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NEURONAL MORPHOLOGY AND EXCITATION/INHIBITION BALANCE IN A MOUSE MODEL OF AUTISM: CORRELATION WITH BEHAVIORAL PHENOTYPES

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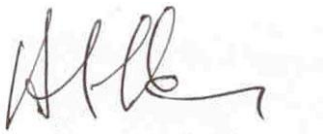

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ABBREVIATIONS

Arc	activity-regulated cytoskeleton-associated protein
ASD	autism spectrum disorder
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
bp	base pair
CaMKII	calmodulin-dependent protein kinase ii
CNS	central nervous system
Ct	treshold cycle
D2R	dopamine d2 receptor
DIV	days *in vitro*
DLS	dorsal lateral striatum
DSM-V	Diagnostic and Statistical Manual of Mental Disorders-V
E/I	excitatory/inhibitory
E18	embrionic day 18
eEF2	eukaryotic translation elongation factor 2
E-I	excitatory-inhibitory
ES	environmental stimulus
Fmr1	fragile X mental retardation 1 gene
FXS	fragile X symdrome
GABA	gamma-aminobutyric acid
GABAAR	GABA receptor type A
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
Gi	G protein inhibitory
Gi/o	G protein i/o subtype
GIRK	G protein-coupled inwardly-rectifying potassium channel
GPCR	G protein coupled receptor
Gq/11	G protein q/11 subtype
GRK	G protein-coupled receptor kinase
Gα	G protein subunit alpha
Gβ	G protein subunit beta
HEK293	human embryonic kidney 293 cells
HGF	hepatocyte growth factor
Hprt1	hypoxanthine phosphoribosyltransferase 1
IGR	intergenic region
IQ	intelligence quotient
IRK	inwardly-rectifying potassium channel
KCC2	potassium-chloride cotransporter 2
Kir	inwardly rectifying potassium channels gene
KO	knock-out
MAF	minor allele frequency

MCNs	magno cellular neuron
mRNA	messenger ribonucleic acid
MSN	medium spiny neuron
Nacc	nucleus accumbens
nAchR	nicotinic acetylcholine receptors
NKCC1	sodium- potassium-chloride cotransporter 1
OFC	orbitofrontal cortex
Oprd1	opioid receptor, delta 1 gene
OXT	oxytocin
OXTR	oxytocin receptor
PCR	polymerase chain reaction
p-eEF2	eukaryotic translation elongation factor 2 phosphorylated form
PLCβ	phospholipase C β
PTZ	pentylentetrazol
PVN	paraventricular nucleus
rAVV	recombinant *adeno*-associated virus
RT-PCR	reverse transcription polymerase chain reaction
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SON	supraoptic *nucleus*
TGOT	[Thr_4,Gly_7]-oxytocin
TP	two-photon
TSC	tuberous sclerosis complex
V1aR	vasopressin receptor type 1a
V1bR	vasopressin receptor type 1b
V2R	vasopressin receptor type 2
WT	wild type

ABSTRACT

The role of oxytocin (OXT) in controlling social behavior suggests a link to neuropsychiatric conditions in which social behavior behavior is aberrant or even absent, such as autism.
Mice lacking the OXT receptor (*Oxtr*$^{-/-}$) display an autistic-like phenotype, including deficits in social interaction, impaired cognitive flexibility (murine correlates of autism core symptoms), increased aggression and susceptibility to seizure (common co-occurring conditions).
The deficit in cognitive flexibility is particularly interesting, because it is present in few animal model of autism. For this reason we decided to investigate its underlying neurobiological and molecular mechanisms.
First, we compared *Oxtr*$^{+/+}$and *Oxtr*$^{-/-}$ neuronal morphology and spine remodeling following a cognitive behavioral test. Interestingly, we highlighted, in the *Oxtr*$^{-/-}$ mice, an enhanced connectivity and overuse of the dorsolateral striatum, possibly arising from an hippocampal dysfunction, and we proposed it as substrate for habit-like symptoms and cognitive rigidity.
Second, we investigated, at the molecular level, possible sources of this hippocampal dysfunction. In particular, we analyzed *Oxtr*$^{-/-}$ hippocampal neurons for the expression of proteins involved in the setting and maintenance of excitatio-inhibition (E-I) balance. We found an upregulation of several inwardly-rectifying K+ channels (belonging to Kir2 and Kir3 families), which could alter membrane excitability, and a lack of the physiological upregulation of the chloride transporter KCC2 during development, that may lead to aberrant GABAergic signaling in mature neurons. These data give important indications that the E-I balance is altered at multiple levels in *Oxtr*$^{-/-}$ hippocampal neurons, as an altered ratio between Glutamatergic and GABAergic synapses was also previously observed in these cultures. These observations are particularly intriguing, because an E-I imbalance has been frequently associated with several neurodevelopmental disorders such as autism.
Third, we disclosed an OXTR-mediated pathway modulating KCC2 expression that may restore a correct E-I balance in hippocampal neurons.
All this information could be useful to understand the pathophysiology of cognitive rigidity and to develop new therapies addressing specific symptoms of autism.

INTRODUCTION

I. OXYTOCIN SYSTEM

Oxytocin (OXT) derives its name from the Greek words ὀξύς, *oxys*, and τόκος, *tokos*, meaning "quick birth", since Sir Hanry Dale in 1906 found that extracts of the posterior pituitary have uterotonic activity . Few years later OXT was also found to induce milk ejection, but its role on the central nervous system (CNS) was far from being identified. Indeed, only in the late 1970's the OXT's action in coordinating a plethora of behavioral changes in the mother, necessary for the survival of the offspring (maternal behavior), started to emerge. From that time on, the involvement of OXT system in the modulation of many aspects of behavior, dealing with sociality, became clear (Dale 1906, Ott & Scott 1910, Ross & Young 2009). More recently broader effects of OXT on the CNS have been identified comprising also cognitive aspects, such as modulation of learning and memory.
Nowadays great interest is emerging around the therapeutic potential of OXT for the treatment of mental disorders characterized by social deficits, such as social anxiety, borderline personality disorders, schizophrenia and autism (Meyer-Lindenberg et al 2011).
For this reason, a greater understanding of oxytocinergic mechanisms modulating social/cognitive behaviors and of the neurobiological basis for its involvement in neuropsychiatric disorders is strongly needed.

This section will introduce the oxytocin system with particular attention to its role and functioning at the central level.

Oxytocin and its receptor

Oxytocin is a nine-aminoacid neuropeptide in which the presence of a disulfide bond between cysteine residues in position 1 and 6 forms a cyclic structure with a 3-aminoacid C-terminal tail(Du Vigneaud et al 1953).
OXT shares 7 out of 9 residues with vasopressin (AVP), the other neurohypophysial peptide. They differ in position 3 (Ile vs Phe) and in position 8 (Leu vs Arg), which justifies binding and

activation of different receptors with slightly different affinities and efficacies (Barberis et al 1998, Caldwell et al 2008).

Their genes also have very similar structures (see fig.1), and reside on the same chromosome (2 in mice, 20 in humans), but with opposite orientation. It is thus believed that they originated from the duplication of a single ancestral gene, as long as 500 million years ago, followed by the inversion of one of the duplicates (Archer 1974).

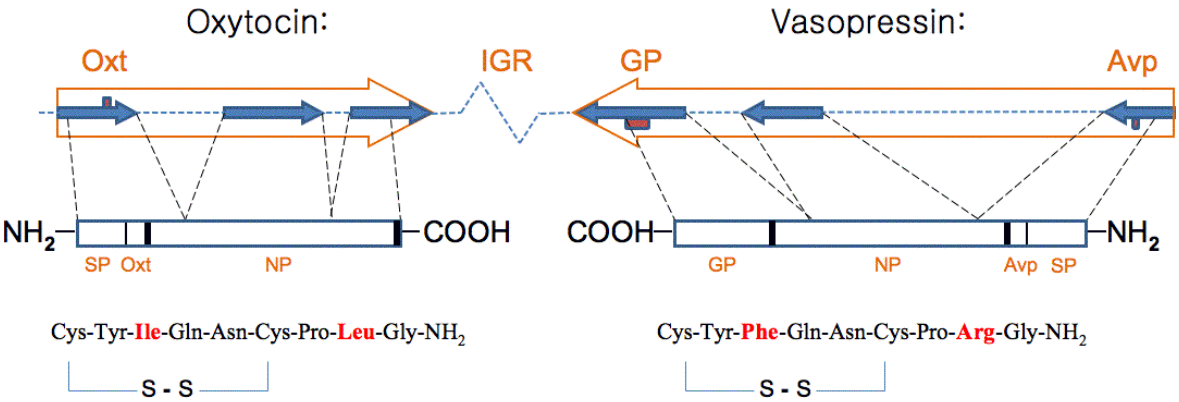

Figure 1. Oxt and Avp: genes organization, structures of precursor proteins and aminoacidic sequence of mature peptides (modified from Caldwell et al., 2008).

The two genes are separated by an intergenic region (IGR), which differs in length among species and they show the same three-exons organization (Hara et al 1990). Exon 1 codifies for a signal peptide, for the nonapeptide and for the first 9 residues on Neurophysin. It also contains a GRK sequence (Gly-Lys-Arg), a signal involved in post-translational processing, where the glycine is important for the C-terminal amidation and the two basic aminoacids constitute a typical cleavage signal found also in other hormonal precursor. Exon 2 codifies for the central portion of neurophysin, highly conserved among species; finally exon 3 codifies for the c-terminal region of neurophysin and in the AVP gene also for a short glicoprotein (Richter 1983, Rose et al 1996, Sausville et al 1985).

Oxytocin receptors (OXTR) are G-protein coupled receptors (GPCR) belonging to the oxytocin/vasopressin receptor subfamily (Barberis et al 1998). Three vasopressin receptors (V1a, V1b and V2) and one oxytocin receptor (OXTR) have been distinguished on the basis of their binding properties and signal transduction.

Actually, the great homology between the OXT/AVP receptors and peptides leads to low binding selectivity. Indeed, AVP binds to OXTR with very similar affinity to that of OXT itself, and OXT binds to the AVP receptor subtypes expressed in the brain (V1AR and V1BR) with slightly lower affinity than AVP itself (Manning et al 2012, Mouillac et al 1995).

Combined evidence from studies involving site-directed mutagenesis, photoaffinity labeling and molecular modeling indicate that the cyclic part of the OXT molecule interacts with transmembrane domains 3, 4 and 6, whereas the linear C-terminal part of the OXT molecule remains closer to the surface and interacts with transmembrane domains 2 and 3, in addition to the first extracellular loop (Chini & Fanelli 2000).

The intracellular C-terminal domain includes several phosphorylation sites and it is important for signal transduction and internalization. In the extracellular N-terminal domain instead there are two (mouse, rat) or three (human, pig, sheep, bovine) potential N glycosilation sites.

As many other GPCRs, oxytocin receptors and V1a and V2 receptors are able to form homo- and heterodimers in transfected HEK 293 and COS 7 cells (Devost & Zingg 2003, Terrillon et al 2003).

Moreover, OXTRs have a promiscuous coupling to $G\alpha_{q/11}$ and $G\alpha_{I}$, which lead to the activation of different signaling pathways, having synergistic or opposing effects. In myometrial cells, OXTR coupling to both $G\alpha_{q/11}$ and the small G proteins of Rho family induces uterine contraction (Sanborn 2001), whereas in neuronal cells OXT can inhibit or stimulate inward rectifier K^+ currents, depending on which G protein pathway (PTX-resistant or sensitive, respectively) is activated (Gravati et al 2010). Similarly, in HEK 293 cells stably transfected with human OXTRs, the receptor coupling to Gαi is responsible for inhibiting cell growth, whereas receptor coupling to a different Gα subunit (possibly $G\alpha_{q}$) has been linked to cell growth stimulation (Guzzi et al 2002, Rimoldi et al 2003).

This divergent pathway activation has been found to be influenced by the receptor localization in lipid rafts, specialized plasma membrane domains enriched in cholesterol, glycosphyngolipids, and lipid-anchored proteins (Rimoldi et al 2003), but also by the ligand itself. Indeed, it has been recently shown that atosiban, a peptidic OXT derivative, is at the same time an OXTR antagonist because of its antagonistic effect on OXTR-$G\alpha_{q}$ coupling, and an OXTR- $G\alpha_{I}$ agonist, thus representing the first pharmacologically coupling-selective agonist of the human OTR (Reversi et al 2006). This phenomenon, called "functional selectivity", has been recently described also for other OXTR ligands (Busnelli et al 2012).

Oxytocin synthesis and release: where and when

Oxytocin synthesis

OXT and AVP are both synthesized as inactive pre-pro-hormones on the rough endoplasmic reticulum and immediately after their transcription they undergo a first proteolitic cleavage, which removes the signal peptide. Resulting pro-hormones are then glycosilated in the Golgi apparatus and accumulated in secretory vesicles in which they are transported down the axon toward the synapse, the site of release. During the transport inside vesicles pro-hormones are further cleaved originating the active nonapeptide and a carrier protein, Neurophysin (Brownstein et al 1980, Ivell & Richter 1984).

Both neurohormones are mainly produced in the supraoptical (SON) and paraventricular (PVN) nuclei of the hypothalamus (for AVP the suprachiasmatic nucleus is also involved) by distinct populations of neurons (see fig.2). Two class of OXT-producing neurons have been identified: magnocellular neurons, residing in both SON and PVN, produce either nonapetides; parvocelluar neurons, which are located only in the PVN, synthesize exclusively OXT (Swanson & Sawchenko 1983).

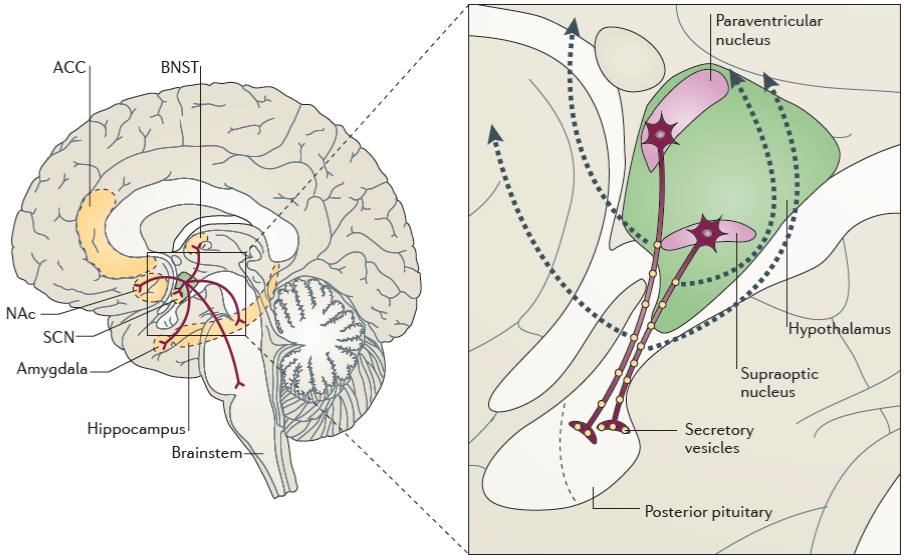

Figure 2. The oxytocin system. Oxytocin is mainly produced by magnocellular and parvocellular neurons in the hypothalamus. Magnocellular neurons reside in both SON and PVN and send their axonal projections to the posterior pituitary, where they release OXT in the systemic circulation, to exert its peripheral effects. They are also able of dendritic OXT release which is known to reach by diffusion distant target in the brain (dotted arrows). Parvocellular neurons are located exclusively in the PVN and projects to many region of the brain (red pathwways).(Meyer-Lindenberg et al 2011)

These two different populations of neurons are responsible for very different effects of OXT. OXT produced by magnocellular neurons is transported to the posterior pituitary and released in the systemic circulation in response to proper stimuli, thus mediating its well-known peripheral effects (Brownstein et al 1980, Poulain & Wakerley 1982). Conversely, parvocellular neurons spread their projections throughout the brain and the spinal cord, where OXT behaves like a classic neurotransmitter, by binding to its G-protein-coupled receptor OXTR (Buijs et al 1985, Swaab et al 1975).

Moreover magnocellular neurons are able to release OXT from their dendrites within PVN and SON themselves. Dendritically released OXT has been hypothesized to reach by diffusion the entire CNS with a decreasing gradient of concentration (Ludwig 1998, Ludwig & Pittman 2003). This intranuclear oxytocin release in magnocellular nuclei is involved in a critical neuroendocrine feedback regulation of oxytocin secretion itself (Moos & Richard 1989, Neumann et al 1996).

Where: Site of oxytocin release and OXTR expression

Sites of OXT-release in the CNS, as described by microdialysis experiments, are the septum (Demotes-Mainard et al 1986, Neumann & Landgraf 1989), the hippocampus (Landgraf et al 1988, Neumann & Landgraf 1989), the central amygdala (Ebner et al 2005) and the brain stem (Landgraf et al 1990). Recently, Knobloch (Knobloch et al 2012) described in greater detail the localization of OXT-neurons' axons in the forebrain of rats exploiting a rAVV-expressing Venus under an OXT gene promoter injected in the PVN and SON. The most intensely innervated structures appeared to be the lateral septum, the paraventricular thalamic nucleus, the bed nucleus of the stria terminalis, the nucleus of the horizontal limb of the diagonal band and the island of Calleja in the olfactory system. Many OXT-fibers do also appear in the rest of the olfactory system as well as in many cortical areas (especially in the frontal association cortex), in the nucleus accumbens, in the medial and central amygdala and in the ventral hippocampus. Notably, no OXT-fibers have been found in the dorsal part of the hippocampus.
The localization of OXT-fibers described by Knobloch is quite in accordance with precedent autoradiographic studies analyzing the localization of the oxytocin receptor (OXTR) in rat brain (Tribollet et al 1988). However, a high expression of the receptor does not always correspond to dense fiber innervation. In the lateral septum, for example, few binding sites were found in autoradiographic experiments (Tribollet et al 1989), but very dense innervation has been

observed in this area (Knobloch et al 2012). In the hippocampus the distribution of receptors seems to resemble that of fibers, at least the enrichment in the ventral part of this structure, even though autoradiographic experiments distinguish less substructures (De Kloet et al 1985, Snijdewint et al 1989, Tribollet et al 1988).

Notably, AVP-fibers in the hippocampus target mainly the ventral part as well (Zhang & Hernandez 2013), where AVP-binding is more abundant too (Brinton et al 1984, Hernando et al 2001).

When: Stimuli to Oxytocin release

It is a long known fact that labor, suckling, iperosmolarity and stressful events are the major triggers to oxytocin release (Neumann 2007). Each of these physiological processes raises a different neuronal activity pattern, determining a distinct pattern of neuropeptide secretion. For example, hyperosmolarity produces a small increase in the spontaneous firing rate of MCNs, whereas lactation induces explosive synchronized bursts of activity in these cells, which causes a pulsatile release of OXT into the circulation (Bourque & Renaud 1991).

