells, present in the cerebellum. Cortical GABAergic neurons are generated in the progenitor zones of the basal ganglia, the lateral and medial ganglionic eminences (LGE, MGE)(Anderson et al., 2001) and from here they migrate in every district of the brain. In postnatal stages, GABA is expressed in developing cortex and the migration of these cells has been found in multiple forebrain regions (Marín et al., 2003).

All cortical regions, including the olfactory bulb, olfactory cortex, cortex and hippocampus are populated by the GABAergic neurons originating from the basal telencephalon (Anderson et al., 2001). Glutamatergic neurons innervate both principal cells and GABAergic interneurons, whereas GABAergic cells project to major extra hippocampal target and multiples areas, playing a vital role in modulation of the activity of principal cells (Jinno et al., 2007) (Jinno et al., 2009). Vesicular inhibitory amino acid transporter (VIAAT) load GABA in synaptic vesicles: following release, GABA transporter GAT1 reuptake extracellular GABA into the presynaptic interneurons (lesser related GAT2 and GAT3 transporters). Following reuptake the breakdown of GABA into glutamate is performed by GABA transaminase (GABA-T). Genes coding for GABA receptor subunits are site for mutations in ASDs (reviewed in Sgadò et al., 2011). GABA activates three major classes of receptors, $GABA_A$, $GABA_B$ and $GABA_C$ receptors, which have different characteristics. $GABA_A$ and $GABA_C$ receptors are ionotropic, while the $GABA_B$ receptors are metabotropic. The $GABA_A$ and $GABA_C$ receptors are Cl- channels that mediate fast synaptic inhibition (Chebib et al., 1999). These receptors are members of a superfamily of transmitter-gated ion channels composed of five subunits that arrange together to form an ion channel. Each subunit has four transmembrane domains. All five subunits arrange forming the wall of the channel pore with their transmembrane domains. Whereas, $GABA_B$ metabotropic receptors are seven transmembrane receptors that activate a second messenger systems mediated by phospholipase C and adenylate cyclase and activate K+ and Ca2+ ion

channels. These receptors produce slow, prolonged inhibitory signals and function to modulate the release of neurotransmitters (Chebib et al., 1999).

During the embryonic period, $GABA_A$ depolarizes the target cells (i.e., excitation) due to a relatively high concentration of intracellular Cl^-; this characteristic is due to low expression of the K-Cl cotransporter KCC2 compared to the expression of the Na-K-2Cl cotransporter NKCC1 in immature neurons. Activation of GABA-gated Cl^- channels receptors by GABA ($GABA_A$ receptors) causes the depolarization of the target postsynaptic neurons and a GABA-mediated calcium influx that increases the intracellular Ca^+ concentration (Cellot et al., 2013) (Pizzarelli et al., 2011). GABA excitation may be important for plasticity, synaptic connections, and for establishing neural networks.

During maturation, GABA acts in a opposite effect: Cl^- ions are pumped out of maturing neurons, such that the binding of GABA to its $GABA_A$ receptors cause the hyperpolarization of the membrane, therefore GABA switch during development from an excitatory to an inhibitory neurotransmission (Ben-Ari et al., 2002) (Schmitz et al., 2005). GABA regulates a variety of different developmental processes from cell proliferation migration, differentiation, synapse maturation, and cell death (Pizzarelli et al., 2011).

The balance of excitation to inhibition is controlled by the regulated activities of glutamatergic and GABAergic neurons: activity levels are regulated by extrinsic excitatory (glutamatergic afferents), mixed excitatory/inhibitory (serotonin, norepinephrine, dopamine and acetylcholine afferents from the hindbrain, midbrain and basal forebrain, respectively) and GABA inhibitory inputs (Rubenstein et al., 2003).

An important property of GABAergic innervation is the control of subcellular organization of glutamatergic synapses (Huang et al., 2007): alteration in GABAergic synapses may affect cortical processing and plasticity. Interneuron subtypes provide specific functions in the innervation of distinct domains of the postsynaptic glutamatergic cells (Huang et al., 2007).

Several studies showed that impaired maturation of the GABAergic circuitry results in an immature structure and function of the cerebral cortex that remains more plastic in particular in the modulation sensory inputs. An immature structure and function of the cerebral cortex is considered a major feature of neurodevelopmental disorders (Sgadò et al.,2011). Indeed, disruption of the balance of neural circuits is a cause of neuropsychiatric disorders, such as epilepsy, autism spectrum disorders, and intellectual disabilities (Kelsom et al., 2013). Disturbances in the development of GABAergic interneurons by genetic or epigenetic factors might alter the assembly or plasticity of neural circuits and lead to aberrant information processing (Huang et al., 2007).

Recent studies have found new strategies and new approaches, which could reestablish cortical plasticity by manipulating GABA signaling in adult patients with

neurodevelopmental disorders (Le Magueresse et al., 2013). Pharmacological tools based on the identification of altered molecular pathways are the starting point of these studies.

1.2.1 Specification of GABAergic interneuron subtypes

GABAergic neurotransmission contributes to the development of the brain from early embryonic stage to adulthood, mediating the shaping and functional maturation of cortical networks (Le Magueresse et al., 2013). The site of origin of glutamatergic and GABAergic cells is different, whereas the generation of interneurons overlaps with the generation of excitatory neurons (Le Magueresse et al., 2013). Murine pyramidal cells originate in the ventricular zone of the dorsal telencephalon from E11 to E17, whereas interneurons derived from the ganglionic eminences of the ventral telencephalon from E11 to E15 (Mitsuhashi et al., 2009). GABA immunoreactivity exists at E14 in the cortical plate but exhibits its mature pattern only postnatally; the pattern of GABA immunoreactivity is similar to that of the adult brain around P16–P21 time (Le Magueresse et al., 2013).The maturation of the GABAergic circuitry starts earlier than the maturation of the glutamatergic system, and continues for a longer period, proceeding in distinct steps.

GABAergic neurons have different spatial and temporal origins and belong to three groups defined by the expression of parvalbumin (PV), somatostatin (SST), and the ionotropic serotonin receptor 5HT3a (5HT3aR) (Rossignol et al., 2011)(Rudy et al.,2011). Interneuron specialization also arises from their ability to innervate specific subcellular regions of their target cells.

Parvalbumin interneurons (PV)

There are two anatomically distinct subgroups of PV neurons: basket cells and chandelier cells and these cells has been associated with the fast-spiking (FS) firing pattern (Rudy et al., 2011). Multipolar bursting (MB) neurons define a possible subgroup of non-FS, PV-expressing neurons (Rossignol, 2011) (Kelsom et al., 2013). Within the CA1 region of the hippocampal formation, basket cells and chandelier cells make up approximately 75% of the total interneuron population (Szilágyi et al., 2011). PV cells play an important role in regulating the delicate balance between excitatory and inhibitory inputs in the cerebral cortex (Kelsom et al., 2013). Basket cells selectively innervate the soma and proximal dendrites, whereas, chandelier cells the axon initial segment of pyramidal cells (Huang et al., 2007).

Basket cells have been involved in two important roles: the initiation of a critical period for cortical plasticity and the generation and synchronization of gamma (γ)-oscillations in the hippocampus and neocortex (Gogolla et al., 2009). Both phenomena have been suggested

to be impaired in the cognitive developmental disorders, such as ASD (Gogolla et al., 2009). Plasticity and cortical connections in autism disorder may be impaired by disruption of PV cell, indeed, different studies demonstrate that PV interneurons have a role in the etiology of autism spectrum disorder and other neurodevelopmental disorder during development (Chattopadhyaya et al., 2012)(Le Magueresse et al., 2013) (Sgadò et al., 2011). It is crucial to study the physiological morphological maturation of PV positive interneurons in animal models of ASDs (Le Magueresse et al., 2013).

During late gestation, Chandelier cells (ChCs) derive from the ventral germinal zone of the lateral ventricle and require the homeodomain protein Nkx2.1 for their specification (Taniguchi et al., 2013). Both basket cells and chandelier cells are fast-spiking, but they differ in electrophysiological properties: at present, the function of chandelier cells is controversial (Kelsom et al., 2013) (Woodruff et al., 2009). ChCs are characterized morphologically by their cartridges forming synapses on the axon initial segment (AIS) of pyramidal cells. A single ChC innervates hundreds of pyramidal cells and may exert influential control over the spiking of a pyramidal cells (Taniguchi et al., 2013). PV-cells form numerous synapses onto the somata of pyramidal cells, which in turn enrich these sites with $GABA_A$ receptors and this makes PV-cells perfectly situated to detect changes in sensory input, to regulate the spiking of excitatory pyramidal cells, and to synchronize brain regions.

Somatostatin interneurons (SOM)

Somatostatin interneurons originate from the medial ganglion eminence (MGE) and are characterized by two subgroups: Martinotti cells, which account for the majority of SST neurons and, less frequently, express other neuropeptides and calcium binding proteins, including CR (expressed in layer IV) (Wang Y., et al., 2004)(Marín et al., 2012). The colocalization of SST and CR is common in mouse cortical inhibitory neurons and there are two subtypes of SST cells: SST/CR+ and SST/CR- cells. Although both SST/CR+ cells and SST/CR- cells exhibit similar anatomical features and have similar adapting spike-firing patterns, they differ in the horizontal extension of their dendritic fields and number of primary processes (Xu et al., 2006). These informations suggest that CR cells are a distinct subtype of inhibitory neuron present in mouse cortex, or are a distinct variant of a similar cell type, likely Martinotti cells. SST/CR+ SST/CR- cells could be defined as a subpopulation of non-pyramidal cells (Rudy et al., 2011) (Batista-Brito et al., 2009). SST expressing interneurons selectively innervate distal dendrites of target neurons (Huang et al., 2007).

Neuropeptide Y (NPY)

NPY is a 36-amino acid neuropeptide that acts as a neurotransmitter in the brain and is widely expressed in neural tissue of nervous system (Lundberg et al., 1984). In the brain, it is produced by GABAergic system and it is presents in various locations including the hypothalamus (Freund et al., 1996). NPY have several functions, including: reducing anxiety and stress, reducing pain perception, affecting the circadian rhythm, in the neurodegenerative disease and in controlling epileptic seizures (Benarroch, 2009) (Cheung et al., 2012) (Kovac et al., 2013). NPY-positive neurons are located both in the principal cell layers and the basal dendritic layers.

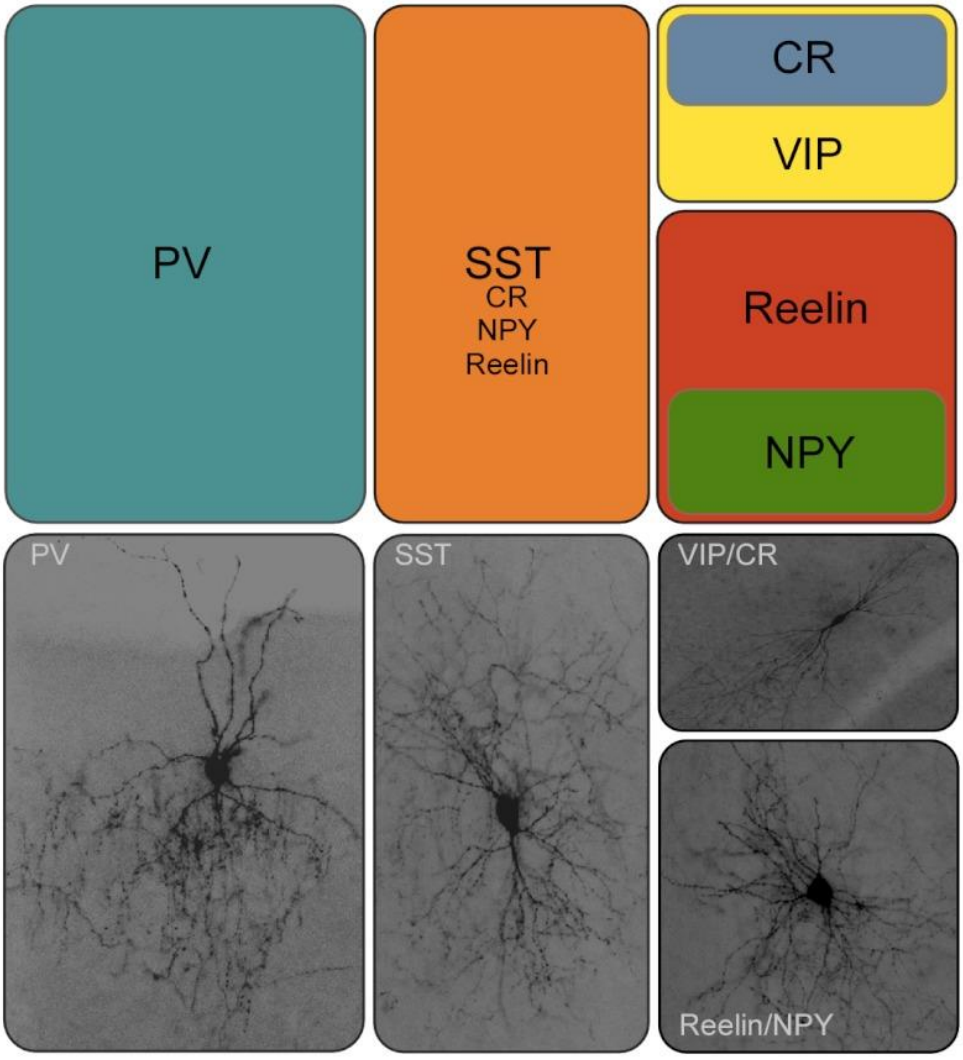


Fig1.1. *Simplify representation of four GABAergic interneurons in mouse cerebral cortex.*
Parvalbumin (PV) containing basket and chandelier cells. In orange, Somatostatin (SST) containing interneurons: many of these neurons have the morphology of Martinotti cells. This population includes several classes of interneurons that may also express Reelin, CR and/or NPY. Then, there are cells expressing very frequently VIP, and many of them also contain CR. Rapidly adapting interneurons with multipolar morphologies. Most of these cells express Reelin but not SST, and many also express NPY (Marín, 2012).

1.2.2 GABAergic dysfunction in the pathophysiology of ASD

GABA is the only neurotransmitter in the brain, that has an important role in early neuronal development (Palmen et al., 2004). During the early neonatal period, GABA provides most of the excitatory drive to developing neurons rather than being an inhibitory neurotransmitter (Ben-Ari, 2002)(Cherubini et al., 1991; Barker et al., 1998)(Rubenstein & Merzenich, 2003).

Blatt and colleagues (Blatt et al., 2001) investigated four neurotransmitter systems (i.e. the GABAergic, serotonergic, cholinergic and glutamatergic system) in the hippocampus of autistic adult male and control cases. The result obtained was that GABAergic system was the only neurotransmitter system found to be significantly reduced in autism (Blatt et al., 2001).

Studies on human postmortem brain tissue have described alterations in different aspects of GABA synthesis and release and also in the expression of particular subtypes of $GABA_A$ receptors (Pizzarelli & Cherubini, 2011). An altered excitation/inhibition (E/I) balance may be caused not only by a decreased $GABA_A$-mediated inhibition but also by an enhanced $GABA_A$-mediated excitation (Selby et al. 2007).

Cortical GABAergic neurons in rodents are largely produced in the MGE and migrate tangentially to the cortex (Nadarajah & Parnavelas, 2002)(Wonders & Anderson, 2006). Defects in the production or migration of cortical GABAergic neurons lead to decreased numbers of GABAergic neurons, leading to a hyper-excitable state (Pleasure et al., 2000) (Mostofsky et al., 2009). The balance of excitatory vs. inhibitory cells is controlled by the relative numbers and activities of glutamatergic and GABAergic neurons (Rubenstein & Merzenich, 2003). Interestingly, immunocytochemical data obtained from human autistic brain have revealed an increased density of calbindin-, calretinin-, and parvalbumin-positive interneurons in the hippocampus, a condition that would alter neuronal signaling and synchronization leading to cognitive dysfunctions (Lawrence et al. 2010).

The disruption of inhibitory circuits may delay critical periods in specific ASDs brain regions, thus perturbing *γ*-oscillations implicated in high cognitive functions (Hensch, 2005). GABA synthesis and signaling has been shown to regulate the maturation of GABAergic innervation also in visual cortex and the onset of critical period plasticity suggest that alteration of GABA synthesis, either due to genetic or environmental causes, can potentially affect nearly all stages of cortical circuit formation, thereby leading to impaired brain development (Chattopadhyaya et al., 2011). Recent studies have demonstrated that some drugs may act on GABAergic synapses rescuing behavioral deficits in animal models of autism and ameliorating some of the symptoms observed in ASD patients (Cellot & Cherubini, 2014).

Several genes regulate the process of GABAergic neurons development and an alteration in these genetic factors may affect ASD patients (Sgadò et al., 2011). Mutations affecting the GABAergic system concern the homeobox genes *Dlx1* and *Dlx2* (Wonders & Anderson, 2006), which, encode homeodomain-containing transcription factors. These genes are located on chromosome 2q31, a region previously associated to autism susceptibility in several genome-wide linkage studies. A strong association with ASD has been demonstrated with the genetic variation of these genes which are associated with abnormalities in the development of GABAergic neurons (Chang et al.2011) (Schmitz et al., 2005). Among the numerous ASD associated genes, *En2* (coding for the homeobox-containing transcription factor Engrailed-2) was originally shown to be involved in posterior brain (mesencephalon/hindbrain) embryonic development (Tripathi et al., 2009).

GABAergic and synaptic alterations in ASD mouse models.

Mouse models of neuropsychiatric diseases are designed to have comparable human symptoms and possess similarity to the underlying causes of the disease. Results of treatments on these animals offer the responses of effect in the human disease. Behavioral phenotype provides an approach to the validity of these mouse models for the autistic disorder (Moy et al., 2006)(Llaneza et al., 2010): individuals with ASD have life-long difficulties and environmental stressors and may suffer from seizures. There are distinct differences between high- and low-functioning individuals and different interventions may be used to improve behaviors (Crawley, 2004) (Paul, 2008). In detail, higher-functioning individuals diagnosed with ASD have minor issues with communication and sociability (Crawley, 2004). Whereas, lower-functioning individuals demonstrate obstacle to communicate that it impairs their ability to obtain basic needs (Paul, 2008).

ASD severity is based on social communication impairments and restrictive, repetitive patterns of behavior; other behavioral alterations include increased anxiety, stereotypic forelimb movements and progressive motor dysfunction with, in particular, impaired balance and coordination, tremors and impaired postural reflexes (Santos et al. 2007)(Moretti & Zoghbi, 2006)(Katz et al., 2012) (Stearns et al., 2007) (Gadad et al., 2013) (Katz et al., 2012) (Downs et al., 2010).

In recent years several mouse strains with targeted mutation of specific genes related to ASD have been generated that show a number of phenotypical alterations relevant for ASD (Marín, 2012)(Bauman & Kemper, 2005; Chattopadhyaya et al., 2012; Rossignol et al., 2011) (Provenzano et al.2012). A comprehensive list of the available mouse models for ASD is present in SFARIgene database (https:// gene.sfari.org). Different studies have been conducted to define the degree of correspondence between ASD and corresponding mouse models. These studies indicate that ASD mice generally have profound

impairments in inhibition, information processing and seizures susceptibility (Sgadò et al., 2011) In accordance with human observations, animal studies have revealed important dysfunctions in GABAergic signaling occurring at different locations of the GABAergic synapses (Paluszkiewicz et al. 2011). Defects in the development and function of GABAergic interneurons are associated to a pathogenic mechanism of ASD syndromes.
GABAergic alteration represents a common aspect in different neurodevelopmental disorders, such as, in Rett syndrome and FXS models. Different variations existing in patients have been found in *Mecp2* mouse model (Neul, 2012). These animals exhibit reduction in glutamic acid decarboxylase 1 (Gad1) and glutamic acid decarboxylase 2 (Gad2) mRNA encoding for GAD67 and GAD65 (Dani et al., 2005)(Chao et al., 2010). Therefore, an excitation/inhibition imbalance has been demonstrated in this mouse model and it may be due to the depression of GABAergic transmission at both pre- and post-synaptic levels (Medrihan et al., 2008)(Cellot & Cherubini, 2014). In detail, in *MecP2* mice, synaptic plasticity processes are correlated with a decrease in GABA and inhibitory neurotransmission, demonstrated also by the impairment of long-term potentiation (LTP) (Pizzarelli & Cherubini, 2011). Mouse models based on a null allele of the Mecp2 locus demonstrate a phenotype consistent with the human disease Rett syndrome, characterized by intellectual disability and elevated incidences of ASD (Dani et al., 2005). These results indicate that defects in GABAergic neurons reproduce many of the clinical features of Rett's syndrome (Marín, 2012).

We can assume that GABAergic dysfunction has been implicated in a number of disease states involving synaptic and circuit alterations: the parallelism between FXS and other developmental disorders is particularly interesting (Paluszkiewicz et al., 2011). Impairment in synaptic and GABAergic circuitry has been reported in animal model of fragile X syndrome (FXS). These defects contribute to the hyperexcitable circuit phenotype (Paluszkiewicz et al., 2011). Studies on *Fmr1* knock-out mice show anatomical alteration, such as, abnormally cortical dendritic spines, and also altered social interactions, all features similar to those observed in autism (Pizzarelli & Cherubini, 2011) (Selby et al., 2007) (Chonchaiya et al., 2012) (Gadad et al., 2013). Improper synaptic pruning and maturation suggest an important role of FMRP at the synaptic level during development (Churchill et al., 2002). *Fmr1* null mice present, also, alteration in neuron and glia differentiation and an abnormal glutamatergic neurogenesis (Castrén et al. 2012).

Mice carrying the R451C mutation in the *Nlgn3* gene show behavioral phenotypes related to ASD mice. They present an increase in the number of GABAergic synapses and in the amplitude of inhibitory currents (Tabuchi et al., 2007). Studies on GABAergic circuit in the

hippocampus of these mice demonstrate a decreased GABA release of parvalbumin-positive (PV) basket cells, which determines an alteration of the excitatory/inhibitory balance (Földy et al. 2013). These altered aspects could be identified also in autistic children (Curatolo et al., 2014). As Neuroligins, in association with neurorexins, controls the formation of both excitatory and inhibitory synapses. They may contribute to alter the balance between excitatory synapses and inhibitory synapses that in turn could affect cognition and social behavior (Gogolla et al., 2009).

SHANK mutations account for ~1% of patients with ASD with a severity in cognitive impairment; in particular, a high frequency and penetrance of SHANK3 mutations has been observed in individuals with ASD (Leblond et al., 2014) (Marín, 2012). *SHANK3* has an important role in development and function of the glutamatergic neurotransmission onto GABAergic neurons (Peça et al., 2011) (Marín, 2012). In cultured hippocampal neurons, knockdown of *SHANK3* leads to a reduced number and of dendritic spines, decreasing spiny density, whereas transfection of *SHANK3* in neurons induces the formation of dendritic spines with functional synapses (Chen et al., 2014)(Betancur et al., 2009).

1.3 Brain-derived Neurotrophic factor (BDNF)

1.3.1 Neurotrophins

Levi-Montalcini and Hamburger discovered that a mouse sarcoma tumor implanted close to the spinal cord of developing chicken secreted a soluble factor that induced the hypertrophy and fiber outgrowth of sympathetic neurons. The factor identified by in vitro studies was the nerve growth factor (NGF) (Cohen & Levi-Montalcini, 1956)(Lessmann et al., 2003). Subsequent studies by Barde and colleagues discovered a neuron specific survival factor from pig brain, which was named brain-derived neurotrophic factor (BDNF) (Barde et al., 1982). BDNF has a highly homologous in protein sequence to NGF (Reichardt, 2006) (Leibrock et al., 1989).

Since then, other members of the family of neurotrophins (NTs) have been identified: neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7), these latter have been found only in fish (Lai et al. 1998).

Neurotrophins have different roles in development and function of the nervous system. They promote the control of cell fate, axon growth and guidance, dendrite structure and pruning, synapse formation, synaptic plasticity and survival of neurons during the development of the central and peripheral nervous systems (Poo, 2001) (Reichardt, 2006).

In rodents there are 22 BDNF transcripts all encoding the same protein: eleven different 5'UTRs are generated by nine exons (1–9a). Each 5'UTR is alternatively spliced to a common downstream exon 9 containing the coding region and a 3'UTR with two polyadenylation sites (Aid et al. 2007) (Baj et al., 2013). Tongiorgi and colleagues demonstrated that BDNF mRNAs are targeted to the distal dendrites in response to tetanic electrical activity or other stimuli as BDNF itself (Tongiorgi et al. 1997). Interestingly, it was noticed that the translation of BDNF in the soma, proximal, or distal dendrite is due to differential trafficking of BDNF mRNA variants to define a ''spatial code hypothesis of BDNF splice variants" (Baj et al., 2013) (Chiaruttini et al. 2008). Different transcripts have different spatial and temporal localization in the neuronal districts suggesting different localizations and biological functions. Indeed, studies demonstrated that some elements of the translational machinery (rough endoplasmic reticulum and the Golgi apparatus) have been found within dendrites, leading an evidence of local protein synthesis in postsynaptic dendrites (Poo, 2001).

All NTs are generated as pre-pro-neurotrophin precursors, which are processed and secreted as mature homodimeric proteins into the extracellular space (Lessmann et al., 2003). BDNF mRNA is designate to ER and the synthesized pro-protein is then packed in transport vesicles and accumulates in the membrane stacks of the *trans*-Golgi network (TGN). NTs can be sorted to the constitutive pathway, where secretory vesicles are transported by default to cell periphery, or to the regulated pathway, dependent upon calcium regulation (Malcangio & Lessmann, 2003). After cleavage the resulting protein is a 32-kDa proBDNF protein. ProBDNF is cleaved intracellularly by enzymes: furin (the abundant protein convertase present in all cells) or pro-convertases. Secreted as the 14 kDa mature BDNF (mBDNF), or secreted as proBDNF and it is then cleaved by extracellular proteases, such as metalloproteinases and plasmin, to mBDNF (Lessmann et al., 2003)(Cunha et al. 2010) (Fig. 1.2).

In the mammalian brain there are four neurotrophins receptors: Trk tyrosine kinases (Trk A, Trk B and Trk C) and p75 (p75NTR). p75NTR, is a low-affinity receptor for NGF, constituted by an extracellular domain, a single transmembrane domain and a cytoplasmic domain (Reichardt, 2006). p75NTR binds each of the neurotrophins with a similar affinity to NGF (Dechant & Barde, 2002).

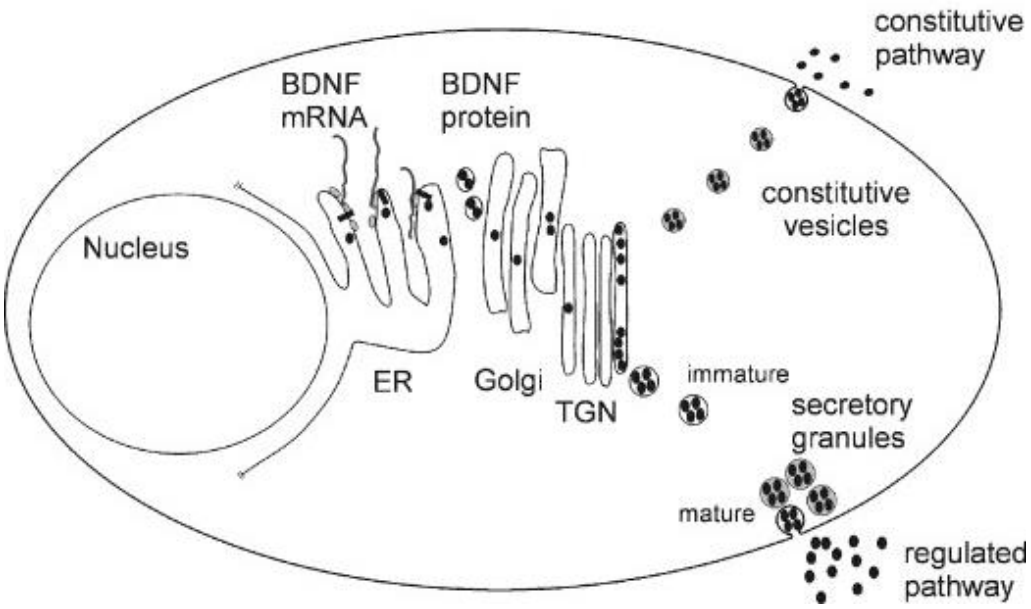


Fig 1.2. Schematic representation of BDNF route from synthesis to secretion (Lessmann et al., 2003).

Trk receptors are activated by one or more NTs and are responsible for mediating most cellular responses: in detail, TrkA is activated by NGF, TrkB by BDNF and NT-4/5, and TrkC by NT-3.

p75 can be activated by the binding of all four neurotrophins with approximately equal affinity, even if it is lower than Trk receptors (Reichardt, 2006). The most abundant TrkB isoforms in the brain are the full-length TrkB receptor and two truncated isoforms of this receptor, TrkB. T1 and TrkB.T2, generated by alternative splicing. The truncated isoforms lack the intracellular catalytic domain (Baxter et al., 1997) (Wong et al. 2013). Trk receptors control the activity of several intracellular signaling pathways, including those of the Ras/ERK and PI3K/Akt kinase cascades (Patapoutian & Reichardt, 2001).

NTs bind to axonal Trk and p75 receptors, becoming endocytosed and retrogradely transported from the axon to the cell soma where they can activate survival promoting cellular signaling cascades (Lessmann et al., 2003) (Poo, 2001). BDNF is involved in the cell proliferation and cell survival and have an important role during CNS repair (Zhang et al., 2003). The level of NTs at synaptic sites may in principle be regulated by two mechanisms: the transport and targeting of NT-containing vesicles to the synapse and the synaptic level of NTs regulated by local translation of NT mRNAs.

1.3.2 BDNF receptors and signaling

There is evidence that all the synaptic effects of BDNF are attributed to TrkB activation: however, proBDNF binds p75 receptor preferentially, which has distinct functional consequences. The binding of BDNF to TrkB truncated variant receptors can act as a dominant-negative inhibitor of BDNF signalling by forming heterodimers with the full-length TrkB, and by internalizing BDNF to function as a clearance receptor. Therefore, ProBDNF, p75 and truncated TrkB isoforms may be considered as negative regulatory mechanisms, with consequent effects for synaptic plasticity and perhaps learning and memory (Cunha et al., 2010).

The binding of BDNF to full-length TrkB leads to TrkB autophosphorylation and subsequent activation of a series of intracellular signaling pathways: phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), and the cascade governed by extracellular signal-regulated kinases (ERK), member of the mitogen-activated protein kinase (MAPK) family (Cunha et al., 2010) (Hartmann et al., 2004) (Patapoutian & Reichardt, 2001). A more relevant mechanism is the dendritic trafficking and local translation of trkB mRNA promoted by neuronal activity both *in vitro* and *in vivo* (Tongiorgi et al., 1997). BDNF and TrkB are widely expressed in the developing and adult hippocampus and neocortex: BDNF mRNA is present in excitatory pyramidal neurons, but not in GABAergic inhibitory interneurons (Rutherford et al.1997), whereas, TrkB is expressed by both classes of neurons, although its expression is higher in inhibitory interneurons. This has raised the possibility that BDNF-to-TrkB signaling modulates an autoregulatory circuit between excitatory pyramidal cells and inhibitory interneurons. Neurotrophic effects such as survival and differentiation, require internalization of the ligand–receptor complex, and its retrograde transport toward the cell body (Roux & Barker, 2002). BDNF and TrkB signaling regulate the dendrites stability directly: they control the members of the Rho family of GTPases, which have an important role in regulating formation and stability of dendrites.

Signaling capacity of P75 is dependent on the activation of Trk receptors (Kaplan & Miller, 2000). Ligand engagement of p75 has been shown to promote survival of some cells and apoptosis of others. p75 is present in several central neuronal populations, including spinal motor neurons and brain stem motor nuclei, lateral geniculate nucleus, amygdala, cortical subplate neurons, as well as Purkinje cells, and cerebellum. Postnatally, p75 levels are reduced in most tissues and restricted to neurons of the basal forebrain, caudate/putamen neurons, motor neurons, cerebellar Purkinje cells (Roux & Barker, 2002). P75 is involved in axonal growth via the cytoskeletal affecting protein Rho; p75 activates Rho, which

inhibits axonal elongation. In the absence of TrkB in neuronal culture, p75 promotes neuronal apoptosis (Roux & Barker, 2002).

Binding of BDNF to Trk receptor induce its dimerization and autophosphorylation of its tyrosine residues in the cytoplasmic domain and stimulate intracellular signaling, activating at least three different pathways: the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3-kinase (PI3K) pathway, and the phospholipase C (PLCγ1) pathway (Chao, 2003). In the MAP kinase pathway activation of Shc recruits the G-protein RAS that, through adaptors Grb2 and SOS, starts a sequential activation by phosphorylation of the MAP kinase cascade; whose elements are Raf (a serine threonine kinase), Mek (a mixed specificity kinase) and Erk (a MAP kinase). ERK is translocated into the nucleus, where it activates some transcription factor such as cAMP-response-element binding protein (CREB). The MAP pathway is fundamental for the normal neuronal development, neuronal survival and neurogenesis (Chao, 2003).

In the PLC_Y pathway, PLC_Y catalyzes the breakdown of phosphatidylinositol 4,5-biphosphate (PIP2) to diacyl glycerol (DAG) and inositol (1,4,5) triphosphate (IP3). DAG activates several protein kinase C (PKC) isoforms. IP3 signaling through specific receptors promotes the release of Ca2+ from the intracellular stores. The rise in the intracellular Ca2+ concentration activates Ca2+ sensitive enzymes including Ca-calmoduline kinases II (CaMKII) and phosphatases, MAPKs and PI3K. PLC-Ɣ pathway is important for many brain functions like NT-dependent regulation of synaptic plasticity and learning and memory.

BDNF regulates its own expression through the CaMKII signalling: in the PI3 kinase pathway, activated Shc recruits PI3K via adaptor proteins Grb2 and Gab1; otherwise, PI3K can be activated by Trk receptors through adaptor protein IRS (Patapoutian & Reichardt, 2001). PI3K phosporylates phosphoinositol (PI) lipids, generating 3,4,5-triphosphates (PIP3). PIP3 recruits two proteins to the plasma membrane and these proteins are Akt (PKB) and PDK-1. PI3K pathway is essential for NT-mediated neuronal survival (Kaplan and Miller, 2000).