The activity of MCNs, and thus the OXT release, is regulated from afferents of many neurotransmission systems. Around 60% of the contacts onto MCNs are GABAergic (Decavel & Van den Pol 1990, Theodosis et al 1986) and could probably be negatively modulated by dendrite-released OXT (de Kock et al 2003). This synapses are capable of great plasticity, either short-termed (Baimoukhametova et al 2004) or long-termed (Brussaard & Herbison 2000), in response to particular stimulation and could be significantly increased in number by concomitant stimulation with OXT and estrogens (Theodosis et al 2006).

A recent paper showed that the effects of GABA stimulation on MCNs depend on post-synaptic neurons' phenotype: on OXT-neurons GABA elicits normal inhibitory actions, whereas on AVP-neurons GABA is excitatory (Haam et al 2012).

The principal excitatory neurotransmitter modulating OXT-neurons is Glutamate, which positively modulates OXT (and AVP) release (Gribkoff & Dudek 1990, van den Pol & Trombley 1993). Other connections on MCNs are noradrenergic, around 10% of the total amount, which mainly arise from A1 and A2 cells groups in the medulla oblungata (Sawchenko & Swanson 1982). There is also some evidence of the involvement of dopamine in the modulation of OXT/AVP release, through activation of D4 presynaptic receptors on GABAergic terminals impinging on MCNs (Baimoukhametova et al 2004). The resulting effect is a reduction in GABA

release and therefore an increased excitability of MCNs (Azdad et al 2003). Finally, MCNs are regulated by Acetylcholine which acts on presynaptic nicotinic receptors on both glutamatergic and GABAergic terminals mediating increased and decreased release, respectively (Li & Pan 2001, Li et al 2001), thus mediating an increase in neuropeptides release. Moreover acetylcholine can directly activate MCNs through the activation of postsynaptic a7-containg nAchR (Zaninetti et al 2002).

Roles of oxytocin

Peripheral organs

Peripherally, the role of OXT in reproductive functions is known since the beginning of 1900, when its ability to stimulate the contraction of uterine smooth muscle cells during labour and of myoepithelial cells in mammary glands during milk ejection was discovered (Dale 1906, Ott & Scott 1910). Moreover, in many species, OXT release is involved in penile erection and ejaculation (Argiolas 1992) and in the regulation of prostatic epithelial cell proliferation and migration (Nicholson 1996).

OXT has also important function on other peripheral organs: on the ovary it stimulates estradiol and progesterone release from luteal cells (Wuttke et al 1998); on the kidney it induces water retention (Conrad et al 1993, Verbalis et al 1991); it promotes cardiomyocites differentiation acting on the hearth (Jankowski et al 1998)*;* it regulates matrix deposition and reabsorption in bones (Tamma et al 2009) and in the tymus OXT has a role in non-self T-lymphocytes selection (Geenen et al 1999).

Central nervous system

The central OXT actions range from the modulation of neuroendocrine reflexes to the establishment of complex social behaviours related to reproduction and care of the offspring.

In particular, OXT is involved in the modulation of behavioral and physiological responses to **stress,** acting as an anxiolytic factor both in rodents and in humans (Guzman et al 2013, Kirsch et al 2005, Legros 2001, Mantella et al 2003). This anxiolytic action is probably mediated by the activation of OXTR in the central amygdala, which is the region involved in emotional and fear memory. Indeed, oxytocin release in this area was found to attenuate passive fear responses

(freezing) but to increase active fear behaviors (risk assesment) (Gozzi et al 2010, Huber et al 2005, Knobloch et al 2012).

Moreover, OXT is implicated in **feeding** behavior, acting as a satiety hormone. Both magnocellular and parvocellular oxitocinergic neurons participate to the regulation of food intake and energy balance: the former, releases oxytocin from their dendrites, which then diffuses to hypothalamic targets involved in satiety as the ventromedial hypothalamus; the latter, likely relay leptin action to the caudal brainstem, in order to regulate feeding via autonomic functions including the gastrointestinal vago-vagal reflex (Matarazzo et al 2012, Sabatier et al 2013). In addition oxytocin seems to be involved in modulating food-related reward, limiting the intake of palatable foods both in rodents and in humans (Ott et al 2013, Sabatier et al 2013). Interestingly, recent evidences indicate also an OXT-mediated effect on feeding onset in newborn Magel2 mice (a murine model for Prader-Willi syndrome). Indeed, almost 50% of these mice die within P1, due to impaired feeding behavior, unless they are treated with a single injection of OXT (Schaller et al 2010).

Even though OXT actions on stress response and feeding control are receiving more attention in the past few years, still what currently makes oxytocin most interesting for translational research and medicine is its role on **sociability** and **cognition** (Donaldson & Young 2008).

The implication of OXT on learning and memory has been highlighted quite recently with studies on rodents and humans (Chini et al 2013), and its precise role is still controversial. On the contrary, extensive and long-standing evidence is available on all aspects of sociability that are modulated by oxytocin, i.e. maternal and affiliative behavior, sexual behavior and pair bonding, dominance hierarchy, territorial aggression, and finally social memory (Ross & Young 2009). Interestingly, OXT seems to act as a social reinforcement through a coordinated action of OXTR and serotonin receptor, 5HT1B, in the nucleus accumbens (Dolen et al 2013).
Such a profound implication of OXT system in social behavior, suggests a possible role for this peptide in the pathogenesis and/or as a future therapeutic treatment for social dysfunction in neuropsychiatric disorders such as autism.

II. AUTISM

Autism was first described in the early 40's by the psychiatrist Leo Kanner and by the pediatrician Hans Asperger, who reported, independently, the cases of some children with defective sociability and insistence on sameness (Asperger 1938, Kanner 1943).

Nowadays, Autism is considered a set of pervasive, early-onset neurodevelopmental conditions, most of which probably deriving from the interaction between genetic and environmental factors . It is characterized by deficits in the social sphere (communication and interaction), and by restricted interests and repetitive behaviors (Diagnostic and Statistical Manual of mental Disorders- DSM-V edition). Most autistic patients (more than 70%) present co-occurring neuropsychiatric conditions (Simonoff et al 2008), such as intellectual disability, depression, anxiety, hyperactivity and aggressive behaviors, including self-mutilation. Moreover, they frequently exhibit seizures or gastrointestinal disorders. The high frequency of these concurrent symptoms could be due to a shared pathophysiological mechanism or to a secondary effect of having autism (Lai et al 2013).
Due to the high heterogeneity of clinical forms either in terms of causes, severity of symptoms or diversity of co-morbid features, the broader term of Autism Spectrum Disorder (ASD) is now preferred.

Epidemiology

ASD is not a rare syndrome, as believed in the past decades, the latest surveys indicated a median worldwide **prevalence** of 0.62-0.70%. The inflation in the reported prevalence during the last two decades could be due to improved awareness, earlier recognition of symptoms and broadening of diagnosis criteria; however an actual increase in ASD incidence cannot be ruled out. Moreover, a 2- to 5-fold male predominance has been consistently reported, thus implying an etiological role for sex-associated factors at genetic, environmental, but also endocrine and epigenetic levels(Elsabbagh et al 2012, Mattila et al 2011).

Various **risk factors** emerged by epidemiological studies, such as late reproductive age (Hultman et al 2011, Sandin et al 2012), complications during pregnancy (Gardener et al 2009) and exposure to chemicals, but none has proved to be necessary or sufficient for autism to develop (Rodier 2011).

Disease evolution

The **onset of symptoms**, characterized by a delayed or atypical development of social-communication behaviors, occurs very early, in the age between 6 and 12 months, but is often recognized and diagnosed later, around 24-36 months of age. Early signs of autism are deficits or delay in the emergence of joint attention, pretend play and imitation, reduced affective behavior and attention to human faces, delayed verbal communication and response to own name (Ozonoff et al 2010, Zwaigenbaum et al 2009).
Social-communication deficits of ASD patients are believed to depend, at cognitive level, on difficulties in mentalising, the ability to understand mental states in self and others (Boucher 2012); therefore social deficits in autism arise from reduced ability to process information about others, as well as self-referential ones, limitating the potential of using self as a proxy to understand the social world (Lai et al 2013, Lombardo et al 2010).

The disease **progression** is still a matter of debate and needs further studies using prospective and longitudinal methods (Happe & Charlton 2012). However some long-term follow-up studies of children diagnosed with autism, report a poor outcome in term of independent living, educational achievement, employment and relationships, which is strongly linked to the intelligence quotient (IQ). Moreover social and communication deficits, as well as restricted and repetitive behavior persist in adulthood (Billstedt et al 2007, Howlin et al 2004). Nevertheless, cross-sectional comparisons of younger and older ASD patients, give a more hopeful picture, indicating improvements in many symptoms, including restrictive and repetitive behaviors, with increasing age (Esbensen et al 2009, Seltzer et al 2003).
Still mortality risk of middle-aged adults with ASD appears to be raised 2- to 5-fold, with most deaths associated with concurrent medical conditions, such as epilepsy or intellectual disability (Bilder et al 2013).

Etiology

As previously mentioned, most conditions included in ASD are caused by a complex, often undetermined, interaction between genetic and environmental factors, but some monogenic syndromes are described as well: the Fragile X syndrome, which account for about 3% of ASD cases, tuberous sclerosis (TSC) responsible for 2% of ASD cases, Rett syndrome (1%) and neurofibromatosis type 1 (1%)(Betancur 2011, Kumar & Christian 2009).
Genetic variants linked to ASD are estimated to be up to 1000. Both rare mutations with profound effects, as the ones responsible for syndromic forms of autism, or common polymorphism with lighter effects (fig. 3) have been described (Geschwind 2011, Murdoch & State 2013, State & Levitt 2011). Genome-wide association studies identified some important single nucleotide polymorphisms with high frequency in general population (<5%), but none of them have a such important effect to be deemed causal (Geschwind 2011).

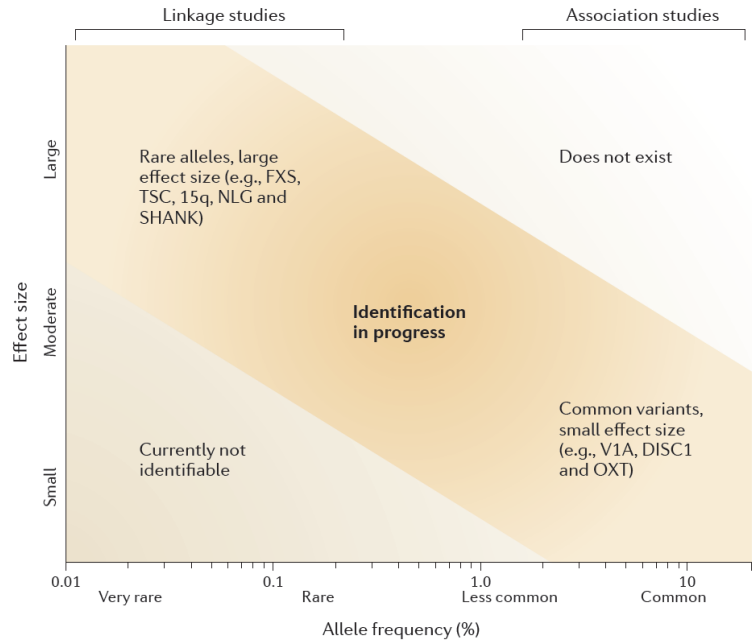

Figure 3. **Towards identification of the genetic basis of ASD.** Genetic studies found a great number of genes associated with ASD, with different incidence and risk effect. Common gene variants, which are defined by a minor allele frequency (MAF) of >5% in the general population, are typically associated with small risk (odds ratio <1.5) to develop ASD whereas rare and highly penetrant mutations carry the largest risk (Ghosh et al 2013).

Nonetheless, several polymorphisms are combined in many simplex and duplex families (with one or more than one member with autism, respectively), and could have an additive effect on risk (Murdoch & State 2013).

Taken together the identified genetic causes of autism explain only about 15% of all cases (Betancur 2011). Nevertheless autism heritability, as calculated from twin studies, is around 80% (Ronald & Hoekstra 2011), vs. about 15% in dizygotic twins, and relatives of ASD probands often show subclinical ASD features referred to as the broader autism phenotype (Happe & Charlton 2012).

Thus, epigenetic mechanisms and specific gene-environment interplay must have a very important role (Corrales & Herbert 2011).

Pathogenesis

Studies using social perception and cognition tasks report consistently an hypoactivity in a network of brain regions, including medial prefrontal cortex, superior temporal sulcus, temporo-parietal junction, amygdala, and fusiform gyrus (Dichter 2012, Pelphrey et al 2011, Philip et al 2012).

ASD patients often display a preference for processing local, rather than global, sensory-perceptual information, which has, as neural base, an enhanced recruitment of primary sensory cortices, a reduced recruitment of association and frontal cortices involved in top-down control, and an enhanced synchronization of parietal-occipital circuits (Minshew & Keller 2010, Samson et al 2012).

Many other abnormalities have been reported in autistic brains, such as reduced Purkinje cells in the cerebellar vermis, amygdala and fusiform gyrus (Schumann et al 2011) accelerated brain growth in infancy (Courchesne et al 2011), neocortical disgenesis (Casanova 2006) and signs of persistent neuroinflammation (Schumann et al 2011).

Moreover, there are promising evidences showing an atypical development of the so-called "social brain" (Pelphrey et al 2011).

However, many evidences from electrophysiological, functional and structural neuroimaging, molecular genetics and information processing suggest that autism is characterized by atypical neural connectivity rather than by alterations in a discrete set of brain regions (Lai et al 2013). The way in which connectivity is altered is still matter of debate, with hypothesis of decreased fronto-posterior and enhanced parietal-occipital connectivity (Just et al 2012, Minshew & Keller 2010) or reduced long-range and increased short-range connectivity (Belmonte et al 2004). Finally, other alterations reported quite consistently in autistic patients are distortion in GABAergic or serotoninergic systems, an imbalanced ratio between excitation and inhibition, which will be the focus of the subsequent chapter, and an atypical synaptogenesis, which will be also described below (Chugani 2011, Rubenstein 2010).

Therapies

Current therapies for people with autism rely greatly on behavioral and educational intervention, aimed at maximizing functional independence, improving social skills and communication and at fulfilling areas of strength (Kasari & Patterson 2012).

The most commonly used drugs are antidepressants, anxyolitic and antipsychotics, but these are effective only to treat co-occuring conditions. Therefore there is a great need for new pharmacologic treatment addressing specifically the core symptoms of ASD (Ghosh et al 2013).

Nowadays promising clinical results come especially from three new lines of research: acetylcholiesterase inhibitors, glutamatergic agents and oxytocin (Farmer et al 2013). In particular, acetylcholiesterase inhibitors seem to have a positive effect on cognition and communication and glutamatergic antagonists show quite reliable improvements on social withdrawal and stereotyped behaviors (Arnold et al 2012, Hardan et al 2012, Niederhofer 2007a, Niederhofer 2007b). Finally, oxytocin, for its role in social and affiliative behavior, is strongly expected to be effective in autism treatment. Indeed, in clinical trials, OXT administration to ASD patients have been shown to increase social awareness and to reduce the severity of repetitive behaviors (Ellenbogen et al 2013, Guastella et al 2010, Hollander et al 2007, Hollander et al 2003).

III. NEURONAL EXCITATION-INHIBITION (E/I) BALANCE

E/I balance in neuropsychiatric disease

Autism, as several other neuropsychiatric disorders, have been hypothesized to arise from an increased ratio between excitation and inhibition (E/I) within neural microcircuitry ((Markram & Markram 2010, Rubenstein 2010, Rubenstein & Merzenich 2003, Vattikuti & Chow 2010). This proposed neurophysiological substrate could be the consequence of a wide range of seemingly unrelated genetic abnormalities and could account for social and cognitive deficits observed in such disorders (Eichler & Meier 2008). Consistently, it is supported by diverse physiopathological findings: many autism-related genes are ion channels or synaptic proteins (Bourgeron 2009), frontal cortical hyperactivity is frequently reported in autistic children and seizures affect ≈ 30% of ASD patients.

Notably, disease-relevant effects of impaired E/I balance could rely both on abnormal circuits development (Ramocki & Zoghbi 2008, Rubenstein 2010) and on real-time functioning (Yizhar et al 2011). Indeed, an elegant work by Yizhar and coworkers (Yizhar et al 2011) demonstrated, by means of combinatorial optogenetics, that acutely inducing an elevated E/I state in prefrontal cortical circuits leads to profound (but reversible) impairments in social and cognitive functions and that increasing cellular inhibition partially rescued the social deficit.

This observation supports the desirable possibility that even E/I imbalances of developmental origin could be treated in adult age with good results.

Setting and maintaining the E/I balance

Physiologically, fluctuations in neuronal firing are means of information transfer in the brain, thus the E/I balance is continuously perturbed by development- or experience-dependent changes. As a consequence our brain needs to preserve functional stability by tightly regulating average firing rates without impairing information flow (Atallah & Scanziani 2009, Pouille et al

2009, Shu et al 2003). This is achieved thanks to an array of homeostatic feedback mechanisms which allows neurons and/or circuits to sense how active they are and to appropriately adjust their excitability (Davis 2006). Interferences with information transfer are prevented by differentiated temporal scale, being compensative mechanisms very much slower (Turrigiano & Nelson 2004).

These homeostatic mechanisms act both at circuitry level, being central neurons embedded in complex networks of excitatory and inhibitory neurons, and at individual neuron or synapse level, by modulating intrinsic membrane excitability or synaptic strength, respectively (Marder & Goaillard 2006, Turrigiano 2011, Turrigiano & Nelson 2004).

Synaptic strength is both modulated at presynaptic level, by controlling neurotransmitter release probability and reuptake mechanisms (Nicoll & Schmitz 2005), and at postsynaptic level, by changing the density and/or quality of neurotransmitter receptors present at the postsynaptic membrane (Bogdanov et al 2006, Borgdorff & Choquet 2002, Ehlers et al 2007).

Intrinsic membrane potential and excitability is determined by electrochemical gradients of ions, mainly Ca^{2+}, Na^{+}, K^{+} and Cl^{-}, which are maintained in turn by several transporters and channels that operate in either directions.

Such a complex network of mechanism needs the correct functioning of a great number of protein. Consequently, dysfunctions in a wide variety of protein, especially during early development, could have detrimental effects on the E-I balance, thus affecting system stability and/or plasticity, (Davis 2006, Turrigiano 2011).

During development, indeed, a complex sequence of events, aimed at the proper formation of neuronal networks, takes place: neurons migrate to their proper location, synaptic contacts are established and stabilized, coherent neuronal activity patterns appear and evolve and the E/I balance is set (Crepel et al 2007, Garaschuk et al 2000, Spitzer 2006).

The switch of GABA

In mature nervous systems the E/I ratio depends strongly on the balance between the prevalent excitatory and inhibitory neurotransmitter systems, Glutamate and GABA, respectively. On the other hand, in the earliest stages of brain maturation, GABAergic transmission have depolarizing effects (Rivera et al 1999), which seem to be extremely

important for the correct neural development (Ben-Ari et al 1989). Therefore, the switch in GABAergic polarity, from depolarizing (excitatory) to hyperpolarizing (inhibitory), and its proper timing is very relevant for the establishment of a correct E/I balance.

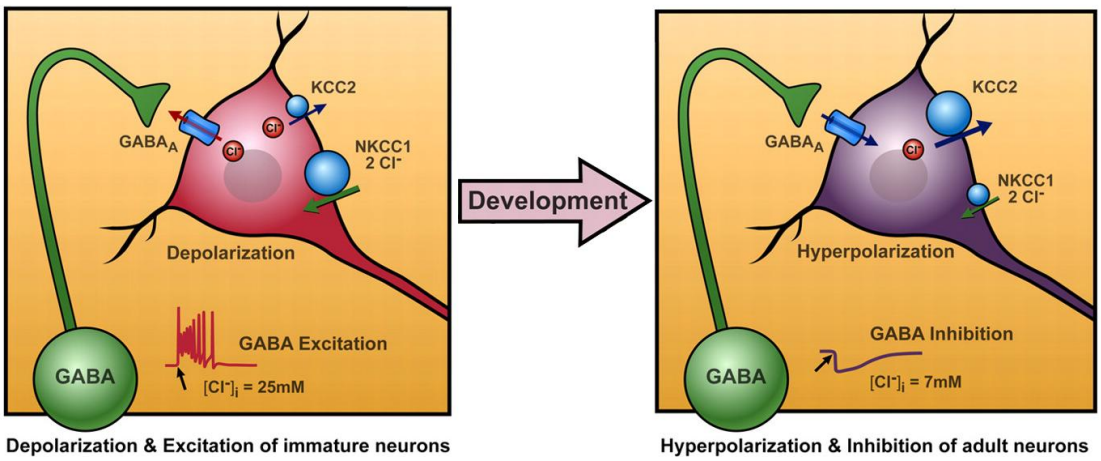

Figure 4. Developmental switch of GABA actions, from excitatory to inhibitory, is due to the modulation of two Cl- transporters, KCC2 and NKCC1. In immature neurons the Cl^- importer NKCC1 is highly expressed, determining elevated intracellular Cl^- concentrations and, consequently, depolarizing GABA actions (left panel). In mature neurons the expression of the Cl^- exporter KCC2 become predominant, determining low intracellular Cl^- concentration and, thus, hyperpolarizing actions of GABA. Modified from (Ben-Ari et al 2007)Ben-Ari et al., 2007.

Two neuronal cathion-chloride cotransporters (CCCs), NKCC1 and KCC2, are responsible for this change, as they are able to modify Cl^- electrochemical gradient by altering its intracellular concentration. In particular, the Cl^- importer NKCC1 is responsible for the high $[Cl^-]_i$ of immature neurons. The upregulation of the Cl^- exporter KCC2, occurring during development gradually decreases $[Cl^-]_i$ leading to the switch in GABA polarity (Rivera et al 1999).
Consistently with their role in the E/I balance, CCCs are getting attention as therapeutic targets for the treatment of neuropsychiatric disorders such as epilepsy and autism (Lemonnier et al 2012, Nardou et al 2011).

IV. DENDRITIC SPINE REMODELING

Dendritic spines are small protrusions of dendritic membranes; they were first identified at the end of the 19th century by Ramon y Cajal thanks to the Golgi staining (Ramon y Cajal 1891) and since then neuroscientists never lost interest in them. Now we know that dendritic spines are the main site of neuronal connections, where about 90% of excitatory synapses are located (Harris 1999). They are present on both excitatory and inhibitory neurons throughout the nervous system and in a wide range of species (Shepherd 1996). Typically, each dendritic spine hosts a single excitatory synapse, located at the head, but they can also receive inhibitory input (Knott et al 2002). However, they are commonly considered the unitary postsynaptic compartment of excitatory inputs (Sala et al 2008).
Spine morphology is highly heterogeneous and is linked to different functions; for instance "*thin*" spines are more responsive to changes in synaptic activity, whereas "*mushroom-shaped*" spines are more stable and contain stronger synapses (Segal 2005). Moreover, shape is not a static feature of dendritc spines, since it changes constitutively and upon stimulation, within hours or even minutes (Parnass et al 2000).

Dendritic spine and neurological disease

Since spine morphology and number are closely connected to neuronal function, alterations in their structure and dynamics may diversely affect neural circuits, thus contributing to the development of diverse cognitive impairments (Bhatt et al 2009). There are, indeed, some psychiatric and neurological diseases which are associated with altered spine morphology or density. For instance, schizophrenic patients display a reduced spine density in neocortical pyramidal neurons (Glantz & Lewis 2000). On the other hand, individuals with Fragile X syndrome (FXS) have greatly increased spine density and altered spine morphology towards a more immature, filopodia-like phenotype (Hinton et al 1991, Irwin et al 2001). Interestingly, *Fmr1* knockout mice, a murine model of FXS, have overlapping alterations (1994, Verkerk et al 1991).

Evidence that repeated exposure to psychostimulants, such as amphetamine and cocaine, can affect spine density at least for animal models has also been reported (Norrholm et al 2003, Robinson & Kolb 1999).
Notably, slight changes in spine dynamics and turnover may accumulate over time and lead to a significant change in spine density only later in life or under specific stressors (Bhatt et al 2009).

Spine remodeling during development

Spinogenesis

Dendritic spines appear on extending dendrites early in postnatal development, when neurons rapidly establish an enormous number of synaptic connections (Dailey & Smith 1996).
During this period, dendrites extend great number of filopodia (long and thin protrusions without bulbous head), which undergo rapid extension and retraction allowing them to sample nearby axons (Portera Cailliau & Yuste 2001). Once they choose proper presynaptic partners, the contacts get stabilized through activity-dependent or -independent signaling and filopodia become shorter, driving the axon terminal closer to the dendritic shaft. Then, the maturation of an active synapse at the head, greatly slows down spine motility, stabilizing the structure (Dailey & Smith 1996, Fiala et al 1998). Not all the filopodia becomes spines with synapse, indeed absence of proper signals or presence of an alternative one, would lead to filopodia withdrawal into dendritic shaft (Jontes & Smith 2000, Marrs et al 2001). Although it is widely believed that filipodia are precursors of dendritic spines, it is still not clear if they are necessary for the formation of all spines (Bhatt et al 2009).
The extremely dynamic properties of spines during development, including fast spine turnover, rapid changes in shape and great motility, are vital for the formation and function of neural circuits (Dailey & Smith 1996).

Pruning

The rapid synaptogenesis occurring during early postnatal development in the cerebral cortex of mammals, including humans, is followed by a remarkable (about 50%) loss of spines/synapses (Huttenlocher 1979, Rakic et al 1986, Rakic et al 1994). This process of pruning and sculpting early established synaptic connections was observed already by Ramon y Cajal in 1899, and it's thought to be fundamental for the maturation of the brain (Bhatt et al 2009).

Taking advantage of new *in vivo* two-photon (TP) imaging techniques spine dynamics during postnatal development have been extensively described in mice. In particular, the period of spine pruning was found to start before P14 (Grutzendler et al 2002, Holtmaat et al 2005, Majewska et al 2006) and to continue until four months of age, when a 25% of net spine loss is reported (Zuo et al 2005). Moreover, rate of spine elimination and formation (17% and 6% over two weeks, respectively) were successfully recorded.

Stability

It has been observed that in mature neurons spines undergo fewer transitions between categories (Dunaevsky et al 1999). However, the degree of spine plasticity in adulthood, once neural circuitry are formed and synaptic contacts stabilized, is still a matter of debate. Recent studies employing TP in vivo imaging to analyze spine stability in mouse cortex indicate that in mature adults (> 4 months age) a large percentage of dendritic spines likely persists throughout an animal's lifetime (Grutzendler et al 2002, Holtmaat et al 2005, Majewska et al 2006, Zuo et al 2005). Although the majority of studies examine layer V pyramidal cells, there are some recent evidences on other cell types, including inhibitory neurons, and in other brain regions, such as hippocampus and olfactory bulb, which analogously reported a high degree of stability of dendritic spines in adulthood (Holtmaat et al 2005, Majewska et al 2006, Mizrahi 2007). Adult dendritic spines can thus represent a physical substrate for long-term information storage (Bhatt et al 2009).

Experience-dependent spine remodeling

Many lines of evidence indicate that experience-induced spine plasticity strongly depends on the stage of development. Indeed malleability of the nervous system seems to progressively diminish, as spine plasticity decreases (Bhatt et al 2009). Nonetheless, even "stable" adult spines undergo a small degree of turnover and maintain a certain level of shape plasticity (Grutzendler et al 2002, Holtmaat et al 2005, Majewska et al 2006, Zuo et al 2005). Therefore, changes in synaptic strength and in spine turnover may underlie various forms of learning and plasticity in the mature brain. The way and the extent to which experience can modify adult spines is still a matter of intense investigation.

RATIONALE AND AIMS

I. RATIONALE

The role of the oxytocin system in controlling social behavior suggests a natural link to neuropsychiatric conditions in which such behavior is aberrant or even absent. Autism is the most common of such disorders.

Even if a strong genetic correlation between oxytocin and autism has not been reported so far, there are compelling reasons for a detailed investigation of OXT/OXTR involvement in autism. In particular, reduced levels of peripheral OXT have been found in ASD patients and OXT treatment is giving promising results in attenuating symptoms.

Moreover, the transgenic mouse line lacking the oxytocin receptor (*Oxtr*$^{-/-}$, generated in Nishimory laboratory (Takanayagi et al., 2005), shows a very interesting autism-like phenotype. This mouse displays strong impairments in many aspects of social behavior, including maternal and affiliative behavior, interest and memory for conspecifics and aggression. These deficits are thought to be murine correlates of the defective sociability and increased aggression observed in autistic patients. In addition, *Oxtr*$^{-/-}$ mice, tested in a T-maze test, show no learning defects, but an impaired reversal of learning, which is considered a sign of cognitive inflexibility (Sala et al 2011). This feature is, in our opinion, particularly interesting, because resistance to change represents one of the core symptoms of autism, but it is rarely described in animal models. Interestingly, the heterozygous *Oxtr*$^{+/-}$ mouse, which express 50% of the receptors, does not display any impairment in reversal of learning, indicating that a partial expression of oxytocin receptor is sufficient to maintain normal cognitive flexibility (Sala et al 2013). It is also relevant to note that the vasopressin V1aR seems to be involved in this process as well, because OXT and AVP, administered i.c.v. are both able to fully revert this phenotype (Sala et al 2011).

Finally, the *Oxtr*$^{-/-}$ mouse displays an enhanced susceptibility to PTZ-induced tonic-clonic seizures, which correlates with the increased ratio between Glutamatergic and GABAergic synapses observed in *Oxtr*$^{-/-}$ cultured hippocampal neurons (Sala et al 2011). This is a another very relevant feature of the autistic-like phenotype of *Oxtr*$^{-/-}$ mice. Indeed, seizures are frequent in autistic patients (Tuchman & Rapin 2002).

Considering all these issues, the *Oxtr*$^{-/-}$ mouse can be considered a valid neurobiological model of autism.

II. AIMS

While the social profile of *Oxtr*$^{-/-}$ mice has been extensively investigated, its cognitive deficits, and, more in general, the cognitive implications of OXT, has received far less attention. Consequently, we decided to clarify the neurobiological basis of cognitive autistic-like symptoms of the *Oxtr*$^{-/-}$ mouse, that is, in particular, its cognitive rigidity.

- Firstly, we compared neuronal morphology of *Oxtr*$^{+/+}$and *Oxtr*$^{-/-}$ mice, taking into account three brain regions, involved in different aspects of cognitive processes: the CA1 of hippocampus (CA1), responsible for spatial (place) learning (Hirsh 1974, Mishkin M. 1984, Moser et al 1994), the dorsolateral striatum (DLS), implicated in procedural (response/habit) learning (Packard M.G. 1987) and the orbitofrontal cortex (OFC), involved in cognitive flexibility (Dias et al 1997, Nonneman et al 1974, Ragozzino 2007).
- Secondly, we analyzed how *Oxtr*$^{+/+}$and *Oxtr*$^{-/-}$ neurons react to different cognitive tests, in term of dendrite and spine remodeling.
- Finally, given the relevance of neuronal E-I imbalance in autism (Rubenstein & Merzenich 2003) and the previously reported imbalance between Glutamatergic and GABAergic synapses in *Oxtr*$^{-/-}$ hippocampal neurons, we investigated how the oxytocin system could be involved in the maintenance of this E-I balance and, thus, in the pathogenesis of ASD and/or in the emergence of autistic symptoms.

MATERIALS AND METHODS

I. ANIMALS

Oxtr$^{+/+}$ and *Oxtr*$^{-/-}$ mice (Takayanagi et al 2005), carrying a C57BL/6 genetic background, were obtained from L. Young (USA) and stabulated in standard conditions, with *ad-libitum* access to food and water. Colony propagation have been carried on by heterozygous mating, and litters have been genotyped by PCR. The behavioral tests were started when the mice were 12 weeks old and completed before the mice reached the age of 17 weeks.

II. ANALYSIS OF BEHAVIOR-RELATED CHANGES IN NEURONAL MORPHOLOGY

T-maze test and Golgi staining

Three months old male *Oxtr*$^{-/-}$and *Oxtr*$^{+/+}$ mice were used in the behavioral studies. Animals were initially moved from the back-up colony to the behavior facility. After a week familiarization to the new environment, they were food deprived to 85% of their *ad-libitum* weight and, for the next 5 days, habituated to explore and find food across the T-maze apparatus (stem length: 41 cm; arm length: 91 cm; walls height: 19 cm; sections width: 11cm). Subsequently, mice started the acquisition phase of the test: they were trained to obtain food placed at the end of one arm (reinforcer) with ten daily sessions. The number of days taken to reach the criterion, i.e. 80% of correct choices for three consecutive days, was measured. Once completed this first phase mice were tested in the reversal procedure: the reiforcer position was switched to the opposite arm, animals were trained as above and again the number of days taken to reach the criterion was measured.

Oxtr$^{-/-}$ and *Oxtr*$^{+/+}$ mice were sacrificed and processed for Golgi staining and subsequent morphological analysis at different time point during behavioral testing (see fig.5), obtaining 5 experimental groups for each genotype:

- "*lab*": age- and gender- matched mice at P90 from the back-up colony
- "*Environmental stimulus*" (ES): *lab* mice moved to the behavioral facility for the familiarization period
- "*Habituation*"(H): *ES* mice after the habituation to the T-maze,
- "*Learning*"(L): *H* mice which completed also the acquisition phase of the T-maze test
- "*Reversa*l"(R): *L* mice after the reversal training in the T-maze

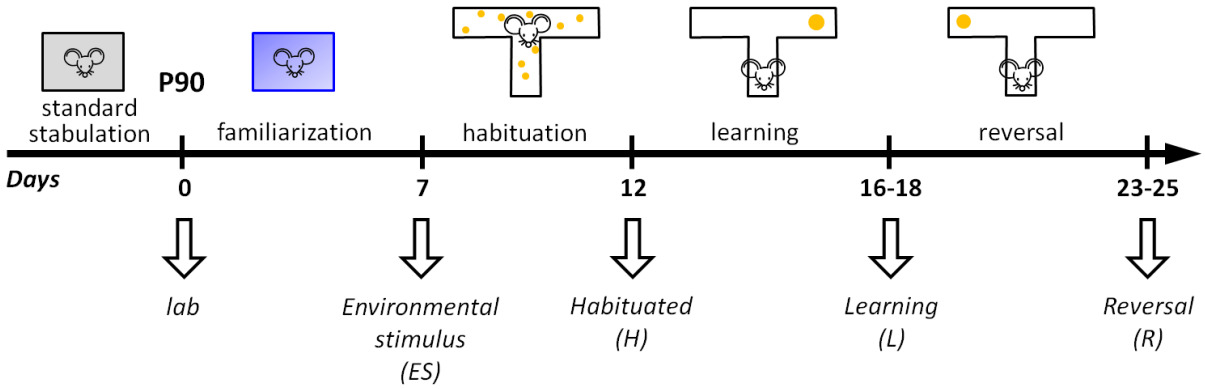

Figure 5. Time-line of behavioral testing and experimental groups obtained (empty arrows). Mice started the behavioral protocol at three month of age (P90). The temporal sequence of behavioral tests is depicted along the time line, with indication of the duration of each phase(days).

Animals of each experimental group (n=3-8) were intracardially perfused, under deep anesthesia, with 0.9% saline solution, 24 hours after the end of their training. Brains were impregnated for 6 days in Golgi-Cox solution and for 2 additional days in a 30% sucrose solution, then 100-µm-thick coronal sections were obtained using a vibratome, mounted on gelatinized slides and finally stained according to the protocol by Gibb and Kolb (Gibb & Kolb 1998).

Using a mouse brain atlas ("*The mouse brain in stereotaxis coordinates*" - Keith B.J. Franklin and Gorge Paxinos), the three brain regions of interest: CA1 of hippocampus (bregma -1,46mm/-3,52mm), orbitofrontal cortex (OFC; bregma 2,58mm/2,00mm) and dorsolateral striatum (DLS; bregma 1,18mm/-0,58mm) were identyfied (fig. 6).

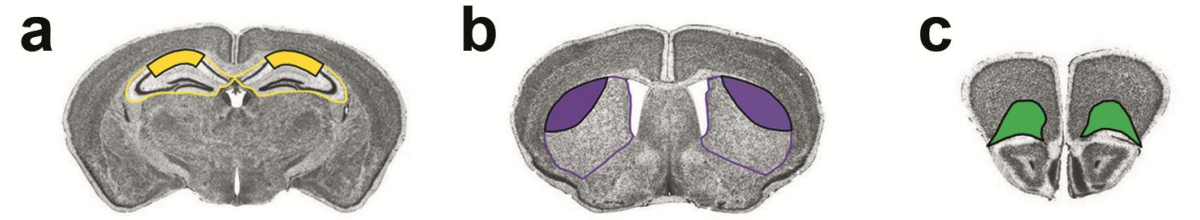

Figure 6. Coronal brain sections highlighting the three brain regions taken into account. (a) The yellow line outlines hippocampal perimeter, the filled region represents the CA1 portion. (b) The whole striatal region is surrounded by a violet line. Violet filling indicates the dorsolateral part of this structure. (c) Orbitofrontalcortex is indicated by the green filling. (Modified from the "High Resolution Mouse Brain Atlas " Sidman et al., http://www.hms.harvard.edu/research/brain/index.html)

Within each region and for each animal, at least 3 Pyramidal (CA1 and OFC) or Medium Spiny neurons (DLS) showing intense and complete impregnation and no truncations were selected and acquired with a Axiovert microscope (Zeiss) equipped with a Digital CCD Camera ORCA-AG (Hamamatsu). Brightfield images were acquired with a 63x magnification objective (NA=1.4) at several focal planes (0,5 µm apart one from the other) and then stacked together to have on focus all the dendrites of interest.