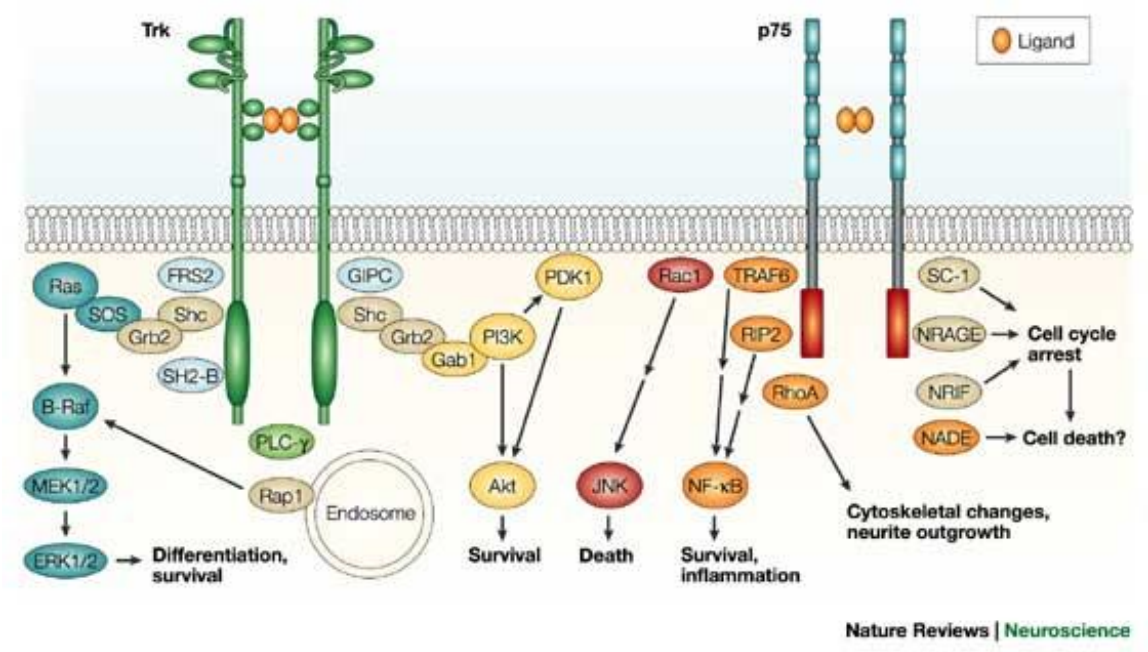


Fig 1.3. Neurotrophin receptors signaling.
Trk receptors mediate differentiation and survival signalling through extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLC-γ) pathways. The p75 receptor predominantly signals to activate NF-κB and Jun N-terminal kinase (JNK), and modulates RhoA activity (Chao, 2003).

1.3.3 Role of BDNF in GABAergic interneurons specification

BDNF is considered as a general modulator of transmitter release, including the release of GABA (Singh et al., 2006). Endogenous BDNF is released from excitatory neurons in activity-dependent manner and transferred from excitatory neurons to postsynaptic GABAergic neurons, promoting dendritic development of the latter neurons (Palizvan et al., 2004). NTs in general play an important role in regulating the level of expression of GABA and the morphology of GABA-containing neurons (Marty & Berninger, 1997).

Several studies have reported increase in the number or size of GAD-positive terminals after BDNF induction (Singh et al., 2006): in neuronal cell culture, a prolonged application of BDNF enhanced the formation and maturation of GABAergic synapses (Gottmann et al. 2009)(Palizvan et al., 2004). In detail, BDNF induces an increased in presynaptic GAD65 content from hippocampal cultures, an overall cellular enhancement of GAD expression (Yamada et al., 2002) and an increase in the size and intensity of GAD positive puncta (Henneberger et al., 2005). The effects of BDNF on the GABAergic system vary with the exposure time in culture and also with the cell types (Wardle & Poo, 2003). Short treatment with BDNF modifies synaptic parameters and induces a depression of inhibitory synaptic

transmissions in hippocampal slices, whereas, a longer exposure supports the maturation of GABAergic neurons, promotes the establishment of GABAergic synaptic connections and increases their strength, upregulating GABA release and $GABA_A$ receptor expression (Henneberger et al., 2005) (Rutherford et al., 1997) (Tanaka et al.1997) (Yamada et al., 2002).

BDNF also regulates $GABA_A$ receptor responses by Trk type receptor located in postsynaptic neurons (Brunig et al.2001). Several studies observed that BDNF promotes GABAergic neuron development, in some cases without influence glutamatergic circuit. Glutamatergic synapses on the other hand are converted from immature into active synapses upon BDNF induction (Yamada et al., 2002).

BDNF may lead to increased synaptogenesis, GABAergic development and GABAergic responses through the regulation of KCC2 expression (Aguado et al., 2003). The BDNF-induced shift of the E/I balance of synaptic input toward will inevitably produce enhanced network activity, which may be relevant to the etiology of neurological disorders with synaptic dysfunction such as epilepsy, schizophrenia and autism. Indeed the inhibitory neurons are the principal target of BDNF action, as GABAergic system appears immature and functionally impaired in BDNF knockout mice (lacking one copy of the BDNF gene) (Abidin et al. 2008). BDNF null mutant mice show reduced expression of NPY, parvalbumin, and calbindin in the cerebral cortex and the hippocampus (Kelsom & Lu, 2013). Overall, both *in vitro* and *in vivo* studies have demonstrated that BDNF may influence the maturation of GABAergic inhibitory interneurons in several ways and that BDNF is required for dendrite growth of GABAergic interneurons (Marty et al.1997)(Jones et al.1994). BDNF can also affects the migration of cortical interneurons, but it is not required during dendritic development of cortical inhibitory interneurons (Berghuis et al., 2006)(Gorski et al. 2003). Transgenic mice overexpressing BDNF in the postnatal forebrain do have an accelerated development of PV interneurons (Huang et al., 1999). Different studies suggest that BDNF also mediates part of the effects of neuronal activity on GABA-containing neurons (Marty et al., 1997). Different data demonstrated that during development the number of GABA synapses is regulated by BDNF expression, which is itself regulated by physiologic synaptic activity (Marty et al., 1997). Downregulation of BDNF expression leads to GABA reduction in layer IV of the barrel cortex, following sensory deprivation. While whisker stimulation, for example, induces a marked increase in *Bdnf* mRNA levels in layer IV of the corresponding barrels (Rocamora et al., 1996).

1.3.4 BDNF in autism spectrum disorder

Recent evidence suggests an involvement of BDNF in autism. Several studies in small population samples showed an involvement of the BDNF/TrkB signaling pathway in autistic children: BDNF levels are increased in the blood, serum and brain, compared with normal controls (Nelson et al., 2001) (Perry et al., 2001). Reduced BDNF in the cerebellum may be an indicator of aberrant brain development and growth in autism. “Recent reviews have shown that the neuroprotective effect of neurotrophic factor therapy appears to be one of the most promising approaches toward effective treatment of neurodegenerative diseases. The obstacle in the use of trophic factors is their delivery into the CNS (Poduslo & Curran, 2005). BDNF proteins do not cross the brain capillary endothelial wall, which forms the blood–brain barrier (Sakane & Pardridge, 1997). Neurotrophins should be considered to enable receptor-mediated transport across an intact blood–brain barrier in vivo, in order to have neuropretective function (Zhang & Pardridge, 2006). Chimeric peptide technology has been suggested as one strategy for the noninvasive targeting of neuroprotection following intravenous administration (Pardridge W.M., 1997) (Pardridge W.M. et al., 2001). Abnormal BDNF expression has been linked to many human diseases, such as Huntington's disease, schizophrenia and depression (Chang et al. 2006). Recent result suggest an association between BDNF and Mecp2 *in vivo* and a possible role of BDNF in RTT syndrome (Chang et al., 2006). *MeCP2* may function in the silencing of *BDNF* basal transcription in activity-suppressed neurons (Chang et al., 2006) and may contribute to defects caused by BDNF deficiency. Indeed, *BDNF* mRNA levels are reduced in *MeCP2* null mice and other studies demonstrate that *BDNF* mRNA levels was lower, also, in brain samples from RTT patients (Chapleau et al.2009) (Hashimoto et al., 2006) (Miyazaki et al., 2004). Another disorder characterized by the decrease of BDNF mRNA levels in the neocortex, is Alzheimer disease (AD).

Studies confirmed the hypothesis that deficits or imbalances in BDNF isoforms may lead to neuropsychiatric disorders: reports indeed showed that a reduction in pro-BDNF processing and BDNF secretion correlates with deficits in memory and with cognitive decline in mild cognitive impairment (Peng et al. 2005)(Zuccato & Cattaneo, 2009).

In subjects with autism, the increase in pro-BDNF may be responsible for reduced neuronal differentiation and dendritic spines, leading to an altered synaptic connectivity and neurotransmitter levels (Garcia et al., 2012). Conversely, a reduction in truncated BDNF isoform correlates with cognitive impairment in schizophrenia (Carlino et al., 2011). In ASD patients, an increase in pro-BDNF and a reduction in truncated BDNF have been reported, leading to serious effects on learning, memory, and attention (Garcia et al., 2012). In healthy controls, it has been verified an increased concentration during the first

years of life that decreases in adulthood (Nickl-Jockschat & Michel, 2011). No alteration in *BDNF* mRNA in fusiform gyrus of subjects with autism, compared with controls (Garcia et al., 2012). Despite extensive research, the full impact of possible change in BDNF isoform ratios has yet to be determined.

1.4 Engrailed-2 knockout (En2-/-): a model for ASD

The *Engrailed* genes, *Engrailed-1* (*En1*) and *Engrailed-2* (*En2*), are homoeobox containing transcription factors, homologue to the Drosophila *engrailed*. In Drosophila the gene is involved in the establishment of the anterior-posterior compartment border in several imaginal discs and in body segments (Guillén et al., 1995)(Joyner & Martin, 1987). In Drosophila and in mammals, the *Engrailed* genes play a dual role: in early steps of development they participate to the specification of the midbrain/hindbrain region (Joyner & Martin, 1987) and regulates cerebellar patterning and connectivity (Sillitoe et al. 2010). During developmental stages *Engrailed* genes contribute to the maintenance of neuronal population in these areas.

Engrailed genes have a high homology and seem to compensate for one another. Indeed, when the *En1* gene is replaced by either *En2* (Hanks et al. 1995) or the Drosophila *engrailed* gene (Hanks et al., 1995) the resulting homozygous mice show virtually no brain defects. In contrast to the single mutation, the combined mutation of the two genes results in dramatic repercussions on the maintenance of neuronal population. *En2* homozygous mutants are viable and fertile and exhibit only a mild phenotype that appears restricted to cerebellum (Joyner et al., 1991). Conversely, homozygous *En1* mutants die at birth, with severe deletion in the cerebellar and dorsal midbrain tissues. *En2-/-* mice display a decrease in cerebellar size, minor abnormalities of patterning foliation with a reduction in the number of Purkinje, granule, deep nuclear, and inferior olive neurons (B Kuemerle, et al.1997). Recent analysis indicated that *En2* is also expressed in the hippocampus and cortex (Tripathi et al., 2009).

1.4.1 Engrailed-2 gene

Animal studies have demonstrated that *En2* is expressed throughout CNS development and regulates numerous cells biological processes implicated in ASD including connectivity, excitatory/inhibitory (E/I) circuit balance, and neurotransmitter development (Benayed et al., 2005)(Benayed et al., 2009). The homology level of *En1* and *En2* genes is more than 90% at the protein level in the homeodomain region, that confers its

transcriptional regulation function (Logan et al., 1992) (Morgan, 2006). There are other four regions of similarity (EHs), EH1-EH5, where EH2 and EH3 bind PBX, a second homeodomain-containing transcription factor that modifies the DNA binding affinity and specificity of Engrailed. The domains EH1 and EH5, however, mediate the transcriptional repression recruiting the corepressor Groucho (Tolkunova et al.1998). The homeodomain EH4 is a highly conserved sequence of about 60 amino acids common to all classes of homeodomain protein, and is composed of three alpha helices, of which the third binds double-stranded DNA (Morgan, 2006). Engrailed proteins have other properties among which the ability to be secreted by cells and internalized and the ability to bind directly to the eukaryotic translation initiation factor 4E (eIF4E), through a sequence localized at the N-terminal (Nédélec et al., 2004).

Two intronic single nucleotide polymorphisms (SNP), rs1861972 and rs1861973, are consistently associated with ASD (Jiyeon Choi, 2011)(Benayed et al., 2005)(Gharani et al., 2004). More recently, evidence was provided that one of these SNPs (rs1861973, A-C haplotype) was functional: when tested with a luciferase reporter assay in rat, mouse and human cell lines, this SNP markedly affected *En2* promoter activity (Benayed et al., 2009). For these reasons and for the anatomical phenotype that the *En2* mouse mutants display, *En2* was considered a candidate gene for ASD. Recent study demonstrated that epigenetic alterations in *En2* promoter are evident in the cerebellum of some individuals with autism (James et al., 2014).

To study the role of the *En2* gene during the development, Joyner and colleagues generated mice homozygous for a targeted deletion of the *En2* homeobox region (Joyner et al., 1991). Mutant mice were viable and showed no obvious defects in embryonic development, suggesting that this might be due to functional redundancy of *En1* with *En2*. Mutant mice had abnormal foliation in the adult cerebellum, where normally only En2 is expressed (Cheng et al., 2010).

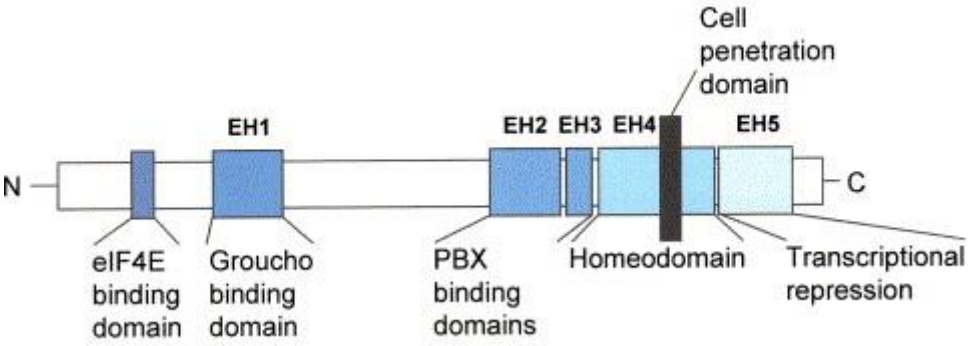


Fig 1.4. Domains within the En protein (Morgan, 2006).

1.4.2 Anatomical and behavioral characterization of En2-/- mice

Other studies defined the *En2* gene as an autism-susceptibility gene based on family linkage studies indicating an increased transmission of *En2* polymorphic variants from parents to affected children (Benayed et al., 2009) (James et al., 2014). *Engrailed-2* (*En2*) is considered to be an autism susceptibility gene based on neuroanatomical parallels between autism and cerebellar developmental abnormalities in rodent models (Benayed et al., 2005).

Anatomically, *En2* mutant mice display defective cerebellar patterning and a reduction of cell types of the olivocerebellar circuit (Purkinje, granule, inferior olive and deep nuclear) (Kuemerle et al., 2007). Reduced numbers of Purkinje cells in the cerebellar hemispheres have been observed in both childhood and adult cases, in individuals with and without a history of seizures or medication usage and appear to be unrelated to cognitive function (Bauman & Kemper, 2005). These studies also demonstrate a variable changes in the volume of the amygdala with an abnormal enlargement and a reduction in the neuronal number (Kuemerle et al., 2007). Amygdaloid regions in *En2* mutant mice exhibited defects: anterior shift of the amygdaloid complex within the temporal lobe positional may have negative effects on cognitive function (Kuemerle et al., 2007). These irregularities in the cerebella development (Purkinje cell deficit and abnormal folia) and in the amygdala (positional shift in cortical location) might resemble some of those reported in ASD patients (Kuemerle et al., 2007).

In the hippocampus, *En2-/-* mice exhibit a reduction in parvalbumin and somatostatin expression: a feature that may be associated with the increased seizure susceptibility shown by these mutants (Tripathi et al., 2009). Recent studies evaluated behavioral phenotypes of *En2* mutant mice, relevant to the diagnostic and associated symptoms of ASD (Brielmaier et al., 2012). Learning and memory deficits are common in ASD, and co-morbid mental retardation has been identified in autistic individuals (Bhattacharya & Klann, 2012) (Cheh et al., 2006).

Brielmaier and colleagues confirmed deficits in cognition in *En2* null mice, such as a sensorimotor gating impairment and a depression-related phenotype (Brielmaier et al., 2012) (Cheh et al., 2006). Interestingly, reduced exploration has been observed in both autistic children and En2 mutant mice, correlated with cerebellar hypoplasia (Pierce & Courchesne, 2001). Their results suggest that *En2* mutations lead to deficits in social behaviors but not in social communication, confirming the first diagnostic symptom of autism, without abnormalities in the communication and repetitive symptom domains

(Brielmaier et al., 2012). There is not a genotype difference in neurobehavioral development, general health, pain sensitivity, open field locomotor activity, anxiety-like behaviors, sensory abilities and pain sensitivity (Brielmaier et al., 2012).

2. AIM OF THE THESIS

En2-/- mice have been proposed as a model for ASD, due to a number of anatomical and behavioral features similar to those reported for ASD patients, including ASD-like behaviors, increased seizure susceptibility and altered GABAergic innervation of limbic circuits. Defects in GABAergic transmission have been hypothesized to underlie the symptoms of ASD. This research project first aimed at investigating the effect of *En2* ablation on the maturation of GABAergic interneurons in the hippocampus and cerebral cortex, hypothesizing a possible role of *En2* in the development and/or maintenance of forebrain GABAergic interneurons. Recent bioinformatics analysis of the molecular signature of *En2-/-* hippocampus revealed a significant convergence of neurobiological pathways previously linked to ASD. In particular, these studies demonstrated that ablation of *En2* induces a prevalence of pathways related to seizure and altered synaptic transmission, indicating altered synaptic development in these mice. A second aim of the project was also to analyze the development of synaptic connections of both GABAergic and glutamatergic neurons in vivo and in vitro, and the effects of Brain derived neurotrophic factor (BDNF; a neurotrophic factor implicated in cell survival, and synaptic connectivity) on the maturation of the GABAergic system in *En2-/-* mice.

3. MATERIALS AND METODHS

3.1 Animals

Experiments were conducted in conformity with the European Community Directive 2010/63/EU and approved by the Italian Ministry of Health. Animals were housed in a 12 hr light/dark cycle with food and water available ad libitum. All surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize suffering. The generation of *En2-/-* mice was previously described (Joyner et al., 1991). The original *En2* mutants (mixed 129Sv x C57BL/6 and outbred genetic background) were crossed at least five times into a C57BL/6 background. Heterozygous mating (En2+/- x En2+/-) was used to generate the En2+/+ (wild type, WT) and En2-/- littermates used in this study. In the knockout the cerebellum is reduced in size and cell counts have determined a ~30-40% reduction in all the major cerebellar cell types including Purkinje cells. In this study, we also used a mouse line GAD67-GFP (line G42) that we crossed with En2 mice. This mouse line selectively express enhanced green fluorescent protein (EGFP) in PV-positive basket interneurons (soma, dendrites, and axons) and also in putative presynaptic boutons (Chattopadhyaya et al., 2004). PCR genotyping, for these mice lines, was performed according to the protocol available on the Jackson Laboratory website http://jaxmice.jax.org/strain/002657.html and http://jaxmice.jax.org/strain/007677.html). For all the experiments, WT and En2-/- age-matched adult littermates (3-5 months old; weight = 25-35 g) and young (p30) mice of both sexes were used.

Table 3.1. *Primer En2 mouse line: PCR genotyping.* Abbreviation as in the text.

Primer	5' Label	Sequence 5' --> 3'	3' Label	Primer Type
12370	-	GCCCACAGACCAAATAGGAG	-	Common
12371	-	TGCAAAGGGGACTGTTTAGG	-	Wild type Forward
12372	-	ACCGCTTCCTCGTGCTTTAC	-	Mutant Forward

Table 3.2. *Primer* GAD67-GFP (line G42) *mouse line: PCR genotyping.* Abbreviation as in the text.

Primer	5' Label	Sequence 5' --> 3'	3' Label	Primer Type
10201	-	AGTGCTTCAGCCGCTACC	-	Transgene Forward
10202	-	GAAGATGGTGCGCTCCTG	-	Transgene Reverse
13731	FAM	TTCAAGTCCGCCATGCCCGAA	ZEN/IBFQ	Tg Probe
oIMR1544	-	CACGTGGGCTCCAGCATT	-	Control Forward
oIMR3580	-	TCACCAGTCATTTCTGCCTTTG	-	Control Reverse
TmoIMR0105	Cy5	CCAATGGTCGGGCACTGCTCAA	BHQ 2	IC Probe

3.2 RNA extraction and quantitative RT-PCR from tissue

Total RNAs were extracted by Trizol© reagent (Invitrogen) from dissected hippocampi, somatosensory cortices, visual cortices and cerebellums from WT and En2-/- adult mice. DNAse-treated RNAs were purified and concentrated with RNeasy Mini Kit (Qiagen). cDNA was synthesized from pooled RNAs (2 µg) using the SuperScript® VILO™ (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a Biorad thermal cycler with real-time detection of fluorescence. Individual PCR reactions were conducted in a volume of 10 µl using the MESA GREEN qPCR kit (Eurogentec SA, Belgium) according to manufacturer's instructions. Mouse mitochondrial ribosomal protein L41 (Mrpl41) was used as a standard for quantification (Tripathi et al., 2009). Primers were designed on different exons to avoid amplification of genomic DNA. Primer sequences are reported in Table 1. Each PCR cycle consisted of denaturation for 10 s at 94 ºC, annealing for 20 s at 60 ºC and extension for 30 s at 72 ºC. The fluorescence intensity of SYBR green I was read and acquired at 72 ºC after completion of the extension step of each cycle. Relative expression values were calculated using the Biorad software. Mean cycle threshold values from triplicate experiments (Ct; cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of the reactions) were calculated for each marker and L41, and corrected for PCR efficiency and inter-run calibration. Ratios of each marker / L41 values were then calculated for WT and En2-/- mice. Each experiment was performed at least three times. Values are expressed as mean ± SEM.

Table 3.3. *List of primers used for quantitative Real time PCR.* Table of different primers used for the experiments, primer forward and primer reverse. L41 is the housekeeping gene, used as reference control during the real time procedure. The other primers are: vGlut (excitatory presynaptic transporter), vGAT (inhibitory presynaptic transporter), GAD67 (glutamate decarboxylase), CALB (calbindin-GABAergic interneuron), PV (Parvalbumin-GABAergic interneuron), NPY (neuropeptide Y-GABAergic subpopulation), SOM (somatostatin- GABAergic interneuron) and the different BDNF Exon primers. BDNF primers (ExonI,II,IV,VI,VII,IXa).

Name	Forward primer (5′-3′)	Reverse primer (5′-3′)
L41	GGTTCTCCCTTTCTCCCTTG	GCACCCCGACTCTTAGTGAA
vGLUT	CACAGAAAGCCCAGTTCAAC	CATGTTTAGGGTGGAGGTAGC
vGAT	TCACGACAAACCCAAGATCAC	GTCTTCGTTCTCCTCGTACAG
GAD67	TCCAAGAACCTGCTTTCCTG	GAGTATGTCTACCACTTCCAGC
CALB	AGATCTGGCTTCATTTCGACG	TTCATTTCCGGTGATAGCTCC
PV	TGCTCATCCAAGTTGCAGG	GCCACTTTTGTCTTTGTCCAG
NPY	TCACAGAGGCACCCAGAG	AGAGATAGAGCGAGGGTCAG
SOM	AGGACGAGATGAGGCTGG	CAGGAGTTAAGGAAGAGATATGGG
ExonI	CTTCCAGCATCTGTTGGGGAGACG	TTCTGGTCCTCATCCAGCAGC
ExonII	CCAGCGGATTTGTCCGAGGTGG	TTCTGGTCCTCATCCAGCAGC
ExonIV	ACCGGTCTTCCCCAGAGCAG	TTCTGGTCCTCATCCAGCAGC
ExonVI	AGGGACCAGGAGCGTGACAA	TTCTGGTCCTCATCCAGCAGC
ExonVII	CTCTGTCCATCCAGCGCACC	TTCTGGTCCTCATCCAGCAGC
ExonIXa	ATGGGCCACATGGTGTCCCCAA	TTCTGGTCCTCATCCAGCAGC

3.3 Morphometric analyses

Bright-field images of the hippocampus and somatosensory cortex were acquired at 10 × primary magnification using a Nikon Eclipse 90i microscope and merged using Adobe Photoshop software. Morphometric analysis of hippocampal and cortical layers was performed according to published protocols (Baj et al., 2012) on 3 to 5 NeuN-stained sections per animal, taken at the level of the dorsal hippocampus (n = 4 WT and 5 *En2-/-*). Brain areas were identified according to Franklin and Paxinos (1997). The total thickness of hippocampus and somatosensory cortex measured in WT mice did not differ from that of C57Bl/6 mice, as from published mouse brain atlases (Franklin and Paxinos, 1997; Allen Mouse Brain Atlas)(hippocampal total thickness: WT=1192 ± 30 µm, C57Bl/6 = 1147 ± 76 µm; somatosensory total thickness: WT = 886 ± 15 µm, C57Bl/6 = 925 ± 50 µm; *t*-test, $p > 0.05$).

3.4 In situ hybridization

For In situ hybridization, brains from 2 WT and 2 En2-/- adult mice were rapidly removed and frozen on dry ice. For digoxygenin (DIG) labelled riboprobes the sagittal cryostat sections (20 m thick) were postfixed in 4% PFA, washed in PBS, digested briefly with Proteinase K (1µg/ml) in PBS, rinsed twice in PBS then treated with a solution of Triethanolamine-HCl/Acetic Anhydride pH 8.0 [0.1M Triethanolamine, 0.25% Acetic Anhydride]. The sections were then permeabilised in a solution of PBS supplemented with 1% Triton-X100 (Merck, Germany) and hybridised in Hybridisation Buffer [50% deionized formamide, 5X Sodium Salt Citrate (SSC), 5X Denhard's solution, 250µg/ml yeast tRNA, 500µg/ml salmon sperm or herring sperm DNA] containing 1µg/ml of DIG labelled riboprobe, hybridized overnight at 65-75_C depending on the riboprobe specificity. The post-hybridization washes consisted of a dilution series of SSC (5X, 2X, 0.2X) at 65_C. The sections were then equilibrated and washed in B1 Buffer [100mM Tris pH 7.5, 150mM NaCl] and incubated overnight with the anti-digoxygenin AP antibody (Roche Diagnostic, Germany), diluted 1:5000 in B1 buffer supplemented with 10% new-born calf serum (NCS). Specimens were developed using a pre-formulated AP substrate, BM-purple (Roche Diagnostic, Germany). Brain areas were identified according to Franklin and Paxinos (1997). For each section, bright-field images of the hippocampus, cerebral cortex and cerebellum were acquired at 10x primary magnification using a Zeiss microscope, Zeiss Observer Z1. In situ hybridizations on brain sections were conducted in contemporary with brain sections from control animals. The DNA template for in vitro retrotranscription was amplified by PCR using specific 5' primers bearing the T7 RNA polymerase sequence that has such a site in its sequence.

Table 3.4 *Primer probes for In situ Hybridization.* List of primers used for in vitro retrotranscriptions. The table shows the T7 sequence, the BDNF sequences and the amplicon measure. NB. +40 is reported because after the first cycle we have the "integration"of t3 and t7 "tails" in the amplicon.

Primer	T7 sequence	Sequence 5' --> 3'	Primer Type	Amplicon
Fw_T7Ex1 1	GCGTAATACG ACTCACTATA GGG	TGGGGGT ACTCTGAAAC TCC	ExonI Forward	515+40
Fw_T7Ex2BC	GCGTAATACG ACTCACTATA GGG	GTTTGGT CCCCTCATTG AGC	ExonII Forward	226+40
Fw T7Ex6 1	GCGTAATACG ACTCACTATA GGG	CGGCTTG GAGAAGGAAA C	ExonVI Forward	267+40
Fw T7Ex9a	GCGTAATACG ACTCACTATA GGG	TCTGGTG ACAGCGTGTG CAG	Exon Ixa Forward	416+40
Rev T3allE	CGCATTAACC CTCACTAAAG GG	ACAACCGA AGTATGAAAT AACC	Common Reverse	

3.5 Immunohistochemistry (IHC)

Immunohistochemical characterization of postnatal En1 and En2 protein expression was performed on four weeks old and adult WT mice. Brains were fixed by transcardial perfusion with 4% paraformaldehyde followed by 1 h post-fixation at 4 °C, and 30 µm thick cryosections were collected. Optimal En1/2 staining was obtained on four week old mice (n=4), whereas a weaker signal was obtained in adult mice (not shown). A total of 13 WT and 14 En2-/- adult (3–5 months old) littermates were used for immunohistochemical identification of GABAergic interneuron subtypes and morphometric analyses of the hippocampus and somatosensory cortex. Brains were fixed by immersion in 4% paraformaldehyde and coronal sections (50 µm thick) were cut on a vibratome. Serial sections at the level of the dorsal hippocampus were incubated overnight with appropriate antibodies as follows: affinity purified rabbit polyclonal anti-En-homeodomain1 that recognizes En1 and En2 proteins (αEnhd1; 1:100 dilution); anti-parvalbumin (PV) mouse monoclonal (Sigma-Aldrich, USA; 1:2000 dilution); anti-somatostatin (SOM) rabbit polyclonal (Bachem, UK; 1:2000 dilution); anti-neuropeptide Y (NPY) rabbit polyclonal (Bachem, UK; 1:2000 dilution); anti-calbindin 28kD (CALB) mouse monoclonal (Swant, Switzerland; 1:5000 dilution); anti-glutamic acid decarboxylase 67 kDa isoform (GAD67) monoclonal (Millipore, USA; 1:500 dilution). Signals were revealed with biotin-conjugated secondary antibody and streptavidin conjugated to appropriate fluorophores (AlexaFluor 488/594, Invitrogen Life Technologies,USA). The immunoistochemical technique in the cerebral cortex of Gad1EGFP; En2-/- (n=2) and Gad1EGFP; En2WT (n=2) mice, has been performed following the previous IHC protocol. The antibodies concentration: anti-inhibitory presynaptic marker (vGAT) rabbit polyclonal (Synaptic System, 1:500 dilution); anti-green fluorescent protein (GFP) chicken polyclonal (Life technologies, 1:1000 dilution); anti-Parvalbumin (PV) mouse monoclonal (Sigma-Aldrich, USA; 1:2000 dilution). For morphometric analysis, sections were probed with anti-NeuN mousemonoclonal (Millipore, USA; 1:500 dilutions) antibody and signal was revealed by diaminobenzidine staining. NeuN staining confirmed cerebellar hypoplasia in En2-/- brains (not shown), according to previous studies (Joyner et al., 1991; Kuemerle et al., 1997).

3.6 Cell counts

Counts of GAD65/67 in situ mRNA positive cells in the hilus and somatosensory cortex were performed on 3 sections per animal (n = 3 WT and 4 *En2-/-* mice) taken at the level of the dorsal hippocampus. Brain areas were identified according to Franklin and Paxinos (1997). For each section, bright-field images of the hilus and somatosensory cortex were

acquired at 10 × primary magnification using a Nikon Eclipse 90i microscope and merged using Adobe Photoshop software. Three to five immunolabelled sections at the level of the dorsal hippocampus and somatosensory cortex were analyzed per animal (7 WT and 9 *En2-/-* mice). For each section, large images of the hippocampus and cortex were acquired at 20 × primary magnification using a Zeiss Axio Observer z1 microscope with a motorized stage. Acquisitions were automatically performed using the MosaiX and Z-Stack modules of the Zeiss AxioVision software (v4.3.1). Five Z-planes for each image were projected into a single sharp one. Fluorescence intensity, exposure time and microscope settings were optimized for each marker and then held constant. Cell counts were then performed on each image using the ImageJ software. To establish a consistent guideline for counting individual cells, only cell densities larger than 5 µm with a clearly visible nucleus were counted. Signals smaller than 5 µm were excluded to avoid counting neurites, nerve terminals, and false signals. The same counting procedures were then followed for both in situ hybridization (GAD65/67) and immunohistochemistry (interneuron subtypes) images. To count positive cells in the hilus, the total hilar area was measured excluding the granule cell layer. The total area of the hilus did not differ between WT and *En2-/-* mice (WT, 109,780 ± 5032 µm^2; *En2-/-*, 100,958 ± 2602 µm^2; *t*-test, $p > 0.05$). Cell densities were then plotted as the number of positive cells/0.1 mm^2. For the somatosensory cortex, cell densities were separately counted in superficial (II–III) and deep (V–VI) layers. A counting frame with an area of 200 µm × 600 µm was used. The sampling field was moved systematically through the cortical layer of interest at least three times for each section. CALB-positive interneurons were identified by double-labeling with GAD67. Cell densities in the cerebral cortex were plotted as the number of positive cells/counting area (200 µm × 600 µm). All counts were performed by two independent experimenters blind of genotypes.

3.7 Analysis of perisomatic innervation.

Brains were fixed by transcardial perfusion with 4% paraformaldehyde followed by 1 h post-fixation at 4 °C, and 75 µm thick sections were collected. Briefly, at least 3 confocal images of the perisomatic cells were taken within using a 63X oil objective (Zeiss, NA 1.4) and a Zeiss 780 LSM Confocal Microscope. Scans from each channel were collected in multiple-track mode and subsequently merged. Care was taken to use the lowest laser power and no bleed through was visible between Alexa647, Alexa594 and Alexa488 channels. Z-stacks were acquired with 1µm steps, exported as TIFF files, and analyzed using Fiji Image software. Green and red channels were separated using custom-made

macros. The analyses were performed on PV and vGAT immunostaining. Synaptic puncta onto GABAergic and glutamatergic soma was selected using a donut-like shape (10 pixels size selection brush). After the subtraction of background, PV and vGAT signal per soma was averaged along with their standard error. Student t-test was used to evaluate the statistical differences.

3.8 Western Blotting analysis

Protein expression was studied using samples homogenized and processed in Ripa buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 2 mM PMSF, 5 ug/mL leupeptin, 5 ug/mL pepstatin) homogenizing tissue samples in 1 ml of Ripa reagent per 50-100 mg. RNA extraction continue with the support of Rneasy Lipid tissue mini kit. Total protein extracts (30 µg) were separated on 4–12 % NuPage Pre-Cast Gels (Invitrogen), transferred to 0.2 mm PVDF membranes. The membranes were washed 10 min in TBS-T, pH 7.4 (0.1 M Tris, 0.15 M NaCl) and blocked in 5% nonfat dry milk, in TBS with 0.1% Tween 20 (TBST) for 1 h. The incubation with the following primary antibodies, BDNF (1:1500 N-20, Santa Cruz), TrkB (1:750- Promega), has been performed at +4 °C O/N. The next step has been washed the membranes in TBS-T, incubating with the secondary goat anti-mouse HRP antibody (1:10000) for 1 h at RT.

Detection was performed using ECL chemiluminescence system (GE Healthcare). Protein levels were detected using chemiluminescence (BioRad) software and quantitative densitometry analysis has been completed using ImageJ software. In some β-Tubulin was used as a standard for protein quantification. In BDNF western blotting analyses, the normalization for loading was not necessary because we calculated the ratio within subject. The specificity of the Western blot results was confirmed by negative controls with lysis buffer and PBS in 2× Laemli buffer without the protein extract.