Spines were counted on at least five II-IV order-dendrites per neuron. Protrusions longer than 2 µm or with no evident connection with the dendritic shaft were excluded from the count.

The software NeuronStudio was used to assess dendrite length, number of branches and to calculate spine density.

III. NEURONAL CULTURES

Primary neuronal cultures were prepared from hippocampi of embryonic day 18 (E18) *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice as described by Kaech and Banker (Kaech & Banker 2006), with slight modifications. Briefly, a pregnant mouse was sacrificed by cervical dislocation, embryos' brains were removed and hippocampi dissected out. Tissue dissociation was carried out with an enzymatic treatment (0.25% trypsin for 20 minutes at 37°C) followed by a mechanic trituration with a fire-smoothed Pasteur pipette. Dissociated cells were plated, at densities ranging from 25 000 to 35 000 cells/cm^2, in poly-L-lysine coated multiwell dishes (or glass coverslips)

containing Neurobasal medium additioned with B27 supplement (2%, v/v), L-glutamine (2 mM), penicillin/streptomycin (100 U/ml) and 25µM Glutamate. Five hours after plating the medium was replaced with a glutamate-free one to avoid excitotoxicity. Neurons were than maintained at 37°C in a humidified atmosphere at 95% air and 5% CO_2 up to DIV17, replacing half of the medium twice a week.

Analysis of morphology

GFP-transfection

Two different preparation of both *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons, seeded on glass coverslips 25 000 cells/cm^2, were transfected at DIV7 with GFP-expressing vector (pLL3.7) using Lipofectamine2000 (LIPO; lifetechnologies).

For each well of a 12-well dish, 2,5 µg of DNA were diluted in 50 µl of Neurobasal medium containing no additives; 2,5 µl of LIPO per well were diluted in equal volume of Neurobasal to maintain a 1:1 ratio (wt:vol).

The DNA and the LIPO solutions were allowed to sit at room temperature (RT) for 5 minutes and then mixed together by gently pipetting. While the resulting mix was sitting at RT, half of the growth medium was removed from each well, transferred to a conical tube and mixed with equal volume of fresh complete Neurobasal medium (half-conditioned medium).

After 15 minutes of RT incubation the DNA-LIPO mix was dropped on neurons, 100µl per well. Transfection medium was completely removed 1 hour after transfection and replaced by previously prepared half-conditioned medium.

Imaging and morphological analysis

Neurons were maintained until DIV17, then fixed with 4% paraformaldheyde-4% sucrose; coverslips were mounted on glass slides and imaged using a Zeiss 510LSM Meta laser scanning confocal microscope (Carl Zeiss). For the analysis of neuronal arborization images of at least three neurons were acquired with a 40x objective (NA = 1.3) on three focal planes (z-step=0,5µm); for the analysis of spine density and morphology at least five neurons per genotype were acquired, a 63x objective was used and the focal planes were stacked together in a projection. Spine count was made manually, whereas dendritic length measurements and Sholl analysis were performed with NeuronStudio software. Spine morphology paramenter

(spine length, head diameter, and neck width) were measured using ImageJ software, then spine were assigned to different subclasses according to NeuronStudio criteria.

Analysis of mRNA expression

To evaluate the expression of OXT/AVP receptors, NKCC1/KCC2 transporters and members of the inward-rectifying K^+ channel (IRK) family in our primary hippocampal neurons, total RNA was isolated, treated with DNAse, reverse transcribed into cDNA using a reverse transcriptase and, finally, resulting cDNA was used as templates for subsequent quantitative (Real-time PCR) and semi-quantitative PCR analysis.

RNA isolation and purification

Neurons' RNA content was isolated from $2,5*10^5$ neurons, at the indicated DIV, using the Zymo microRNA extraction kit (Euroclone), following manufacturer instruction.
RNA was eluted in 12µl of RNAse-free water and it's concentration and purity was assessed by measuring the optical density at 260 and at 280 nm wavelength with a nanodrop spectrophotometer.
In each experiment equal amounts of RNA per sample (typically around 200 ng) were used. Samples were diluted in DEPC-water to an equal volume (8µl), then 1µl of 10x DNAse reaction buffer (400mM Tris-HCl, pH 8.0; 100mM $MgSO_4$ and 10mM $CaCl_2$; Promega) and 1µl of RNase-free DNase (Promega) were added and samples were incubated for 30 minutes at 37°C. The reaction was stopped by adding 1 µl of STOP solution (20mM EGTA, pH 8.0; Promega) and incubating 10 minutes at 65°C. This step ensured no genomic contamination would be carried on.

cDNA synthesis

RNA samples were firstly incubated at 65°C for 5 minutes with 500 ng oligo$(dT)_{12-18}$ and 1mM of each dNTP to denature RNA secondary structure and then quickly chilled on ice to let primers anneal to RNA. Secondly, reaction mix was completed by adding to the tube: 5x First-Strand buffer (250mM Tris-HCl. pH 8.3; 375 mM KCl; 15mM $MgCl_2$; Life Technologies), 5mM DTT, 40 Units of Recombinant Rnase Inhibitor (RNase OUT; Life Technologies) and 200 Units of the enzyme Reverse Transcriptase (SuperScript III; Life Technologies). Retrotranscription was

carried out in a termalcycler (PTC-100, MJ Research) with the following protocol: 50 minutes at 50°C for the extension and 15 minutes at 85°C to inactivate the enzyme.

Finally, the obtained cDNA was cleaned from RNA complementary to the cDNA by adding 2 Units of E.coli RNase H and incubating 20 minutes at 37°C.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For the analysis of OXT/AVP receptors and inward-rectifying K^+ channels expression cDNA samples were amplified by PCR using appropriate intron-spanning primers (see table 1).

Gene	Primer (Forward e Reverse)	Amplicon length (Bp)	Tm (°C)	$MgCl_2$
Oxtr	5'-TGTGCTGGACGAATTTCTTC-3' 5'-GCCCGTGAACAGCATGTAGA-3'	150	60 °C	2,5 mM
V1ar	5'-ATTGCTGGGCTACCTTCATCC-3' 5'-CCTTGGCGAATTTCTGCGCT-3'	532	60 °C	5 mM
V1br	5'-TCACCTGGACCACCATGGCC-3' 5'-TAAGACGGAGAGTAGATGGACC-3'	567	60 °C	5 mM
V2r	5'-TTCGTGCCTATGTCACCTGG-3' 5'-TCAGGAGGGTGTATCCTTCAT-3'	700 pre-mRNA 512 V2aR 455 V2bR	62 °C	5 mM
GAPDH	5'-GCCATCAACGACCCCTTCATTG-3' 5'-TGCCAGTGAGCTTCCCGTTC-3'	598	63,9 °C	2,5 mM
Kir 2.1	5'-GAGTAAGCAGGACATTGACAATG-3' 5'-GATTCTCGCCTTAAGGGCC-3'	431	58 °C	2,5 mM
Kir 2.2	5'-CTTCCTGAGCAGAGATGAG-3' 5'-GGACATGGGACCTATTGTGG-3'	193	62 °C	2,5 mM
Kir 2.3	5'-ATGGGCAAGGAGGAGCTGG-3' 5'-GCATGCGCTCCAGATCCA-3'	466	60 °C	3,5 mM
Kir 3.1	5'-ATCGAAGCTGCAGAAAATTACG-3' 5'-CCTAAAGGGGTGTTTTGCTATGT-3'	323	58 °C	2,5 mM
Kir 3.2	5'-AGTGGCCATTCACCAGC-3' 5'-GTGGGTGGAAAAGACCAG-3'	552	62 °C	2,5 mM
Kir 3.3	5'-GCCTCGATGCCCATCTCTA-3' 5'-TGCCTGCCTCCTCTTCTTCCA-3'	242	62 °C	2,5 mM
Kir 3.4	5'-TCTGAAACAGCACTTCTTGC-3' 5'-CCATGTCTTGATTCATAGCATT-3'	122	60 °C	3,5 mM

Table 1. RT-PCR primers sequences, amplicon length and reaction conditions used for each gene of interest.

In parallel, as internal control, the cDNA of an housekeeping gene, the GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) has been amplified using a couple of specific primers that produce a PCR product of 598 bp (see table 1).
The PCR reaction solution and amplification cycles were adjusted for each gene in terms of melting temperature and Magnesium concentration in order to improve specificity and efficiency (see table 1).
PCR products were loaded on 2% agarose 0,05% Ethidium Bromide gel and their size was determined by comparison with appropriate markers (BenchTop 100bp DNA Ladder; Promega) run on the same gel.

Real-Time Polymerase Chain Reaction (Real-Time PCR)

The expression of KCC2 and NKCC1 cotransporters in primary neuron cultures was quantitatively determined by Real-Time PCR using TaqMan technology (KCC2: Slc12a5 #Mm00803929_m1; NKCC1: Slc12a2 #Mm00436554_m1; Life Technologies).
Specific TaqMan Gene Expression Assays contain specific forward and reverse primers and a TaqMan probe, which binds to a sequence between that recognized by the primers. A fluorescent reporter dye (6-carboxy-fluorescein, FAM) is fused to the 5' end of the TaqMan probes and a quencher fluorescent dye (6-carboxy-tetramethyl-rhodamine, TAMRA) is attached to their 3' ends.
As many amplicon are generated, as much TaqMan probes are degraded by the endonuclease activity of the polymerase and, proportionally, as much reporter fluorophore is distanced from the quencher, thus determining proportional increase in fluorescent signal.
As reference gene, the housekeeping hypoxantine phosphoribosyltransferase 1(HPRT-1) gene was amplified in parallel using its specific TaqMan Assay (Hprt1 #Mm00446968_m1; Life Technologies).
Reaction mixes, for each well of a 96-well reaction plate, were prepared as follows:

- 13 ng cDNA,
- 12,5 µl 2x Universal PCR Mastermix (Applied Biosystems),
- 1 µl di TaqMan Gene Expression (Applied Biosystems),
- Nuclease-free water (Promega) to 25 µl

The reference and target genes of each experimental point were amplified in separate tubes in triplicate.

Real-Time PCR was performed using the PCR System ABI 7000 (Applied Biosystems) equipped with ABI Prism 7900 sequence detection system, using an amplification protocol consisting of one first step at 50°C for 2 minutes followed by 40 cycles of 15 seconds at 95°c and 1 minute at 60°C.

Results were elaborated with ABIPrism1.0.1 software (Applied Biosystems) using the comparative threshold cycle (Ct) method. The Ct is defined as the cycle at which the amplification plot crosses a predefined threshold of fluorescence. The threshold was individuated as the value where all amplification plots begins their exponential phase in the first experiment and then maintained constant over experiments.

Once logarithmic Ct values were obtained, they were normalized by subtracting the relative Ct values of the reference gene (Hprt-1):

$$Ct_{target} - Ct_{reference} = \Delta Ct$$

Since our samples constituted a Time- course of neuronal development for each target gene the ΔCt was further normalized by the ΔCt value of the first time point (DIV1):

$$\Delta Ct - \Delta Ct_{t0} = \Delta\Delta Ct$$

Finally, to bring values back to linearity $2^{-\Delta\Delta Ct}$ value was calculated for each time point and have been graphically indicated as fold increase over DIV1.

Analysis of proteins expression

Pharmacological treatments

Oxytocin, Vasopressin, TGOT were purchased from Bachem. All drugs were pre-diluted in Neurobasal medium and then a applied to neurons at the indicated time and final concentration. Duration of treatments depend on the experiment and is always indicated.

Western-blotting

Neurons at the indicated DIV were lysed in ice-cold RIPA buffer (NaCl 150mM, TrisHCl 50 mM, pH 7.4, EDTA 1mM, Triton X-100 1% and NP-40 1%) with Protease Inhibitor Cocktail (Sigma) and Phosphatase Inhibitor (PhosSTOP, Roche).

Protein concentration was determined with the DC protein Assay kit (Bio-Rad) and samples were diluted in 3x-sample buffer (375mM Tris-HCl, pH6.8, 20% glycerol w/v, 9% SDS w/v, β-mercaptoethanol 10% v/v and Bromophenol Blue 0.05% w/v).
Alternatively neurons were lysed directly in Sample Buffer.
Protein samples were separated in a 9% SDS-PAGE and then transferred onto a nitrocellullose membrane (Bio-Rad). To prevent aspecific binding membranes were incubated in 5% BSA for 1 hour at 37°C, and then probed with the proper primary antibody (anti-KCC2, anti-p-eEF2, anti-GAPDH and anti-β-tubulin) at the indicated dilution (see table 2). Secondary antibody conjugated with infrared-emitting-fluorophore were used (see table 2) and signals were detected and quantified using Odissey-LICOR scanner equipped with ImageStudio software. Kcc2 and p-eEF2 bands signal was normalized by GAPDH and Tubulin bands signal, respectively.

Protein MW (kDa)	Primary Antibody	Secondary Antibody
140	Rabbit Anti-Kcc2 (*Millipore*) 1:250	IRDye® 800CW Goat Anti-Rabbit (*Li-Cor*) 1:5000
95	Rabbit Anti-p-eEF2 (*Cell Signaling Technology*) 1:500	
55	Mouse Anti-Tub (*Sigma-Aldrich*) 1:30000	IRDye® 680RD Goat Anti-Mouse (*Li-Cor*) 1:7500
37	Mouse Anti-GAPDH (*Santa Cruz*) 1:750	

Table 2. Primary and Secondary antibodies used for western blot analysis. The molecular weight (MW) of expected bands is reported in the first column.

RESULTS

I. ENLARGED ARBOR AND PROMINENT SPINE REMODELING OF STRIATAL MSN IN THE *OXTR*$^{-/-}$ MICE

Social and cognitive aspects of the *Oxtr*$^{-/-}$ mouse behavior have been investigated previously by different research groups including ours (Sala et al 2011). The phenotype that emerged from these studies, as described above, recapitulates pretty well autistic-like symptoms.

While the social profile of this mice is extensively investigated, its cognitive deficits, and, more in general, cognitive implication of OXT system alteration, receive far less attention.

In particular, the neurobiological basis of the cognitive flexibility deficit of the *Oxtr*$^{-/-}$ mouse have never been clarified.

In the first part of my PhD project I analyzed morphological changes occurring to neurons after different cognitive tests, comparing *Oxtr*$^{-/-}$ mice with Wild-Type (*Oxtr*$^{+/+}$) ones.

Behavioral tests were organized as a sequence, thus each test represents the starting point and the control (basal level) for the subsequent (fig. 7). A group of animals (*Oxtr*$^{+/+}$and *Oxt*$^{-/-}$) was sacrified 24 hours after the end of each phase and neuronal morphology was assessed by using the Golgi staining.

I focalized my analysis on three brain regions, particularly involved in different aspects of cognitive processes. I chose the CA1 of hippocampus (CA1), as it is responsible for spatial (place) learning (Hirsh 1974, Mishkin M. 1984, Moser et al 1994), the dorsolateral striatum (DLS), because it's implicated in procedural (habit/response) learning (Mishkin M. 1984, Packard M.G. 1987) and the orbitofrontal cortex (OFC), for its role in cognitive flexibility (Dias et al 1997, Nonneman et al 1974, Ragozzino 2007).

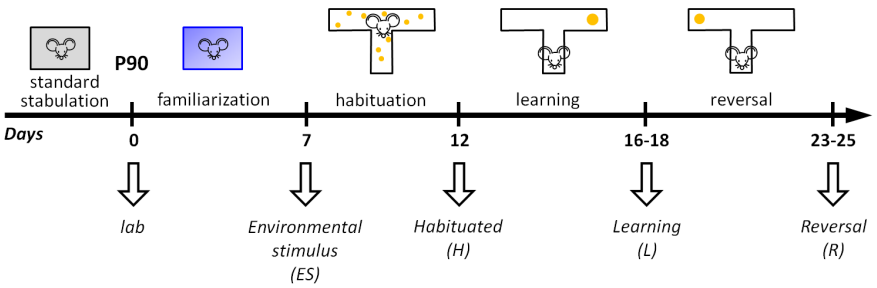

Figure 7. Time-line of behavioral testing and experimental groups obtained (empty arrow).

Oxtr$^{-/-}$ mice have normal spine density in all considered areas, but an increased number of spine/neuron in DLS

Firstly, spine density and dendritic arborization of normally stabulated *Oxtr*$^{+/+}$ e *Oxtr*$^{-/-}$ mice (named "lab") were evaluated to compare basal levels of the two genotypes in term of neuronal morphology.

As shown in the bar graphs in fig. 8 (a, c, j, k), no significant difference in spine density was observed between genotypes in any analyzed area.

Moreover, the analysis of dendritic arborization in CA1 reveals no significant difference either in the overall dendrite length or in the number of branching points (Fig. 8 d-g).
Conversely, in DLS, *Oxtr*$^{-/-}$ dendrites are significantly longer (Fig. 8 l), and have a more complex arborization, displaying an increased number of branching points (Fig.8 m).
Plotting data of dendrite length and branching points in relation with the distance from soma it's possible to appreciate that the difference in dendrites length is evident between 50 and 100 µm from soma (Fig. 8 n) and derives from a greater number of branching point in the first part of the dendritic arbor, closer to the soma (Fig. 8 o).

Environmental Stimuli affect spine density in different brain areas in the two genotypes

Since standard stabulation conditions give rather no stimuli to the animals, even a small change in the environment could represent for them an important alteration of the treadmill and therefore could possibly determine relevant morphological changes in their neurons.
For this reason, we decided to test the effect of a simple change in the external environment on neuronal morphology, by moving mice cages to the behavioral facility of our department for a week and subsequently performing the Golgi staining. We called this step "environmental stimulus" (ES).
Quite surprisingly, ES had important effects on spine density in both genotypes, but it interested different brain areas. In *Oxtr*$^{+/+}$ mice spine density of CA1 apical dendrites raised by almost 50% of its basal value (lab: 0,7956 ± 0,04604 vs ES: 1,181 ± 0,07731 spine/µm) and no other area was affected (Fig.9 a).

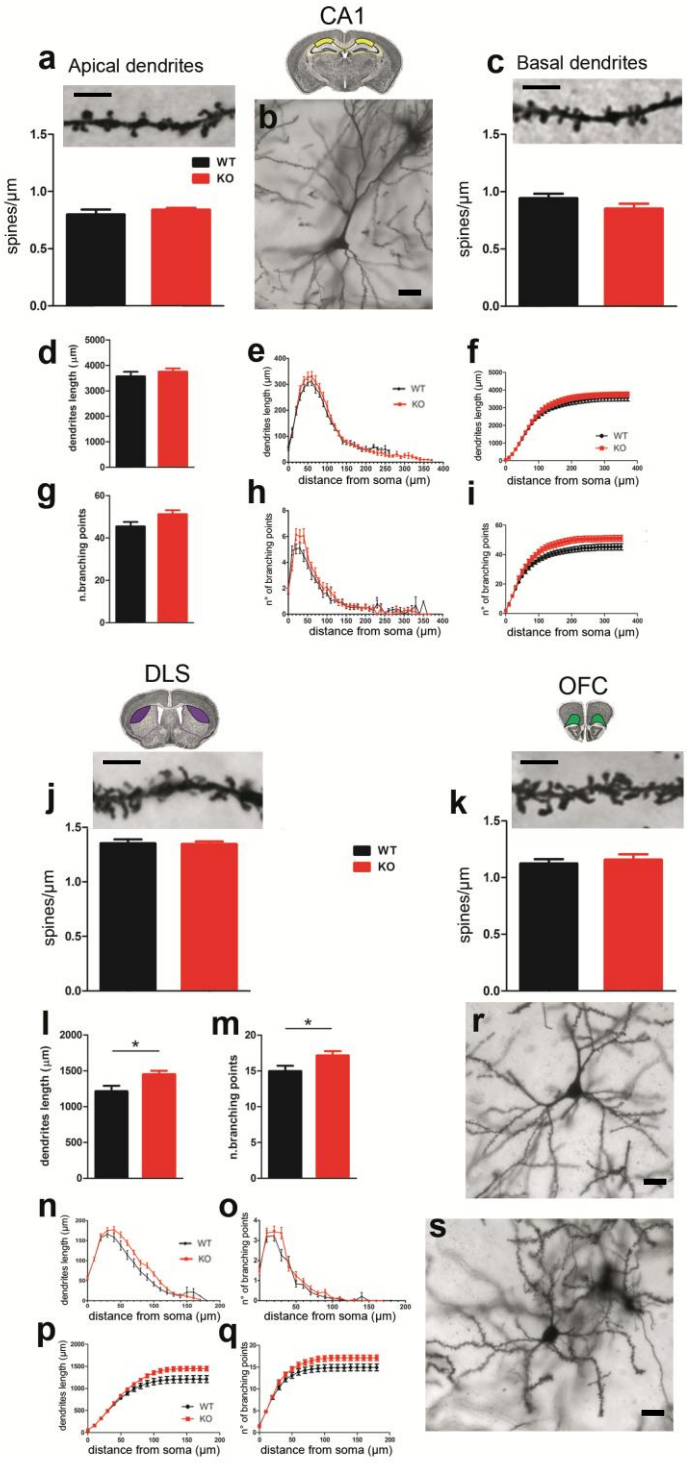

Figure 8. Spine density and dendritic arborization in CA1 (a-i), DLS (j-q, s) and OFC (k, r) in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice. Neuronal morphology was evaluated on at least 3 neurons per area, in 3 brains per group. (a, c) Spine density of hippocampal neurons on apical and basal dendrites, respectively. (j, k) Spine density in striatal medium spiny neurons and in pyramidal cells of OFC, respectively. Images above graphs (in a, c, j, k) are representative dendrites of corresponding areas; scale bars = 5 µm. (b, r, s) Representative images of Golgi-stained CA1 hippocampal pyramidal neuron, OFC pyramidal neuron and DLS medium spiny neuron, respectively. Scale bars = 20 µm. Dendrite length and number of branching point per neuron are presented as mean value± SEM (d, g, l, m). Partial (e, h, n, o) and cumulative (f, i, p, q) dendrite length and branching point are plotted as function of the distance from soma . Statistical analysis was carried out using Student t-test. *p<0.05 versus matched *Oxtr*$^{-/-}$.

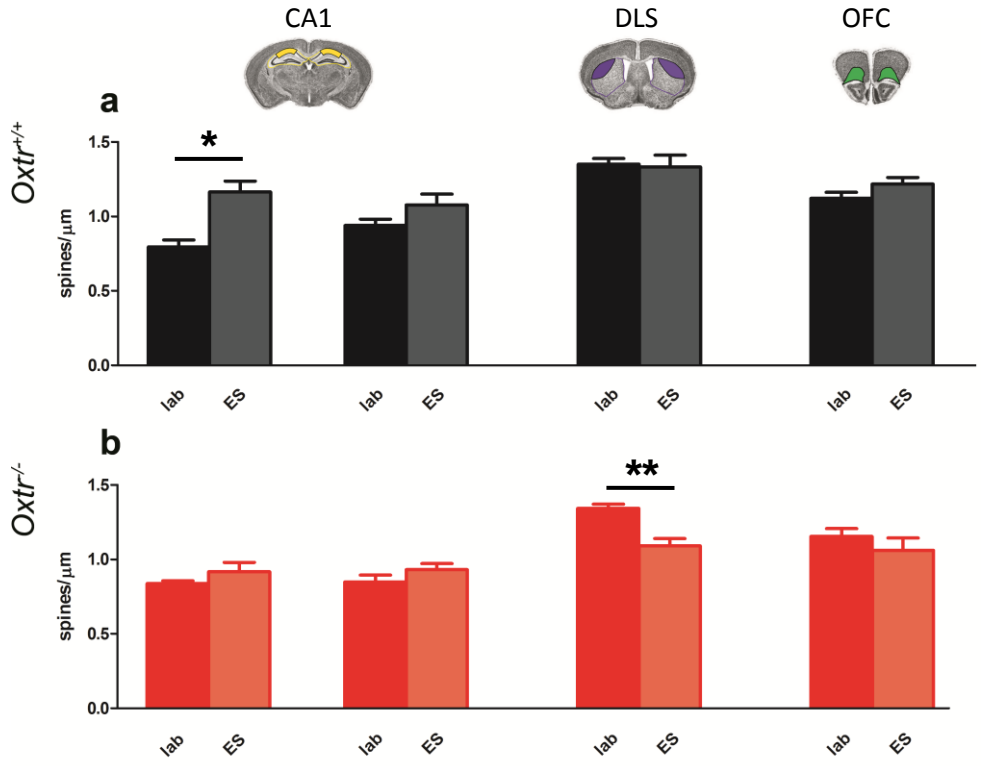

Figure 9. Effects of ES on spine density in *Oxtr*$^{+/+}$ (a) and *Oxtr*$^{-/-}$ (b) mice in CA1 (apical and basal dendrites), DLS and OFC. Values of spine density (n° of spines/µm) are presented as mean value± SEM. Statistical analysis was carried out using Student t-test. *p<0.05; **p<0.01 versus matched *Oxtr*$^{-/-}$. lab: normally stabulated mice at P90 from the back-up colony. ES (*environmental stimulus*): *lab* mice moved to the behavioral facility for one week to familiarize with the new environment.

Conversely, in *Oxtr*$^{-/-}$ mice the only affected region is the DLS (Fig. 9 b), where we found a 20% decrease in spine density (lab: 1,343 ± 0,02769 vs ES: 1,092 ± 0,04826 spine/µm).

Habituation to the T-maze apparatus: more complex stimuli determine extensive effects in *Oxtr*$^{-/-}$ mice

After a week of familiarization to the behavioral facility (ES), the protocol for the cognitive test started with a 5-days period called "habituation"(H). During this phase animals undergo many different stimuli: food restriction, daily handling and exploration of the T-maze apparatus and finally the taste of a new food (subsequently used as reward).

This complex phase produces dramatic effects on spine remodeling in *Oxtr*$^{-/-}$ mice. Indeed, as shown in fig. 10 a, spine density is increased in DLS by about 20% of the value observed after the previous phase (CA1. ES: 0,9400 ± 0,05769 vs H: 1,157 ± 0,05745 spine/µm; DLS. ES: 1,092 ± 0,04826 vs H: 1,324 ± 0,03830 spine/µm) and an increasing trend was present in CA1 (apical dendrites) as well.

No significant effects on spine density were observed in these two areas in *Oxtr*$^{+/+}$ mice (fig. 10 b), which conversely showed a 15% reduction in OFC values (ES: 1,217 ± 0,04455 vs H: 1,018 ± 0,03244 spine/µm).

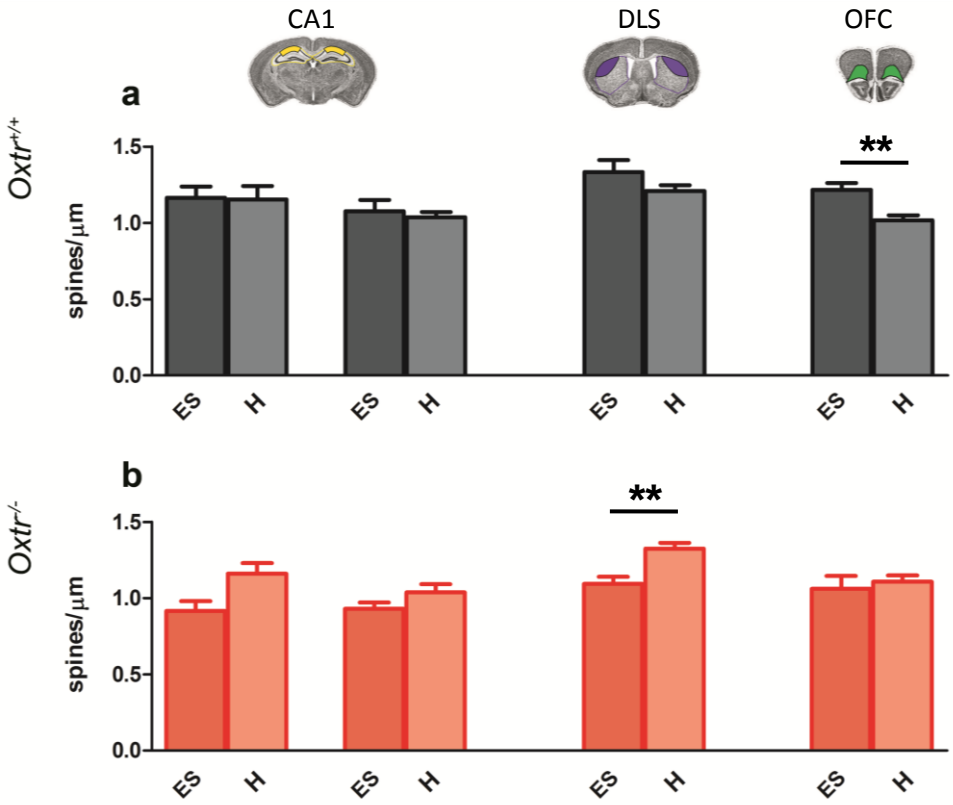

Figure 10. Effects of the habituation period on spine density in *Oxtr*$^{+/+}$ (a) and *Oxtr*$^{-/-}$ (b) mice in CA1 (apical and basal dendrites), DLS and OFC. Values of spine density (n° of spines/µm) are presented as mean value± SEM. Statistical analysis was carried out using Student t-test. **p<0.01 versus matched *Oxtr*$^{-/-}$. ES (*environmental stimulus*): *lab* mice moved to the behavioral facility for one week to familiarize with the new environment. H (*habituation*): *ES* mice after the period of habituation to the T-maze.

Decreasing trend of spine density along the T-maze test period in the two genotypes

Once habituated to the apparatus and to the reward food, animals entered the acquisition phase of the T-maze test (also called learning, or L) which lasts from 4 to 7 days, until animals learn to find food in the baited arm.

As shown in fig. 11 a-b the effect of the learning phase of the test on *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice is similar: we observed a small but generalized decrease in spine density in every brain area.

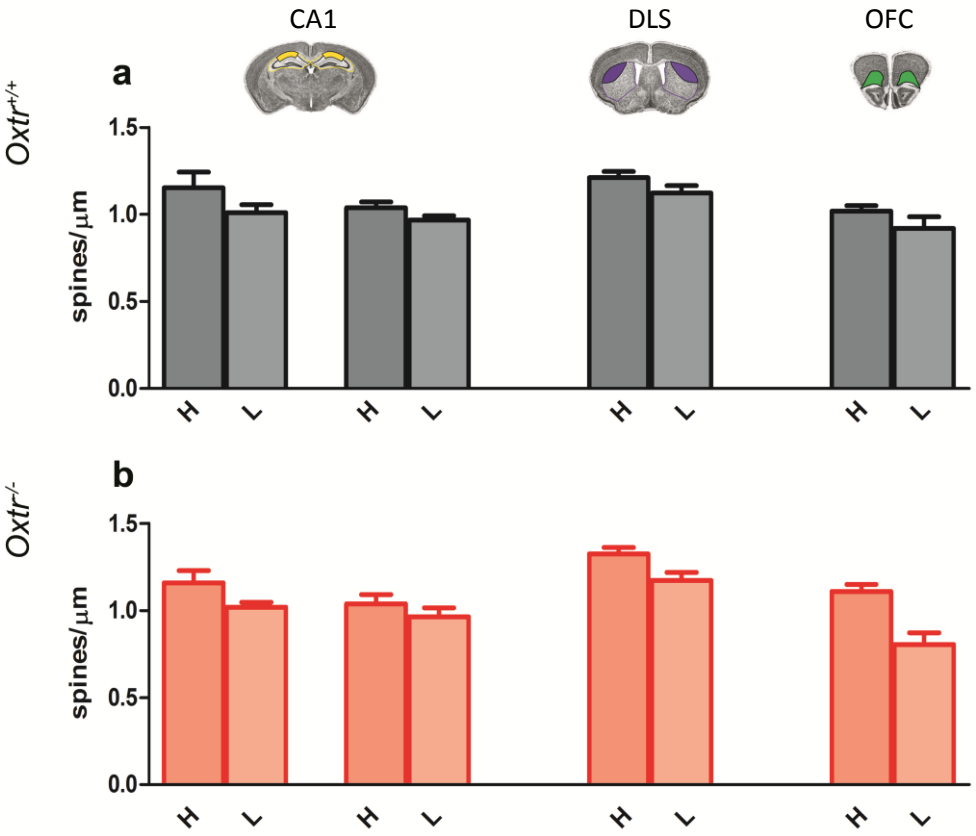

Figure 11. Effects of learning in a T-maze test on spine density in *Oxtr*$^{+/+}$ (a) and *Oxtr*$^{-/-}$ (b) mice in CA1 (apical and basal dendrites), DLS and OFC. Values of spine density (n° of spines/µm) are presented as mean value± SEM. Statistical analysis was carried out using Student t-test. H (*habituation*): *ES* mice after the period of habituation to the T-maze. L (*learning*): *H* mice which completed also the acquisition phase of the T-maze test.

Finally, we analyzed the effect on spine density of the reversal phase of the T-maze test (R), which ends within 7 days, whether or not the animals have reached the criterion.

A significant decrease was highlighted only on apical dendrites of CA1 of *Oxtr*$^{-/-}$ mice (fig. 12 b. R vs L: 0,7547 ± 0,03849 vs 1,019 ± 0,02702 spine/µm). and for *Oxtr*$^{+/+}$ also basal dendrites are similarly affected (fig. 12 a. R vs L: 0,8750 ± 0,03722 vs 0,9675 ± 0,02390 spine/µm).
On the other hand, the reversal training produced no effect on spine density in DLS and OFC, either in *Oxtr*$^{+/+}$ or in *Oxtr*$^{-/-}$ (fig. 12a-b).

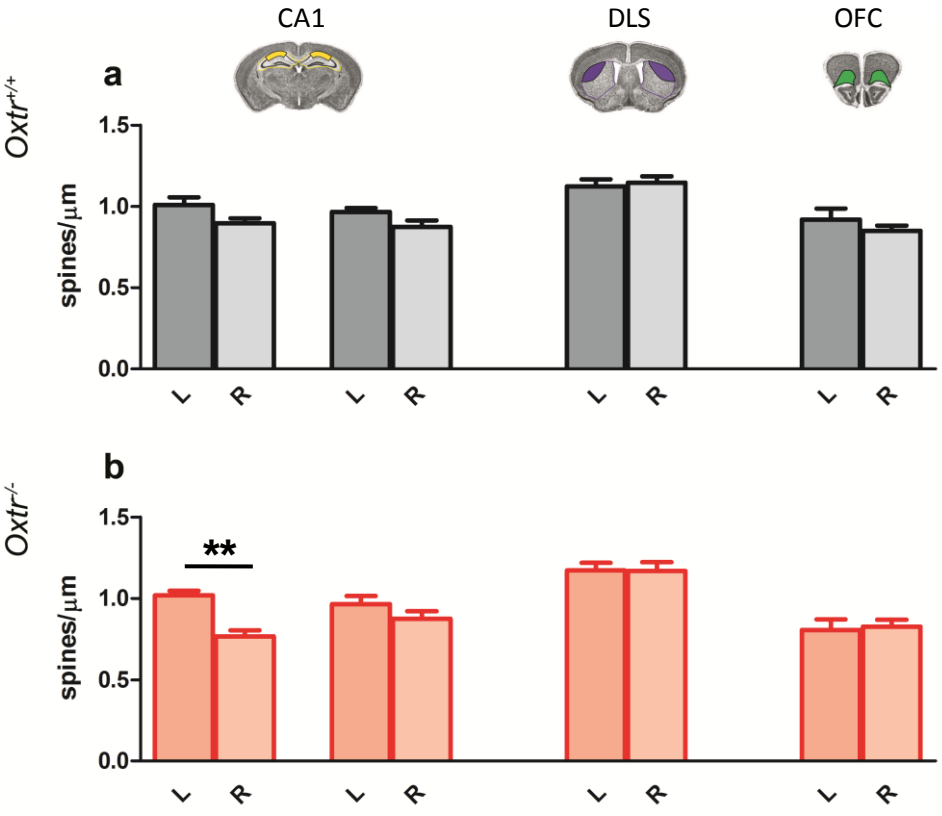

Figure 12. Effects of reversal in a T-maze test on spine density in *Oxtr*$^{+/+}$ (a) and *Oxtr*$^{-/-}$ (b) mice in CA1 (apical and basal dendrites), DLS and OFC. Values of spine density (n° of spines/µm) are presented as mean value± SEM. Statistical analysis was carried out using Student t-test. **p<0.01 versus matched *Oxtr*$^{-/-}$. L (*learning*): *H* mice which completed also the acquisition phase of the T-maze test. R (*reversal*): *L* mice after the reversal training in the T-maze.

Interestingly, the spine density level reached after the reversal phase by both genotype is significantly lower than the basal level of normally stabulated animals both in OFC and in DLS, whereas in CA1, on both basal and apical dendrites, spine density values after the reversal phase gets back to their initial level (Table 3).