3.9 Primary neuronal cell culture

Brains of E18.5 mice were dissected, the meninges were removed and the hippocampi were isolated. Hippocampi were transferred in a 15-ml tissue culture tubes for a pooled mouse culture. The tissue was resuspended in dissection medium and dissociated mechanically in plating medium with a polished tip of glass Pasteur pipettes. Take a 10-µl aliquot of the dissociated cells to estimate viable cell density on a hemocytometer. Cells were plated adding 90,000 cells to an 18-mm coverslip in a 12-well dish (65 cells per mm^2) coated with Poly-L-Lysine. Cells were incubated in the cell culture incubator at 37 °C for

2–6 h. Four hours after plating cells, they were examined under the microscope to ensure that the cells have settled on the substrate. Gently the medium has been aspirated from each well and add 1.5 ml of fresh maintenance medium warmed to 37 °C to each well in the 12-well dish. Cells were incubated in a cell culture incubator at 37 °C and the medium was changed every 3-4 days (Beaudoin et al., 2012).

3.10 Immunocytochemistry analyses

Cells were recovered at day 14 (DIV14) and coverslips were washed for 5 min with PBS and then incubated with paraformaldehyde (PFA) 4% solution in PBS for 20 min. The slices were washed three times with PBS and incubated with blocking solution for 1h. Cells were incubated for 1.5 h with primary antibody in blocking solution and then washed three times for 5 min with PBS containing Triton 0.1%. Cells were then incubated for 1 h with secondary antibodies, washed again, incubated for 5 min with Hoechst and mounted with Aqua Polymount (Polysciences). The primary antibodies used were: vGAT (1:1000, 131 003, Synaptic System), vGlut2 (1:1000, 135 403, Synaptic System), MAP2 (1:200, 556320, BD Bioscience). The secondary antibodies used were: Alexa Fluor 594 Donkey anti-rabbit (1:1000; Jackson ImmunoLab, 711-545-152), Alexa Flour 488 Donkey anti-rabbit (1:1000; life technologies, A-21207). Images were taken using Confocal, Leica.

The number of synapses in a neuron was calculated using Columbus Image Data Storage and Analysis system (PerkinElmer) (v. 2.4.2). The images were uploaded in the system by means of Columbus Importer software (v 2.4), using 0.25 µm as pixel size defaults. Based on MAP2 staining, cell nuclear region was identified by a texture analysis algorithm. This image analysis algorithm or ‘building block’ analyzes the intensity structure of a defined image region for the occurrence of typical patterns. Using a combination of intensity and morphology properties, the population of real cell objects was defined. The cell body was then estimated by creating a region of interest starting from the cell nuclear region. Neurites were detected based on MAP2 staining and assigned to a specific cell body, using the bulding block “Find Neurites”. Automatically it generates a set of neurite properties characterizing each cell’s neurite tree such as total neurite length, length of longest neurite, number of segments, number of extremities, number of roots and number of nodes. Furthermore it generated a set of neurite properties related to the neurite segment population including the segment length in pixel, the branch level which indicates if the segment branched directly from cell body or from another neurite. Based on these properties, segments with length less than 5 pixels were excluded. Synapses were

quantified on neurites by adding the “Select Region” building block to enlarge the neurite region (by choice of pixel) and using the “Find Spots” building block in the selected neurite region exploiting the protein vGAT and vGlut2. The algorithm to identify spots was based on “Detection of local intensity maxima” as first step. Each object of the output population (spots) is characterized by set properties including the corrected spot intensity (the mean spot intensity minus spot background intensity) and spot area (the area of the spot region in pixel units). For each neurite segment, the synaptic contact was calculated as the ratio between the number of spots and the segment length. The differences (“genotype” and “treatment”) were evaluated using two-way ANOVA followed by Tukey’s range test for multiple comparisons.

3.11 Statistical Analysis

Statistical analyses for RT-qPCR were performed by SigmaStat software. Student’s t-test or ANOVA followed by appropriate post-hoc test (as indicated) were used (WT vs *En2*-/-), with statistical significance level set at $p<0.05$. Comparisons between two or more groups were performed with Student’s t-test or one-way/two way ANOVA as appropriate, using GraphPad Prism 6.0. A p-value < 0.05 was considered to be significant.

4. RESULTS

4.1 Cortical Interneuron characterization in En2-/- mice

Engrailed protein expression in the postnatal mouse hippocampus and cerebral cortex.

Neuroanatomical and behavioral studies performed on adult *En2-/-* mice suggest that *En2* might be expressed also in anterior brain structures during adulthood (Cheh et al., 2006) (Kuemerle et al.,2007). Previous studies from our laboratory showed that, in the adult mouse brain, *En2* mRNA is expressed in the hippocampus and cerebral cortex, two regions crucially involved in seizure generation and spread (Tripathi et al., 2009). In order to evaluate the expression pattern of Engrailed proteins, we performed immunohistochemical analysis, using an antibody that recognizes both En1 and En2 proteins (αEnhd1) on serial sagittal sections. This expression profile revealed the pattern of Engrailed proteins (En1/2) in the postnatal mouse forebrain. In primary somatosensory cortex, En1/2 staining was nuclear, according to the transcriptional activity of the Engrailed proteins. Double labeling experiments reveals the presence of En1/2 proteins were present in PV-positive and CALB-positive interneurons, in the hippocampus and cerebral cortex, (Fig.4.1 A, F).

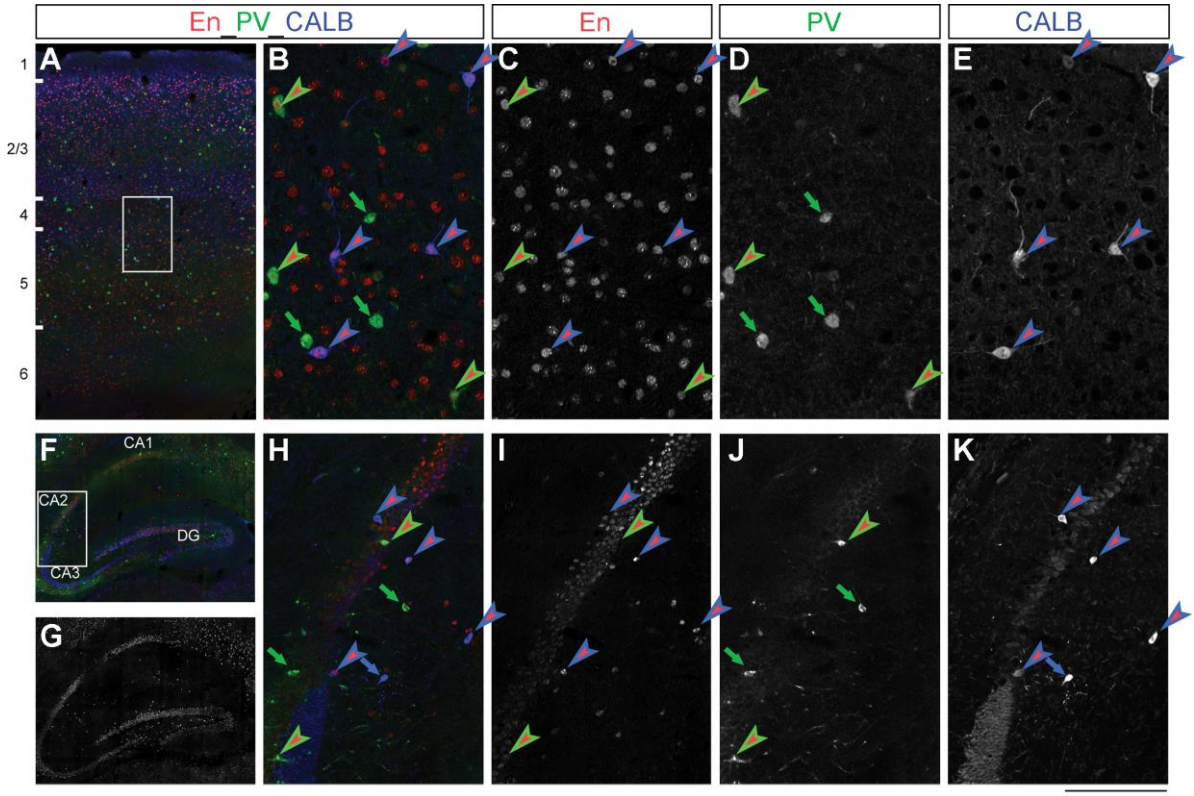


Fig.4.1. *Immunohistochemical data of En1/En2 protein expression in hippocampus and somatosensory cortex.*
A) B) Representative immunostainings of in the somatosensory cortex of adult WT mice (P28). **C) D) E)** Single- channel acquisition of EN (red), CALB (blu) and PV (green) positive interneurons in cortical layer. **F) G) H)** Triple immunostaining showing En (red), PV (green) and CALB (blue) positive cells in the P28 mouse hippocampus**. I) J) K)** Single channel acquisitions of En, PV and CALB immunostainings from the CA2–CA3 hippocampal regions. Scale bar: 400 µm (A), 700 µm (F,G); 95 µm (B-E); 160 µm (H-K).

Cortical integrity and morphometric analysis in En2-/- mice

Given the importance of the Engrailed genes in brain development and patterning of brain areas, we examined the morphology of the *En2-/-* cerebral cortex. NeuN immunohistochemistry revealed no gross layering defects in the somatosensory cortex of adult *En2-/-* mice, as compared to WT littermates. Morphometric analysis showed that the total thickness of the somatosensory cortex (WT, 886 ± 15 µm; *En2-/-*, 938 ± 13 µm) as well as the thickness of each cortical layer (Fig.4.2.A,B) did not differ between WT and *En2-/-* mice ($p > 0.05$, two-way ANOVA followed by Holm–Sidak test; n = 4 WT and 5 *En2-/-*).

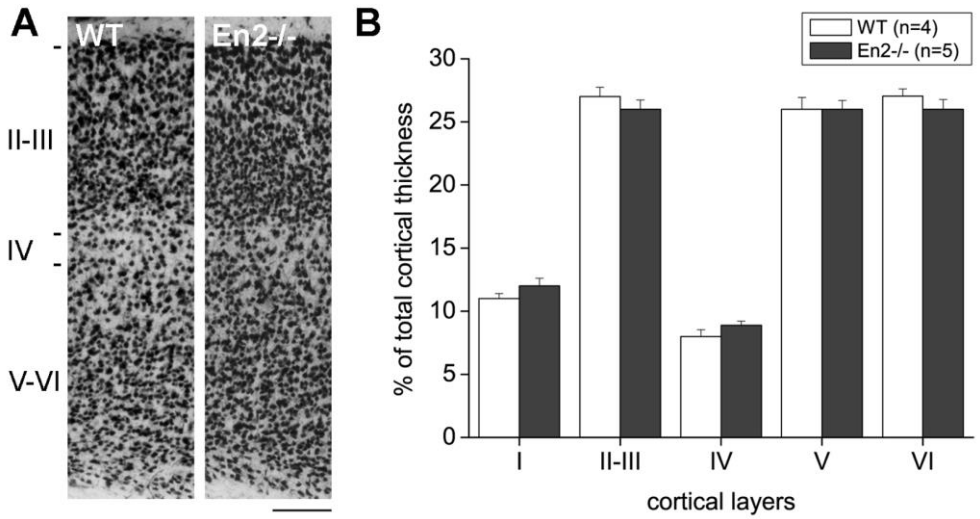


Fig 4.2. *Morphometric analysis in the En2-/- somatosensory cortex.*
Normal layering of the *En2-/-* somatosensory cortex. **A)** Representative NeuN immunostaining of the somatosensory cortex from adult WT and *En2-/-* mice. **B)** Morphometric analysis of cortical in WT and *En2-/-* mice. Layer thickness is plotted as % of total cortical thickness. Layers, animal numbers and genotypes are as indicated. Scale bar: 120 µm.

Interneuron subpopulations in the somatosensory cortex of En2-/- and WT mice.

Several studies showed that alteration of cortical interneuron may play a significant role in the development of neurological disorders (Di Cristo, 2007) (Gogolla et al., 2009) (Kelsom & Lu, 2013). In cerebral cortex, neurons releasing the neurotransmitter GABA have a fundamental role in shaping cortical circuits (Rudy et al., 2011). Disruption of GABA synthesis, affecting migration, maturation and synapse formation may lead to impaired brain development (Rossignol, 2011) (Di Cristo et al., 2011). Malfunction of these neurons has been implicated in a number of diseases, such as epilepsy, schizophrenia, anxiety disorders and autism (Cossart et al., 2001)(Cobos et al., 2005)(Rudy et al., 2011). The link between dysfunction in GABAergic interneurons and the pathophysiology of neurodevelopmental disorders results from an imbalance between excitation and inhibition in local circuit. Defective development of GABAergic interneurons and subsequent reduced inhibition might contribute to the pathogenesis of ASD (Gogolla et al., 2009) (Pizzarelli & Cherubini, 2011)(Chattopadhyaya & Cristo, 2012)(Provenzano, et al., 2012). *En2-/-* mice present a neuroanatomical and behavioral phenotype resembling some features observed in ASD (see references in Introduction). In addition, *En2-/-* mice have an increased susceptibility to KA-induced seizures, displaying more severe and prolonged generalized seizures as compared to WT mice (Tripathi et al., 2009). Hippocampal *En2* mRNA decreased after induced seizures, suggesting that *En2* might also influence the functioning of forebrain areas during adulthood and in response to seizures; more importantly, a reduced staining of parvalbumin and somatostatin was detected in the *En2-/-*

hippocampus, suggestive of an excitation/inhibition imbalance in these mutants (Tripathi et al., 2009). We therefore decided to investigate the different GABAergic subpopulations in somatosensory cortex of *En2-/-* mice, by studying the expression of GABAergic markers at both mRNA and protein level.

We first performed histological experiments to count the number of GAD, PV, NPY, SOM and CALB positive interneurons in the somatosensory cortex of WT and *En2-/-* mice. The number of GAD65/67 positive interneurons was unchanged throughout the somatosensory cortex of *En2-/-* mice, as compared to WT littermates (Fig.4.3, A, B). Following these results, we decided to study the distribution of the different interneuron populations in mutant mice. In the cerebral cortex, PV and SOM interneurons account for about 70% of all interneurons (30% PV, 30% SOM, 10% PV+SOM; (Rudy et al., 2011). The results obtained for the different interneuron markers in the somatosensory cortex of WT and *En2-/-* littermates, showed a reduction in the number of PV and SOM interneurons, in layers II–III of the *En2-/-* cortex, as compared to WT littermates (Fig.4.3, C). In superficial layers, PV and SOM interneurons were significantly reduced (PV: −44%, $p<0.05$; SOM: −25%, $p<0.001$, Student's t-test) (Fig.4.3, D). A significantly reduced number of NPY interneurons was detected in both superficial and deep layers of the *En2-/-* somatosensory cortex, when compared to WT littermates (−30% and −42% in layers II–III and V–VI, respectively; pb0.05, Student's t-test; Figs. 4.3, C, D). CALB-expressing GABAergic interneurons represent a subpopulation of cells present in the cerebral cortex: pyramidal glutamatergic neurons also express CALB (Jinno & Kosaka, 2010). We considered the total number of CALB-expressing neurons throughout all layers of the somatosensory cortex, concluding that there was not difference between WT and *En2-/-* mice (cells/area in layers II/III: WT=178±9, En2-/- =193±6; cells/area in layers V–VI: WT=60±4, En2-/-=57±3; $p>0.05$, Student's t-test). The number of CALB-positive interneurons, identified by GAD67/CALB double staining, was also unchanged between the two genotypes (Figs. 4.3, E,F) $p>0.05$, Student's t-test).

In order to evaluate the mRNA level of different GABAergic markers, we first performed quantitative RT-PCR. RT-PCR experiments revealed a different profile of GABAergic marker mRNA expression in the somatosensory cortex: a statistically significant decrease of PV mRNA expression (−10%) was detected in *En2-/-* mice as compared to WT littermates (Fig.4.3, G; $p<0.05$, Student's t-test). NPY and SOM mRNA expression was unchanged, whereas CALB mRNA expression was significantly decreased (−17%; $p<0.01$, Student's t-test) in mutant mice (Fig.4.3, G). There was no change in mRNA expression

levels for glutamic acid decarboxylase (GAD67), vesicular GABA transporter (vGAT) and vesicular glutamate transporter (vGlut) between the two genotypes.

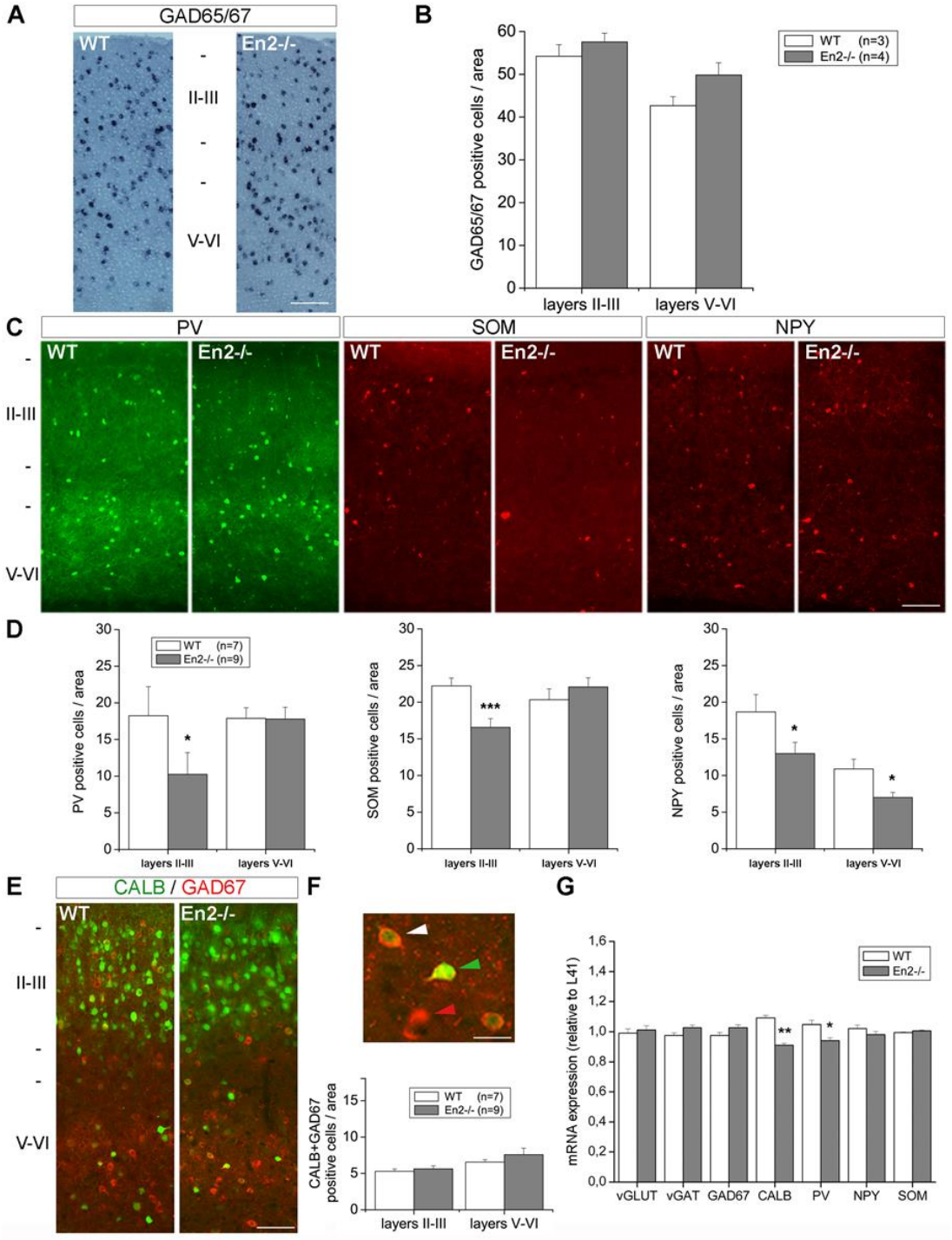


Fig.4.3 *Reduced number of GABAergic interneurons in the En2-/- somatosensory cortex*
A) Representative in situ hybridizations showing GAD65/67 mRNA-positive neurons in the somatosensory cortex of adult WT and *En2-/-* mice. **B)** GAD65/67 mRNA-positive interneuron cell counts. Values are expressed as the mean number (± s.e.m) of positive cells per area (0.12 mm2, see Material and Methods). **C)** Representative immunostainings of PV, NPY and SOM interneurons in the somatosensory cortex of adult WT and *En2-/-* mice. **D)** PV, NPY and SOM interneuron cell counts (values are reported as in B). **E)** Representative immunostainings of CALB (green) and GAD (red)

positive neurons in the somatosensory cortex of adult WT and *En2*-/- mice. **F)** Top: representative GAD (red arrowhead), CALB (green arrowhead) and GAD+CALB (white arrowhead) interneurons in layers V-VI of the *En2*-/- somatosensory cortex. Bottom: cell counts of CALB/GAD double-positive interneurons in the somatosensory cortex of adult WT and *En2*-/- mice (values are reported as in B). **G)** Relative expression of glutamatergic and GABAergic markers mRNAs in the somatosensory cortex of adult WT and *En2*-/- mice (quantitative RT-PCR). Values are plotted as the mean ± s.e.m of 3 replicates from pools of 7 animals per genotype. In all pictures, animal numbers, genotypes and cortical layers are as indicated. In all graphs, asterisks indicate statistical significance (*p< 0.05, ***p< 0.001, Student's t-test, WT *vs. En2*-/-). Scale bars: 200 µm (A), 85 µm (C,E), 20 µm (F).

Our results show a loss of specific interneuron subpopulations detected by immunohistochemistry, which is in agreement with the reduced expression of the corresponding GABAergic markers as revealed by quantitative RT-PCR. Our data demonstrate that loss of En2 results in a decrease of PV, NPY, and SOM interneuron numbers in the somatosensory cortex. Loss of parvalbumin (PV), somatostatin (SST) and neuropeptide Y (NPY) interneurons is not, however, associated with a decrease of the total number of interneurons expressing GAD and with defects in the laminar structure of these areas, indicating that *En2* mutation has a very selective and specific effect on different subpopulation of GABAergic interneurons.

Reduction of interneuron subpopulations in visual cortex.

Previous studies indicated that alterations in visual cortex may be obtained by interfering with the GABAergic function (Hensch, 2005)(Pizzarelli & Cherubini, 2011). In the visual cortex, GABAergic innervation controls the onset of critical periods during which neuronal circuits display a sensitivity to environmental stimuli (Pizzarelli & Cherubini, 2011). In order to evaluate the expression of glutamatergic and GABAergic markers in the visual cortex, we performed immunohistochemistry analyses and quantitative RT-PCR, in adult WT and En2-/- mice. Immunohistochemistry experiments were performed to quantify SOM, NPY, and PV positive cells in the primary visual cortex of adult *WT* and *En2*-/- mice. Immunostaining revealed significant differences in SOM, NPY, and PV positive cell densities between *WT* and *En2*-/- mice, at adult age. *En2*-/- mice showed a lower number (−25%) of SOM cells in layers II–III, and a lower number (−20%) of NPY cells in layers V–VI, as compared to age-matched controls (SOM, $p = 0.024$, Two-Way ANOVA followed by Tukey test; NPY, $p = 0.040$, Two-Way ANOVA followed by multiple *t*-test; $n = 3$ per genotype; Fig. 4.4,A).

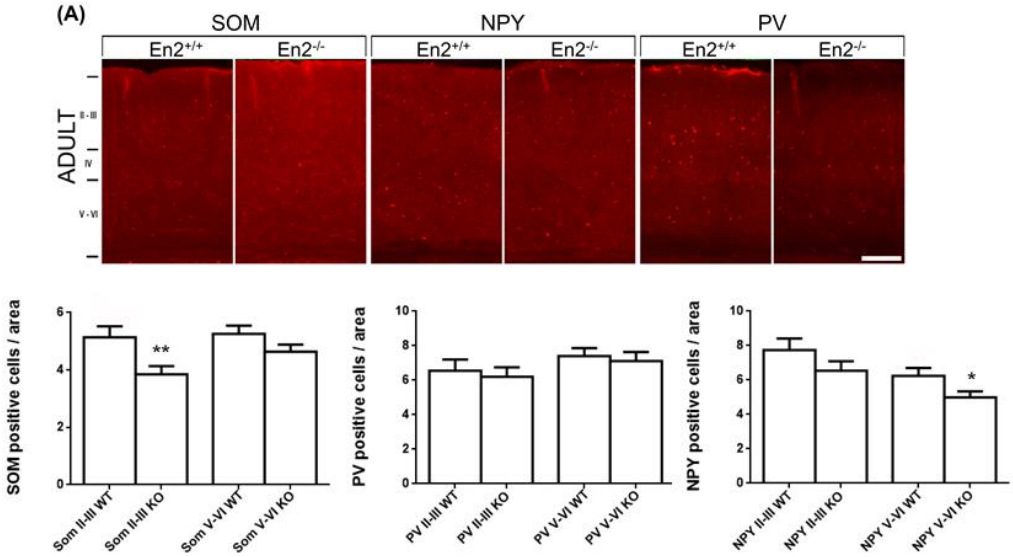


Fig.4.4. *Profile of GABAergic interneurons in visual cortex, at adult age.*
A) Immunostaining of SOM, NPY and PV positive cells, at the level of the primary visual cortex in adult *WT* (white) and *En2-/-* (gray) mice. Immunohistochemistry reveal cell densities in layers II–III and V–VI are plotted as the mean number (± s.e.m.) of cell per area. A lower density of SOM+ cells was detected in the *En2-/-* visual cortex at adult age. Statistical significance: **p* < 0.05; ***p* < 0.01(*WT* vs. *En2-/-*). Scale bar = 100 µm.

The expression profile of GABAergic marker protein in the visual cortex prompted us to investigate the mRNA expression of GABAergic interneurons in the primary visual cortex of *WT* and *En2-/-* mice in adulthood. vGLUT1 mRNA expression significantly lower in *En2-/-* adult mice, as compared to age-matched *WT* controls (–13% in *En2-/-*; Two-Way ANOVA followed by Tukey test, $p < 0.001$, $n = 4$ per genotype). The RT-qPCR revealed a decrease expression also in other GABAergic markers, in particular, in SOM, NPY, vGAT mRNA levels, in En2-/- mice compared to controls (Fig. 4.5).

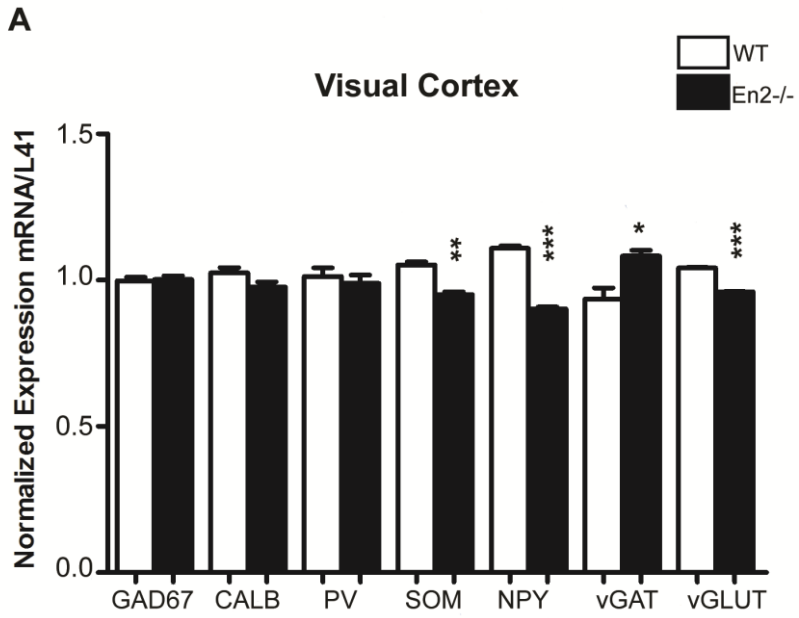


Fig.4.5.*mRNA expression of En2 and glutamatergic/GABAergic markers in the adult visual cortex of WT and En2$^{-/-}$ mice.*

A) Relative mRNA expression level as obtained by quantitative RT-PCR performed on the visual cortex of adult *WT* (white) and *En2-/-* (gray) mice. Values are expressed as each marker/L41 comparative quantitation ratios (mean ± s.e.m.). SOM, NPY, vGLUT, and vGAT mRNA levels were higher in adult WT mice. Statistical significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (*WT* vs.*En2-/-*). Abbreviations as in the text.

Morphometric analysis of the En2-/- visual cortex.

Morphometric analysis did not reveal any significant difference between *WT* and *En2-/-* mice analyzed (Fig. 4.6, A) (*p* > 0.05, Two-Way ANOVA; *n* =3 per genotype) (Fig.4.6, B). NeuN immunohistochemistry also revealed no gross layering defects in the primary visual cortex of adult *En2-/-* mice, as compared to *WT* littermates (Fig.4.6, C). Morphometric analysis showed that at adult age analyzed, the total thickness of the primary visual cortex (adult *WT*, 1018 ± 15.20 μm; adult *En2-/-*, 985.2 ± 8.95 μm) as well as the thickness of each cortical layer (Fig. 4.6, C) did not differ between genotypes (Two-Way ANOVA, *p* > 0.05; *n* = 3 per genotype).

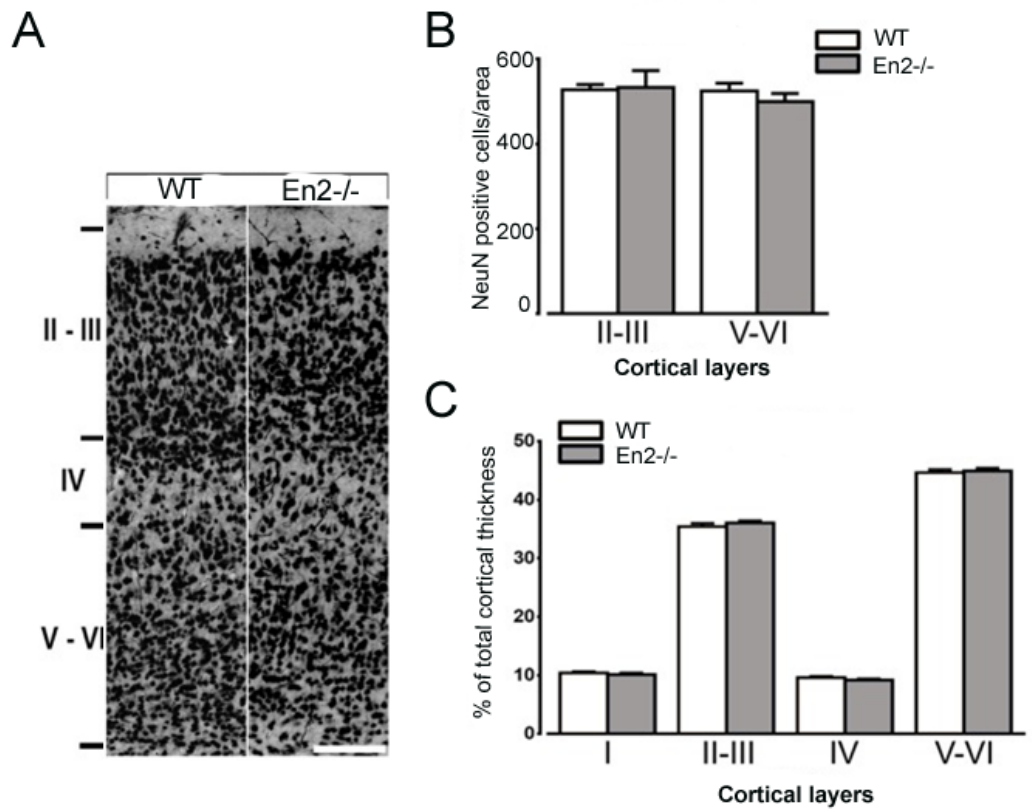


Fig.4.6 *Normal neuronal density and layering in the primary visual cortex of adult En2-/- mice.*
A) Representative NeuN immunostaining of the primary visual cortex, in adult WT (white) and *En2-/-* (gray) mice. **B) C)** Quantification of NeuN immunohistochemistry experiments. Cell densities in layers II–III and V–VI are plotted as the mean number (±s.e.m.) of cell per area (see Materials and Methods). Layer thickness is plotted as % of total cortical thickness. Scale bar=100μm.

4.2 Hippocampal interneuron characterization in En2-/- mice

Previous studies from our laboratory demonstrated *En2* mRNA expression in the adult mouse hippocampus; *En2-/-* mice also showed a staining reduction in PV and SOM interneurons in this brain area (Tripathi et al., 2009). We performed a morphometric analysis on dorsal hippocampal sections stained for the pan-neuronal marker NeuN. A normal anatomical structure of hippocampal layers was detected in WT and *En2-/-* mice (Fig. 4.7, A). Total hippocampal thickness did not differ between WT and *En2-/-* mice (WT: 1192 ± 30 μm, n = 4; *En2-/-*: 1115 ± 28 μm, n = 5; $p > 0.05$, *t*-test), and no difference was detected in hippocampal layer thickness between the two genotypes (Fig.4.7, B, C; $p > 0.05$, two-way ANOVA). Thus, the decreased GABAergic innervation observed in the *En2-/-* hippocampus (Tripathi et al., 2009) is likely due to specific defects of selected interneuron subtypes and not to a general alteration of hippocampal structure.

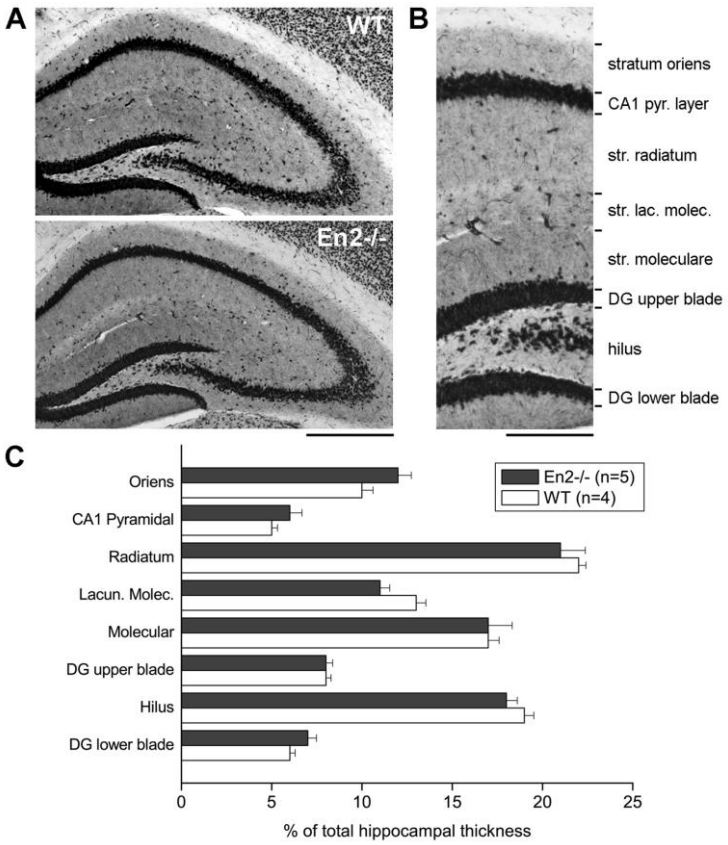


Fig.4.7. *Morphometric analysis in the hippocampus of En2-/- mice.*

A) Representative NeuN immunostaining of the whole dorsal hippocampus from WT and *En2-/-* mice. **B)** Subdivision of hippocampal layers used for morphometric analyses. **C)** Morphometric analysis of hippocampal layers in WT and *En2-/-*mice. Layer thickness is plotted as % of dorsal hippocampus total thickness. Animal numbers and genotypes are as indicated. Abbreviations: DG, dentate gyrus; Lacun. Molec., stratum lacunosum moleculare. NeuN, pan neuronal marker NeuN. Scale bars: 800 µm (A), and 300 µm (B).