		Lab (sp/µm)	Reversal (sp/µm)	P value
CA1	*Oxtr*$^{+/+}$	Apical: 0.7956 ± 0.04604 Basal: 0.9400 ± 0.04173	Apical: 0,8990 ± 0,02773 Basal: 0,8750 ± 0,03722	0,0759 0,2930
	Oxtr$^{-/-}$	Apical: 0.8367 ± 0.02060 Basal: .8483 ± 0.04622	Apical: 0,7547 ± 0,03849 Basal: 0.8740 ± 0.04883	0,1109 0,7613
OFC	*Oxtr*$^{+/+}$	1,120 ± 0,04209	0,8491 ± 0,03186	< 0,0001
	Oxtr$^{-/-}$	1,154 ± 0,05134	0,7960 ± 0,04967	< 0,0001
DLS	*Oxtr*$^{+/+}$	1,350 ± 0,03971	1,147 ± 0,03862	0,0013
	Oxtr$^{-/-}$	1,343 ± 0,02769	1,185 ± 0,04521	0,0114

Table 3. Spine density of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice in CA1 (apical and basal dendrites), DLS and OFC at the first and last step of behavioral protocol. Level of spine density in the indicated brain regions of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice before the beginning of behavioral tests(lab) is compared to the level of spine density at the end of the last behavioral test (reversal) in genotype-matched animals. Values of spine density (n° of spines/µm) are presented as mean value± SEM. P values refers to a Student T-test comparision between these two groups (lab and reversal).

II. HIPPOCAMPAL *OXTR*$^{-/-}$ NEURONS HAVE ALTERED EXPRESSION OF PROTEINS INVOLVED IN THE E/I BALANCE

Another interesting feature that emerged from the behavioral characterization of our autistic mouse model is its increased susceptibility to evoked seizures, which correlates also pretty well with the imbalance between excitatory an inhibitory synapses observed in Oxtr$^{-/-}$ hippocampal neurons in culture (Sala et al 2011).

The alteration of the equilibrium between neuronal excitation and inhibition (E/I balance) have been frequently associated with neuropsychiatric disorders (such as schizophrenia, epilepsy and autism) in many different animal models (Rubenstein & Merzenich 2003, Yizhar et al 2011)

and also in human subjects (Eichler & Meier 2008), but it's neurobiological basis are still unclear.

To investigate how the oxytocin system could be involved in the maintenance of this E/I balance, and thus in the pathogenesis of ASD (or in the control of its symptoms), we used, as a model, *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ primary hippocampal cultures, in which the increased ratio between excitatory/inhibitory synapses was initially highlighted.

Moreover neuronal culture represent a model of the earliest stages of development, when also small alterations could affect the formation of proper connections and the correct evolution of brain circuitry, having potentially dramatic neuropsychiatric consequences. Therefore this time window could be a huge source of information on possible pathologic mechanisms, and could also represent an excellent moment for a promising therapeutic strategy.

Primary hippocampal neurons from *Oxtr*$^{-/-}$ mice show no alteration in spine density and dendrite arborization

Data concerning neuronal morphology in hippocampus at basal level were further investigated on cultured hippocampal neurons.

Neurons from *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ E18 embryos were transfected with a GFP-expression vector (pLL3.7) at DIV7 and then analyzed at DIV17, when they reached an advanced maturation, synaptic contacts are established and spines are formed. The expression of soluble GFP allows a precise visualization of all neuronal processes and dendritic protrusion including spines and filopodia.

The Sholl analysis reveals no difference either in dendritic arborization (Fig.13 a-f) or in spine density (Fig. 14 a), as observed on adult neurons with the Golgi staining. Spine morphology were also evaluated on cultured GFP-expressing neurons but no difference emerged between the two genotypes (Fig.14 b-e).

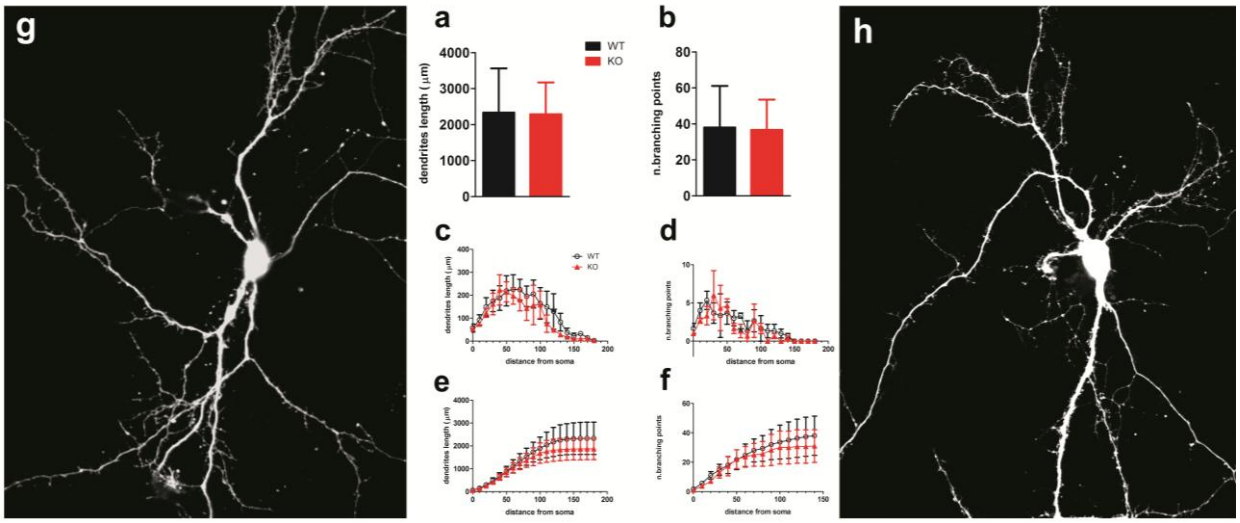

Figure 13. Dendritic arborization of GFP-expressing neurons derived from *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice. In (a, b) dendrite length and n° of branching points are presented as mean values ±SEM. (c-f) Partial (c, d) and cumulative (e, f) dendrite length and branching point are plotted as function of the distance from soma. (g, h) Representative images of GFP-transfected hippocampal neurons from *Oxtr*$^{+/+}$ (g) and *Oxtr*$^{-/-}$ (h) mice. Statistical analysis was carried out using Student t-test.

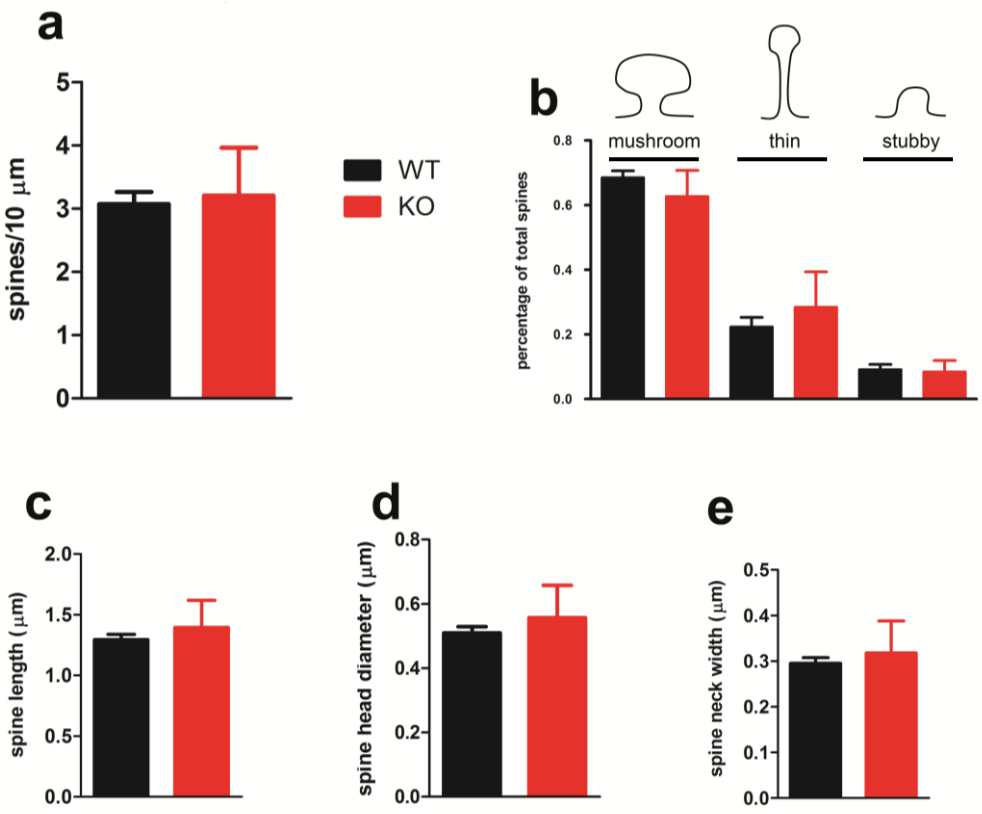

Figure 14. Spine density and morphology of GFP-expressing neurons from *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice. Spine density values (a) are reported as number of spine/10µm of dendrite (mean ±SEM). The classification of spines on morphological basis (b) is shown as percentage of total spines. Morphological parameters: spine length (c), head diameter (d) and neck width (e) are presented as mean values ±SEM. Statistical analysis was carried out using Student t-test.

Primary hippocampal neurons from *Oxtr*$^{-/-}$ mice express differently the receptors of the OXT/AVP family

Firstly, we analyzed the expression of receptors of the OXT/AVP family in our neuronal cultures at three time points: DIV3, DIV6 and DIV11, to map their modulation during development. RT-PCR results (fig. 15) show that in *Oxtr*$^{+/+}$ neuronal cultures Oxtr is already expressed at the earliest stage of development taken into account (DIV3), it is then upregulated until DIV6 and then seem to remain stable until DIV11, when neurons start to reach maturity.
AVP receptors are closely related to oxytocin ones and, due to elevated homology between the two neuhypophyseal peptides, they are poorly selective. Consequently, OXT can bind and activate AVP-receptors, especially the V1a subtype(V1aR), with an affinity not so different from that of AVP. Therefore we cannot rule out the possibility that some physiological effect of OXT are mediated, or can be compensated, by the activation of AVP-receptors.

For these reasons, we analyzed in our neuronal cultures also the developmental profile of AVP-receptor expression, and were not surprised to observe important differences between *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons.

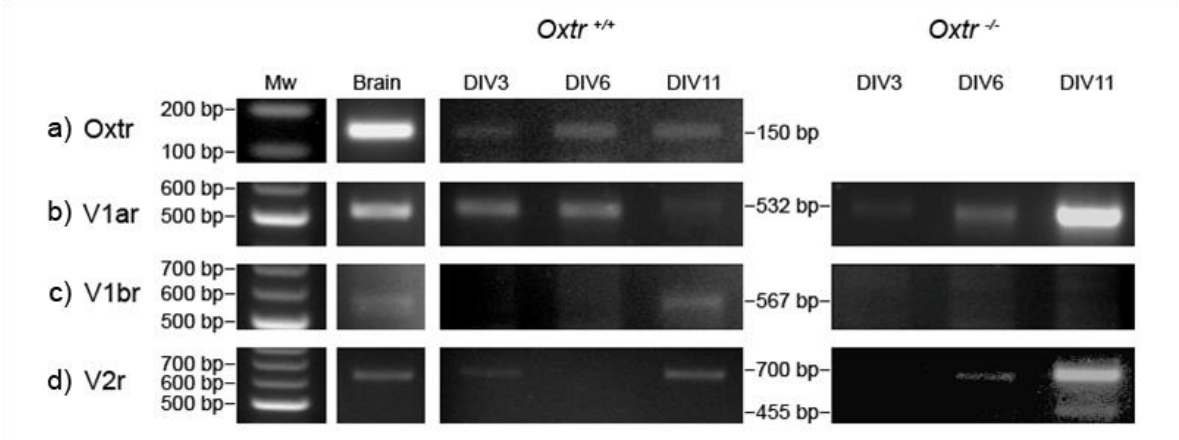

Figure 15. RT-PCR analysis of OXT/AVP receptors expressio in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons during development. Amplification of Oxtr (a), V1ar (b) V1br (c) and V2r (d) was conducted using specific intron-spanning primers' couples. As positive control a whole brain extracts from an adult *Oxtr*$^{+/+}$ mouse was used (Brain). Molecular weight markers (Mw) are shown on the left.

In particular, V1aR is expressed in *Oxtr*$^{+/+}$ neuronal cultures at early stages of *in vitro* development (DIV3 and DIV6), but then is downregulated within DIV11. On the contrary, in *Oxtr*$^{-/-}$ neurons we observed a gradual but very strong increase of V1aR expression during development (fig. 15 b).

Another difference emerged in the expression of V1bR, which appears in *Oxtr*$^{+/+}$ neurons at DIV11, when it's still undetectable in *Oxtr*$^{-/-}$ (fig. 15 c).
Finally, we analyzed the expression of V2R, even if it is described not to be present in CNS (Ostrowski et al 1992), to check eventual ectopic expression due to development in culture. In *Oxtr*$^{+/+}$ neurons we detect only an aspecific band of around 700bp (fig. 15 d) at DIV3 and DIV11, which is compatible with an immature untranslated transcript. The same band appears also in *Oxtr*$^{-/-}$ neurons at all time points considered; however at DIV11 we observed also a labile band at around 450bp, consistent with the transcript coding for a splice variant of the V2 receptor, named V2bR.

IRK channels are upregulated in *Oxtr*$^{-/-}$ neurons

An indication of the role of OXT system in modulating neuronal excitability emerged even before the behavioral characterization of *Oxtr*$^{-/-}$ mice, when in our lab (Gravati et al 2010) was described the OXTR ability to modulate several inwardly-rectifying K^+ channels.

This evidence suggested us to evaluate the expression of these channels in our cultures. In particular we examined the expression of GIRK channels, belonging to the Kir3 class (Kir3.1, Kir3.2 Kir3.3 and Kir3.4) and of three members of IRKs, belonging to the Kir2 class (Kir2.1, Kir2.2 and Kir2,3). GIRKs are closed at resting membrane potential and get activated by certain GPCR, including OXTR, through a signaling pathway which involves the Gβϒ subunits released from a Gi/o tetrameric complex (Dascal 1997, Logothetis et al 1987, Reuveny et al 1994). Conversely, Kir2-class IRKs are constitutively open and can be closed by the phospholipase C β (PLCβ), activated by a Gq/11-coupled GPCR (Firth & Jones 2001, Uchimura & North 1990).

Interestingly, as shown in fig. 16, our RT-PCR analysis reveal a generalized upregulation of all channels taken into account.

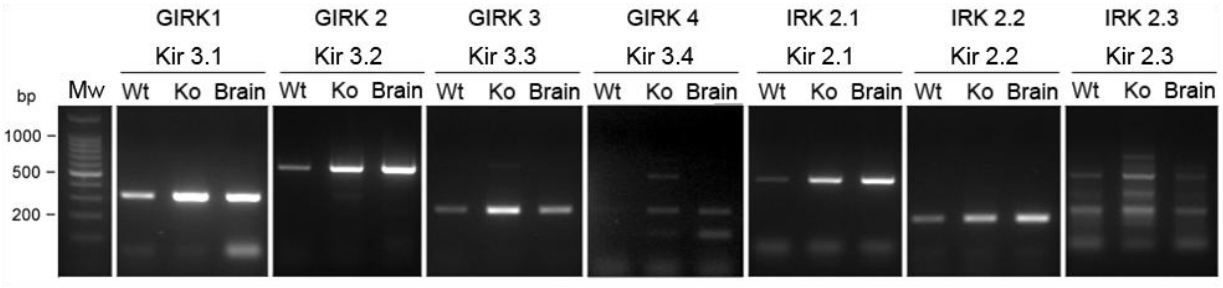

Figure 16. RT-PCR of Kir 3.1 (GIRK1), 3.2 (GIRK2), 3.3 (GIRK3), 3.4 (GIRK4), 2.1 (IRK 2.1), 2.2 (IRK 2.2), e 2.3 (IRK 2.3) channels in Oxtr$^{+/+}$and Oxtr$^{-/-}$ neurons. Total RNA of hippocampal neurons from *Oxtr*$^{+/+}$ (WT) e *Oxtr*$^{-/-}$ (KO) mice was isolated at DIV11, retrotranscribed and amplified with specific intron-spanning couples of primers (see Table 1 in Material and methods section). As positive control a whole brain extracts from an adult *Oxtr*$^{+/+}$ mouse was used (Brain). Molecular weight markers (Mw) are shown on the left.

Developmental increase of KCC2 transcript and protein is lower in *Oxtr*$^{-/-}$ hippocampal cultures

Early neuronal development is characterized by a complex sequence of events aimed at controlling synapse maturation, excitability and coordination of neuronal activity, which guarantees the proper formation of neuronal networks and circuitries (Spitzer 2006).
One of the most relevant events that happens to hippocampal neurons during this phase is the excitatory-to-inhibitory switch in GABA actions. This switch takes place by the end of the second postnatal week in rodents (Ben-Ari et al 1989, Cherubini et al 1991) and it is described also in neuronal cultures, around DIV11 (Ganguly et al 2001). Two neuronal cathion-chloride cotransporters (CCCs), NKCC1 and KCC2, are responsible for this change, since they are able to modify Cl- electrochemical gradient by altering its intracellular concentration. In particular, the ratio between NKCC1 and KCC2 expression (NKCC1/KCC2 ratio) is high in the first phase of in utero development, then, few days after birth, it gradually decreases producing the aforementioned switch in GABA polarity (Rivera et al 1999).

Interestingly, some years ago it was observed in rats that the oxytocin released during labor is able to induce in the fetal hippocampus a temporary switch in GABA polarity (Tyzio et al 2006), but the underlying mechanism remain uninvestigated.

For this reason we analyzed the developmental profile of the expression of these two CCCs, NKCC1 and KCC2 in neuronal cultures from *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice. Real Time PCR results displayed in fig.10a show that the decreasing trend in the expression of NKCC1 observed in *Oxtr*$^{+/+}$ neurons was maintained in *Oxtr*$^{-/-}$ ones. On the contrary, the developmental upregulation of KCC2 transcript, which is massive in *Oxtr*$^{+/+}$ cultures, as described (Rivera et al., 1999), is lower and not significant in *Oxtr*$^{-/-}$ ones (fig. 17 b).