Hippocampal interneuron characterization in En2-/- mice.

We then investigated the anatomical profile of GABAergic interneurons in the hippocampus of WT and *En2-/-* mice. We studied the total number of GABAergic neurons using GAD65/67 mRNA in situ hybridization. The GABAergic number was unchanged in the hilus (Figs.4.8, A, B) of *En2-/-* mice, as compared to WT littermates.

Immunohistochemistry experiments revealed a reduction of PV-, NPY-, SOM- but not CALB-positive interneurons in the hilus of *En2-/-* mice, as compared to WT littermates (Fig.4.8,A). Quantification of immunohistochemistry experiments confirmed a statistically significant reduction of PV, NPY and SOM interneurons in the *En2-/-* hilus (− 38%, − 14% and − 20%, respectively; $p < 0.05$, Student's *t*-test; (Fig.4.8, C), whereas no difference was detected in the number of CALB interneurons (Fig.4.8, C).

RT-qPCR analysis demonstrated a statistically significant decrease of NPY (− 48%), PV (− 25%), SOM (− 24%), but not CALB mRNA expression was detected in *En2-/-* mice as compared to WT controls (Fig.4.8, D; $p < 0.05$, Student's *t*-test).

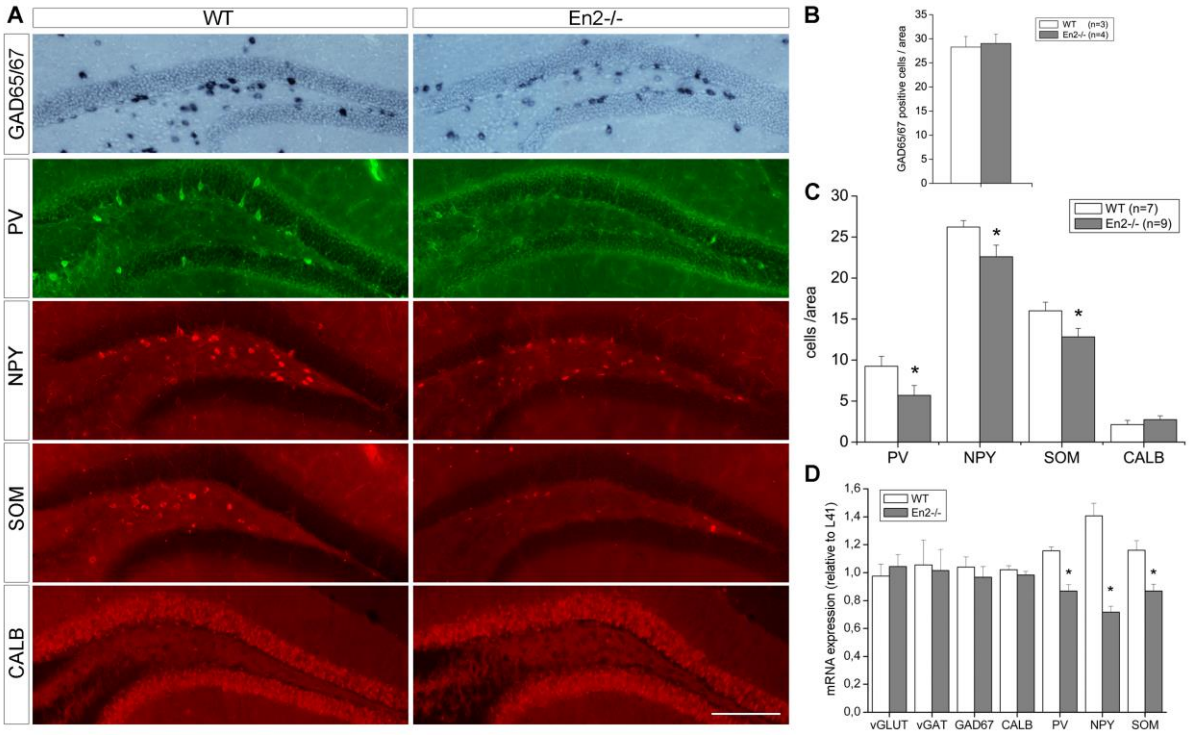


Fig.4.8. *Reduced number of GABAergic interneurons in the En2-/- hilus*

A) Representative pictures showing GAD-, PV-,NPY-, SOM- and CALB-positive interneurons in the hilus of adult WT and *En2-/-* mice. **B)** GAD-positive hilus interneuron cell counts. Values are expressed as the mean number (± s.e.m) of positive cells per area (0.1 mm2, see Materials and methods). **C)** Subtype-specific hilus interneuron cell counts. Values are reported as in B). Animal numbers and genotypes are as indicated. Asterisk indicates statistical significance (*$p < 0.05$, Student's *t*-test, WT vs. *En2−/−*). **D)** Relative mRNA expression level of glutamatergic and GABAergic markers, as obtained by quantitative RT-PCR performed on the whole hippocampus of adult WT and *En2-/-* mice. Values are expressed as each marker/L41 comparative quantitation ratios (mean ± s.e.m of 3 replicates from pools of 7 animals per genotype; *$p < 0.05$, **$p < 0.01$, Student's *t*-test, WT vs. *En2-/-*). Abbreviations: vGLUT, vesicular glutamate transporter; vGAT, vesicular GABA transporter; GAD65/67, glutamic acid decarboxylase (65/67 kD isoform); PV, parvalbumin; CALB, calbindin; NPY, neuropeptide Y; SOM, somatostatin. Scale bar: 300 µm.

We observed that mRNA expression levels for pan-GABAergic (glutamic acid decarboxylase, GAD67; vesicular GABA transporter, vGAT) and pan-glutamatergic (vesicular glutamate transporter, vGLUT) markers did not change between the two genotypes (Fig.4.8, D).

These data indicate that, in the absence of major hippocampal morphological alterations, the *En2* null mutation results in the selective reduction of PV, NPY, SOM but not CALB hilus interneurons.

4.3 Synaptic connectivity and dendritic development in En2-/- mice

Given the GABAergic interneurons dysfunctions in cerebral cortex and hippocampus of our mouse model and the importance of synaptic development and connectivity in ASD, we studied the interneurons synaptic connections on cortical principal neurons (glutamatergic). In particular, we focused our attention on the synaptic connections that PV GABAergic interneurons generate with layer V-VI pyramidal neurons. To this purpose, we crossed En2 mice with Gad1-EGFP (line G42) mice that selectively express enhanced green fluorescent protein (EGFP) in PV-positive basket interneurons (soma, dendrites, and axons) and also in putative presynaptic boutons (Chattopadhyaya et al., 2004). However, the GFP transgene expression in the synaptic boutons was less intense in our mice compared to the original strain, therefore the perisomatic innervation was analyzed using PV antibody (Chattopadhyaya et al., 2004).

We performed immunohistochemical technique in the cerebral cortex of Gad1EGFP; En2-/- (Fig. 4.9, A) and Gad1EGFP; WT mice (Fig. 4.9,B). The immunostaining revealed PV-positive interneurons labeled in red, vGAT (inhibitory presynaptic marker) in blue (Fig. 4.9) and GFP (expressed in basket cells) in green (not shown in this analysis). We analyzed PV and vGAT spine density, considering the intensity of puncta staining of the two antibodies, in layer V-VI of the somatosensory cortex (Fig. 4.10, A). The results obtained show a little variation, not statistically significant, in puncta density of the two GABAergic markers, in En2-/- and WT mice (Fig.4.10, A). Interestingly, these differences became significant once the PV/vGAT ratio is taken into account (Fig.4.10, B).

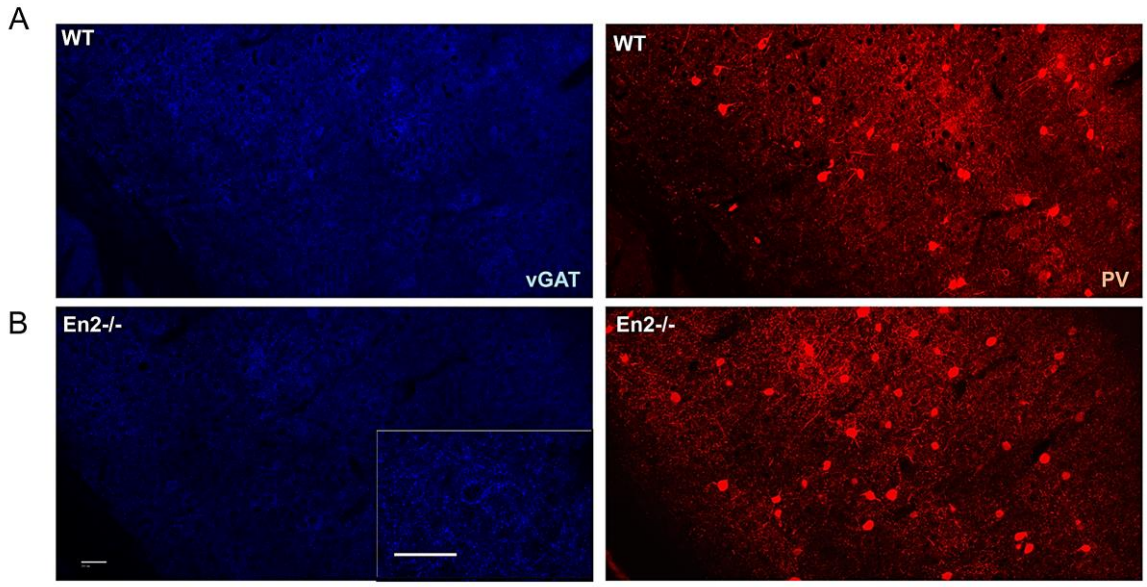


Fig.4.9 *Synaptic density in En2-/- and WT mice, in somatosensory cortex*.
A) Representative images of PV and vGAT staining in cerebral cortex of WT mice. It has been considered layers V-VI in somatosensory cortex, in both the genotype. **B)** Images represent PV and vGAT staining in the cortex of *En2-/-* mice. In vGAT image, it is possible to observe a high magnification of a perisomatic vGAT ring. Scale bar: 200 µm.

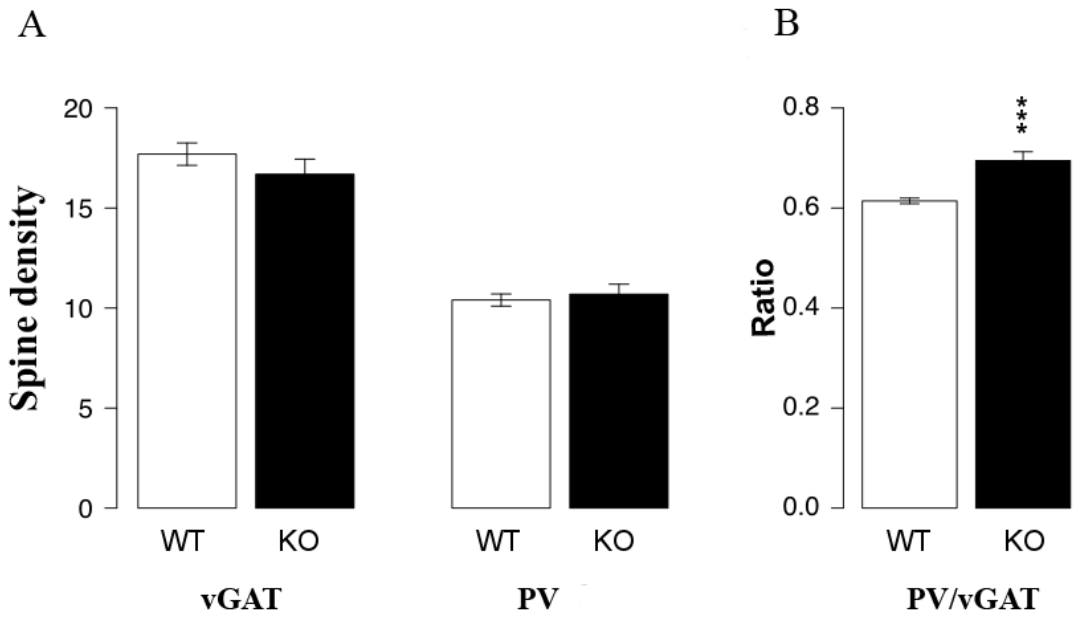


Fig.4.10. *Distribution of synaptic puncta in the somatosensory cortex of En2 mutant and WT mice.*
A) The graphs show the **s**pine density of two GABAergic marker, vGAT and PV, in En2-/- and WT mice. **B)** The PV/vGAT spine density ratio shows an increased expression in Parvabumin, compared to vGAT level, in En2-/- and WT mice. p-value: ***p < 0.001.

Dendritic spine analysis in the En2-/- hippocampus.

Anatomical pathology of autism may involve abnormal spine generation or deficits in spine reorganization, elimination, and pruning (Hutsler & Zhang, 2010)(Gadad et al., 2013). The development of dendritic spines and synapses has been studied in several neuropathologies. Alterations in dendritic spines have been studied in a large number of brain disorders and may suggest the common cause for many neuropsychiatric syndromes, involving deficits in information processing (Penzes et al., 2011). Some animal models resemble these conditions: in fragile-X syndrome there is an abnormal abundance of long, thin protrusions and an increased spine density that apparently persists throughout life (Irwin et al., 2001)(Fiala et al.,2002). FMR1 KO mice exhibit elevated cortical spine densities, similar to those observed in autism, therefore, an overabundance of immature dendritic spines in cortical pyramidal neurons (Grossman et al., 2010)(Portera-Cailliau, 2012)(Gadad et al., 2013). Mice deficient in *MeCP2* also exhibit decreases in the number of functional, excitatory synapses (Chao et al.,2007)(Penzes et al., 2011); the Angelman syndrome mouse, which lacks the maternal *UBE3A* gene, displays altered spine density on hippocampal and cortical pyramidal neurons (Dindot et al., 2008)(Penzes et al., 2011). It has been postulated that GABA signaling plays an important role in regulating synapse development (Chattopadhyaya et al., 2007).

We performed Golgi staining on young (p30) and adult (>5 months) *En2-/-* and WT mice. Brains were processed for Golgi staining and we collected images from basal and apical dendrites of CA1 pyramidal cell and from dendrites of granular cell layer in the dentate gyrus (described in *materials and methods*). In CA1 region, the apical dendrites occupy the stratum radiatum and stratum lacunosum molecolare, whereas the basal dendrites occupy the stratum oriens. The results showed an increased in spine density in the basal dendrites in hippocampus of adult *En2-/-* mice. In young mice (p30) the number of spines was reduced in apical and dentate gyrus, confirming that *Engrailed* could controls spinogenesis (Figs.4.11, A, B).

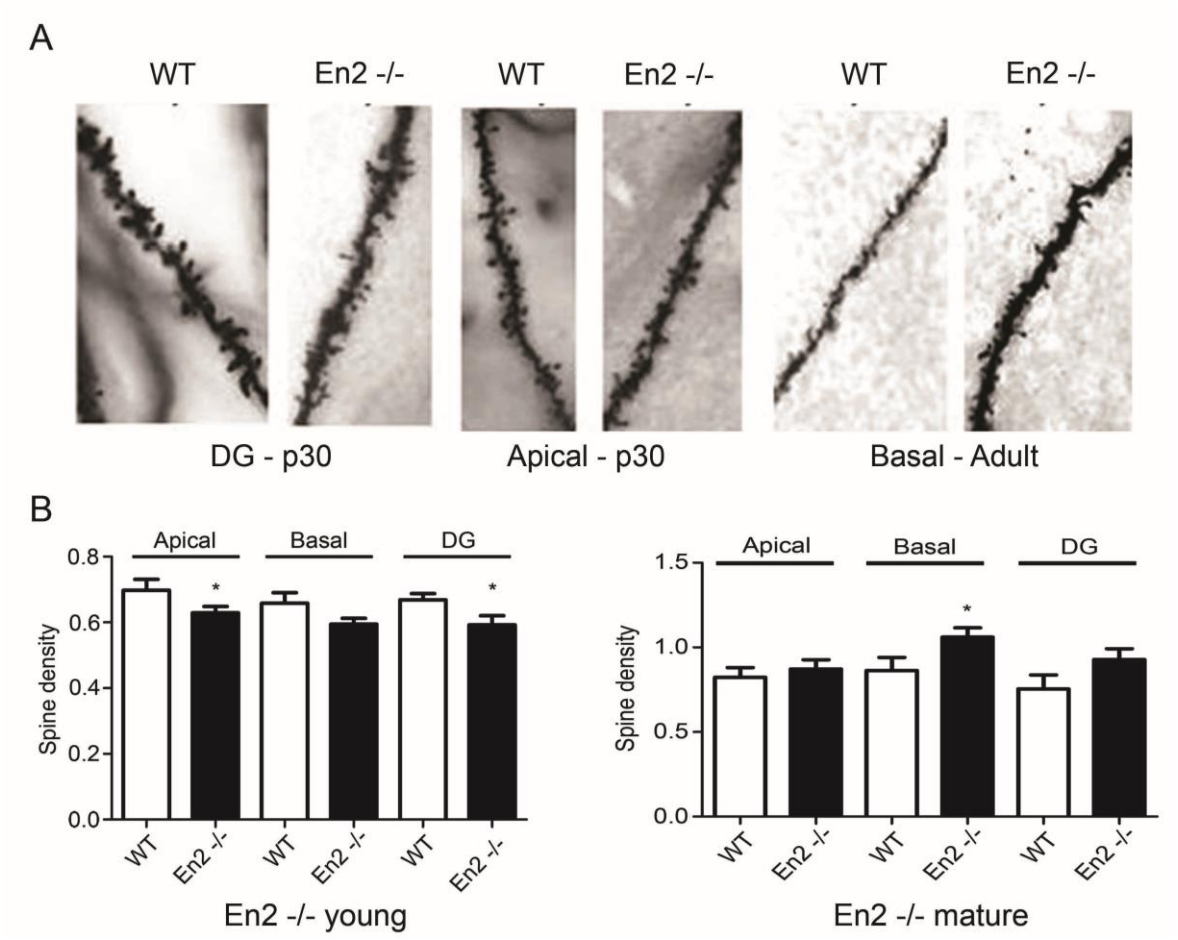


Fig.4.11. *Spinogenesis in the hippocampus of En2-/- mice, at P30 and adult age.*
A) Representative images obtained after Golgi staining of mice hippocampi. Shown are apical and basal dendrites of WT and En2-/- adult mice. **B)** Quantification of spine density (per µm) in young and adult mice in WT and En2-/- mice. In B, *, p=0.0221 for "apical" or p=0.0254 for "DG" (t-test). In E, *, p=0.0219 (t-test). Values are the mean +/- s.e.m. of spine densities measured from a total of 18-25 dendritic segments photographed from 2-3 mice per genotype and age group. Bar in A, 5µm.

4.4 Brain derived neurotrophic factor and receptors

BDNF RNA splicing variants in hippocampus and cerebral cortex of En2-/- mice.

Brain-derived neurotrophic factor (BDNF) is a crucial factor for the postnatal development of forebrain GABAergic neurons (Huang et al.,2007)(Marín, 2012). BDNF, released from pyramidal axons, could signal to many GABAergic neurons and influence inhibitory innervation globally (Kohara et al. 2001) (Huang et al., 2007).

Here we investigate whether altered BDNF expression may be related to the altered development of GABAergic interneurons described in *En2-/-* mice. We first performed a quantitative RT-PCR of the different BDNF mRNA isoforms in the cerebral cortex of *En2-/-* mice and WT controls. RT-PCR results demonstrated a remarkable reduction of different BDNF transcript variants in the cerebral cortex of *En2-/-* mice respect to controls, after

normalization to the mRNA of the housekeeping gene (ribosomal protein, L41) (Fig.4.12, A). Only ExonVII mRNA presented a comparable expression in WT and mutant mice. Conversely, in the En2-/- hippocampus, only ExonVII transcript variant was increased in *En2-/-* mice compared to WT, whereas the other BDNF mRNA level did not show any significant alteration (Fig.4.12, B)

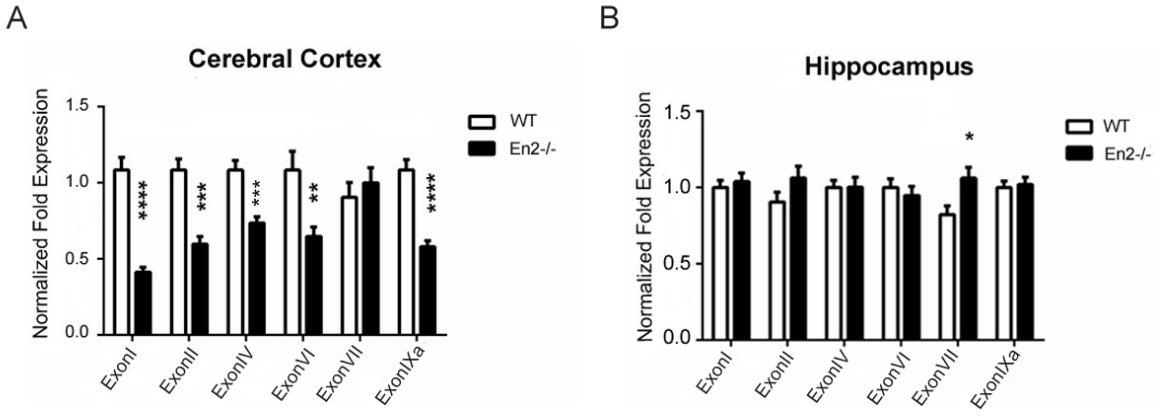


Fig. 4.12.*Analysis of BDNF mRNA in brain regions of En2-/- mice and WT control:*
A) RT-PCR on BDNF transcript variants of cerebral cortex in En2-/- mice. There is a significant alteration in different BDNF transcript variants. **B)** Expression profile of different BDNF transcript variants in hippocampus of *En2-/-* mice compared to WT. The graph reveals an increase in ExonVII expression in mutant mice compared to WT. There is no significant alteration in the other mRNA variants between both the genotype. Values are expressed as each marker/L41 comparative quantitation ratios (mean ± s.e.m of 3 replicates, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, Student's t-test, WT *vs. En2-/-*).

In situ hybridization of BDNF mRNA levels in different brain regions.

In this study we analyzed the distribution of BDNF splice variants, in particular BDNF I, II, VI, and IXa, using cDNA probes. We verified the distribution of BDNF mRNAs in adult WT and mutant mice. Exon I-containing BDNF mRNA is expressed in the whole hippocampus and in detail, in the CA1, CA2, CA3 and granule cell layer of the dentate gyrus (DG) (Fig.4.13, A). Exon II BDNF splice variants [which include exon IIB and exon IIC] seems to be expressed with the same intensity in mutant and WT mice (Fig.4.13, B). Their expression remains strictly somatic: an observation in hippocampus areas supports a somatic localization of this transcript in both exon I and II transcripts, which were constitutively expressed in all principal hippocampal neurons. All cortical layers present a homogenous distribution of BDNF variants, without any difference between *En2-/-* and WT mice. These analyses demonstrate that there is no significant difference in the spatial expression of the different BDNF variants (BDNF I, II) in *En2-/-* brains. In the cerebellum,

the ISH show a reduction in the Purkinje cells in *En2-/-* mice, however any significant change in the spatial distribution of BDNF variants has been observed (Fig.4.13).

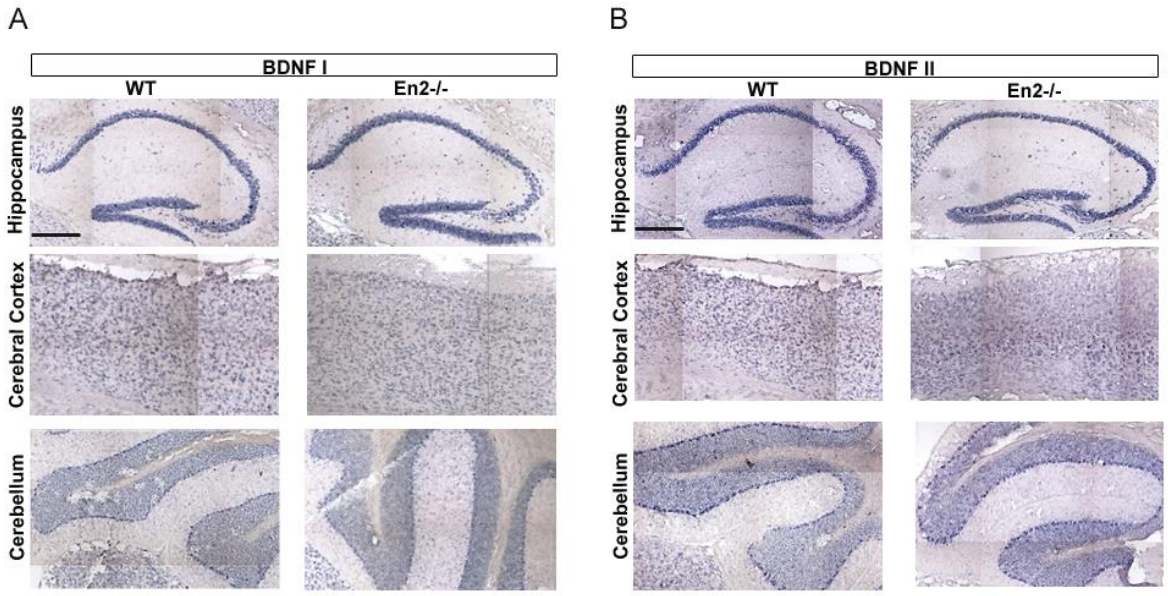


Fig.4.13 *In situ hybridization (ISH) of BDNF varinats in En2-/- and WT mice.*
A) B) Localization of exon I and II (IIB and IIC) BDNF splice variants. Images shown are representative sagittal sections of hippocampus, cerebral cortex and cerebellum of En2-/- mice compared to WT. Scale bar: 500 µm.

We investigated also the subcellular distribution of Exon VI and Exon IXa BDNF in *En2-/-* mice compared to controls. These BDNF variants are constitutively expressed in the entire hippocampus, in cerebral cortex and cerebellum of *En2-/-* mice and WT controls. As compared to Exon I and II, BDNF Exon IXa mRNA showed a similar expresion pattern in the *En2-/-* brain, with no detectable differences respect to WT mice (Fig.4.14). Exon VI mRNA instead showed a different profile (Fig.4.14, A). According to RT-qPCR data, BDNF Exon VI mRNA was downregulated in the *En2-/-* cerebral cortex compared to WT. Contrary to what detected by RT-qPCR, down-regulation of BDNF Exon VI mRNA was also detected in the *En2-/-* hippocampus and cerebellum (Fig.4.14, B).

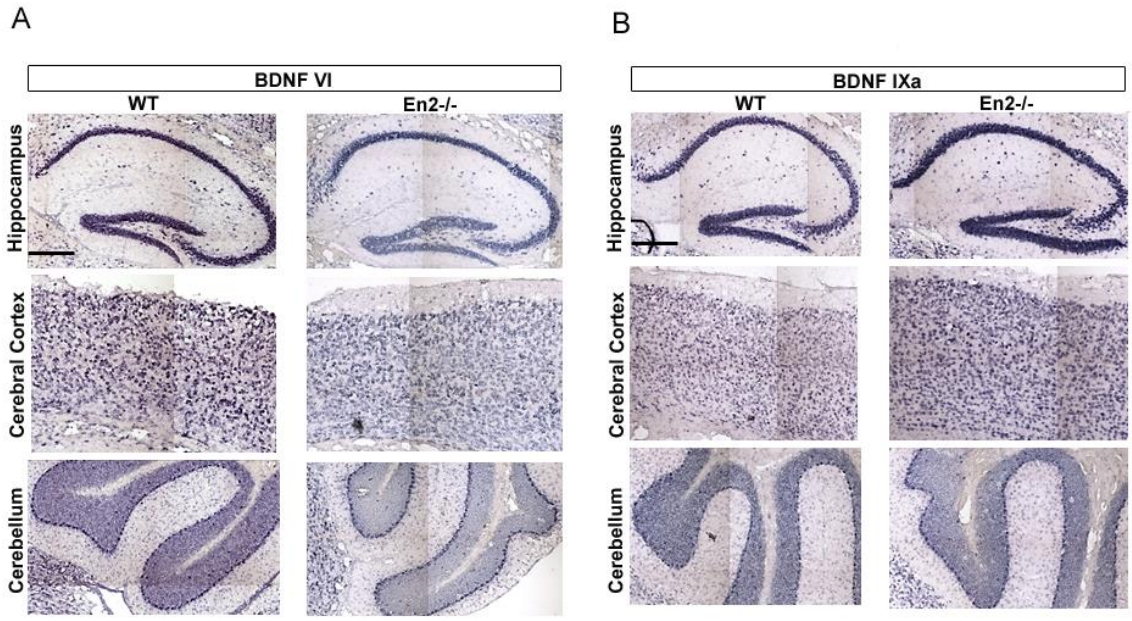


Fig.4.14 *In situ hybridization (ISH) of BDNF varinats in En2-/- and WT mice*
Localization of exon VI and IXa BDNF splice variants.
A) B) Images shown are representative sagittal sections of hippocampus, cerebral cortex and cerebellum of En2-/-mice compared to WT. Scale bar: 500 µm.

BDNF protein isoforms in hippocampus and cerebral cortex of En2-/- mice:

Yan and colleagues presented consistent results of subcellular localization of BDNF in the rat brain, confirming the idea that this neurotrophic factor has an important role in the CNS (Yan et al., 1997). They described in detail, by immunohistochemical analyses, that BDNF is expressed in the hippocampus, claustrum, basolateral amygdala and neocortex of adult rat brains (Yan et al., 1997). Following previous studies, we decided to performed immunohistochemical technique, in order to verify the BDNF protein expression in *En2-/-* mice and WT controls.

The results presented here reveal a widespread localization of BDNF protein throughout the cerebral cortex and hippocampus areas, in both *En2* mutant and WT mice. The level of BDNF proteins seems to be comparable in both genotypes: a heavy staining may be observed in the CA1, CA2, CA3 and dentate gyrus regions. An intense staining is present also at the level of the cerebral cortex (Fig.4.15).

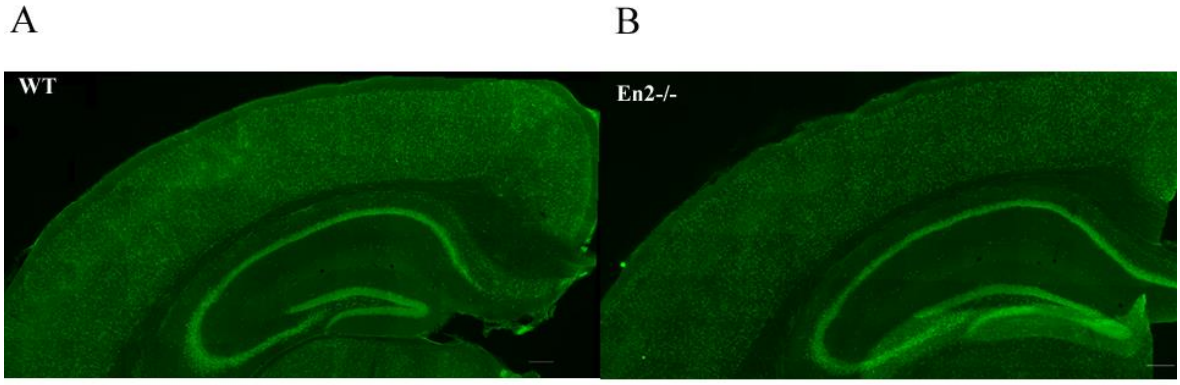


Fig.4.15. *BDNF protein expression in En2-/- mice compared to WT.*

A) B) Representative immunofluorescence images of BDNF immunostaining. These are coronal sections of hippocampus and cerebral cortex, in *En2-/-* mice and WT controls. There is no significant difference in the BDNF protein expression in both the genotype. Scale bar 200um.

Quantification of BDNF protein isoforms in hippocampus:

In order to elucidate whether there is an alteration in BDNF protein expression in *En2* mutant mice, we quantified the different BDNF isoforms in two brain regions, in which we observed an alteration in BDNF mRNA level. Previous results reveal an alteration in BDNF ExonVII expression in hippocampus and a significant decrease in different BDNF splice variants in cerebral cortex of *En2* mutant mice, compared to WT.

We therefore analyzed, by western blotting analysis, the expression of truncated-, pro-, and mature BDNF isoforms in hippocampus and cerebral cortex of *En2-/-* mice, compared to WT. The antibody used detected three immunoreactive bands: the 35-kDa band corresponds to the pro-BDNF molecular weight (pro-BDNF), the 28-kDa band corresponds to truncated form of pro-BDNF and with 14-kDa band was identified the mature BDNF isoform. Band densitometry allowed us to measure the relative percentage of the three isoforms compared to total BDNF, expressed as the ratio of the densitometric value of a given BDNF isoform and the sum of the densitometric values of all isoforms (pro-BDNF + truncated-BDNF + mature-BDNF)(Fig.4.16,A). The isoforms relative percentage of the two genotypes was then compared. Our results demonstrate a low expression of truncated BDNF isoform (Fig.4.16, A), revealing a significant decrease in the truncated BDNF/total BDNF ratio in mutant mice versus controls. The ratio of mat-BDNF and pro-BDNF/total BDNF was comparable between WT and *En2-/-* mice (Fig.4.16, B).

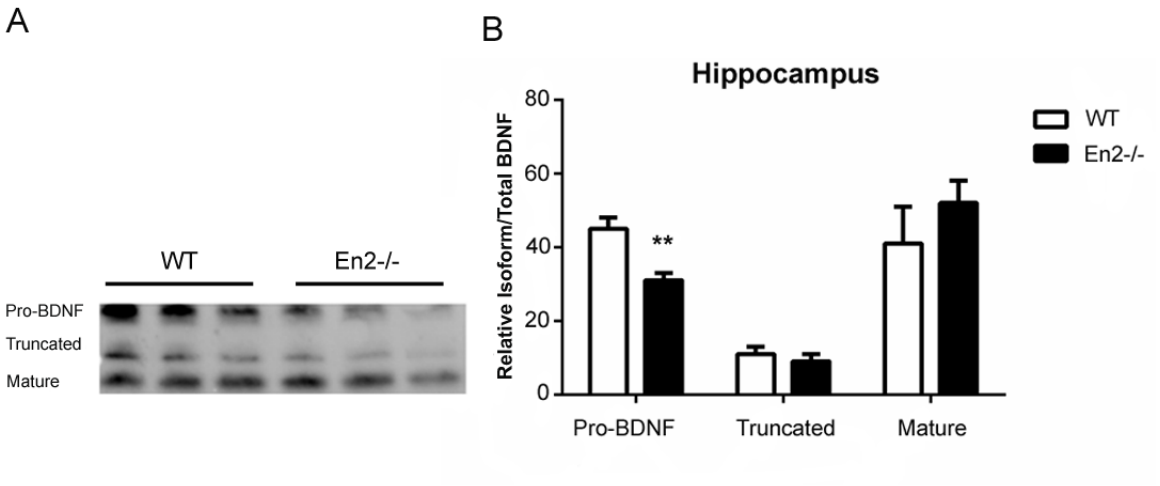


Fig.4.16 *Quantification of BDNF in En2-/- and control samples by Western blotting:*

A) Representative western blot of BDNF isoforms. Housekeeping is representing with B-Tubulin bands, in the upper part of the membrane. **B)** Western Blot graph indicated a significant down-regulation of pro-BDNF isoform, whereas, the other isoforms remain unaltered. For each sample, the pixel value of each BDNF isoform was expressed as a ratio to total BDNF pixel values, where total BDNF is the sum of pro-BDNF + 28-kDa truncated BDNF + mature BDNF. P value: * $p < 0.05$, 2-taile d *t* test. Bars represent mean ± SE. (sample n=4).