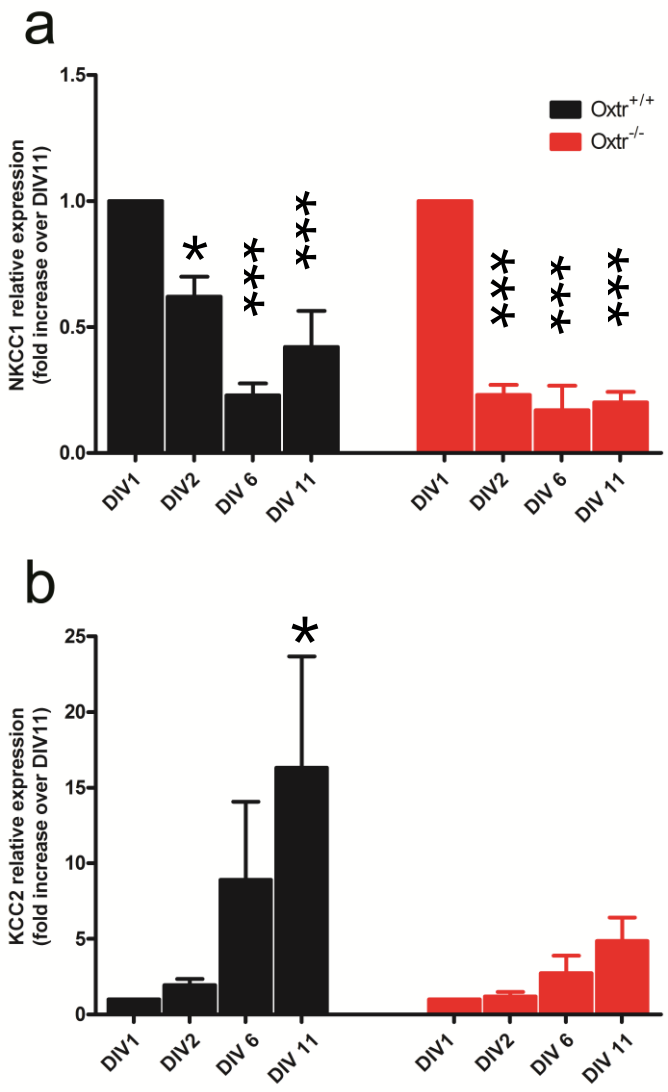

Figure 17. Real Time analysis of NKCC1(a) and KCC2 (b) expression during in vitro development of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons. Abundance of transcripts for NKCC1 (a) and KCC2 (b) is normalized on that of the reference gene, *Hprt1*. Values of relative expression are presented on graphs as fold increase (mean ±SEM) over the level at DIV1. Three different neuronal preparations were analyzed for each genotype. Statistical analysis were performed using One-Way ANOVA with a Tukey post-hoc test. *p<0.05, ***p<0.001 versus DIV1 value.

The difference in the size of the KCC2 upregulation between *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neuronal cultures was further confirmed by Western blot analysis as shown in fig.18.
Unfortunately, there are no commercially available specific NKCC1 antibody (Blaesse et al 2009), therefore we didn't perform Western blot analysis of NKCC1 expression.

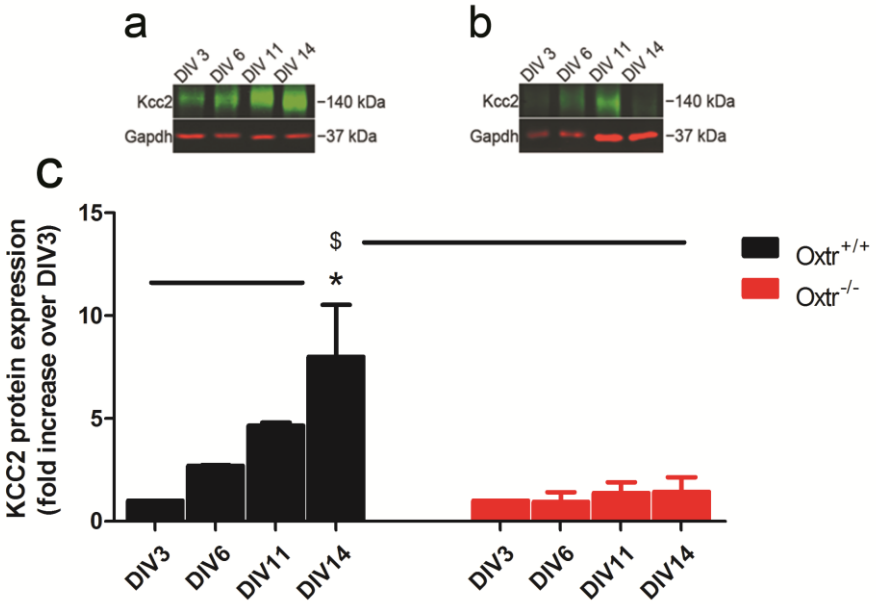

Figure 18. Western Blot analysis of KCC2 expression during in vitro development of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons. Representative KCC2 western blot analysis of neuronal lysates from *Oxtr*$^{+/+}$ (a) and *Oxtr*$^{-/-}$ (b) neurons at the indicated DIV are presented in the upper panels. Values are normalized on corresponding GAPDH levels. Quantification of KCC2 protein expression (c) is presented as fold increase (mean ±SEM) over the level at DIV3. Two different neuronal preparations for each genotype. Statistical analysis were performed using One-Way ANOVA with a Tukey post-hoc test . *p<0.05 versus DIV3 value.

OXT administration early during in vitro development increase KCC2 protein level in *Oxtr*$^{+/+}$ but not in *Oxtr*$^{-/-}$ hippocampal neurons

To further examine the possible involvement of OXT in the modulation of KCC2 expression in developing neurons in culture we designed a treatment protocol which mimic the burst of maternal oxytocin release that takes place during labor.
This protocol, hereafter called "Burst", consist in daily treatments with OXT (final concentration 100 nM) during three days, from DIV3 to DIV5 (fig. 19 a).

We planned also a stronger treatment protocol, called "Chronic", consisting in daily treatment with 100 nM OXT, starting from DIV3 and continuing until the day of the lysis (fig. 19 b). The rationale of this second protocol is to either evaluate possible effects of a chronic therapy with OXT or take into account also the OXT produced in the newborn brain that could possibly reach the hippocampus.

Neurons undergoing Burst and Chronic OXT treatment were lysed at different time points during in vitro development as indicated (fig. 19 a, b), and KCC2 expression was analyzed by western blot.

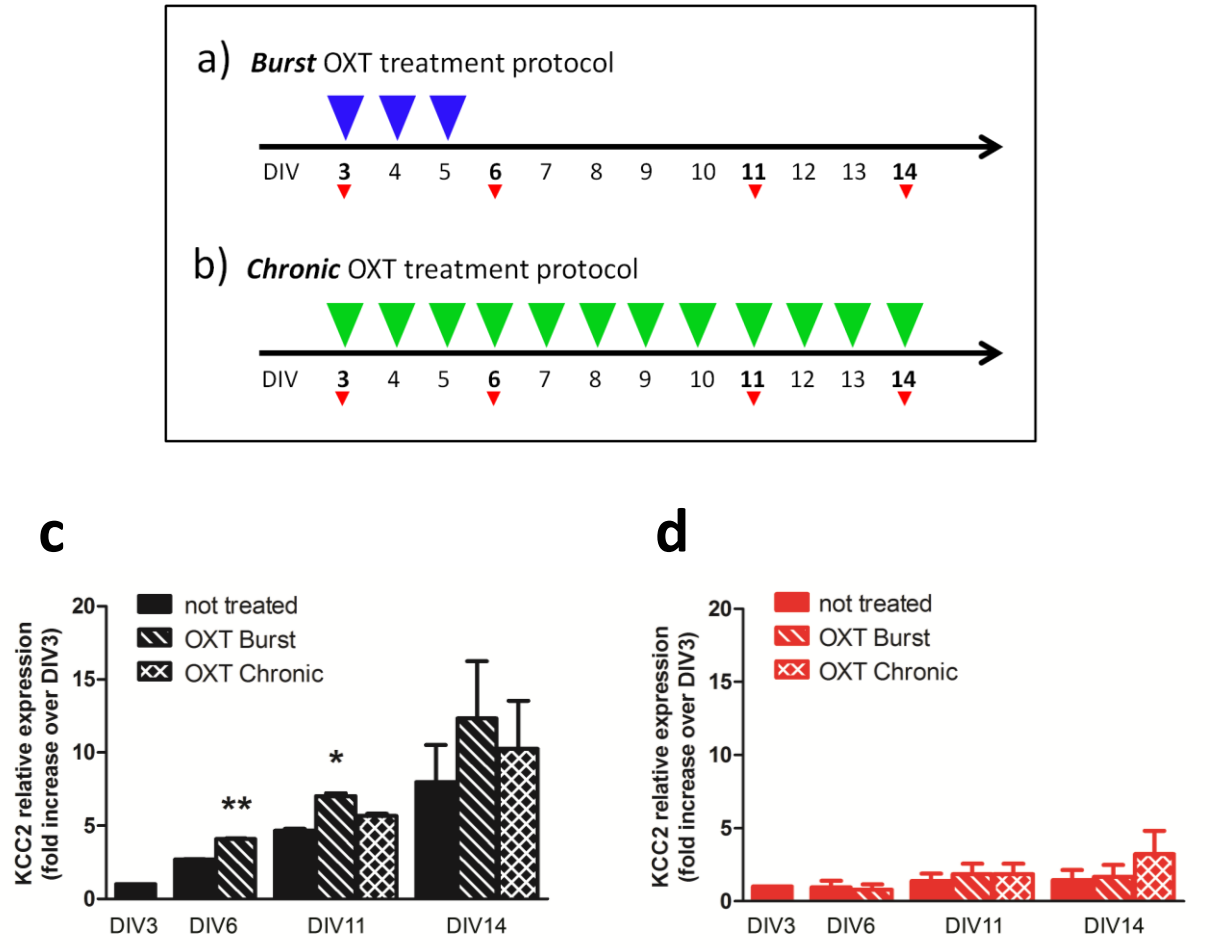

Figure 19. Western Blot analysis of KCC2 expression during in vitro development of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons upon different OXT treatment protocols. A schematic representation of *bust* (a) and *chronic* (b) treatment protocols is shown in the upper panel. Blue arrow-heads (in a) indicate daily treatment with OXT (100nM) in the *burst* protocol; green arrow-heads (in b) represent daily treatments with OXT (100nM) in the *chronic* protocol. Red triangle below DIV numbers (both in a and in b) indicate the moments of lysates collection for subsequent KCC2 western blot analysis. Quantification of KCC2 expression in *burst*- and *chronic*-treated *Oxtr*$^{+/+}$ (a) and *Oxtr*$^{-/-}$ (b) neurons is shown in the lower panels. Values, normalized on corresponding GAPDH levels, are presented as fold increase (mean ±SEM) over DIV3. Two different neuronal preparations for each genotype were quantified. Statistical analysis were performed using unpaired Student t-test. *p<0.05 versus corresponding not treated sample.

In fig.19 (c) is represented the effect of the two treatment protocols on *Oxtr*$^{+/+}$ neurons. Burst OXT treatment protocol produce a 50% increase in the KCC2 protein level at DIV6, 24 hours after the end of the treatment, which is maintained also later on (DIV11, DIV 14). On the contrary, Chronic OXT administration wasn't able to produce any significant effect on KCC2 expression.

Conversely, in *Oxtr*$^{-/-}$ neurons neither Burst nor Chronic OXT treatment were effective on the modulation of KCC2 protein level (fig.19 d).

An acute treatment with OXT determines an increase of KCC2 protein level within 3 days

Finally, we decided to verify if an acute treatment with OXT was sufficient to produce the increase in KCC2 level in *Oxtr*$^{+/+}$ neurons. Preliminary experiments indicate that, at least at an intermediate stadius of maturation (DIV8), an acute administration of OXT (100 nM) determines an increase in KCC2 level, which is not detectable after 24 hours, but become significant within 72 hours after treatment (fig. 20).

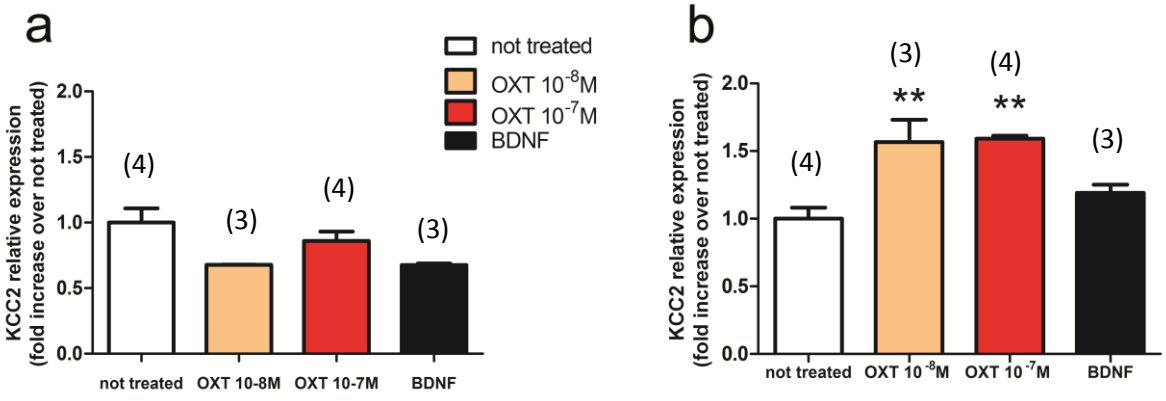

Figure 20. Western Blot analysis of KCC2 expression in *Oxtr*$^{+/+}$ neurons upon OXT or BDNF treatment. *Oxtr*$^{+/+}$ hippocampal neurons were treated with OXT (10 or 100 nM) or with BDNF (50ng/ml) at DIV8 and then were lysed 24 (a) or 72 (b) hours later. Replicates of each samples (number displayed in brackets) were obtained within the same preparation. KCC2 values were normalized to corresponding GAPDH ones and are presented as fold increase (mean ±SEM) over not treated sample. Statistical analysis were performed using One-Way ANOVA with a Dunnet post-hoc test . *p<0.05 versus not treated; **p<0.01 versus not treated.

OXT treatment is able to increase the phosphorylation level of eEF2

To better understand how the lack of OXTR could affect the expression of proteins involved in neuronal E/I imbalance we investigated the signaling pathways activated by OXT treatment in neuronal cultures from *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice.
We focused our attention on eEF2, an important regulator of protein synthesis, since it has been demonstrated that OXTR is able to induce its activation in myometrial cells. Moreover, several evidences suggested that eEF2 is able to regulate local dendritic protein synthesis in an activity-dependent manner (Chotiner et al 2003, Park et al 2008, Sutton et al 2007).

The activation of eEF2 can be evaluated by monitoring its phosphorylation status, indeed eEF2 phosphorylation at Thr56 inhibits general protein translation and elongation (Ryazanov et al 1991, Ryazanov et al 1988). However an opposite effect of eEF2 phosphorylation has been described for some dendritic mRNAs such asCaMKII and Arc (Belelovsky et al 2005, Chotiner et al 2003, Marin et al 1997, Park et al 2008, Scheetz et al 2000).

To test the ability of OXTR to modulate eEF2 activation we treated mature (DIV14) neurons, either *Oxtr*$^{+/+}$ or *Oxtr*$^{-/-}$, with OXT at different doses to obtain a dose-response curve. After 30 minutes we lysed the cells and performed a western blot analysis of p-eEF2 level.
In *Oxtr* $^{+/+}$ neurons we observed a significant increase in the phosphorylation level of eEF2 after treatment with 10 nM OXT, wherease higher or lower dose failed to produce any significant change (fig 21 a). Conversely, in *Oxtr*$^{-/-}$ neurons OXT affect significantly p-eEF2 levels only at hogher doses, 1-10 µM, probably acting on other receptorf of the OXT/AVP family (Fig. 21 b).
Since OXTR is a rather unselective receptor, we tested if also AVP could elicit an analogous enhancing effect on eEF2 phosphorylation in *Oxtr*$^{+/+}$ neurons and, interestingly, that was exactly the case. Indeed, as shown in fig.21 c, 10 and 100 nM AVP increased eEF2 phosphorylation just as OXT 100nM did. Moreover, AVP treatment increases the phosphorylation of eEF2 on *Oxtr*$^{-/-}$ neurons as well, confirming the involvement of other receptors of the OXTR/AVP family in the induction of eEF2 phosphorylation (Fig.21 d).

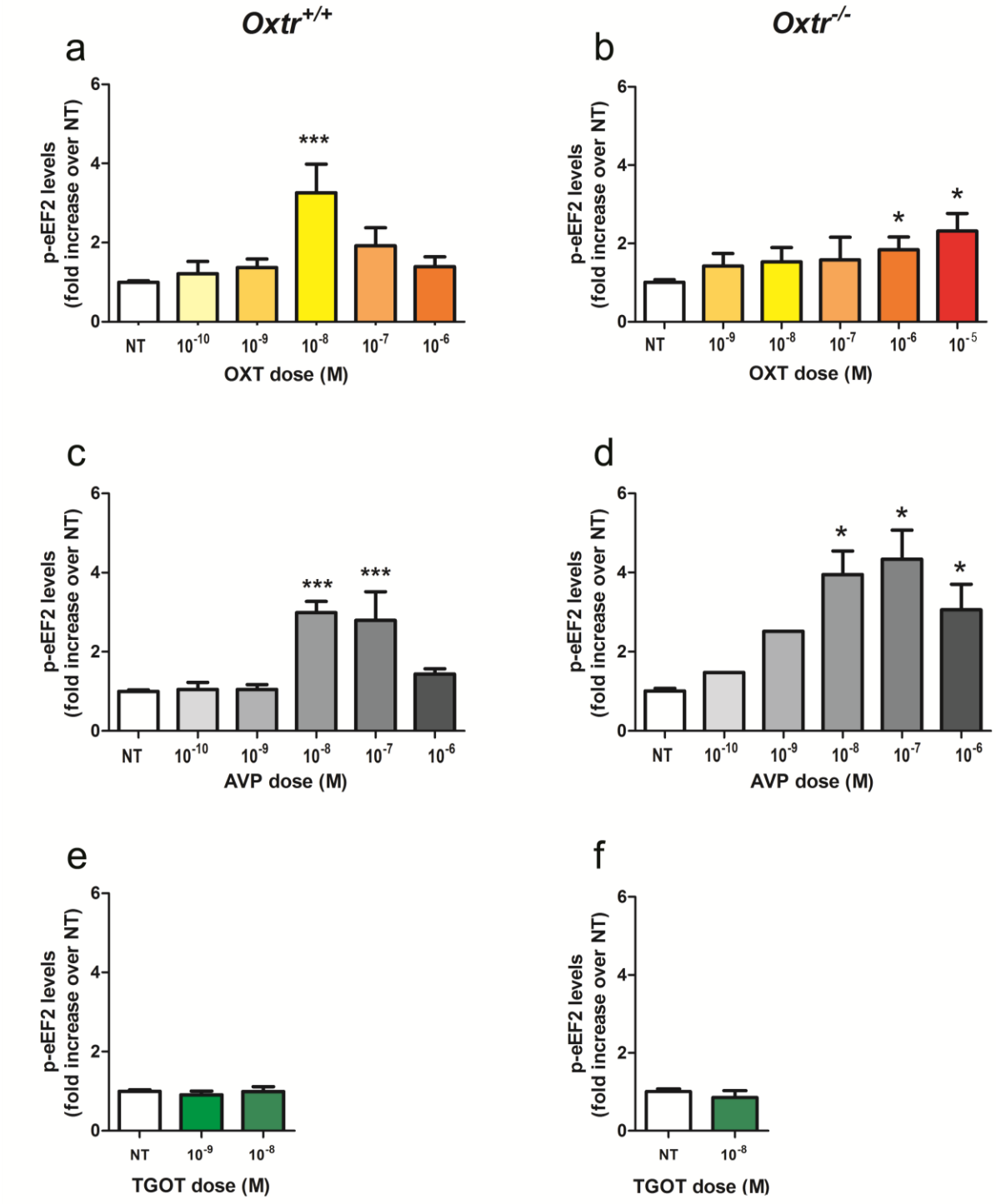

Figure 21. Western Blot analysis of p-eEF2 levels in Oxtr$^{+/+}$ and Oxtr$^{-/-}$ neurons upon treatment with different OXTR agonists. Hippocampal neurons at DIV14 were treated with OXT, AVP or TGOT at the indicated doses and lysed 30 min later. Data were obtained from at least three different preparations. p-eEF2 values are normalized on corresponding β-tubulin ones and presented as fold increase (mean ±SEM) over corresponding not treated sample (NT). Statistical analysis were performed using One-Way ANOVA with a Dunnett post-hoc test . *p<0.05 versus not treated; *** p<0.001 versus not treated.