We next extended our quantification analysis to this brain area and investigated the BDNF protein expression in cerebral cortex of *En2-/-* mice compared to WT controls. Western blotting revealed no significant difference in the BDNF isoforms in both *En2-/-* and control group regions. Mutant mice present a significant up-regulation of pro-BDNF isoform. No significant alteration was observed for the expression of truncated and mature BDNF isoforms in *En2-/-* versus WT mice. (Fig.4.17, B).

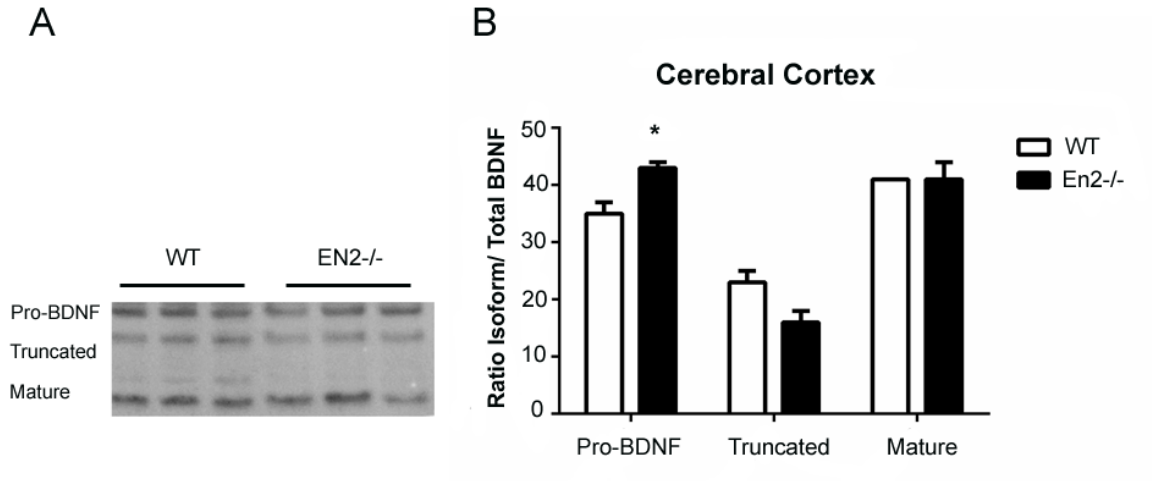


Fig.***4.17*** *Quantification of BDNF in En2-/- and control samples by Western blotting in cerebral cortex:* **A)** Representative western blotting showing the relative expression of BDNF isoform, pro-, truncated and mature BDNF isoforms. **B)** Graph representing relative percentage of pro-BDNF, truncated or mature BDNF with respect to the total amount of serum BDNF (total BDNF is calculated as the sum of the densitometric values of the three BDNF isoforms in each sample. Bars represent mean ± SE (sample n=3). P value *p < 0.05, 2-tailed *t* test.

4.4.1 P75 and TrkB receptors expression

In order to complete the BDNF expression analysis we extended our study to BDNF receptors. We performed quantitative real time PCR experiments, which showed a significant downregulation of p75 receptor in the hippocampus and cerebral cortex of *En2-/-* mice, compared to controls. The TrkB expression is unchanged, with the only exception in TrkB-full length, in the hippocampus of *En2-/-* mice versus WT groups (Fig.4.18, B).

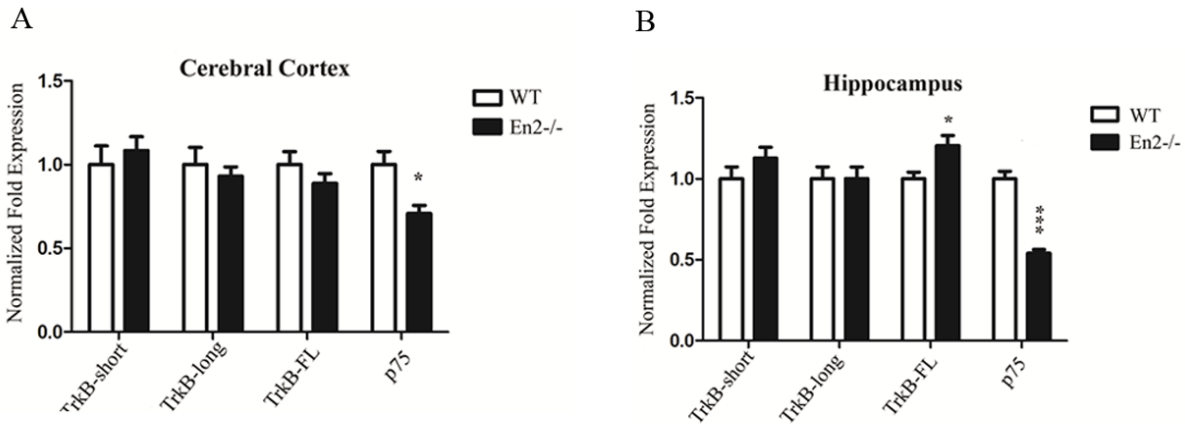


Fig.4.18. *mRNA expression of BDNF receptors:*

A) Quantitative RT-PCR analysis in cerebral cortex of BDNF receptors markers: mRNA expression reveals a down-regulation of p75 marker in *En2-/-* mice. TrkB mRNA levels are comparable in both genotypes **B)** The full length of TrkB results increased in mutant mice, whereas, the truncated isoforms have no significant difference in En2-/- and control mice. Reduced p75 expressions in hippocampus of En2-/- mice compared to WT. Values are plotted as the mean ± s.e.m of 3 replicates from pools of 3 animals per genotype. In all graphs, asterisks indicate statistical significance (*p < 0.05, ***p < 0.001, Student's *t*-test, WT vs. *En2-/-*)

Following these results, we decided to investigate the expression of the high-affinity BDNF receptor, TrkB, using western blotting experiment. The results obtained demonstrate an upregulation of TrkB receptor in the hippocampus of *En2-/-* mice, compared to control group (Fig.4.19).

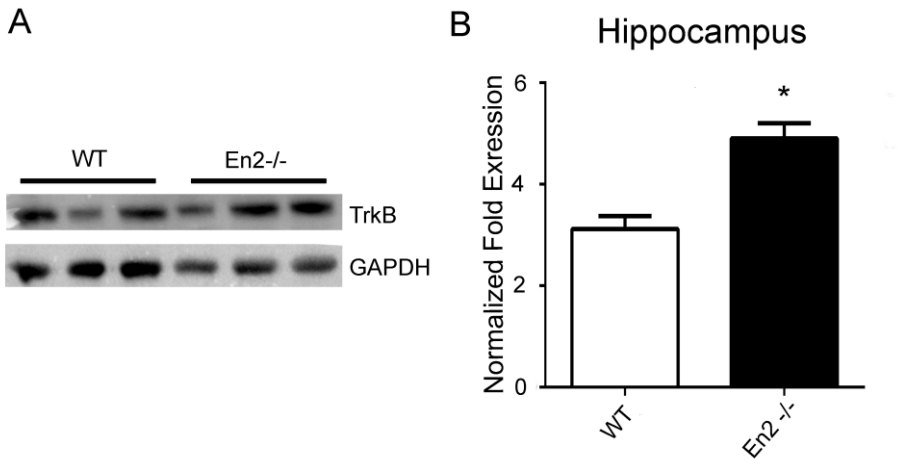


Fig.4.19. *Protein expression of TrkB receptor in hippocampus of En2-/- mice:*
A) Immunoblot on PVDF membrane showing TrkB expression: GAPDH is included as control. **B)** Western blotting analysis reveals an increase of TrkB level in *En2-/-* mice compared to wild types (n=3 per group). Unpaired t-test: *En2-/-* vs WT controls, p-value * p < 0.05. (Sample n=3 per genotype).

4.5 BDNF treatment in *En2-/-* hippocampal cell cultures

BDNF is known to be required for proper development and survival of dopaminergic, GABAergic, cholinergic, and serotonergic neurons. Recent study demonstrated that BDNF promotes the formation of GABAergic synapses in hippocampal and cortical culture and can potentially adjust the number and strength of GABAergic synapses onto targeted glutamatergic cells (Huang et al., 2007). BDNF is produced mainly by cortical glutamatergic cells and its signaling is activity-dependent. Indeed, previous immunohistochemical analyses revealed that *En2* is expressed also in pyramidal neurons), suggesting that En2 ablation could affect BDNF expression in glutamatergic neurons. The decrease expression in different BDNF splicing variants in *En2* mutant mice support this hypothesis, suggesting that alteration of BDNF might play a role in the aberrant GABAergic interneuron development observed in the *En2-/-* mice. For this purpose, we analyzed the effect of BDNF treatment in postnatal hippocampal cell cultures.

Morphology of primary hippocampal neuron in En2-/- mice vs WT.

In order to establish a mature hippocampal cell culture, we used long-term hippocampal cultured neurons (2 weeks), which tend to have synapse maturity (Fig.4.20, A). Neurons in culture have distinct stages of development, starting with extension of lamellopodia, followed by axon and dendrite specification and extension and synapse formation and maturation (Beaudoin et al., 2012). The peak of dendritic growth and synapse formation occurs during the second and third weeks in culture, with dendritic spines first appearing at about 2 weeks in culture. At this stage, hippocampal cultures have formed such a dense network.

A

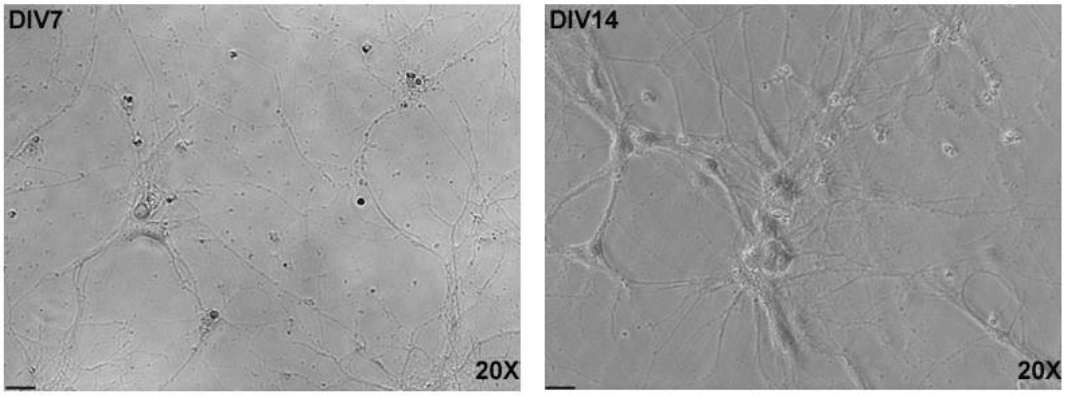


Fig.4.20 *Representative images of cultured hippocampal neurons*.
A) Images of neurons at different stages during culture—DIV 7 and DIV14. Note how neuronal morphology and arborization develop over time. Scale bar 25µm.

Primary hippocampal neurons were cultured in the continuous presence of BDNF protein in the medium (20ng/ml). To test how BDNF acts at dendritic level and to determine the possible alterations in spine density, we co-stained neurons with anti-vGAT (vescicular GABA transporter), vGlut-2 (vescicular Glutamate transporter-2) and anti-MAP2 antibodies. MAP2 is known to label the somatodendritic contour of neurons, but not the presynaptic compartments such as dendritic spines or postsynaptic densities (Bernhardt & Matus, 1984), whereas vGAT is highly concentrated in the nerve endings of GABAergic neurons in the brain.

We performed a confocal analysis of cultured hippocampal neurons labeled anti-vGlut-2 (vesicular glutamate transporter) and anti-vGAT (inhibitory vesicular transporter). In particular, we stained for vGAT, vGlut2 and MAP2, acquiring at high-resolution (63X) with confocal microscope (Fig.4.21) (Fig.4.22). Cultured neurons extend neurites that have characteristic morphologies (Fig. 4.21, a,b,c) and express appropriate dendritic (MAP2) marker. They possess excitatory synapses, as indicated by staining for vGlut2 (excitatory presynaptic marker) and inhibitory synapses, as indicated by vGAT (inhibithory presynaptic marker) as previously reported (Yamada et al., 2002). We performed immunostaining on hippocampal neurons treated and untreated (control cells) with BDNF protein after two weeks in culture. On these cells, we studied spine density, visualizing the typical “spot” representing the synaptic puncta, stained with vGAT or vGlut2 antibodies, and the “fragments” representing the dendrites length. Synaptic density was assessed using Columbus software that allows the determination of the number of synaptic puncta on dendrites length.

No significant differences were observed between control and BDNF-treated cultured neurons, in *En2-/-* and WT mice. All cell cultures present a normal growth efficiency and adherence to the slide. Observing the MAP2 staining, it seems that BDNF treatment leads to a decrease in cell density, in the level of branching and the number of connections between neurons. The connections present in the treated cells, although less numerous, appear less branched. This effect can be seen looking at the pictures with MAP2 staining (Figs.4.21 c, d,) (Figs.4.22 c, d). Conversely, in the control is observed a high degree of branching, with a higher number of connections (Figs.4.21 a, b) (Fig.4.22 a, b). The puncta staining characterizes both the GABAergic and glutamatergic marker, but vGAT has a lower signal compared to vGlut2. In En2-/- treated neurons the vGAT and MAP2 staining seems to be damage, suggesting a possible suffering of neurons after the treatment.

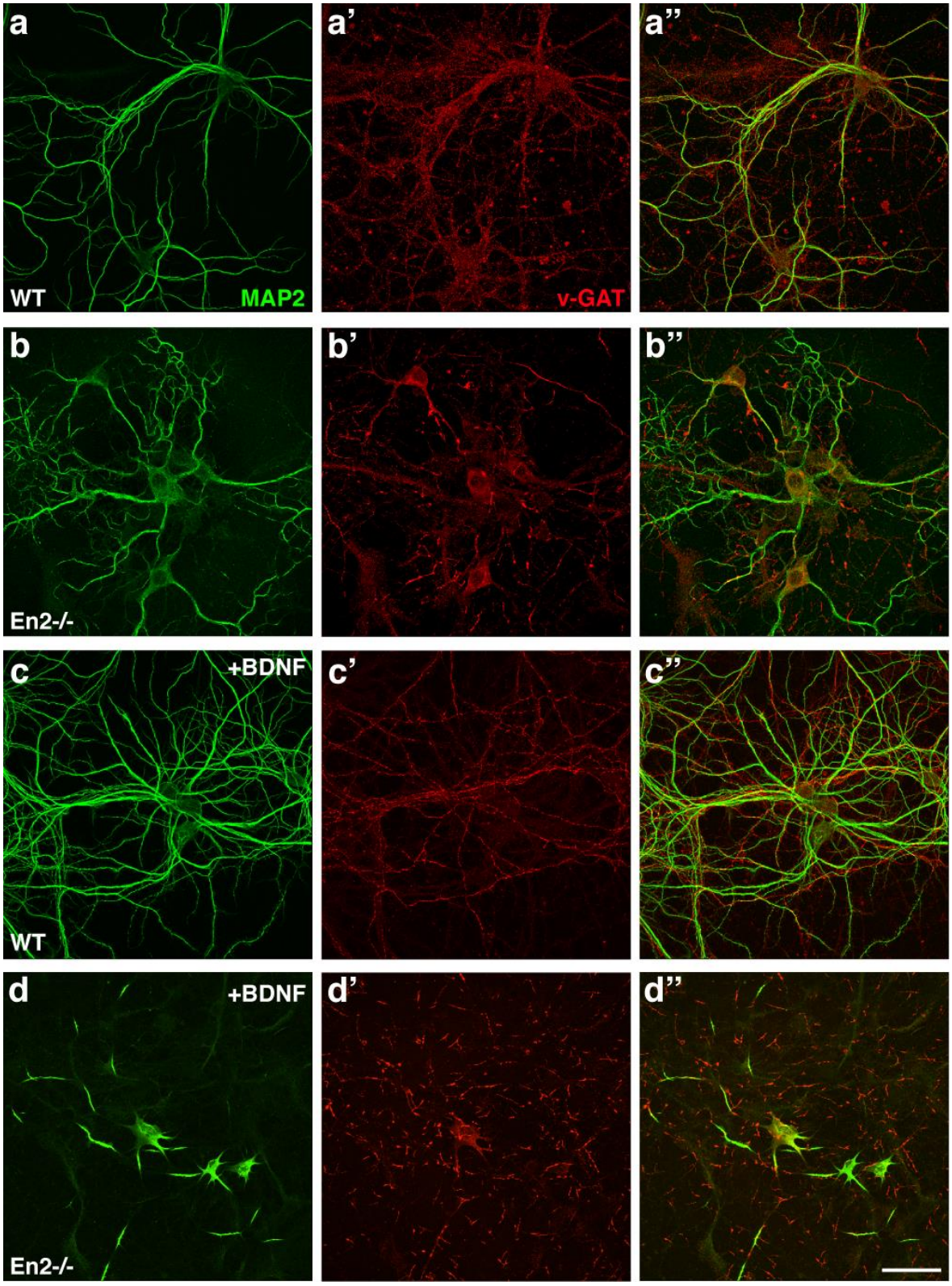


Fig.4.21. *vGAT staining of primary postnatal hippocampal cell culture.*
a) a') a") Images show MAP2 and vGAT staining in WT hippocampal neurons at DIV14. **b) b') b")** Representative images of postnatal cell culture neurons of *En2* mutant mice. It is possible to observe a vGAT staining (red) and MAP2 expression (green). **c) c') c")** BDNF treatment in WT hippocampal neurons, stained at DIV14 with MAP2 (green) and vGAT (red) antibodies. **d) d') d"**) Images show *En2-/-* cell culture neurons treated with BDNF. Immunofluorescence performed using MAP2 and vGAT staining. Scale bar: 50 µm.

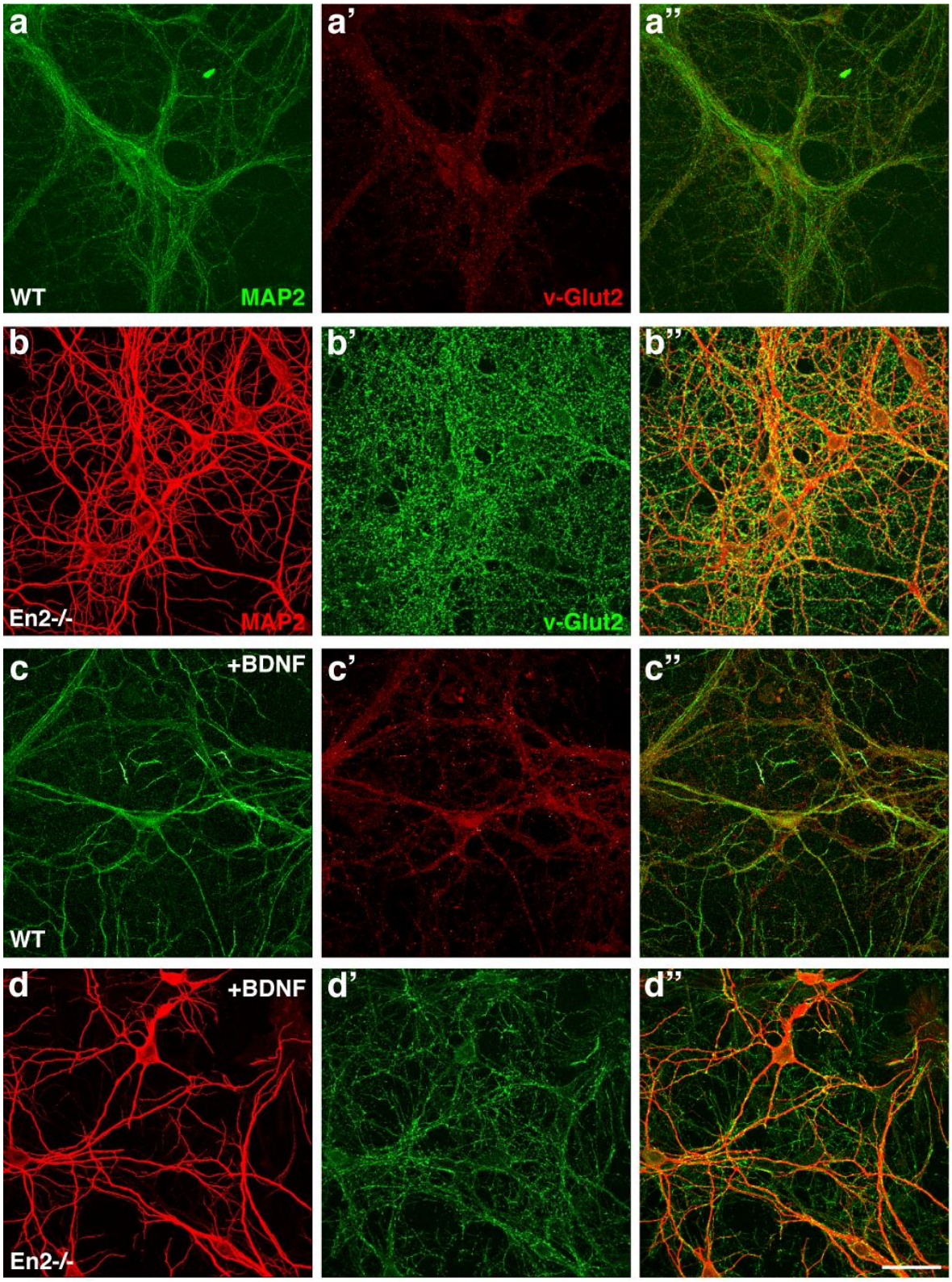


Fig.4.22. *vGlut2 staining of primary postnatal hippocampal cell culture.*
a) a') a") Images show MAP2 and vGlut2 staining in WT hippocampal neurons at DIV14. **b) b') b")** Representative images of postnatal cell culture neurons of En2 mutant mice. It is possible to observe a vGlut2 staining (green) and MAP2 expression (red). **c) c') c")** BDNF treatment in WT hippocampal neurons, stained at DIV14 with MAP2 (green) and vGlut2 (red) antibodies. **d) d') d"**) Images show *En2-/-* cell culture neurons treated with BDNF. Immunofluorescence performed using MAP2 (red) and vGLut2 (green) staining. Scale bar: 50µm.

GABAergic synapse analysis in postnatal hippocampal neurons.

We used double immunostainings for MAP2 and vGAT to quantitatively analyze GABAergic synapse in WT and *En2-/-* hippocampal cultures treated and untreated with BDNF. Double stainingg were analyzed by confocal imaging. The resolution obtained with the confocal microscope allowed the study of individual synapses. However, Z stack images with a step interval of 0.10 µm supplemented each XY image for accurate synapse quantification. The results obtained from postnatal hippocampal cell cultures at DIV14 exhibited a significant difference between WT and *En2-/-* untreated cultures (no BDNF treatment) (Fig.4.23). A significantly decreased number of vGAT-positive puncta was detected between BDNF-treated and BDNF-untreated WT neurons. These results showed a significant difference in spine density between the genotype.

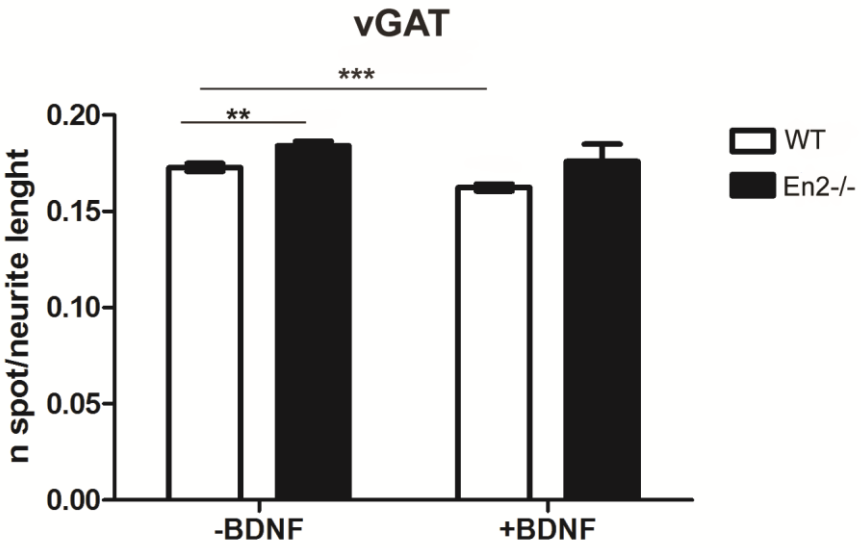


Fig.4.23 *Statistical analyses of vGAT synapse density.*
The graph shows GABAergic synapse density in *En2-/-* and WT hippocampal cultures, evaluated by vGAT staining. ***p<0.001, two-way ANOVA followed by Tukey's multiple comparisons test.

We also investigated the effect of BDNF on glutamatergic synapse density in WT and *En2-/-* hippocampal cultures using immunostaining for the glutamatergic marker vGlut2. The analyses revealed a significant decrease of vGlut2 puncta in *En2-/-* cultures in the absence of BDNF treatment. BDNF treatment significantly decreased vGlut2 puncta in WT neurons, while increasing the number of vGlut2 synapses in *En2-/-* hippocampal neurons (Fig. 4.24).

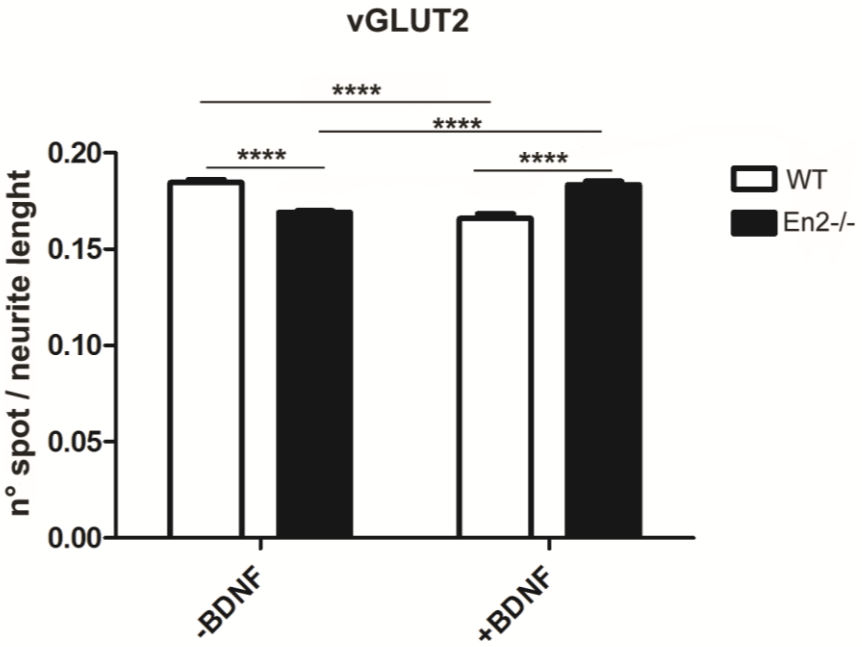


Fig.4.24 *Statistical analyses of vGlut2 synapse density.*
The graph shows glutamatergic synapse density in *En2-/-* and WT hippocampal cultures, evaluated by vGlut2 staining. ***p<0.001,****p<0.0001, two-way ANOVA followed by Tukey's multiple comparisons test.

5. DISCUSSION

5.1 Brief summary of results

In this study, we showed that GABAergic interneurons are partially lost in the cerebral cortex and hippocampus of adult *En2-/-* mice, a well-recognized model of ASD. Interneuron loss was accompanied by altered spine density and increased number of PV-positive puncta in the En2-/- forebrain. Considering the crucial role of BDNF in forebrain GABAergic interneuron development, we sought to determine whether interneuron loss in En2-/- forebrain might be related to altered expression of BDNF and its signaling receptors. Quantitative RT-PCR experiments revealed a markedly decreased expression of various splicing variants of BDNF mRNA in the cerebral cortex but not hippocampus of adult En2-/- mice, as compared to WT controls. Immunoblot analyses instead revealed increased levels of mature BDNF, and reduced levels of truncated- and pro-BDNF isoforms in the hippocampus of En2-/-, as compared to WT controls. These variations were accompanied by reduced levels of p75 and increased levels of TrkB receptors in the En2-/- forebrain. We next used primary cultures of hippocampal neurons from WT and En2-/- mice to investigate the effect of BDNF on GABAergic and glutamatergic neuron connectivity. Preliminary results indicate that BDNF is able to increase the number of GABAergic and glutamatergic synaptic contacts in En2-/- hippocampal neurons. Our results suggest a beneficial role of BDNF in rescuing synaptic defects of GABAergic and glutamatergic neurons in En2-/- mice.

5.2 En2, GABAergic interneurons, and postnatal forebrain development

Loss of forebrain GABAergic interneurons in En2-/- mice.

Previous studies from our laboratory showed that *En2* mRNA is expressed in the adult mouse hippocampus and cerebral cortex, suggesting that *En2* may also function in these brain areas (Tripathi et al., 2009). Since then, other studies confirmed that *En2* transcripts are expressed at low but significant levels in several telencephalic structures, such as the cerebral cortex, the hippocampus, and other areas of the limbic system (i.e. the amygdala) (Brielmaier et al., 2012) confirming and expanding our mRNA expression analysis. mRNA expression does not however indicate the presence of a functional protein; we therefore expanded this data demonstrating the presence of both Engrailed proteins in cortical and hippocampal GABAergic interneurons and reported alterations in the development and

maintenance of GABAergic interneurons also in forebrain areas in the absence of *En2* (see Results, Fig.4.1). We investigated the expression of both Engrailed proteins, describing their expression in a subsets of PV- and CALB-expressing GABAergic interneurons in the postnatal mouse hippocampus and somatosensory cortex. In accordance, *En2* ablation results in a partial loss of PV, NPY and SOM (but not CALB) interneurons in the somatosensory cortex and in the hippocampus, without affecting the total number of GAD-expressing interneurons (see Results, Fig.4.3). In the visual cortex, instead, *En2*-/- mice show a decreased number of SOM and NPY interneurons, but not of PV interneurons, without significant alterations in the total number of interneurons, as measured by GAD-espression. In both somatosensory and visual cortex the pattern of GABAergic interneuron reduction appears layer-specific, being confined for superficial layers in the case of SOM interneurons. The PV-positive cell population seems also less affected in the visual cortex as compared to the somatosensory cortex, suggesting that different sensory cortices display a distinct profile of interneuron maturation in *En2*-/- mice. Indeed, the somatosensory cortex has a greater number of PV+ interneurons as compared to the visual cortex (Tanahira et al., 2009). Overall, these data indicate that the *En2* mutation has a very selective, region-specific effect on different subpopulations of cortical GABAergic interneurons. Previous studies showed that loss of *En2* markedly reduces neurogenesis in posterior brain areas, including development of inhibitory neurons in the cerebellum. Reduction in number and a delay in maturation of CALB-expressing Purkinjie neurons has been described In the cerebellum of *En2-/-* mice (Kuemerle et al.,1997)(Kuemerle et al.,2007) (Sudarov & Joyner, 2007), suggesting that the *En2* gene may have a direct effect on the development of this inhibitory neurons.

As for the expression of the GABAergic interneurons markers at mRNA level, discordances have arisen with the cell counting experiments. PV mRNA levels and PV interneurons were both reduced in the somatosensory cortex of *En2* mutants; conversely, a loss of NPY and SOM neurons was not paralleled by lower mRNA expression levels. This discordance could be due to up-regulation of NPY and SOM mRNAs in the remaining interneurons of the *En2*-/- somatosensory cortex to compensate for the loss of their input. It would be interesting to explore this hypothesis analyzing the SOM and NPY innervation on principal neurons. The downregulation of CALB mRNA in the absence of a reduced number of CALB-positive neurons in the *En2*-/- cortex might be related to hyperexcitability (Sonnenberg et al., 1991). It is important to point out that *En2-/-* mice do display hippocampal and cortical hyperexcitability, as determined by increased susceptibility to KA-induced seizures (Tripathi et al., 2009). This study also demonstrated that the expression of *En2* mRNA in the adult hippocampus was downregulated by seizure activity, indicating that *En2* may be also involved in the functioning of adult brain areas and in their

response to seizures (Tripathi et al., 2009). Recent transcriptome study, from our laboratory, performed in the En2-/- hippocampus, supports this hypothesis. Bioinformatic analysis of enriched gene ontology terms and functional pathways in the differentially expressed genes revealed that ablation of *En2* induces a prevalence of terms and pathways related to seizure, altered synaptic transmission, as well as neuronal activities and calcium mediated signal (Sgadò et al., 2013).

Overall, these findings are in line with the hypothesis that structural and functional abnormalities of GABAergic interneurons might represent the anatomical substrate of an unbalanced excitation/inhibition ratio in the cerebral cortex and other brain areas, which has been postulated to occur in the autistic brain (Rubenstein and Merzenich, 2003). Other studies suggested this hypothesis. Gogolla and colleagues confirmed, by a meta-analysis study, a reduction of PV-positive cells in the neocortex of ASD mouse models as compared to control mice (Gogolla et al., 2009) (Medrihan et al., 2008). Moreover, a marked loss of PV-expressing interneurons has been detected in the somatosensory cortex of *Fmrp1* and *Nlgn3* mutants (Durand et al., 2012).
Altered or delayed development of GABAergic inhibition has been proposed as a pathogenic mechanism of multiple neurodevelopmental disorders, including ASD (Di Cristo, 2007). Lawrence and colleagues showed an abnormal density of Calretinin-, CALB- and PV-immunoreactive interneurons in the post-mortem studies in hippocampus of ASD patients, compared to control samples (Lawrence et al., 2010). They proposed that GABAergic interneurons dysfunction may have a crucial role in the brains of individual with autism (Lawrence et al., 2010). A GABAergic signaling dysfunction has been investigated in ASD patients in several instances: a reduction in GAD65/67 proteins levels has been shown in post-mortem tissues in the cerebellum and cerebral cortex from autistic patients (Fatemi et al., 2002)(Pizzarelli & Cherubini, 2011) (Chattopadhyaya et al., 2012).

Delay in visual cortex circuit development.

To analyze possible alterations of GABAergic interneurons in the visual cortex of *En2-/-* mice and its possible impact on cortical function we investigated, in collaboration with the CNR Institute of Neuroscience, the functional features of the mouse visual system to elucidate the relationship between GABAergic dysfunction and maturation of cortical function in *En2-/-* mice (Allegra et al., 2014). In parallel with the a lower number of SOM- and NPY- (but not PV-) positive cells detected in the adult visual cortex of *En2-/-* mice, we also found a developmental decrease of SOM- and PV- cells in the visual cortex of *En2-/-* as well as in *WT* mice, though less pronounced than that observed in *En2-/-* animals (Allegra et al. 2014). The visual cortex has been used as a model system to test the

physiological consequences of an altered development of the GABAergic system. As an assay of the potential for plasticity, many groups employed the paradigm of monocular deprivation. The anatomical abnormalities, in the visual cortex, were accompanied by a significant increase of binocularity in juvenile and adult *En2*-/- mice, and by the inability to respond to a brief monocular deprivation performed during the critical period and in adulthood (Allegra et al., 2014). In addition, the electrophysiological analysis demonstrated that several basic physiological properties (visual acuity, latency and amplitude of the visual response, receptive field size, spontaneous discharge) were within the normal range in both juvenile and adult *En2*-/- animals (Allegra et al., 2014). Thus, lack of *En2* selectively affects balance of eye-specific drive in the primary visual cortex while leaving the development of other functional properties unaffected. The enhanced binocularity of *En2*-/- mice is likely of cortical origin and not secondary to alterations in retinogeniculate afferents, as a normal segregation of eye-specific inputs was found in the dLGN (Allegra et al., 2014).

Moreover, application of a brief period of monocular occlusion in juvenile mice lacking *En2* failed to produce an ocular dominance shift while it was extremely effective in aged-matched WT animals. Response to monocular deprivation was also not detectable in adult *En2*-/- mice. Furthermore electrophysiological recordings in the visual cortex ipsilateral to the deprivation were able to demonstrate a genuine deficit in plasticity. Thus, lack of *En2* impairs activity-dependent modifications at the peak of the “normal” critical period.

Previous studies have examined the role of GABAergic inhibition in the control of visual plasticity, showing that the proper maturation of the GABAergic system is a crucial determinant of cortical plasticity during postnatal development (Hensch et al., 1998). Susceptibility to monocular deprivation has recently been used to assess plasticity of cortical networks in models of mental retardation and ASD such as *Fmr1* (Dölen et al., 2007) and *MeCP2* (Tropea et al., 2009b) mutant mice, which are known to present anatomical and functional deficits of cortical GABAergic interneurons (Centonze et al., 2008) (Chao et al., 2010).

Developmental defects in GABAergic interneurons.

A set of preliminary data, obtained from our laboratory, demonstrated a significant reduction in SOM- and PV- positive interneurons at different stages of postnatal development (P10, P30): the considered areas were the somatosensory cortex and the hippocampus of *En2-/-* mice and WT. These results, obtained at early postnatal stages, support our theory that *En2* might be involved in the development and migration of interneurons subpopulations. This phenomenon could be explained in terms of distribution: the maturation and stabilization of the total number of these interneurons occurs at around

P30-P40, therefore, it may still be subject to modification at P30 age, when the interneurons are not fully mature and stable (Huang et al., 1999). This suggests that the *En2* gene, delaying the maturation of these cells, may also be involved in their late maturation. We hypothesize that the loss of *En2* may have a specific effect, direct or indirect, in the postnatal maturation of GABAergic cells. This defect might indicate an immature and unbalanced connectivity in the cortex that underlies the complex behavioral phenotype of *En2-/-* mice, a mouse model that mimics the autistic phenotype. GABAergic interneurons are crucial for activity-dependent remodeling of cortical circuits; alterations of these cells during development could produce non cell-autonomous changes and alter brain connectivity in general.

The next step will be to understand how and at which stage of development En2 protein is crucial for the correct development of these classes of interneurons. To this purpose, we could make use of *En2* conditional mutant mice with specific ablation of *En2* in GABAergic interneurons or glutamatergic neurons. These conditional mice could elucidate other aspects on the origin of GABAergic alteration observed in the En2-/- forebrain. It might be interesting to understand if these interneurons defects are due to altered specification, migration or during the differentiation of these cells populations. The generation of conditional mutants specifically lacking *En2* in forebrain interneurons is currently ongoing in our laboratory.

5.3 Synaptic development in the En2-/- forebrain

Dendritic spine density in En2-/- mice.

Several genome-wide association studies have identified polymorphic variants in genes encoding synaptic proteins as important determinants of the risk of developing ASD (van Spronsen & Hoogenraad, 2010). Recent transcriptome profiles from three different brain regions (frontal cortex, temporal cortex and cerebellum) have demonstrated a downregulation of genes involved in synaptic function and vesicular transport in ASD patients (Voineagu et al., 2011). These data have been confirmed also in our mouse model, using a transcriptome analysis (Sgadò et al., 2013). In particular, our bioinformatics analysis of the differentially expressed genes in the hippocampus of *En2-/-* mice found significant enrichment for terms related to seizure and altered synaptic transmission (Sgadò et al., 2013). These data lead us to hypothesize defects in synaptogenesis and spine density alterations in our *En2* mouse model. To this purpose, Golgi staining was performed on the hippocampus of *En2-/-* mice compared to WT, in juvenile and adult mice.

En2-/- mice displayed a significant decrease in spine density on apical dendrites of CA1 region and on dendrites of granule cells in DG, at p30 age. We found also an increased spine density in the basal dendrites of CA1 region, in adult age. These results might be consistent with the idea that *En2* promotes spinogenesis. In collaboration with the "Laboratoire de dynamique membranaire et maladies neurologiques" in Paris (Prof. Olivier Stettler), we investigated the role of Engrailed in cell culture medium during hippocampal cell differentiation. Engrailed protein treatment in culture produces an increase of dendritic complexity and an excess of spinogenesis, increasing immature and plastic spines. This data support the hypothesis that Engrailed genes may participate to the fine tuning of synaptic formation in the hippocampal network (Soltani et al., in preparation).

Increased spinogenesis has been observed also in other ASD mouse models. In adult Fmr1 KO mice, Golgi staining in the cerebral cortex reported a higher density of cortical spines than in controls (Comery et al., 1997) (Dölen et al., 2007). This data has been recently contradicted, reporting a normal spine density in juvenile and adult Fmr1KO mice (Pan et al., 2010), arguing that this result could be due to a technical artifact of the Golgi method. However, in the hippocampal cultures of *Fmr1* mutant mice have been reported to have a lower spine density (Braun & Segal, 2000), which would be in accord with what has been seen in our hippocampal cultures treated with Engrailed (Soltani et al., in preparation). We also observed an opposite result of the spine density between young and adult *En2-/-* mice. This discrepancy might be correlated to a high vitality of spine turnover in young animals, which is altered /arrested with the adult age, leading to an increase of immature spines. The immaturity of spine is correlated with the stabilization of the dendritic spines, which is independent of the spine density measured by Golgi and could be analyzed by other means (Portera-Cailliau, 2012). Therefore, an increase spine density in the adult *En2* mutant mice might suggest a compensatory mechanism, due to a possible aberrant morphology of spine in adult *En2-/-* mice.

Following our recent experiments, the next goal will be to detect alterations in spine size and morphology, considering the importance of the dynamics of spine in the healthy brain. It could be significant to study the morphology of the spines, in order to understand in which way the instability of the spine influence the proper balance between stable and dynamic connections, this latter fundamental to define mature synapses (Portera-Cailliau, 2012).

Synaptic connectivity in En2-/- mice.

Considering the potential effect of *En2* on spine formation, we examined the possible role of *En2* in synaptic formation and maturation, in our mouse model. We approached this

hypothesis analyzing the perisomatic innervation of GABAergic synapses in fixed tissue and evaluating the synaptic puncta *in vitro*.

Given the importance of GABAergic function in the formation of synaptic circuits, reduced GABAergic inhibition and its link to synaptic development may be considered a recurrent pathophysiological mechanisms for ASD and for many other neurodevelopmental disorders (Hussman, 2001) (Bateup et al., 2013). Following previous analyses on the study of spine density in hippocampal area of *En2-/-* mice, we extended our research on the possible alteration of GABAergic synapse density in *En2-/-* mice and WT controls. The failure of spine to stabilize during development strongly suggests that *En2-/-* mice could have problems in maintaining a proper balance in the brain connectivity therefore failing to establish stable and/or dynamic connections in the brain.

To study the interneurons synaptic connections on cortical principal pyramidal neurons, we crossed *En2* mice with Gad1-EGFP (line G42) mice, selectively expressing enhanced green fluorescent protein (EGFP) in PV-positive basket interneurons and in putative presynaptic boutons (Chattopadhyaya et al., 2004). Previous studies showed a higher distribution of GFP-positive neurons in infragranular layers V and VI, in the visual cortex (Chattopadhyaya et al., 2004). In our case however, the GFP transgene expression in the synaptic boutons was lower compared to the original strain, therefore the perisomatic innervation was analyzed using PV antibody. We anyway focused our attention on the synaptic connections that GABAergic interneurons generate with layer V-VI pyramidal neurons, where a higher concentration of transgene expression had been reported, using PV and vGAT antibody. The results show a small change, not statistically significant, in synapse density of the two GABAergic markers, in *En2-/-* mice compared to WT. By observing the trends of vGAT and PV in both the genotypes, we hypothesized an increase in PV interneurons expression and a decrease in vGAT number, to be further characterized. Interestingly, these differences became significant among genotypes once the PV/vGAT ratio was taken into account. This ratio measures the mean intensity of PV and vGAT staining, without considering the area of the cells included in the analysis. Indeed, we found an increase in the area of pyramidal cells in *En2-/-* mice compared to WT and this result affected, negatively, the trend of GABAergic synaptic markers expression. Whether the area difference represents a technical artifact or an actual effect of *En2* on pyramidal neurons needs to be established.

As a future perspective, we might consider extending our study to the visual cortex, where we observed developmental alterations in GABAergic interneurons, expanding the analysis to other layer of the somatosensory cortex, for examples, layer II-III and IV.

Synaptic density in hippocampal cell cultures.

To further characterize the synaptic density phenotype in our model we examined synaptic formation in cell cultures. We performed double fluorescent immunostaining of vGAT-MAP2 and vGlut-MAP2 antibodies to be able to analyze the dendritic development in culture. The results obtained from hippocampal cell culture after 14 days in culture showed a significant alteration between WT and *En2-/-* neurons. We observed a significant increase number of vGAT dendritic puncta in *En2-/-* cultures compared to WT. This is in agreement with previous RT-qPCR results performed on visual cortex of *En2-/-* mice. Indeed, we have shown an increase mRNA expression in visual cortex of *En2-/-* mice, *in vivo*, but we have not analyzed the spine density (perisomatic puncta) in this area. Furthermore, our *in vitro* analyses revealed an increase protein expression in this GABAergic marker only on the dendritic tree, excluding the soma. Our aim was also to consider the glutamatergic spine density in the cultured neurons, using vGlut2. Also in this case we found a significant decrease of dendritic glutamatergic puncta, confirming our *in vivo* analyses of vGlut mRNA level in the visual cortex of *En2-/-* mice. This data suggest an altered maturation of synaptic connection and dendritic development in *En2-/-* mice.

The sequential development of GABA- and glutamate-mediated connections is independent on the arrival of afferent dendritic inputs but is related to the degree of maturation of the targeted cells (i.e. dendritic length, somatic size) (Tyzio et al., 1999). Hence, GABAergic synapses development requires the presence of small apical dendrites in stratum radiatum of the hippocampus. Our Golgi analysis indicated a decreased spine density in the stratum radiatum of the CA1 of young *En2-/-* mice that could lead to a loss of GABAergic synapses and GABAergic neurons. We might also hypothesize that the loss of GABAergic input induces the increased number of spine in CA1 hippocampal region in the adult, contributing to establish immature synaptic circuits.

5.4 BDNF expression in the En2-/- forebrain

Expression of different BDNF splicing variants in En2 brain.

Previous studies showed that some BDNF transcript variants are implicated in cell survival, while other are involved in the regulation of dendritogenesis and spinogenesis (Xu et al., 2000)(Berghuis et al., 2006). BDNF, released from pyramidal axons, could signal to GABAergic neurons and influence inhibitory innervation globally (Kohara et al., 2001) (Huang et al.,2007). In our study, we investigated whether altered BDNF expression might be related to the altered development and synaptic connectivity of GABAergic interneurons

detected in the *En2-/-* forebrain. According to previous studies, we focused our attention on BDNF mRNA splice variants, using in situ hybridization and RT-qPCR techniques. Firstly, in order to understand the expression of BDNF splice variants in the hippocampus and cerebral cortex of *En2-/-* mouse model, we analyzed the mRNA level by RT-qPCR of different splicing variants (in detail, Exon I, II, IV, VI, VII, IXa). RT-PCR results demonstrated a significant reduction of different BDNF transcript variants in cerebral cortex of *En2-/-* mice: all BDNF splice variants showed an important decrease in *En2-/-* mice compared to the control (see Results, Fig. 4.15). This is in agreement with previous studies, which showed the decrease level of BDNF mRNA in individuals with Alzheimer disease (Phillips et al., 1991) and Huntington disease (HD) (Ferrer et al., 2000) (Zuccato et al., 2005). In the cerebral cortex of HD mouse model, a decrease in mRNA level from early pre-symptomatic stages was reported (Zuccato et al., 2005). A quite widespread reduction in cortical BDNF mRNA has been observed also in schizophrenia (Ray et al., 2014). Thus, BDNF splice variants decrease may underlie a general decrease in cortical BDNF expression that may participate to the defects observed in GABAergic interneurons in our model.

Analyzing the hippocampus of *En2-/-* mice, we instead found a significant increase in Exon VII transcripts variant, in the hippocampus of *En2-/-* mice, compared to WT, while no other BDNF splice variant showed substantial alteration. This might be in agreement with previous study on autistic patients, which showed no significant difference in BDNF mRNA levels in the fusiform gyrus of control and autism samples (Garcia et al., 2012). Preliminary results obtained by specific laser microdissection analyses (LMD) from our laboratory confirmed the increased of BDNF Exon VII transcript variants in the hippocampus of *En2-/-* mice compared to WT. We collected somatic and dendritic areas of the hippocampal region and demonstrated an increase of BDNF Exon VII expression in somatic CA3 area. LMD revealed a general trend of BDNF somatic expression compared to dendritic level, in the hippocampus of *En2-/-* mice.

Performing in situ hybridization analyses, we demonstrated a widespread level of the mRNA expression in the hippocampus, cerebral cortex and cerebellum of our mouse model and WT control, at adult age. These data confirmed that a large amount of BDNF mRNA has been found in these three important brain regions, without particular difference in the distribution between the genotype. In situ hybridization confirmed the decreased expression in Exon VI BDNF variants in the cerebral cortex. Contrary to what detected by RT-qPCR, down-regulation of BDNF Exon VI mRNA by in situ hybridization was also detected in the *En2-/-* hippocampus and cerebellum.

Expression of different BDNF isoform in En2-/- brain.

The presence of BDNF mRNA may be taken as an indication of the distribution of the BDNF protein. However, the expression of mRNA does not reflect the rate of protein synthesized because of the post-translational modifications. In the present study, we also examined BDNF protein expression, using Western blotting and immunohistochemistry analyses, in the cerebral cortex and hippocampus of *En2-/-* and WT mice. Immunofluorescence experiments revealed that there is a widespread distribution of BDNF protein in the hippocampus and cerebral cortex of *En2-/-* mice. We next used western blot to investigate the presence of the different BDNF isoforms in our *En2* mouse model (Marcinkiewicz et al., 1998) (Michalski & Fahnestock, 2003). Western blot detection revealed a decreased expression in pro-BDNF signal in hippocampus of *En2* mutant mice compared to WT. Conversely, we demonstrated a marked increase of pro-BDNF level and a decrease of truncated isoform in cerebral cortex of *En2-/-* mice compared to WT. The total number of the three BDNF isoforms did not change in our mouse model compared to WT (data not shown). Therefore, the balance among all three BDNF isoforms is very important: the increased pro-BDNF and reduced truncated BDNF isoform, in the cerebral cortex of En2 mutant mice, suggests that this isoform may be responsible for reduced neuronal differentiation and dendritic spines. Indeed, previous analyses performed on autistic patients (Pang et al., 2004)(Teng et al., 2005) (Garcia et al., 2012) demonstrate an alteration in pro-BDNF isoform, suggesting that this might lead to alteration in synaptic connectivity at neurotransmitter levels in autistic patients (Garcia et al., 2012). Translation or mRNA stability could not account for these differences in BDNF isoforms; the biological properties of pro-BDNF suggest that increases in this isoform may be responsible for reduced neuronal differentiation and dendritic spines and altered synaptic connectivity. These notions have been previously hypothesized (Teng et al., 2005) (Koshimizu et al., 2009): Chang and colleagues showed *in vivo* data demonstrating a decreased expression of BDNF protein in the brain of *MeCP2* knockout mice. These data suggest that *MeCP2* knockout mice have a reduced neuronal activity correlated with reduced BDNF brain levels (Chang et al., 2006) (Calfa et al., 2011)(Moretti & Zoghbi, 2006).

BDNF receptors analysis.

In order to complete our analysis of alteration in BDNF signaling in the brain of *En2-/-* mice, we extended our study at BDNF receptors. An increasing number of studies have suggested the involvement of BDNF/TrkB in various pathological conditions. BDNF/TrkB dysregulation leads to cellular proliferative changes and degenerative and behavioral changes in the brain (Gupta et al., 2013).

Therefore, we decided to investigate the mRNA expression of BDNF receptors: in particular, we performed RT-qPCR experiments, which showed a significant downregulation of p75 receptor in the hippocampus and cerebral cortex of *En2-/-* mice, compared to controls. TrkB expression was unchanged, with the only exception of TrkB full length (TrkB.FL) isoform, which was increased in the hippocampus of *En2-/-* mice as compared to WT controls (see Results. Fig.4.18). TrkB.FL plays a more prominent role in increasing proximal dendritic branching, whereas truncated TrkB isoforms are involved in the elongation of distal dendrites (Gupta et al., 2013). Recent study demonstrated that and increased TrkB expression leads to impair synaptic plasticity in a mouse model of *Fmr1* (Correia et al., 2010) (Louhivuori et al., 2011)(Gupta et al., 2013). Therefore, alterations in TrkB signaling might contribute to an increased vulnerability to autism also in our mouse model. It has been demonstrated that TrkB expression changes have a role in the development of Rett syndrome pathology as well (Abuhatzira et al., 2007).
As for the neurotrophin receptor, p75 promotes distinct signaling pathways in the cells; in most cases these pathways are opposed, but sometimes they coordinate with TrkB promoted pathways (Gupta et al., 2013). Previous studies demonstrated that the dose of *Engrailed* is inversely correlated to the expression level of p75 (Alavian et al., 2009). Therefore, observing the decrease expression of mRNA p75 level in hippocampus and cerebral cortex of *En2-/-* mice, we might hypothesize that the reduction of p75 receptor might be caused by the loss of *En2* in our mouse model.

We also investigate the expression of the high-affinity BDNF receptor, TrkB, at protein level using western blotting experiment. The results obtained demonstrate an up-regulation of TrkB receptor in the hippocampus of *En2-/-* mice, compared to control group. Increase in TrkB expression has been associated with increased seizure susceptibility and epileptogenesis (Weskamp & Reichardt, 1991). Indeed, increase susceptibility to seizures has been previously shown in *En2-/-* mice (Tripathi et al., 2009; Sgadò et al., 2013). Neurotrophin receptors may act as a point of convergence, involved in the integration of many environmental inputs: this can lead to alterations in neuronal circuitry and, ultimately, in behavior (Chao, 2003).

Effect of BDNF on En2-/- hippocampal neurons in culture.

Previous studies demonstrated that BDNF promotes the formation of GABAergic synapses in hippocampal and cortical cultures and can potentially adjust the number and strength of GABAergic synapses onto targeted glutamatergic cells (Huang et al., 2007). We therefore analyzed the effect of BDNF treatment in primary hippocampal cell culture neurons.

Synaptic vGAT puncta were increased in *En2-/-* untreated cells, with no statistically significant increase in *En2-/-* vs WT neurons treated with BDNF. We could hypothesize that the transcriptional factor En2 might influence the number of vGAT synaptic puncta; indeed, we can observe that in *En2-/-* neurons vGAT expression tends to increase. In order to investigate the effect of BDNF on glutamatergic synapse density in WT and *En2-/-* hippocampal cultures, we performed the immunostaining using vGlut2 antibody. The analyses revealed a significant decrease in the number of vGlut2 synaptic boutons in *En2-/-* cells, compared to WT. This result appears different in the cell culture treated with BDNF protein, in which we observed an increase of vGlut2 synaptic puncta in *En2-/-* treated cells. Observing this data, it is possible to hypothesize that the treatment with BDNF protein in cell culture neurons might leads to a complete rescue of vGlut2 synaptic puncta in *En2-/-* treated cells, reaching the WT untreated cells expression in our mouse model.

5.5 Conclusions and future perspectives

In this study, we showed marked defects in forebrain GABAergic interneurons in *En2-/-* mice. Interneuron loss was accompanied by defects of synaptic connectivity (i.e. dendritic spines and synaptic puncta), which might depend on marked deficits of BDNF expression detected in the *En2-/-* forebrain. Future studies will be aimed at investigating the causal relationship between BDNF deficits and interneuron loss in the *En2-/-* forebrain. Specifically, we will try to determine whether transgenic overexpression of BDNF in the forebrain is able to rescue GABAergic interneuron defects in *En2-/-* mice. To this purpose, the generation of WT and *En2-/-* mice overexpressing BDNF is currently ongoing in our laboratory.

REFERENCES

Abidin, I., Eysel, U. T., Lessmann, V., & Mittmann, T. (2008). Impaired GABAergic inhibition in the visual cortex of brain-derived neurotrophic factor heterozygous knockout mice. *The Journal of Physiology*, *586*(7), 1885–901. doi:10.1113/jphysiol.2007.148627

Abuhatzira, L., Makedonski, K., Kaufman, Y., Razin, A., & Shemer, R. (n.d.). MeCP2 deficiency in the brain decreases BDNF levels by REST/CoREST-mediated repression and increases TRKB production. *Epigenetics : Official Journal of the DNA Methylation Society*, *2*(4), 214–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18075316

Agnes Cristina Fett-Conte, A. L. B.-M. and P. P.-N. (2013). *Recent Advances in Autism Spectrum Disorders - Volume I*. (M. Fitzgerald, Ed.). InTech. doi:10.5772/46001

Aguado, F., Carmona, M. A., Pozas, E., Aguiló, A., Martínez-Guijarro, F. J., Alcantara, S., … Soriano, E. (2003). BDNF regulates spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K+/Cl- co-transporter KCC2. *Development (Cambridge, England)*, *130*(7), 1267–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12588844

Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., & Timmusk, T. (2007). Mouse and rat BDNF gene structure and expression revisited. *Journal of Neuroscience Research*, *85*(3), 525–35. doi:10.1002/jnr.21139

Alavian, K. N., Sgadò, P., Alberi, L., Subramaniam, S., & Simon, H. H. (2009). Elevated P75NTR expression causes death of engrailed-deficient midbrain dopaminergic neurons by Erk1/2 suppression. *Neural Development*, *4*, 11. doi:10.1186/1749-8104-4-11

Allegra, M., Genovesi, S., Maggia, M., Cenni, M. C., Zunino, G., Sgadò, P., … Bozzi, Y. (2014). Altered GABAergic markers, increased binocularity and reduced plasticity in the visual cortex of Engrailed-2 knockout mice. *Frontiers in Cellular Neuroscience*, *8*, 163. doi:10.3389/fncel.2014.00163

Anderson, S. A., Marín, O., Horn, C., Jennings, K., & Rubenstein, J. L. (2001). Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development (Cambridge, England)*, *128*(3), 353–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11152634

Arndt, T. L., Stodgell, C. J., & Rodier, P. M. (2004). The teratology of autism. *International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience*, *23*(2-3), 189–99. doi:10.1016/j.ijdevneu.2004.11.001

Ascoli, G. A., Alonso-Nanclares, L., Anderson, S. A., Barrionuevo, G., Benavides-Piccione, R., Burkhalter, A., … Yuste, R. (2008). Petilla terminology: nomenclature of features of

GABAergic interneurons of the cerebral cortex. *Nature Reviews. Neuroscience*, *9*(7), 557–68. doi:10.1038/nrn2402

Baj, G., D'Alessandro, V., Musazzi, L., Mallei, A., Sartori, C. R., Sciancalepore, M., … Tongiorgi, E. (2012). Physical exercise and antidepressants enhance BDNF targeting in hippocampal CA3 dendrites: further evidence of a spatial code for BDNF splice variants. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *37*(7), 1600–11. doi:10.1038/npp.2012.5

Baj, G., Del Turco, D., Schlaudraff, J., Torelli, L., Deller, T., & Tongiorgi, E. (2013). Regulation of the spatial code for BDNF mRNA isoforms in the rat hippocampus following pilocarpine-treatment: a systematic analysis using laser microdissection and quantitative real-time PCR. *Hippocampus*, *23*(5), 413–23. doi:10.1002/hipo.22100

Barde, Y. A., Edgar, D., & Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *The EMBO Journal*, *1*(5), 549–53. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=553086&tool=pmcentrez&rendertype=abstract

Baribeau, D. A., & Anagnostou, E. (2013). A comparison of neuroimaging findings in childhood onset schizophrenia and autism spectrum disorder: a review of the literature. *Frontiers in Psychiatry*, *4*, 175. doi:10.3389/fpsyt.2013.00175

Bassell, G. J., & Warren, S. T. (2008). Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. *Neuron*, *60*(2), 201–14. doi:10.1016/j.neuron.2008.10.004

Bateup, H. S., Johnson, C. A., Denefrio, C. L., Saulnier, J. L., Kornacker, K., & Sabatini, B. L. (2013). Excitatory/inhibitory synaptic imbalance leads to hippocampal hyperexcitability in mouse models of tuberous sclerosis. *Neuron*, *78*(3), 510–22. doi:10.1016/j.neuron.2013.03.017

Batista-Brito, R., & Fishell, G. (2009). The developmental integration of cortical interneurons into a functional network. *Current Topics in Developmental Biology*, *87*, 81–118. doi:10.1016/S0070-2153(09)01203-4

Baudouin, S. J. (2014). Heterogeneity and convergence: the synaptic pathophysiology of autism. *The European Journal of Neuroscience*, *39*(7), 1107–13. doi:10.1111/ejn.12498

Bauman, M. L., & Kemper, T. L. (2005). Neuroanatomic observations of the brain in autism: a review and future directions. *International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience*, *23*(2-3), 183–7. doi:10.1016/j.ijdevneu.2004.09.006

Baxter, G. T., Radeke, M. J., Kuo, R. C., Makrides, V., Hinkle, B., Hoang, R., … Feinstein, S. C. (1997). Signal Transduction Mediated by the Truncated trkB Receptor Isoforms,

trkB.T1 and trkB.T2. *J. Neurosci.*, *17*(8), 2683–2690. Retrieved from http://www.jneurosci.org/content/17/8/2683.full

Beaudoin, G. M. J., Lee, S.-H., Singh, D., Yuan, Y., Ng, Y.-G., Reichardt, L. F., & Arikkath, J. (2012). Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. *Nature Protocols*, *7*(9), 1741–54. doi:10.1038/nprot.2012.099

Ben-Ari, Y. (2002). Excitatory actions of gaba during development: the nature of the nurture. *Nature Reviews. Neuroscience*, *3*(9), 728–39. doi:10.1038/nrn920

Ben-Ari, Y., Khalilov, I., Kahle, K. T., & Cherubini, E. (2012). The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *18*(5), 467–86. doi:10.1177/1073858412438697

Benarroch, E. E. (2009). Neuropeptide Y: its multiple effects in the CNS and potential clinical significance. *Neurology*, *72*(11), 1016–20. doi:10.1212/01.wnl.0000345258.18071.54

Benayed, R., Choi, J., Matteson, P. G., Gharani, N., Kamdar, S., Brzustowicz, L. M., & Millonig, J. H. (2009). Autism-associated haplotype affects the regulation of the homeobox gene, ENGRAILED 2. *Biological Psychiatry*, *66*(10), 911–7. doi:10.1016/j.biopsych.2009.05.027

Benayed, R., Gharani, N., Rossman, I., Mancuso, V., Lazar, G., Kamdar, S., … Millonig, J. H. (2005). Support for the homeobox transcription factor gene ENGRAILED 2 as an autism spectrum disorder susceptibility locus. *American Journal of Human Genetics*, *77*(5), 851–68. doi:10.1086/497705

Berghuis, P., Agerman, K., Dobszay, M. B., Minichiello, L., Harkany, T., & Ernfors, P. (2006). Brain-derived neurotrophic factor selectively regulates dendritogenesis of parvalbumin-containing interneurons in the main olfactory bulb through the PLCgamma pathway. *Journal of Neurobiology*, *66*(13), 1437–51. doi:10.1002/neu.20319

Berkel, S., Marshall, C. R., Weiss, B., Howe, J., Roeth, R., Moog, U., … Rappold, G. A. (2010). Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum disorder and mental retardation. *Nature Genetics*, *42*(6), 489–91. doi:10.1038/ng.589

Bernhardt, R., & Matus, A. (1984). Light and electron microscopic studies of the distribution of microtubule-associated protein 2 in rat brain: a difference between dendritic and axonal cytoskeletons. *The Journal of Comparative Neurology*, *226*(2), 203–21. doi:10.1002/cne.902260205

Betancur, C. (2011). Etiological heterogeneity in autism spectrum disorders: more than 100 genetic and genomic disorders and still counting. *Brain Research*, *1380*, 42–77. doi:10.1016/j.brainres.2010.11.078

Betancur, C., Sakurai, T., & Buxbaum, J. D. (2009). The emerging role of synaptic cell-adhesion pathways in the pathogenesis of autism spectrum disorders. *Trends in Neurosciences*, *32*(7), 402–12. doi:10.1016/j.tins.2009.04.003

Bhattacharya, A., & Klann, E. (2012). The molecular basis of cognitive deficits in pervasive developmental disorders. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *19*(9), 434–43. doi:10.1101/lm.025007.111

Blatt, G. J., Fitzgerald, C. M., Guptill, J. T., Booker, A. B., Kemper, T. L., & Bauman, M. L. (2001). Density and distribution of hippocampal neurotransmitter receptors in autism: an autoradiographic study. *Journal of Autism and Developmental Disorders*, *31*(6), 537–43.

Blumberg, S. J., Bramlett, M. D., Kogan, M. D., Schieve, L. A., Jones, J. R., & Lu, M. C. (2013). Changes in prevalence of parent-reported autism spectrum disorder in school-aged U.S. children: 2007 to 2011-2012. *National Health Statistics Reports*, (65), 1–11, 1 p following 11.

Bonati, M. T., Russo, S., Finelli, P., Valsecchi, M. R., Cogliati, F., Cavalleri, F., … Larizza, L. (2007). Evaluation of autism traits in Angelman syndrome: a resource to unfold autism genes. *Neurogenetics*, *8*(3), 169–78. doi:10.1007/s10048-007-0086-0

Braun, K., & Segal, M. (2000). FMRP involvement in formation of synapses among cultured hippocampal neurons. *Cerebral Cortex (New York, N.Y. : 1991)*, *10*(10), 1045–52.

Brielmaier, J., Matteson, P. G., Silverman, J. L., Senerth, J. M., Kelly, S., Genestine, M., … Crawley, J. N. (2012). Autism-relevant social abnormalities and cognitive deficits in engrailed-2 knockout mice. *PloS One*, *7*(7), e40914. doi:10.1371/journal.pone.0040914

Briggs, S. W., & Galanopoulou, A. S. (2011). Altered GABA signaling in early life epilepsies. *Neural Plasticity*, *2011*, 527605. doi:10.1155/2011/527605

Bruining, H., Eijkemans, M. J., Kas, M. J., Curran, S. R., Vorstman, J. A., & Bolton, P. F. (2014). Behavioral signatures related to genetic disorders in autism. *Molecular Autism*, *5*(1), 11. doi:10.1186/2040-2392-5-11

Brunig, I., Penschuck, S., Berninger, B., Benson, J., & Fritschy, J.-M. (2001). BDNF reduces miniature inhibitory postsynaptic currents by rapid downregulation of GABAA receptor surface expression. *European Journal of Neuroscience*, *13*(7), 1320–1328. doi:10.1046/j.0953-816x.2001.01506.x

Butler, M. G., Dasouki, M. J., Zhou, X.-P., Talebizadeh, Z., Brown, M., Takahashi, T. N., … Eng, C. (2005). Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations. *Journal of Medical Genetics*, *42*(4), 318–21. doi:10.1136/jmg.2004.024646

Caglayan, A. O. (2010). Genetic causes of syndromic and non-syndromic autism. *Developmental Medicine and Child Neurology*, *52*(2), 130–8. doi:10.1111/j.1469-8749.2009.03523.x

Calfa, G., Percy, A. K., & Pozzo-Miller, L. (2011). Experimental models of Rett syndrome based on Mecp2 dysfunction. *Experimental Biology and Medicine (Maywood, N.J.)*, *236*(1), 3–19. doi:10.1258/ebm.2010.010261

Carlino, D., Leone, E., Di Cola, F., Baj, G., Marin, R., Dinelli, G., … De Vanna, M. (2011). Low serum truncated-BDNF isoform correlates with higher cognitive impairment in schizophrenia. *Journal of Psychiatric Research*, *45*(2), 273–9. doi:10.1016/j.jpsychires.2010.06.012

Castrén, E., Elgersma, Y., Maffei, L., & Hagerman, R. (2012). Treatment of neurodevelopmental disorders in adulthood. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *32*(41), 14074–9. doi:10.1523/JNEUROSCI.3287-12.2012

Cellot, G., & Cherubini, E. (2013). Functional role of ambient GABA in refining neuronal circuits early in postnatal development. *Frontiers in Neural Circuits*, *7*, 136. doi:10.3389/fncir.2013.00136

Cellot, G., & Cherubini, E. (2014). GABAergic signaling as therapeutic target for autism spectrum disorders. *Frontiers in Pediatrics*, *2*, 70. doi:10.3389/fped.2014.00070

Centonze, D., Rossi, S., Mercaldo, V., Napoli, I., Ciotti, M. T., De Chiara, V., … Bagni, C. (2008). Abnormal striatal GABA transmission in the mouse model for the fragile X syndrome. *Biological Psychiatry*, *63*(10), 963–73. doi:10.1016/j.biopsych.2007.09.008

Chang, Q., Khare, G., Dani, V., Nelson, S., & Jaenisch, R. (2006). The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. *Neuron*, *49*(3), 341–8. doi:10.1016/j.neuron.2005.12.027

Chang, S.-C., Pauls, D. L., Lange, C., Sasanfar, R., & Santangelo, S. L. (2011). Common genetic variation in the GAD1 gene and the entire family of DLX homeobox genes and autism spectrum disorders. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics*, *156*(2), 233–9. doi:10.1002/ajmg.b.31148

Chao, H.-T., Chen, H., Samaco, R. C., Xue, M., Chahrour, M., Yoo, J., … Zoghbi, H. Y. (2010). Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. *Nature*, *468*(7321), 263–9. doi:10.1038/nature09582

Chao, H.-T., Zoghbi, H. Y., & Rosenmund, C. (2007). MeCP2 controls excitatory synaptic strength by regulating glutamatergic synapse number. *Neuron*, *56*(1), 58–65. doi:10.1016/j.neuron.2007.08.018

Chao, M. V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nature Reviews. Neuroscience*, *4*(4), 299–309. doi:10.1038/nrn1078

Chapleau, C. A., Larimore, J. L., Theibert, A., & Pozzo-Miller, L. (2009). Modulation of dendritic spine development and plasticity by BDNF and vesicular trafficking: fundamental roles in neurodevelopmental disorders associated with mental retardation and autism. *Journal of Neurodevelopmental Disorders*, *1*(3), 185–96. doi:10.1007/s11689-009-9027-6

Chattopadhyaya, B. (2011). Molecular mechanisms underlying activity-dependent GABAergic synapse development and plasticity and its implications for neurodevelopmental disorders. *Neural Plasticity*, *2011*, 734231. doi:10.1155/2011/734231

Chattopadhyaya, B., & Cristo, G. Di. (2012a). GABAergic Circuit Dysfunctions in Neurodevelopmental Disorders. *Frontiers in Psychiatry*, *3*. doi:10.3389/fpsyt.2012.00051

Chattopadhyaya, B., & Cristo, G. Di. (2012b). GABAergic circuit dysfunctions in neurodevelopmental disorders. *Frontiers in Psychiatry*, *3*, 51. doi:10.3389/fpsyt.2012.00051

Chattopadhyaya, B., Di Cristo, G., Higashiyama, H., Knott, G. W., Kuhlman, S. J., Welker, E., & Huang, Z. J. (2004). Experience and activity-dependent maturation of perisomatic GABAergic innervation in primary visual cortex during a postnatal critical period. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *24*(43), 9598–611. doi:10.1523/JNEUROSCI.1851-04.2004

Chattopadhyaya, B., Di Cristo, G., Wu, C. Z., Knott, G., Kuhlman, S., Fu, Y., … Huang, Z. J. (2007). GAD67-mediated GABA synthesis and signaling regulate inhibitory synaptic innervation in the visual cortex. *Neuron*, *54*(6), 889–903. doi:10.1016/j.neuron.2007.05.015

Chebib, M., & Johnston, G. A. (1999). The “ABC” of GABA receptors: a brief review. *Clinical and Experimental Pharmacology & Physiology*, *26*(11), 937–40.

Cheh, M. A., Millonig, J. H., Roselli, L. M., Ming, X., Jacobsen, E., Kamdar, S., & Wagner, G. C. (2006). En2 knockout mice display neurobehavioral and neurochemical alterations relevant to autism spectrum disorder. *Brain Research*, *1116*(1), 166–76. doi:10.1016/j.brainres.2006.07.086

Chen, J., Yu, S., Fu, Y., & Li, X. (2014). Synaptic proteins and receptors defects in autism spectrum disorders. *Frontiers in Cellular Neuroscience*, *8*, 276. doi:10.3389/fncel.2014.00276

Cheng, Y., Sudarov, A., Szulc, K. U., Sgaier, S. K., Stephen, D., Turnbull, D. H., & Joyner, A. L. (2010). The Engrailed homeobox genes determine the different foliation patterns in the vermis and hemispheres of the mammalian cerebellum. *Development (Cambridge, England)*, *137*(3), 519–29. doi:10.1242/dev.027045

Cheung, A., Newland, P. L., Zaben, M., Attard, G. S., & Gray, W. P. (2012). Intracellular nitric oxide mediates neuroproliferative effect of neuropeptide y on postnatal hippocampal precursor cells. *The Journal of Biological Chemistry*, *287*(24), 20187–96. doi:10.1074/jbc.M112.346783

Chiaruttini, C., Sonego, M., Baj, G., Simonato, M., & Tongiorgi, E. (2008). BDNF mRNA splice variants display activity-dependent targeting to distinct hippocampal laminae. *Molecular and Cellular Neurosciences*, *37*(1), 11–9. doi:10.1016/j.mcn.2007.08.011

Chonchaiya, W., Au, J., Schneider, A., Hessl, D., Harris, S. W., Laird, M., … Hagerman, R. J. (2012). Increased prevalence of seizures in boys who were probands with the FMR1 premutation and co-morbid autism spectrum disorder. *Human Genetics*, *131*(4), 581–9. doi:10.1007/s00439-011-1106-6

Churchill, J. D., Beckel-Mitchener, A., Weiler, I. J., & Greenough, W. T. (2002). Effects of Fragile X syndrome and an FMR1 knockout mouse model on forebrain neuronal cell biology. *Microscopy Research and Technique*, *57*(3), 156–8. doi:10.1002/jemt.10062

Cobos, I., Calcagnotto, M. E., Vilaythong, A. J., Thwin, M. T., Noebels, J. L., Baraban, S. C., & Rubenstein, J. L. R. (2005). Mice lacking Dlx1 show subtype-specific loss of interneurons, reduced inhibition and epilepsy. *Nature Neuroscience*, *8*(8), 1059–68. doi:10.1038/nn1499

Cohen, S., & Levi-Montalcini, R. (1956). A NERVE GROWTH-STIMULATING FACTOR ISOLATED FROM SNAKE VENOM. *Proceedings of the National Academy of Sciences of the United States of America*, *42*(9), 571–4.

Comery, T. A., Harris, J. B., Willems, P. J., Oostra, B. A., Irwin, S. A., Weiler, I. J., & Greenough, W. T. (1997). Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(10), 5401–4.

Correia, C. T., Coutinho, A. M., Sequeira, A. F., Sousa, I. G., Lourenço Venda, L., Almeida, J. P., … Vicente, A. M. (2010). Increased BDNF levels and NTRK2 gene association suggest a disruption of BDNF/TrkB signaling in autism. *Genes, Brain, and Behavior*, *9*(7), 841–8. doi:10.1111/j.1601-183X.2010.00627.x

Cossart, R., Dinocourt, C., Hirsch, J. C., Merchan-Perez, A., De Felipe, J., Ben-Ari, Y., … Bernard, C. (2001). Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. *Nature Neuroscience*, *4*(1), 52–62. doi:10.1038/82900

Crawley, J. N. (2004). Designing mouse behavioral tasks relevant to autistic-like behaviors. *Mental Retardation and Developmental Disabilities Research Reviews*, *10*(4), 248–58. doi:10.1002/mrdd.20039

Cunha, C., Brambilla, R., & Thomas, K. L. (2010). A simple role for BDNF in learning and memory? *Frontiers in Molecular Neuroscience*, *3*, 1. doi:10.3389/neuro.02.001.2010

Curatolo, P., Ben-Ari, Y., Bozzi, Y., Catania, M. V., D'Angelo, E., Mapelli, L., … Cherubini, E. (2014). Synapses as therapeutic targets for autism spectrum disorders: an international symposium held in pavia on july 4th, 2014. *Frontiers in Cellular Neuroscience*, *8*, 309. doi:10.3389/fncel.2014.00309

Dani, V. S., Chang, Q., Maffei, A., Turrigiano, G. G., Jaenisch, R., & Nelson, S. B. (2005). Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(35), 12560–5. doi:10.1073/pnas.0506071102

Darnell, J. C., & Richter, J. D. (2012). Cytoplasmic RNA-binding proteins and the control of complex brain function. *Cold Spring Harbor Perspectives in Biology*, *4*(8), a012344. doi:10.1101/cshperspect.a012344

Marco García, N. V, Karayannis, T., & Fishell, G. (2011). Neuronal activity is required for the development of specific cortical interneuron subtypes. *Nature*, *472*(7343), 351–5. doi:10.1038/nature09865

De Rubeis, S., Fernández, E., Buzzi, A., Di Marino, D., & Bagni, C. (2012). Molecular and cellular aspects of mental retardation in the Fragile X syndrome: from gene mutation/s to spine dysmorphogenesis. *Advances in Experimental Medicine and Biology*, *970*, 517–51. doi:10.1007/978-3-7091-0932-8_23

Dechant, G., & Barde, Y.-A. (2002). The neurotrophin receptor p75 (NTR): novel functions and implications for diseases of the nervous system. *Nature Neuroscience*, *5*(11), 1131–6. doi:10.1038/nn1102-1131

Di Cristo, G. (2007). Development of cortical GABAergic circuits and its implications for neurodevelopmental disorders. *Clinical Genetics*, *72*(1), 1–8. doi:10.1111/j.1399-0004.2007.00822.x

Di Cristo, G., Chattopadhyaya, B., Kuhlman, S. J., Fu, Y., Bélanger, M.-C., Wu, C. Z., … Huang, Z. J. (2007). Activity-dependent PSA expression regulates inhibitory maturation and onset of critical period plasticity. *Nature Neuroscience*, *10*(12), 1569–77. doi:10.1038/nn2008

Di Cristo, G., Pizzorusso, T., Cancedda, L., & Sernagor, E. (2011). GABAergic circuit development and its implication for CNS disorders. *Neural Plasticity*, *2011*, 623705. doi:10.1155/2011/623705

DiCicco-Bloom, E., Lord, C., Zwaigenbaum, L., Courchesne, E., Dager, S. R., Schmitz, C., … Young, L. J. (2006a). The developmental neurobiology of autism spectrum disorder. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(26), 6897–906. doi:10.1523/JNEUROSCI.1712-06.2006

DiCicco-Bloom, E., Lord, C., Zwaigenbaum, L., Courchesne, E., Dager, S. R., Schmitz, C., … Young, L. J. (2006b). The developmental neurobiology of autism spectrum disorder. *The*

Journal of Neuroscience : The Official Journal of the Society for Neuroscience, *26*(26), 6897–906. doi:10.1523/JNEUROSCI.1712-06.2006

Dindot, S. V, Antalffy, B. A., Bhattacharjee, M. B., & Beaudet, A. L. (2008). The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. *Human Molecular Genetics*, *17*(1), 111–8. doi:10.1093/hmg/ddm288

Dölen, G., Osterweil, E., Rao, B. S. S., Smith, G. B., Auerbach, B. D., Chattarji, S., & Bear, M. F. (2007). Correction of fragile X syndrome in mice. *Neuron*, *56*(6), 955–62. doi:10.1016/j.neuron.2007.12.001

Downs, J., Géranton, S. M., Bebbington, A., Jacoby, P., Bahi-Buisson, N., Ravine, D., & Leonard, H. (2010). Linking MECP2 and pain sensitivity: the example of Rett syndrome. *American Journal of Medical Genetics. Part A*, *152A*(5), 1197–205. doi:10.1002/ajmg.a.33314

Durand, C. M., Betancur, C., Boeckers, T. M., Bockmann, J., Chaste, P., Fauchereau, F., … Bourgeron, T. (2007). Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nature Genetics*, *39*(1), 25–7. doi:10.1038/ng1933

Durand, S., Patrizi, A., Quast, K. B., Hachigian, L., Pavlyuk, R., Saxena, A., … Fagiolini, M. (2012). NMDA receptor regulation prevents regression of visual cortical function in the absence of Mecp2. *Neuron*, *76*(6), 1078–90. doi:10.1016/j.neuron.2012.12.004

Fagiolini, M., Pizzorusso, T., Berardi, N., Domenici, L., & Maffei, L. (1994). Functional postnatal development of the rat primary visual cortex and the role of visual experience: dark rearing and monocular deprivation. *Vision Research*, *34*(6), 709–20.

Faras, H., Al Ateeqi, N., & Tidmarsh, L. (n.d.). Autism spectrum disorders. *Annals of Saudi Medicine*, *30*(4), 295–300. doi:10.4103/0256-4947.65261

Fatemi, S. H., Halt, A. R., Stary, J. M., Kanodia, R., Schulz, S. C., & Realmuto, G. R. (2002). Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices. *Biological Psychiatry*, *52*(8), 805–10.

Fatemi, S. H., Stary, J. M., Halt, A. R., & Realmuto, G. R. (2001). Dysregulation of Reelin and Bcl-2 proteins in autistic cerebellum. *Journal of Autism and Developmental Disorders*, *31*(6), 529–35.

Ferrer, I., Goutan, E., Marín, C., Rey, M. J., & Ribalta, T. (2000). Brain-derived neurotrophic factor in Huntington disease. *Brain Research*, *866*(1-2), 257–61.

Fiala, J. C., Spacek, J., & Harris, K. M. (2002). Dendritic spine pathology: cause or consequence of neurological disorders? *Brain Research. Brain Research Reviews*, *39*(1), 29–54.

Földy, C., Malenka, R. C., & Südhof, T. C. (2013). Autism-associated neuroligin-3 mutations commonly disrupt tonic endocannabinoid signaling. *Neuron*, *78*(3), 498–509. doi:10.1016/j.neuron.2013.02.036

Fombonne, E. (1999). The epidemiology of autism: a review. *Psychological Medicine*, *29*(4), 769–86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10473304

Fombonne, E. (2009). Epidemiology of pervasive developmental disorders. *Pediatric Research*, *65*(6), 591–8. doi:10.1203/PDR.0b013e31819e7203

Freund, T. F., & Buzsáki, G. (1996). Interneurons of the hippocampus. *Hippocampus*, *6*(4), 347–470. doi:10.1002/(SICI)1098-1063(1996)6:4<347::AID-HIPO1>3.0.CO;2-I

Gadad, B. S., Hewitson, L., Young, K. A., & German, D. C. (2013). Neuropathology and animal models of autism: genetic and environmental factors. *Autism Research and Treatment*, *2013*, 731935. doi:10.1155/2013/731935

Garcia, K. L. P., Yu, G., Nicolini, C., Michalski, B., Garzon, D. J., Chiu, V. S., … Fahnestock, M. (2012). Altered balance of proteolytic isoforms of pro-brain-derived neurotrophic factor in autism. *Journal of Neuropathology and Experimental Neurology*, *71*(4), 289–97. doi:10.1097/NEN.0b013e31824b27e4

Geschwind, D. H. (2011). Genetics of autism spectrum disorders. *Trends in Cognitive Sciences*, *15*(9), 409–16. doi:10.1016/j.tics.2011.07.003

Geschwind, D. H., & Levitt, P. (2007). Autism spectrum disorders: developmental disconnection syndromes. *Current Opinion in Neurobiology*, *17*(1), 103–11. doi:10.1016/j.conb.2007.01.009

Gharani, N., Benayed, R., Mancuso, V., Brzustowicz, L. M., & Millonig, J. H. (2004). Association of the homeobox transcription factor, ENGRAILED 2, 3, with autism spectrum disorder. *Molecular Psychiatry*, *9*(5), 474–84. doi:10.1038/sj.mp.4001498

Gherbassi, D., & Simon, H. H. (2006). The engrailed transcription factors and the mesencephalic dopaminergic neurons. *Journal of Neural Transmission. Supplementum*, (70), 47–55.

Gillberg, C., & Forsell, C. (1984). Childhood psychosis and neurofibromatosis--more than a coincidence? *Journal of Autism and Developmental Disorders*, *14*(1), 1–8.

Gilman, S. R., Iossifov, I., Levy, D., Ronemus, M., Wigler, M., & Vitkup, D. (2011). Rare de novo variants associated with autism implicate a large functional network of genes involved in formation and function of synapses. *Neuron*, *70*(5), 898–907. doi:10.1016/j.neuron.2011.05.021

Gogolla, N., Leblanc, J. J., Quast, K. B., Südhof, T. C., Fagiolini, M., & Hensch, T. K. (2009). Common circuit defect of excitatory-inhibitory balance in mouse models of autism. *Journal of Neurodevelopmental Disorders*, *1*(2), 172–81. doi:10.1007/s11689-009-9023-x

Gorski, J. A., Zeiler, S. R., Tamowski, S., & Jones, K. R. (2003). Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *23*(17), 6856–65.

Gottmann, K., Mittmann, T., & Lessmann, V. (2009). BDNF signaling in the formation, maturation and plasticity of glutamatergic and GABAergic synapses. *Experimental Brain Research*, *199*(3-4), 203–34. doi:10.1007/s00221-009-1994-z

Grossman, A. W., Aldridge, G. M., Lee, K. J., Zeman, M. K., Jun, C. S., Azam, H. S., … Rhyu, I. J. (2010). Developmental characteristics of dendritic spines in the dentate gyrus of Fmr1 knockout mice. *Brain Research*, *1355*, 221–7. doi:10.1016/j.brainres.2010.07.090

Guidotti, A., Auta, J., Davis, J. M., Di-Giorgi-Gerevini, V., Dwivedi, Y., Grayson, D. R., … DiGiorgi Gerevini, V. (2000). Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. *Archives of General Psychiatry*, *57*(11), 1061–9.

Guillén, I., Mullor, J. L., Capdevila, J., Sánchez-Herrero, E., Morata, G., & Guerrero, I. (1995). The function of engrailed and the specification of Drosophila wing pattern. *Development (Cambridge, England)*, *121*(10), 3447–56.

Guilmatre, A., Huguet, G., Delorme, R., & Bourgeron, T. (2014). The emerging role of SHANK genes in neuropsychiatric disorders. *Developmental Neurobiology*, *74*(2), 113–22. doi:10.1002/dneu.22128

Gupta, V. K., You, Y., Gupta, V. B., Klistorner, A., & Graham, S. L. (2013). TrkB Receptor Signalling: Implications in Neurodegenerative, Psychiatric and Proliferative Disorders. *International Journal of Molecular Sciences*, *14*(5), 10122–42. doi:10.3390/ijms140510122

Hall, S. S., Lightbody, A. A., Hirt, M., Rezvani, A., & Reiss, A. L. (2010). Autism in fragile X syndrome: a category mistake? *Journal of the American Academy of Child and Adolescent Psychiatry*, *49*(9), 921–33. doi:10.1016/j.jaac.2010.07.001

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B., & Joyner, A. L. (1995). Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. *Science (New York, N.Y.)*, *269*(5224), 679–82.

Hartmann, M., Brigadski, T., Erdmann, K. S., Holtmann, B., Sendtner, M., Narz, F., & Lessmann, V. (2004). Truncated TrkB receptor-induced outgrowth of dendritic filopodia involves the p75 neurotrophin receptor. *Journal of Cell Science*, *117*(Pt 24), 5803–14. doi:10.1242/jcs.01511

Hashimoto, K., Iwata, Y., Nakamura, K., Tsujii, M., Tsuchiya, K. J., Sekine, Y., … Mori, N. (2006). Reduced serum levels of brain-derived neurotrophic factor in adult male patients with autism. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *30*(8), 1529–31. doi:10.1016/j.pnpbp.2006.06.018

Hatton, D. D., Sideris, J., Skinner, M., Mankowski, J., Bailey, D. B., Roberts, J., & Mirrett, P. (2006). Autistic behavior in children with fragile X syndrome: prevalence, stability, and the impact of FMRP. *American Journal of Medical Genetics. Part A*, *140A*(17), 1804–13. doi:10.1002/ajmg.a.31286

Haznedar, M. M., Buchsbaum, M. S., Wei, T. C., Hof, P. R., Cartwright, C., Bienstock, C. A., & Hollander, E. (2000). Limbic circuitry in patients with autism spectrum disorders studied with positron emission tomography and magnetic resonance imaging. *The American Journal of Psychiatry*, *157*(12), 1994–2001. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11097966

Heimel, J. A., van Versendaal, D., & Levelt, C. N. (2011). The role of GABAergic inhibition in ocular dominance plasticity. *Neural Plasticity*, *2011*, 391763. doi:10.1155/2011/391763

Henneberger, C., Kirischuk, S., & Grantyn, R. (2005). Brain-derived neurotrophic factor modulates GABAergic synaptic transmission by enhancing presynaptic glutamic acid decarboxylase 65 levels, promoting asynchronous release and reducing the number of activated postsynaptic receptors. *Neuroscience*, *135*(3), 749–63. doi:10.1016/j.neuroscience.2005.06.044

Hensch, T. K. (2005). Critical period plasticity in local cortical circuits. *Nature Reviews. Neuroscience*, *6*(11), 877–88. doi:10.1038/nrn1787

Hensch, T. K., Fagiolini, M., Mataga, N., Stryker, M. P., Baekkeskov, S., & Kash, S. F. (1998). Local GABA circuit control of experience-dependent plasticity in developing visual cortex. *Science (New York, N.Y.)*, *282*(5393), 1504–8.

Herman, G. E., Butter, E., Enrile, B., Pastore, M., Prior, T. W., & Sommer, A. (2007). Increasing knowledge of PTEN germline mutations: Two additional patients with autism and macrocephaly. *American Journal of Medical Genetics. Part A*, *143A*(6), 589–93. doi:10.1002/ajmg.a.31619

Huang, Z. J., Di Cristo, G., & Ango, F. (2007). Development of GABA innervation in the cerebral and cerebellar cortices. *Nature Reviews. Neuroscience*, *8*(9), 673–86. doi:10.1038/nrn2188

Huang, Z. J., Kirkwood, A., Pizzorusso, T., Porciatti, V., Morales, B., Bear, M. F., … Tonegawa, S. (1999). BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. *Cell*, *98*(6), 739–55.

Hussman, J. P. (2001). Suppressed GABAergic inhibition as a common factor in suspected etiologies of autism. *Journal of Autism and Developmental Disorders*, *31*(2), 247–8.

Hutsler, J. J., & Zhang, H. (2010). Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. *Brain Research*, *1309*, 83–94. doi:10.1016/j.brainres.2009.09.120

Irwin, S. A., Patel, B., Idupulapati, M., Harris, J. B., Crisostomo, R. A., Larsen, B. P., … Greenough, W. T. (2001). Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. *American Journal of Medical Genetics*, *98*(2), 161–7.

Jamain, S., Quach, H., Betancur, C., Råstam, M., Colineaux, C., Gillberg, I. C., … Bourgeron, T. (2003). Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nature Genetics*, *34*(1), 27–9. doi:10.1038/ng1136

James, S. J., Shpyleva, S., Melnyk, S., Pavliv, O., & Pogribny, I. P. (2014). Elevated 5-hydroxymethylcytosine in the Engrailed-2 (EN-2) promoter is associated with increased gene expression and decreased MeCP2 binding in autism cerebellum. *Translational Psychiatry*, *4*, e460. doi:10.1038/tp.2014.87

Jinno, S., Klausberger, T., Marton, LF., Dalezios, Y., Roberts, JD., Fuentealba, P., Bushong, EA., Henze, D., Buzsáki, G., Somogyi, P. (2007). Neuronal diversity in GABAergic long-range projections from the hippocampus. *Journal Neuroscience,* 27(33):8790-804.

Jinno, S. (2009). Structural organization of long-range GABAergic projection system of the hippocampus. *Frontiers in Neuroanatomy,* 3:13. doi: 10.3389/neuro.05.013.2009.

Jinno, S., & Kosaka, T. (2010). Stereological estimation of numerical densities of glutamatergic principal neurons in the mouse hippocampus. *Hippocampus*, *20*(7), 829–40. doi:10.1002/hipo.20685

Jiyeon Choi, S. K. T. R. P. G. M. and J. H. M. (2011). *Autism Spectrum Disorders - From Genes to Environment*. (T. Williams, Ed.). InTech. doi:10.5772/747

Jones, J. R., Skinner, C., Friez, M. J., Schwartz, C. E., & Stevenson, R. E. (2008). Hypothesis: dysregulation of methylation of brain-expressed genes on the X chromosome and autism spectrum disorders. *American Journal of Medical Genetics. Part A*, *146A*(17), 2213–20. doi:10.1002/ajmg.a.32396

Jones, K. R., Fariñas, I., Backus, C., & Reichardt, L. F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell*, *76*(6), 989–99.

Joyner, A. L. (1996). Engrailed, Wnt and Pax genes regulate midbrain--hindbrain development. *Trends in Genetics : TIG*, *12*(1), 15–20.

Joyner, A. L., Herrup, K., Auerbach, B. A., Davis, C. A., & Rossant, J. (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the En-2 homeobox. *Science (New York, N.Y.)*, *251*(4998), 1239–43.

Joyner, A. L., & Martin, G. R. (1987). En-1 and En-2, two mouse genes with sequence homology to the Drosophila engrailed gene: expression during embryogenesis. *Genes & Development*, *1*(1), 29–38.

Kaplan, D. R., & Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Current Opinion in Neurobiology*, *10*(3), 381–91.

Katz, D. M., Berger-Sweeney, J. E., Eubanks, J. H., Justice, M. J., Neul, J. L., Pozzo-Miller, L., … Mamounas, L. A. (2012). Preclinical research in Rett syndrome: setting the foundation for translational success. *Disease Models & Mechanisms*, *5*(6), 733–45. doi:10.1242/dmm.011007

Kelsom, C., & Lu, W. (2013). Development and specification of GABAergic cortical interneurons. *Cell & Bioscience*, *3*(1), 19. doi:10.1186/2045-3701-3-19

Kemper, T. L., & Bauman, M. (1998). Neuropathology of infantile autism. *Journal of Neuropathology and Experimental Neurology*, *57*(7), 645–52.

Kishino, T., Lalande, M., & Wagstaff, J. (1997). UBE3A/E6-AP mutations cause Angelman syndrome. *Nature Genetics*, *15*(1), 70–3. doi:10.1038/ng0197-70

Kohara, K., Kitamura, A., Morishima, M., & Tsumoto, T. (2001). Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. *Science (New York, N.Y.)*, *291*(5512), 2419–23. doi:10.1126/science.1057415

Koshimizu, H., Kiyosue, K., Hara, T., Hazama, S., Suzuki, S., Uegaki, K., … Kojima, M. (2009). Multiple functions of precursor BDNF to CNS neurons: negative regulation of neurite growth, spine formation and cell survival. *Molecular Brain*, *2*, 27. doi:10.1186/1756-6606-2-27

Kovac, S., & Walker, M. C. (2013). Neuropeptides in epilepsy. *Neuropeptides*, *47*(6), 467–75. doi:10.1016/j.npep.2013.10.015

Kriaucionis, S., & Bird, A. (2004). The major form of MeCP2 has a novel N-terminus generated by alternative splicing. *Nucleic Acids Research*, *32*(5), 1818–23. doi:10.1093/nar/gkh349

Kuemerle, B., Gulden, F., Cherosky, N., Williams, E., & Herrup, K. (2007). The mouse Engrailed genes: a window into autism. *Behavioural Brain Research*, *176*(1), 121–32. doi:10.1016/j.bbr.2006.09.009

Kuemerle, B., Zanjani, H., Joyner, A., & Herrup, K. (1997). Pattern deformities and cell loss in Engrailed-2 mutant mice suggest two separate patterning events during cerebellar development. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(20), 7881–9.

Lai, K. O., Fu, W. Y., Ip, F. C., & Ip, N. Y. (1998). Cloning and expression of a novel neurotrophin, NT-7, from carp. *Molecular and Cellular Neurosciences*, *11*(1-2), 64–76. doi:10.1006/mcne.1998.0666

Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M.-P., … Briault, S. (2004). X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *American Journal of Human Genetics*, *74*(3), 552–7. doi:10.1086/382137

Lawrence, Y. A., Kemper, T. L., Bauman, M. L., & Blatt, G. J. (2010). Parvalbumin-, calbindin-, and calretinin-immunoreactive hippocampal interneuron density in autism. *Acta Neurologica Scandinavica*, *121*(2), 99–108. doi:10.1111/j.1600-0404.2009.01234.x

Le Magueresse, C., & Monyer, H. (2013). GABAergic interneurons shape the functional maturation of the cortex. *Neuron*, *77*(3), 388–405. doi:10.1016/j.neuron.2013.01.011

Leblond, C. S., Nava, C., Polge, A., Gauthier, J., Huguet, G., Lumbroso, S., … Bourgeron, T. (2014). Meta-analysis of SHANK Mutations in Autism Spectrum Disorders: A Gradient of Severity in Cognitive Impairments. *PLoS Genetics*, *10*(9), e1004580. doi:10.1371/journal.pgen.1004580

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., … Barde, Y. A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature*, *341*(6238), 149–52. doi:10.1038/341149a0

Lessmann, V., Gottmann, K., & Malcangio, M. (2003). Neurotrophin secretion: current facts and future prospects. *Progress in Neurobiology*, *69*(5), 341–74. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12787574

Llaneza, D. C., DeLuke, S. V, Batista, M., Crawley, J. N., Christodulu, K. V, & Frye, C. A. (2010). Communication, interventions, and scientific advances in autism: a commentary. *Physiology & Behavior*, *100*(3), 268–76. doi:10.1016/j.physbeh.2010.01.003

Logan, C., Hanks, M. C., Noble-Topham, S., Nallainathan, D., Provart, N. J., & Joyner, A. L. (1992). Cloning and sequence comparison of the mouse, human, and chicken engrailed genes reveal potential functional domains and regulatory regions. *Developmental Genetics*, *13*(5), 345–58. doi:10.1002/dvg.1020130505

London, E. A. (2000). The environment as an etiologic factor in autism: a new direction for research. *Environmental Health Perspectives*, *108 Suppl* , 401–4.

Lotter, V. (1966). Epidemiology of autistic conditions in young children. *Social Psychiatry*, *1*(3), 124–135. doi:10.1007/BF00584048

Louhivuori, V., Vicario, A., Uutela, M., Rantamäki, T., Louhivuori, L. M., Castrén, E., … Castrén, M. L. (2011). BDNF and TrkB in neuronal differentiation of Fmr1-knockout mouse. *Neurobiology of Disease*, *41*(2), 469–80. doi:10.1016/j.nbd.2010.10.018

Lundberg, J. M., Terenius, L., Hökfelt, T., & Tatemoto, K. (1984). Comparative immunohistochemical and biochemical analysis of pancreatic polypeptide-like peptides with special reference to presence of neuropeptide Y in central and peripheral neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *4*(9), 2376–86. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6548255

Malcangio, M., & Lessmann, V. (2003). A common thread for pain and memory synapses? Brain-derived neurotrophic factor and trkB receptors. *Trends in Pharmacological Sciences*, *24*(3), 116–21. doi:10.1016/S0165-6147(03)00025-7

Marcinkiewicz, M., Savaria, D., & Marcinkiewicz, J. (1998). The pro-protein convertase PC1 is induced in the transected sciatic nerve and is present in cultured Schwann cells: comparison with PC5, furin and PC7, implication in pro-BDNF processing. *Brain Research. Molecular Brain Research*, *59*(2), 229–46. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9729404

Marín, O. (2012). Interneuron dysfunction in psychiatric disorders. *Nature Reviews. Neuroscience*, *13*(2), 107–20. doi:10.1038/nrn3155

Marín, O., & Rubenstein, J. L. R. (2003). Cell migration in the forebrain. *Annual Review of Neuroscience*, *26*, 441–83. doi:10.1146/annurev.neuro.26.041002.131058

Markram, H., Toledo-Rodriguez, M., Wang, Y., Gupta, A., Silberberg, G., & Wu, C. (2004). Interneurons of the neocortical inhibitory system. *Nature Reviews. Neuroscience*, *5*(10), 793–807. doi:10.1038/nrn1519

Martin, G. A., Viskochil, D., Bollag, G., McCabe, P. C., Crosier, W. J., Haubruck, H., … Cawthon, R. M. (1990). The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell*, *63*(4), 843–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2121370

Marty, S., Berzaghi MdaP, & Berninger, B. (1997). Neurotrophins and activity-dependent plasticity of cortical interneurons. *Trends in Neurosciences*, *20*(5), 198–202.

Marty, S., da, M., & Berninger, B. (1997). Neurotrophins and activity-dependent plasticity of cortical interneurons. *Trends in Neurosciences*, *20*(5), 198–202. doi:10.1016/S0166-2236(96)01026-0

McBride, K. L., Varga, E. A., Pastore, M. T., Prior, T. W., Manickam, K., Atkin, J. F., & Herman, G. E. (2010). Confirmation study of PTEN mutations among individuals with autism or developmental delays/mental retardation and macrocephaly. *Autism Research : Official Journal of the International Society for Autism Research*, *3*(3), 137–41. doi:10.1002/aur.132

Medrihan, L., Tantalaki, E., Aramuni, G., Sargsyan, V., Dudanova, I., Missler, M., & Zhang, W. (2008). Early defects of GABAergic synapses in the brain stem of a MeCP2 mouse model of Rett syndrome. *Journal of Neurophysiology*, *99*(1), 112–21. doi:10.1152/jn.00826.2007

Michalski, B., & Fahnestock, M. (2003). Pro-brain-derived neurotrophic factor is decreased in parietal cortex in Alzheimer's disease. *Brain Research. Molecular Brain Research*, *111*(1-2), 148–54.

Miles, J. H. (2011). Autism spectrum disorders--a genetics review. *Genetics in Medicine : Official Journal of the American College of Medical Genetics*, *13*(4), 278–94. doi:10.1097/GIM.0b013e3181ff67ba

Minshew, N. J., & Williams, D. L. (2007). The new neurobiology of autism: cortex, connectivity, and neuronal organization. *Archives of Neurology*, *64*(7), 945–50. doi:10.1001/archneur.64.7.945

Mitsuhashi, T., & Takahashi, T. (2009). Genetic regulation of proliferation/differentiation characteristics of neural progenitor cells in the developing neocortex. *Brain & Development*, *31*(7), 553–7. doi:10.1016/j.braindev.2009.05.002

Miyazaki, K., Narita, N., Sakuta, R., Miyahara, T., Naruse, H., Okado, N., & Narita, M. (2004). Serum neurotrophin concentrations in autism and mental retardation: a pilot study. *Brain & Development*, *26*(5), 292–5. doi:10.1016/S0387-7604(03)00168-2

Moretti, P., & Zoghbi, H. Y. (2006). MeCP2 dysfunction in Rett syndrome and related disorders. *Current Opinion in Genetics & Development*, *16*(3), 276–81. doi:10.1016/j.gde.2006.04.009

Morgan, R. (2006). Engrailed: complexity and economy of a multi-functional transcription factor. *FEBS Letters*, *580*(11), 2531–3. doi:10.1016/j.febslet.2006.04.053

Mostofsky, S. H., Powell, S. K., Simmonds, D. J., Goldberg, M. C., Caffo, B., & Pekar, J. J. (2009). Decreased connectivity and cerebellar activity in autism during motor task performance. *Brain : A Journal of Neurology*, *132*(Pt 9), 2413–25. doi:10.1093/brain/awp088

Moy, S. S., Nadler, J. J., Magnuson, T. R., & Crawley, J. N. (2006). Mouse models of autism spectrum disorders: the challenge for behavioral genetics. *American Journal of Medical Genetics. Part C, Seminars in Medical Genetics*, *142C*(1), 40–51. doi:10.1002/ajmg.c.30081

Muhle, R., Trentacoste, S. V, & Rapin, I. (2004). The genetics of autism. *Pediatrics*, *113*(5), e472–86.

Nadarajah, B., & Parnavelas, J. G. (2002). Modes of neuronal migration in the developing cerebral cortex. *Nature Reviews. Neuroscience*, *3*(6), 423–32. doi:10.1038/nrn845

Neale, B. M., Kou, Y., Liu, L., Ma'ayan, A., Samocha, K. E., Sabo, A., Daly, M. J. (2012). Patterns and rates of exonic de novo mutations in autism spectrum disorders. *Nature*, *485*(7397), 242–5. doi:10.1038/nature11011

Nédélec, S., Foucher, I., Brunet, I., Bouillot, C., Prochiantz, A., & Trembleau, A. (2004). Emx2 homeodomain transcription factor interacts with eukaryotic translation initiation factor 4E (eIF4E) in the axons of olfactory sensory neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(29), 10815–20. doi:10.1073/pnas.0403824101

Nelson, K. B., Grether, J. K., Croen, L. A., Dambrosia, J. M., Dickens, B. F., Jelliffe, L. L. Phillips, T. M. (2001). Neuropeptides and neurotrophins in neonatal blood of children with autism or mental retardation. *Annals of Neurology*, *49*(5), 597–606. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11357950

Neul, J. L. (2012). The relationship of Rett syndrome and MECP2 disorders to autism. *Dialogues in Clinical Neuroscience*, *14*(3), 253–62.

Nickl-Jockschat, T., & Michel, T. M. (2011). The role of neurotrophic factors in autism. *Molecular Psychiatry*, *16*(5), 478–90. doi:10.1038/mp.2010.103

Palizvan, M. R., Sohya, K., Kohara, K., Maruyama, A., Yasuda, H., Kimura, F., & Tsumoto, T. (2004). Brain-derived neurotrophic factor increases inhibitory synapses, revealed in solitary neurons cultured from rat visual cortex. *Neuroscience*, *126*(4), 955–66. doi:10.1016/j.neuroscience.2004.03.053

Palmen, S. J. M. C., van Engeland, H., Hof, P. R., & Schmitz, C. (2004). Neuropathological findings in autism. *Brain : A Journal of Neurology*, *127*(Pt 12), 2572–83. doi:10.1093/brain/awh287

Paluszkiewicz, S. M., Martin, B. S., & Huntsman, M. M. (2011). Fragile X syndrome: the GABAergic system and circuit dysfunction. *Developmental Neuroscience*, *33*(5), 349–64. doi:10.1159/000329420

Pan, F., Aldridge, G. M., Greenough, W. T., & Gan, W.-B. (2010). Dendritic spine instability and insensitivity to modulation by sensory experience in a mouse model of fragile X syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(41), 17768–73. doi:10.1073/pnas.1012496107

Pang, P. T., Teng, H. K., Zaitsev, E., Woo, N. T., Sakata, K., Zhen, S., Lu, B. (2004). Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science (New York, N.Y.)*, *306*(5695), 487–91. doi:10.1126/science.1100135

Pardo, C. A., & Eberhart, C. G. (2007). The neurobiology of autism. *Brain Pathology (Zurich, Switzerland)*, *17*(4), 434–47. doi:10.1111/j.1750-3639.2007.00102.x

Pardridge, WM. (1997). Drug delivery to the brain. *Journal of Cerebral Blood Flow & Metabolism – Nature, 17(7):713-31.*

Patapoutian, A., & Reichardt, L. F. (2001). Trk receptors: mediators of neurotrophin action. *Current Opinion in Neurobiology*, *11*(3), 272–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11399424

Paul, R. (2008). Interventions to improve communication in autism. *Child and Adolescent Psychiatric Clinics of North America*, *17*(4), 835–56, ix–x. doi:10.1016/j.chc.2008.06.011

Payakachat, N., Tilford, J. M., Kovacs, E., & Kuhlthau, K. (2012). Autism spectrum disorders: a review of measures for clinical, health services and cost-effectiveness applications. *Expert Review of Pharmacoeconomics & Outcomes Research*, *12*(4), 485–503. doi:10.1586/erp.12.29

Peça, J., Feliciano, C., Ting, J. T., Wang, W., Wells, M. F., Venkatraman, T. N., Feng, G. (2011). Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. *Nature*, *472*(7344), 437–42. doi:10.1038/nature09965

Peng, S., Wuu, J., Mufson, E. J., & Fahnestock, M. (2005). Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. *Journal of Neurochemistry*, *93*(6), 1412–21. doi:10.1111/j.1471-4159.2005.03135.x

Penzes, P., Cahill, M. E., Jones, K. A., VanLeeuwen, J.-E., & Woolfrey, K. M. (2011). Dendritic spine pathology in neuropsychiatric disorders. *Nature Neuroscience*, *14*(3), 285–93. doi:10.1038/nn.2741

Perry, E. K., Lee, M. L., Martin-Ruiz, C. M., Court, J. A., Volsen, S. G., Merrit, J., Wenk, G. L. (2001). Cholinergic activity in autism: abnormalities in the cerebral cortex and basal forebrain. *The American Journal of Psychiatry*, *158*(7), 1058–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11431227

Phillips, H. S., Hains, J. M., Armanini, M., Laramee, G. R., Johnson, S. A., & Winslow, J. W. (1991). BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. *Neuron*, *7*(5), 695–702.

Pierce, K., & Courchesne, E. (2001). Evidence for a cerebellar role in reduced exploration and stereotyped behavior in autism. *Biological Psychiatry*, *49*(8), 655–64.

Pillai, A., Veeranan-Karmegam, R., Dhandapani, K. M., & Mahadik, S. P. (2008). Cystamine prevents haloperidol-induced decrease of BDNF/TrkB signaling in mouse frontal cortex. *Journal of Neurochemistry*, *107*(4), 941–51. doi:10.1111/j.1471-4159.2008.05665.x

Pizzarelli, R., & Cherubini, E. (2011). Alterations of GABAergic signaling in autism spectrum disorders. *Neural Plasticity*, *2011*, 297153. doi:10.1155/2011/297153

Pleasure, S. J., Anderson, S., Hevner, R., Bagri, A., Marin, O., Lowenstein, D. H., & Rubenstein, J. L. (2000). Cell migration from the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. *Neuron*, *28*(3), 727–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11163262

Poduslo JF, Curran GL.(2005). Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. *Brain Research.Molecular Brain Research, 36(2):280-6.*

Poo, M. M. (2001). Neurotrophins as synaptic modulators. *Nature Reviews. Neuroscience*, *2*(1), 24–32. doi:10.1038/35049004

Portera-Cailliau, C. (2012). Which comes first in fragile X syndrome, dendritic spine dysgenesis or defects in circuit plasticity? *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *18*(1), 28–44. doi:10.1177/1073858410395322

Pride, N. A., Payne, J. M., & North, K. N. (2012). The impact of ADHD on the cognitive and academic functioning of children with NF1. *Developmental Neuropsychology*, *37*(7), 590–600. doi:10.1080/87565641.2012.695831

Provenzano, G., Zunino, G., Genovesi, S., Sgadó, P., & Bozzi, Y. (2012). Mutant mouse models of autism spectrum disorders. *Disease Markers*, *33*(5), 225–39. doi:10.3233/DMA-2012-0917

Ramocki, M. B., Peters, S. U., Tavyev, Y. J., Zhang, F., Carvalho, C. M. B., Schaaf, C. P., Zoghbi, H. Y. (2009). Autism and other neuropsychiatric symptoms are prevalent in individuals with MeCP2 duplication syndrome. *Annals of Neurology*, *66*(6), 771–82. doi:10.1002/ana.21715

Ray, M. T., Shannon Weickert, C., & Webster, M. J. (2014). Decreased BDNF and TrkB mRNA expression in multiple cortical areas of patients with schizophrenia and mood disorders. *Translational Psychiatry*, *4*, e389. doi:10.1038/tp.2014.26

Reichardt, L. F. (2006). Neurotrophin-regulated signalling pathways. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *361*(1473), 1545–64. doi:10.1098/rstb.2006.1894

Reiss, A. L., Abrams, M. T., Greenlaw, R., Freund, L., & Denckla, M. B. (1995). Neurodevelopmental effects of the FMR-1 full mutation in humans. *Nature Medicine*, *1*(2), 159–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7585014

Rocamora, N., Welker, E., Pascual, M., & Soriano, E. (1996). Upregulation of BDNF mRNA expression in the barrel cortex of adult mice after sensory stimulation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *16*(14), 4411–9.

Romero-Munguía, M. Á. (2011). *Mnesic Imbalance and the Neuroanatomy of Autism Spectrum Disorders*.

Rossignol, E. (2011). Genetics and function of neocortical GABAergic interneurons in neurodevelopmental disorders. *Neural Plasticity*, *2011*, 649325. doi:10.1155/2011/649325

Roux, P. P., & Barker, P. A. (2002). Neurotrophin signaling through the p75 neurotrophin receptor. *Progress in Neurobiology*, *67*(3), 203–33.

Rubenstein, J. L. R., & Merzenich, M. M. (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes, Brain and Behavior*, *2*(5), 255–267. doi:10.1034/j.1601-183X.2003.00037.x

Rubenstein, J. L. R., & Merzenich, M. M. (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes, Brain, and Behavior*, *2*(5), 255–67.

Rudy, B., Fishell, G., Lee, S., & Hjerling-Leffler, J. (2011). Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Developmental Neurobiology*, *71*(1), 45–61. doi:10.1002/dneu.20853

Rutherford, L. C., DeWan, A., Lauer, H. M., & Turrigiano, G. G. (1997). Brain-derived neurotrophic factor mediates the activity-dependent regulation of inhibition in neocortical cultures. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(12), 4527–35.

Sakai, Y., Shaw, C. A., Dawson, B. C., Dugas, D. V, Al-Mohtaseb, Z., Hill, D. E., & Zoghbi, H. Y. (2011). Protein interactome reveals converging molecular pathways among autism disorders. *Science Translational Medicine*, *3*(86), 86ra49. doi:10.1126/scitranslmed.3002166

Sakane, T., Pardridge, WM. (1997). Carboxyl-directed pegylation of brain-derived neurotrophic factor markedly reduces systemic clearance with minimal loss of biologic activity. Pharmaceutical research, 14(8):1085-91.

Santos, M., Silva-Fernandes, A., Oliveira, P., Sousa, N., & Maciel, P. (2007). Evidence for abnormal early development in a mouse model of Rett syndrome. *Genes, Brain, and Behavior*, *6*(3), 277–86. doi:10.1111/j.1601-183X.2006.00258.x

Schmahmann, J. D. (2004). Disorders of the cerebellum: ataxia, dysmetria of thought, and the cerebellar cognitive affective syndrome. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *16*(3), 367–78. doi:10.1176/appi.neuropsych.16.3.367

Schmitz, C., van Kooten, I. A. J., Hof, P. R., van Engeland, H., Patterson, P. H., & Steinbusch, H. W. M. (2005). Autism: neuropathology, alterations of the GABAergic system, and animal models. *International Review of Neurobiology*, *71*, 1–26.

Schumann, C. M., Barnes, C. C., Lord, C., & Courchesne, E. (2009). Amygdala enlargement in toddlers with autism related to severity of social and communication impairments. *Biological Psychiatry*, *66*(10), 942–9. doi:10.1016/j.biopsych.2009.07.007

Selby, L., Zhang, C., & Sun, Q.-Q. (2007). Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein. *Neuroscience Letters*, *412*(3), 227–32. doi:10.1016/j.neulet.2006.11.062

Sgadò, P., Dunleavy, M., Genovesi, S., Provenzano, G., & Bozzi, Y. (2011). The role of GABAergic system in neurodevelopmental disorders: a focus on autism and epilepsy. *International Journal of Physiology, Pathophysiology and Pharmacology*, *3*(3), 223–35.

Sgadò, P., Genovesi, S., Kalinovsky, A., Zunino, G., Macchi, F., Allegra, M., …Bozzi, Y. (2013). Loss of GABAergic neurons in the hippocampus and cerebral cortex of Engrailed-2 null mutant mice: implications for autism spectrum disorders. *Experimental Neurology*, *247*, 496–505. doi:10.1016/j.expneurol.2013.01.021

Sgadò, P., Provenzano, G., Dassi, E., Adami, V., Zunino, G., Genovesi, S., … Bozzi, Y. (2013a/b). Transcriptome profiling in engrailed-2 mutant mice reveals common molecular pathways associated with autism spectrum disorders. *Molecular Autism*, *4*(1), 51. doi:10.1186/2040-2392-4-51

Sillitoe, R. V, Vogel, M. W., & Joyner, A. L. (2010). Engrailed homeobox genes regulate establishment of the cerebellar afferent circuit map. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *30*(30), 10015–24. doi:10.1523/JNEUROSCI.0653-10.2010

Singh, B., Henneberger, C., Betances, D., Arevalo, M. A., Rodríguez-Tébar, A., Meier, J. C., & Grantyn, R. (2006). Altered balance of glutamatergic/GABAergic synaptic input and associated changes in dendrite morphology after BDNF expression in BDNF-deficient hippocampal neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(27), 7189–200. doi:10.1523/JNEUROSCI.5474-05.2006

Singh, S. K., & Eroglu, C. (2013). Neuroligins provide molecular links between syndromic and nonsyndromic autism. *Science Signaling*, *6*(283), re4. doi:10.1126/scisignal.2004102

Soghomonian, J. J., & Martin, D. L. (1998). Two isoforms of glutamate decarboxylase: why? *Trends in Pharmacological Sciences*, *19*(12), 500–5.

Sonnenberg, J. L., Frantz, G. D., Lee, S., Heick, A., Chu, C., Tobin, A. J., & Christakos, S. (1991). Calcium binding protein (calbindin-D28k) and glutamate decarboxylase gene expression after kindling induced seizures. *Brain Research. Molecular Brain Research*, *9*(3), 179–90.

Stearns, N. A., Schaevitz, L. R., Bowling, H., Nag, N., Berger, U. V, & Berger-Sweeney, J. (2007). Behavioral and anatomical abnormalities in Mecp2 mutant mice: a model for Rett syndrome. *Neuroscience*, *146*(3), 907–21. doi:10.1016/j.neuroscience.2007.02.009

Sudarov, A., & Joyner, A. L. (2007). Cerebellum morphogenesis: the foliation pattern is orchestrated by multi-cellular anchoring centers. *Neural Development*, *2*, 26. doi:10.1186/1749-8104-2-26

Südhof, T. C. (2008). Neuroligins and neurexins link synaptic function to cognitive disease. *Nature*, *455*(7215), 903–11. doi:10.1038/nature07456

Szilágyi, T., Orbán-Kis, K., Horváth, E., Metz, J., Pap, Z., & Pávai, Z. (2011). Morphological identification of neuron types in the rat hippocampus. *Romanian Journal of Morphology and Embryology = Revue Roumaine de Morphologie et Embryologie*, *52*(1), 15–20.

Tabuchi, K., Blundell, J., Etherton, M. R., Hammer, R. E., Liu, X., Powell, C. M., & Südhof, T. C. (2007). A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science (New York, N.Y.)*, *318*(5847), 71–6. doi:10.1126/science.1146221

Tanahira, C., Higo, S., Watanabe, K., Tomioka, R., Ebihara, S., Kaneko, T., & Tamamaki, N. (2009). Parvalbumin neurons in the forebrain as revealed by parvalbumin-Cre transgenic mice. *Neuroscience Research*, *63*(3), 213–23. doi:10.1016/j.neures.2008.12.007

Tanaka, T., Saito, H., & Matsuki, N. (1997). Inhibition of GABAA Synaptic Responses by Brain-Derived Neurotrophic Factor (BDNF) in Rat Hippocampus. *J. Neurosci.*, *17*(9), 2959–2966.

Taniguchi, H., Lu, J., & Huang, Z. J. (2013). The spatial and temporal origin of chandelier cells in mouse neocortex. *Science (New York, N.Y.)*, *339*(6115), 70–4. doi:10.1126/science.1227622

Teng, H. K., Teng, K. K., Lee, R., Wright, S., Tevar, S., Almeida, R. D., … Hempstead, B. L. (2005). ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(22), 5455–63. doi:10.1523/JNEUROSCI.5123-04.2005

Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D., & Jaynes, J. B. (1998). Two distinct types of repression domain in engrailed: one interacts with the groucho corepressor and is preferentially active on integrated target genes. *Molecular and Cellular Biology*, *18*(5), 2804–14.

Tongiorgi, E., Righi, M., & Cattaneo, A. (1997). Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(24), 9492–505.

Tonsgard, J. H., Yelavarthi, K. K., Cushner, S., Short, M. P., & Lindgren, V. (1997). Do NF1 gene deletions result in a characteristic phenotype? *American Journal of Medical Genetics*, *73*(1), 80–6.

Tripathi, P. P., Sgadò, P., Scali, M., Viaggi, C., Casarosa, S., Simon, H. H., … Bozzi, Y. (2009). Increased susceptibility to kainic acid-induced seizures in Engrailed-2 knockout mice. *Neuroscience*, *159*(2), 842–9. doi:10.1016/j.neuroscience.2009.01.007

Tropea, D., Giacometti, E., Wilson, N. R., Beard, C., McCurry, C., Fu, D. D., … Sur, M. (2009). Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(6), 2029–34. doi:10.1073/pnas.0812394106

Trottier, G., Srivastava, L., & Walker, C. D. (1999). Etiology of infantile autism: a review of recent advances in genetic and neurobiological research. *Journal of Psychiatry & Neuroscience : JPN*, *24*(2), 103–15.

Tyzio, R., Represa, A., Jorquera, I., Ben-Ari, Y., Gozlan, H., & Aniksztejn, L. (1999). The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *19*(23), 10372–82. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10575034

Van Spronsen, M., & Hoogenraad, C. C. (2010). Synapse pathology in psychiatric and neurologic disease. *Current Neurology and Neuroscience Reports*, *10*(3), 207–14. doi:10.1007/s11910-010-0104-8

Veenstra-Vanderweele, J., Christian, S. L., & Cook, E. H. (2004). Autism as a paradigmatic complex genetic disorder. *Annual Review of Genomics and Human Genetics*, *5*, 379–405. doi:10.1146/annurev.genom.5.061903.180050

Voineagu, I., Wang, X., Johnston, P., Lowe, J. K., Tian, Y., Horvath, S., … Geschwind, D. H. (2011). Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature*, *474*(7351), 380–4. doi:10.1038/nature10110

Waite, K. A., & Eng, C. (2002). Protean PTEN: form and function. *American Journal of Human Genetics*, *70*(4), 829–44. doi:10.1086/340026

Wang Y, Toledo-Rodriguez M, Gupta A, Wu C, Silberberg G, Luo J, Markram H.(2004). Anatomical, physiological and molecular properties of Martinotti cells in the somatosensory cortex of the juvenile rat. *The Journal of Physiology*, 561(Pt 1):65-90.

Wang, D. D., & Kriegstein, A. R. (2011). Blocking early GABA depolarization with bumetanide results in permanent alterations in cortical circuits and sensorimotor gating deficits. *Cerebral Cortex (New York, N.Y. : 1991)*, *21*(3), 574–87. doi:10.1093/cercor/bhq124

Wardle, R. A., & Poo, M. (2003). Brain-derived neurotrophic factor modulation of GABAergic synapses by postsynaptic regulation of chloride transport. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *23*(25), 8722–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14507972

Weskamp, G., & Reichardt, L. F. (1991). Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. *Neuron*, *6*(4), 649–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1849725

Wing, L., & Potter, D. (2002). The epidemiology of autistic spectrum disorders: is the prevalence rising? *Mental Retardation and Developmental Disabilities Research Reviews*, *8*(3), 151–61. doi:10.1002/mrdd.10029

Wonders, C. P., & Anderson, S. A. (2006). The origin and specification of cortical interneurons. *Nature Reviews. Neuroscience*, *7*(9), 687–96. doi:10.1038/nrn1954

Wong, J., Rothmond, D. A., Webster, M. J., & Weickert, C. S. (2013). Increases in two truncated TrkB isoforms in the prefrontal cortex of people with schizophrenia. *Schizophrenia Bulletin*, *39*(1), 130–40. doi:10.1093/schbul/sbr070

Woodruff, A., Xu, Q., Anderson, S. A., & Yuste, R. (2009). Depolarizing effect of neocortical chandelier neurons. *Frontiers in Neural Circuits*, *3*, 15. doi:10.3389/neuro.04.015.2009

Xu, B., Zang, K., Ruff, N. L., Zhang, Y. A., McConnell, S. K., Stryker, M. P., & Reichardt, L. F. (2000). Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. *Neuron*, *26*(1), 233–45.

Xu, Y., Roby, KD., Callaway, EM. (2006). Mouse cortical inhibitory neuron type that coexpresses somatostatin and calretinin. *The Journal of Comparative neurology*, *499(1):144-60.*

Yamada, M. K., Nakanishi, K., Ohba, S., Nakamura, T., Ikegaya, Y., Nishiyama, N., & Matsuki, N. (2002). Brain-Derived Neurotrophic Factor Promotes the Maturation of GABAergic Mechanisms in Cultured Hippocampal Neurons. *J. Neurosci.*, *22*(17), 7580–7585.

Yan, Q., Rosenfeld, R. D., Matheson, C. R., Hawkins, N., Lopez, O. T., Bennett, L., & Welcher, A. A. (1997). Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. *Neuroscience*, *78*(2), 431–48.

Yip, J., Soghomonian, J.-J., & Blatt, G. J. (2008). Increased GAD67 mRNA expression in cerebellar interneurons in autism: implications for Purkinje cell dysfunction. *Journal of Neuroscience Research*, *86*(3), 525–30. doi:10.1002/jnr.21520

Young, J. I., & Zoghbi, H. Y. (2004). X-chromosome inactivation patterns are unbalanced and affect the phenotypic outcome in a mouse model of rett syndrome. *American Journal of Human Genetics*, *74*(3), 511–20. doi:10.1086/382228

Zhang, J., Geula, C., Lu, C., Koziel, H., Hatcher, L. M., & Roisen, F. J. (2003). Neurotrophins regulate proliferation and survival of two microglial cell lines in vitro. *Experimental Neurology*, *183*(2), 469–81. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14552887

Zhang, Y, Pardridge, WM. (2001). Conjugation of brain-derived neurotrophic factor to a blood-brain barrier drug targeting system enablesneuroprotection in regional brain ischemia follo wing intravenous injection of the neurotrophin. *Brain Research*, 889(1-2):49-56.

Zhang, Y., Pardridge, WM. (2006). Blood-brain barrier targeting of BDNF improves motor function in rats with middle cerebral artery occlusion. *Brain Research*, 1111(1):227-9.

Zuccato, C., & Cattaneo, E. (2009). Brain-derived neurotrophic factor in neurodegenerative diseases. *Nature Reviews. Neurology*, *5*(6), 311–22. doi:10.1038/nrneurol.2009.54

Zuccato, C., Liber, D., Ramos, C., Tarditi, A., Rigamonti, D., Tartari, M., … Cattaneo, E. (2005). Progressive loss of BDNF in a mouse model of Huntington's disease and rescue by BDNF delivery. *Pharmacological Research : The Official Journal of the Italian Pharmacological Society*, *52*(2), 133–9. doi:10.1016/j.phrs.2005.01.001

APPENDIX

Published Papers containing results present in this Thesis:

1) **Loss of GABAergic neurons in the hippocampus and cerebral cortex of Engrailed-2 null mutant mice: Implications for autism spectrum disorders.**
   Sgadò P, Genovesi S*, Kalinovsky A*, Zunino G*, Macchi F, Allegra M, Murenu E, Provenzano G, Tripathi P.P, Casarosa S, Joyner A.L, Bozzi Y.
   Experimental Neurology, 247, 496–505. doi:10.1016/j.expneurol.2013.01.021;2013.

** equal contribution as second authors*

In this article my contribution has been to perform morphometric and immunohistochemical analyses, cell counting and RT-qPCR analyses. I performed a morphometric analysis on dorsal hippocampal and cerebral cortex sections stained for the pan-neuronal marker NeuN to carefully investigate hippocampal and cortical layer structure in WT and En2-/- mice. I participated to the study of the reduced expression of GABAergic marker mRNAs in these brain areas in adult En2-/- mice. In addition, I contributed to the experiments showing reduction in parvalbumin (PV), somatostatin (SOM) and neuropeptide Y (NPY) expressing interneurons, in the hippocampus and cerebral cortex of adult En2-/- mice. The images correlated to this contribution are present in this thesis (see *Results)*:

- Fig 4.1 Immunohistochemical data of En1/En2 protein expression in hippocampus and somatosensory cortex.
- Fig 4.2 Morphometric analysis in the En2-/- somatosensory cortex.
- Fig.4.3 Reduced number of GABAergic interneurons in the En2-/- somatosensory cortex
- Fig.4.7. Morphometric analysis in the hippocampus of En2-/- mice.
- Fig.4.8. Reduced number of GABAergic interneurons in the En2-/- hilus.

2) **Altered GABAergic markers, increased binocularity and reduced plasticity in the visual cortex of Engrailed-2 knockout mice.**
   Allegra M, Genovesi S, Maggia M, Cenni MC, Zunino G, Sgadò P, Caleo M, Bozzi Y.
   Frontiers in Cellular Neuroscience, *8*, 163. doi:10.3389/fncel.2014.00163; 2014.

I participated to this study, performing immunostaining analyses, cell counts and qPCR, which revealed significant differences in SOM-, NPY-, and PV- positive cell densities between *WT* and *En2*-/- mice, at adult age. I also contributed to the expression profile of GABAergic marker protein in the visual cortex that prompted us to investigate the mRNA expression of GABAergic interneurons in the primary visual cortex of *WT* and *En2*-/- mice

in adulthood. The result obtained revealed a decrease expression in GABAergic markers, in detail, in SOM, NPY, vGAT mRNA levels, and also in glutamatergic marker, vGlut1, in *En2-/-* mice compared to controls.

The images correlated to this contribution are present in this thesis (see *Results*):

- Fig.4.4. Profile of GABAergic interneurons in visual cortex, at adult age.
- Fig.4.5. mRNA expression of En2 and glutamatergic/GABAergic markers in the juvenile and adult visual cortex of WT and *En2-/-* mice.
- Fig.4.6 Normal neuronal density and layering in the primary visual cortex of adult *En2-/-* mice.

Other Published Papers containing results not presented in this Thesis:

3) Transcriptome profiling in engrailed-2 mutant mice reveals common molecular pathways associated with autism spectrum disorders.

Sgadò P, Provenzano G, Dassi E, Adami V, Zunino G, Genovesi S, Casarosa S, Bozzi Y.

Molecular Autism. 19;4(1):51; 2013.

I participated to this study, setting up PCR conditions for differentially expressed genes of interest in the En2-/- hippocampus (Fig. 5 of the published paper).

4) Hippocampal dysregulation of neurofibromin-dependent pathways is associated with impaired spatial learning in engrailed 2 knock-out mice.

Provenzano G, Pangrazzi L, Poli A, Pernigo M, Sgadò P, Genovesi S, Zunino G, Berardi N, Casarosa S, Bozzi Y.

The Journal of Neuroscience. 1;34(40):13281-8.;2014.

I participated to this study, setting up immunostaining conditions of En2 in the hippocampus (Fig. 3 of the published paper).

5) Mutant mouse models of autism spectrum disorders.

Provenzano G, Zunino G, Genovesi S, Sgadó P, Bozzi Y.

Disease Markers. 33(5):225-39.; 2012.

I participated in writing some paragraphs of this review, regarding mouse ASD models; part of the content of this review has been used to write the Introduction of this thesis.

6) Early *depolarizing* GABA controls critical period plasticity in the rat visual cortex

Deidda G, Allegra M, Cerri C, Naskar S, Bony G, Zunino G, Bozzi Y, Caleo M, Cancedda L. *Nature Neuroscience*, 18(1):87-96. doi: 10.1038/nn.3890. 2015

In addition to my thesis project, I collaborated with Laura Cancedda's group at the Italian Institute of Technology (IIT_Genova), in a project regarding the interference of depolarizing GABA during early postnatal development on visual cortex plasticity. Bumetanide-treated animals present an extension of critical period of plasticity in the visual cortex, without affecting the overall development of the visual system. This effect on plasticity is accompanied by changes in fundamental regulators of plasticity and it is rescued by increasing BDNF signaling during depolarizing-GABA interference. Here I describe my specific contribution to this publication:

Depolarizing GABAergic signaling is fundamental for early physiological maturation of various brain areas (Wang & Kriegstein, 2011). Inhibitory GABAergic transmission is a well-known regulator of visual system development and plasticity; here we studied the role of depolarizing GABA in the regulation of cortical plasticity.

In order to evaluate the perinatal interference with depolarizing GABA on the critical period for visual cortex plasticity, we used a classical paradigm of experience-dependent plasticity (i.e., a brief period of 3 days of monocular deprivation, MD) in bumetanide- and vehicle-treated rats. During the critical period, brief MD results in a dramatic shift of ocular dominance (OD, assessed at the end of the MD period) of cortical neurons in favor of the non-deprived eye (Heimel, van Versendaal, & Levelt, 2011). We performed the MD experiments at P17 (opening of critical period plasticity), P26 (peak of plasticity), P35 (closure of critical period plasticity) and P75, when animals are considered fully adult and do not show plasticity.

I participated in the study of the GABAergic interneurons development; examining their laminar distribution by immunostaining for the GABA biosynthetic enzyme GAD67 in cortical sections from bumetanide- and vehicle treated rats at P35. Count of GAD67 cells were performed on 3 sections for each experimental animal. For each section, images of the visual cortex were acquired with a 20X objective (air, 0.5 NA) by a Zeiss AxioObserver z1 microscope with a motorized stage. Acquisitions were automatically performed using the MosaiX and Z-Stack modules of the Zeiss AxioVision software (v4.3.1). I collected five Z-series for each image, which were projected to two-dimensional representations. Results showed no significant differences in the distribution of interneurons between the two groups of animals, treated and vehicle.

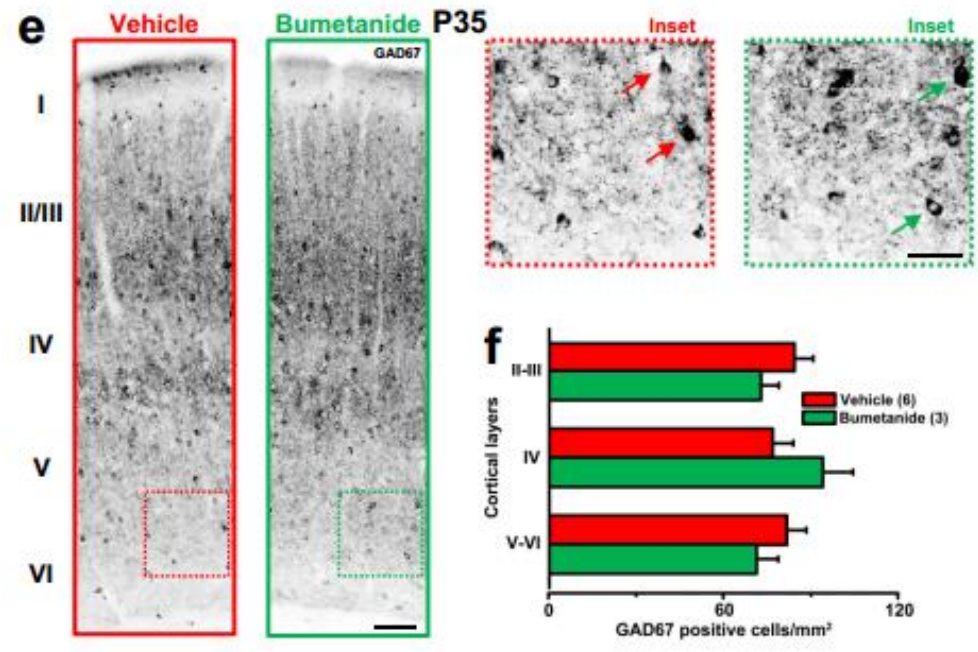


Fig A1. *Early GABAergic interference does not alter the overall structural development of the visual system:*
e) Representative images acquired from coronal sections of the visual cortex from vehicle- and bumetanide-treated animals at P35 labeled for GAD67. Scale bar, 100 µm. Insets: higher magnification images from fields as reported on the left. Arrows point to examples of GAD67-positive cells. Scale bar, 50 µm. **f)** Quantification of the density of GAD67-positive cells across cortical layers revealed no significant differences (Two Way ANOVA on ranks, $P > 0.05$). The histogram depicts average ± SEM. Numbers in parentheses: animals processed.

Brain derived neurotrophic factor (BDNF) controls the closure of the critical period in the visual system, influencing inhibitory circuits (Heimel et al., 2011)(Fagiolini et al.,1994). In this work has been investigated its expression in bumetanide- and vehicle-treated animals at P35, founding a significantly lower levels of BDNF in visual cortices from bumetanide-treated animals. In order to test the hypothesis that BDNF signaling is reduced in response to GABAA receptor activation, we collected visual cortex samples from rat pups perinatally treated with bumetanide or vehicle (from P3 to P8) and systemically challenged at P8 by acute injection of a GABAA receptor agonist. I performed quantitative RT-PCR and we found a significant up-regulation of Bdnf mRNA expression in vehicle-treated animals injected with midazolam (Mid), in agreement with the literature. Interestingly, animals perinatally treated with bumetanide showed no significant upregulation of Bdnf mRNA expression upon midazolam treatment.

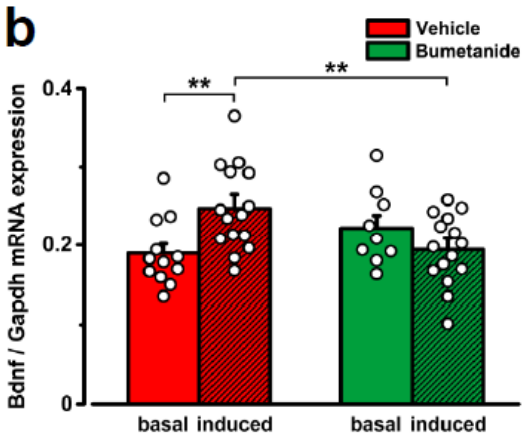
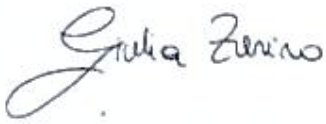


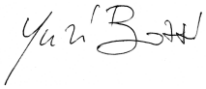
Figure A2. *BDNF mRNA expression*:
b) *BDNF* mRNA expression measured at P8 in vehicle- (red) and bumetanide-treated pups (green) either in basal condition or after induction by i.p. injection of $GABA_A$ receptor agonist Midazolam in rat pups. *Gapdh* was used as an internal standard. Two Way ANOVA analysis of variance showed that bumetanide treatment blocked (post Tukey test, $P = 0.004$) the Midazolam-induced increase (post Tukey test, $P = 0.003$) of *Bdnf* mRNA expression. The histogram depicts average ± SEM, whereas circles indicate single data points.

Declaration

I Giulia Zunino confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledge.



Signature of the PhD candidate



Signature of the Tutor

6. ACKNOLEDGEMENTS

First of all I would like to thank my PhD tutor, Professor Yuri Bozzi for his guidance, support and encouragement during these years. He helped me to appreciate science even when times become hard and unfair.

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This thesis would not have been possible without the help, support and patience of Andrea and Angela, bright minds and true friends.

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I would like express appreciation to Michele, who spent sleepless nights with me and was always my support in every moment. We've laughed, cried and cursed together, you changed me for the better, thank you love.

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