Consequently, we decided to verify if the increase in p-eEF2 level in *Oxtr*$^{+/+}$ neurons was really OXTR-dependent by treating these neurons with TGOT, a selective OXTR agonist (see table 4). Unexpectedly, this treatment failed to produce any effect on eEF2 phosphorylation (fig. 21 e), thus excluding OXTR from being able to activate this pathway.

Similarly, as shown in fig. 21 f, no effect was observed also when *Oxt*$^{-/-}$ neurons were treated with a selective dose of TGOT (10 nM).

Peptide	**Sequence**	**Ki for (nM ± %CV):**		
		mOTR	**mV1a**	**mV1b**
OXT	Cys^{1}, Tyr^{2}, Ile^{3}, Gln^{4}, Asn^{5}, Cys^{6}, Pro^{7}, Leu^{8}, Gly-NH2^{9}	0.83 ± 17	20.38 ± 26	36.32 ± 7
AVP	Cys^{1}, Tyr^{2}, **Phe^{3}**, Gln^{4}, Asn^{5}, Cys^{6}, Pro^{7}, **Arg^{8}**, Gly-NH2^{9}	0.87 ± 8	1.11 ± 27	0.43 ± 12
TGOT	Cys^{1}, Tyr^{2}, Ile^{3}, **Thr^{4}**, Asn^{5}, Cys^{6}, **Gly^{7}**, Leu^{8}, Gly-NH2^{9}	0.04 ± 32	>1,000	>10,000

Table 4. Nonapeptides used for treatments: aminoacid sequences and affinity for murine receptors expressed in the brain. Aminoacidic substitution with respect to the sequence of OXT are indicated in bold. Data are from (Busnelli et al 2013).

DISCUSSION

I. GENERAL ISSUES

Social and cognitive aspects of the *Oxtr*$^{-/-}$ mouse behavior recapitulate the key features of the autistic phenotype, including both core symptoms (social interaction and communication deficits and repetitive behaviors) and some common co-occurring conditions (aggression and susceptibility to seizures) (Sala et al 2011). Even though a strong genetic correlation between oxytocin system and ASD has not been highlighted to date (with the exception of a common polymorphism with weak impact; (Wu et al 2005)), our model could be useful to understand the underlying mechanism of shared patophysiological aspects and to design proper treatments. Indeed, intranasal OXT treatment is currently tested on ASD patients, giving quite promising results (Ellenbogen et al 2013, Ellenbogen et al 2012, Guastella et al 2010).

Given the well-known involvement of OXT in sociability, *Oxtr*$^{-/-}$ mice have been extensively characterized at the social level, highlighting deficits in a wide variety of behaviors, including social interaction, social memory, aggression and affiliative behavior (Sala et al 2011, Takayanagi et al 2005).
On the contrary, less attention have been paid to the **cognitive aspects** of *Oxtr*$^{-/-}$ mice behavior, as, more in general, to the implication of OXT in cognitive processes.
Our previous analysis of the *Oxtr*$^{-/-}$ mice revealed an important deficit in **cognitive flexibility**, that is the ability of modifying the behavior in response to changes in the environment. This aspect of the *Oxtr*$^{-/-}$ mouse phenotype is very relevant, since, in humans, impairments in flexibility, e.i. repetitive/compulsive behaviors, or as resistance to change, are typical symptoms of ASD, schizophrenia, anorexia nervosa, obsessive-compulsive disorders and attention deficit hyperactivity disorder (ADHD). Different forms of behavioral flexibility can be evaluated: in our mouse, in particular, we analyzed the *reversal of learning*, which is the ability of switching the stimulus-reinforcement association within a single dimension. This process could be tested in rodents with a T-maze test task, as we did for our experiments, by switching the previously learned position of the food reward on the other arm of the maze (Floresco & Jentsch 2011, Ragozzino 2002). A more complex version of the reversal of learning is the *set-shifting*. This cognitive process requires a change in the strategy used or a switch in the attention to the

variable of the stimulus which is relevant for the association with the reward (Dias et al 1996). More fundamental forms of flexibility exist as well, the *response inhibition* and the *extinction learning*. Response inhibition is the control and rapid suppression of an output action and is required, at some point, for all forms of behavioral flexibility (Eagle et al 2008); the process of extinction consist of the inhibition of a previously acquired association and imply a new learning rather than the erasure of the previous one (Bouton et al 2006, Robbins 1990). Anyway, most forms of flexibility seems to be stricktly connected to the **learning strategy** used. (Kleinknecht et al 2012).

Oxtr$^{-/-}$ mice were tested in an appetitive-based T maze test. To solve this spatial learning task, mice have to learn the location of food in either arm of the maze (acquisition phase) and then they have to revert this learning, when food location is switched in the other arm (reversal phase). In the acquisition phase mice can use either an allocentric **place strategy**, based on spatial cues and orientation, or an egocentric **procedural strategy**, based on habits or motor sequences (Packard & McGaugh 1996, Restle 1957). The place learning was found to rely mostly on hippocampal funtion, whereas the dorsolateral portion of striatum mediate procedural learning (Hirsh 1974, Mishkin M. 1984, Packard M.G. 1987). *Oxtr*$^{-/-}$ mice did not display any deficit in the acquisition phase but were severely impaired in the reversal phase.

The analysis of dendritic spines during the different phases of this test highlighted an enhanced connectivity and **overuse of the dorsolateral striatum** in *Oxtr*$^{-/-}$ mice, which could possibly mediate the emergence of habit-like symptoms and cognitive rigidity. On the other hand, such an overreliance on striatal function could represent a compensative mechanism for an **impaired hippocampus**, as it was demonstrated for Oprd1$^{-/-}$ mice (Le Merrer et al 2013). Discriminating the contribution of the hippocampal versus striatal strategy to the spatial learning of our mice could help us to clarify this issue.

This could be done at the **behavioral level** using, for instance, a cross-maze test task supplied with cues, by switching the starting point (north to south) after learning takes place and then observing the trajectory followed by the animal. An animal who learned using a place strategy will orientate in the maze and reach the arm that was baited before; the one who used a procedural strategy will follow the same route he learned in the previous phase (i.e.: turn right), thus reaching the opposite arm (Block et al 2007). Alternatively, either approach could be highlighted, by using more specific learning tests in which the contribution of other cognitive

process is limited. For example, the hippocampal place learning greatly prevail in the object recognition test, while the accelerated rotarod test rely more on striatal motor learning (Le Merrer et al 2013).

Furthermore, the presence of an hippocampal dysfunction could be confirmed at the **molecular level**, as well. The imbalance between excitatory an inhibitory synapses observed in *Oxtr*$^{-/-}$ hippocampal neurons (Sala et al 2011), represents, indeed, the first neurobiological substrate of such dysfunction. Present work highlights further aberrations of *Oxtr*$^{-/-}$ hippocampal neurons, concerning the expression of Kir channels, which are involved in membrane excitability, and KCC2, which is responsible for the switch of GABA action. Although electrophysiological confirmations are still needed, these data give important indications that the **E-I balance** is altered at multiple levels in *Oxtr*$^{-/-}$ hippocampal neurons, thus possibly impacting hippocampal functions. Importantly, we identified an OXTR-mediated pathway modulating KCC2 expression with long-term effect, thus possibly being able to restore a correct E-I balance. This observation is particularly intriguing, since E-I imbalance have been frequently associated with several neuropsychiatric disorders, such as autism, schizophrenia and epilepsy (Markram & Markram 2010, Rubenstein 2010, Rubenstein & Merzenich 2003, Vattikuti & Chow 2010, Yizhar et al 2011). This proposed neurophysiological substrate could be the consequence of a wide range of seemingly unrelated genetic abnormalities and could account for social and cognitive deficits observed in patients and in animal models of such disorders.

II. STRIATAL OVERRESPONSIVENESS OF *OXTR*$^{-/-}$ MICE:

The source of habit-like symptoms?

Oxtr$^{-/-}$ mice didn't show any significant constitutive alteration in dendritic spine density in any of the region analyzed. However, neuronal morphology appeared to be altered in the dorsolateral striatum (DLS), where the MNS of *Oxtr*$^{-/-}$ mice have 20% longer dendrites compared to *Oxtr*$^{+/+}$ ones. As spine density in this area is not different between the two genotypes, longer dendrites imply a greater number of spine/neuron in *Oxtr-/-* mice.
The dorsolateral portion of striatum is involved in procedural (or response/habit) learning and memory (Lovinger 2010, Yin et al 2009). Thus, alterations in neuronal morphology in this area

could reflect modifications in motor learning skills and could be linked to repetitive behaviors typical of autistic patients (Langen et al 2013).

This enlargement of MSN dendritic arbor may be likely attributable to alterations occurred during circuitry development and/or to compensatory mechanisms balancing alterations occurred in other areas. Consistently with our observation, an enlargement in MSN dendritic arbors was described also in the Nucleus Accumbens (NAcc; the ventral portion of striatum) of prenatally-Valproic acid-exposed rats, a typical ASD model (Bringas et al 2013). Interestingly, also the $Emx^{Cre}/Met^{fx/fx}$ mouse, another model of ASD, showed an analogous alteration of MSN morphology in the DLS. This mouse have forebrain-restricted deficiencies in the signaling of Met, the HGF (hepatocyte growth factor) receptor, which is, indeed, associated with ASD (Campbell et al 2008, Campbell et al 2006, Jackson et al 2009).

The consistency of striatal neurons alteration among different ASD models suggests a prominent role of this region in the emergence of autistic-like symptoms.

In particular, in the abovementioned studies the altered MSN arborization was interpreted, as a sign of the local hyperconnectivity, frequently reported in autism (Casanova 2006, Casanova et al 2002, Wass 2011).

We hypothesized that the enlargement of MSN arbors in this area reflects an enhanced aptitude of this mice for procedural approaches to experience. Indeed, our subsequent observations, indicate a prominent use of DLS by *Oxtr*$^{-/-}$ mice in different behavioral paradigm. In particular, *Oxtr*$^{-/-}$ mice didn't show the expected hippocampal spine remodeling in response to an environmental stimulus (Jenkins et al 2004, Rinaldi et al 2010); on the contrary, they display only a striatal response. Notably, also the subsequent exposition to a new complex environment, during the habituation phase, produced a net change of spine density in striatum only in *Oxtr*$^{-/-}$ mice, confirming an overresponsiveness of this region in the knockout mice.

The consequence of an hippocampal deficit?

Recently, a similar "striatal preference" was described in mice lacking the delta opioid receptor (Le Merrer et al 2013). These mice were more prone to solve tasks using a response strategy and showed an enhanced motor skill learning; on the contrary, they were impaired in spatial learning tasks. Since the two systems (hippocampus and striatum) compete in driving learning behavior (Middei et al 2004, Schroeder et al 2002), authors hypothesize that compromised

hippocampal functions in Oprd1$^{-/-}$ mice facilitated the striatal-based procedural learning (Le Merrer et al 2013).

Considering this issue, we postulate that the striatal overuse of our knock-out mouse could possibly arise from an hippocampal deficit, that we identified as the absence of an hippocampal response to spatial novelty exposition. The lack of impairment they showed in the acquisition phase of the T-maze task (Sala et al 2011) is not conflicting with this hypothesis, since the protocol used allows the use of either a place (hippocampal) or a response (striatal) strategy**.** To verify decisively the presence of an hippocampal impairment we are currently performing an object recognition task relying exclusively on hippocampal functions. This task has also the additional advantage of not being appetitive-driven. The lack of food or other kind of reward, indeed, prevents possible confounding effects on striatal circuits. As expected, preliminary results confirm the deficit in hippocampal functions, as *Oxtr*$^{-/-}$ mice perform worse than *Oxtr*$^{+/+}$ ones in this learning task.

Or both?

A striatal compensation of hippocampal learning deficiencies could also represent the neurobiological basis for reduced cognitive flexibility of our mouse model. Indeed, it has been recently demonstrated that in mice (C57BL6) spatial cognitive flexibility requires a place learning strategy, which in turn needs an intact hippocampus (Kleinknecht et al 2012). Findings supporting this evidence have been reported also for rats, even though these animals with a prolonged training, manage to reverse a response learning (McDonald et al 2001, Ragozzino et al 1999).

In humans the involvement of both hippocampal and striatal memory systems have been postulated to contribute to the emergence of habit-like symptoms in neuropsychiatric disorders (such as Tourette syndrome, eating disorders and ASD). A particularly interesting hypothesis is that functional and anatomical abnormalities in the hippocampus may be responsible, not only for deficits in hippocampal-dependent memory, but also for maladaptive and habitual symptoms of these disorders, by contributing to an overreliance on the habit learning system (Goodman et al 2013). Importantly, this would be in perfect agreement with what we observed in our ASD model.

III. ABERRATIONS IN HIPPOCAMPAL *OXTR*$^{-/-}$ NEURONS:

Since in *Oxtr*$^{-/-}$ hippocampal neurons an imbalance between Glutamatergic and GABAergic synapses was previously described, we decided to verify if hippocampal dysfunctions could arise from alterations of the E-I balance in hippocampal neurons.

A matter of E-I balance?

Since **dendritic spines** are the main site of excitatory synapses, we started the evaluation of *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ primary hippocampal cultures from the morphological characterization of dendritc arbors and spines. This analysis reveal no evident alterations in the morphology of neurons, confirming what we observed in adult neurons with the Golgi-staining.

Developing and adult hippocampi do not express a high level of OXTR; consistently, we found low **levels of OXTR** in our wild-type hippocampal cultures. Nonetheless, the deletion of the OXTR was able to induce an hippocampal upregulation of V1aR in embryonal neurons as well as in the adult brain, as we previously reported (Sala et al 2011).

Subsequently, we focused our attention on some of the proteins involved in the setting and maintenance of the excitation-inhibition balance. The first relevant finding observed is a generalized **upregulation of Kir2 and Kir3** channels in *Oxtr*$^{-/-}$ hippocampal neurons. Indeed, these channels are important regulators of membrane excitability and resting potential.
In *Oxtr*$^{-/-}$ cultures both Kir2 and Kir3 channels are concomitantly upregulated; since these channels are in different conformations (closed and open, respectively) at resting potential, we cannot guess the prevaling effect on cell excitability without performing electrophysiological recordings. Several relevant neuronal features, other than excitability, have been correlated with Kir channels alterations (Ciruela et al 2010, Kleene et al 2010). Recently, for example, a Kir2 downregulation has been reported in the striatum of mice overexpressing the D2 dopamine receptor(D2R); interestingly, these mice exhibit also reduced arborization of MSN in the dorsolateral striatum. Moreover, the authors demonstrated that the decrease in dendritic arborization is a direct consequence of reduced Kir2.1 expression, because the overexpression

of a dominant-negative form of this channel was sufficient to produce morphological alterations observed in the D2R-overexpressing mice (Cazorla et al 2012). This suggests that the enlargement of striatal MSN's arbor observed in *Oxtr*$^{-/-}$ mice could be a consequence of Kir2 overexpression. However, to confirm this hypothesis we would need to repeat the analysis of Kir channels expression in the same structure and developmental stage in which we observed the morphological alterations, i.e. in the adult striatum.
Furthermore, the evidence of D2R overexpression causing Kir2 downregulation, is relevant also because dopamine receptors have been found to dimerize with oxytocin receptor (Romero-Fernandez et al 2013). Consequently, the absence of OXTR in our model could possibly impact also dopamine receptor expression and, vice versa, D2R overexpression could affect OXTR density, giving a reasonable explanation to consistent finding in term of neuronal morphology and Kir channels expression.

Proceeding with the evaluation of excitation-inhibition balance, we analyzed one of the most relevant events that happens in neurons during early development, the excitatory-to-inhibitory switch in GABA actions. This switch takes place also in hippocampal cultures, around DIV11 (Ganguly et al 2001) and is mediated predominantly by an increased membrane expression of KCC2 protein (Rivera et al 1999).
Consistently, *Oxtr*$^{+/+}$ neurons showed a developmental increase in **KCC2 expression**, both at transcriptional and translational level. On the contrary, *Oxtr*$^{-/-}$ display only a partial non significant upregulation of this transporter during in vitro development. These data could indicate a delayed and/or incomplete switch of GABA actions, that needs electrophysiological confirmations. At least two different scenarios could be imagined: one in which only few hippocampal neurons display inhibitory action of GABA at the proper developmental stage; another one in which most neurons have an intermediate electrochemical gradient for chloride such that neither depolarization nor hyperpolarization of plasma membrane could be achieved upon $GABA_AR$ activation. Clearly, both scenarios imply important alteration in excitability and transmission among developing neurons, having potentially a great impact on brain circuitry formation. Indeed, on one hand, the excitatory action of GABA during development is necessary for system maturation (Ben-Ari 2002), on the other hand GABAergic inhibition in mature neurons is essential to prevent hyperexcitability and excitotoxicity. Moreover, the onset of GABAergic inhibition participates in the regulation of later stages of neuronal development (Liu et al 2006).

Noteworthy, KCC2 has been frequently associated with epilepsy (Huberfeld et al 2007, Munoz et al 2007, Palma et al 2006). Mice with heterozygous deletion of KCC2 gene have increased susceptibility to seizures (Woo et al 2002) and KCC2 reduction is often observed in *ex vivo* tissues from epileptic patients (Huberfeld et al 2007, Palma et al 2006). Therefore, our findings of dysregulated KCC2 expression in neurons from *Oxtr*$^{-/-}$ mice is consistent with the previously identified increase in seizure susceptibility of this mouse model (Sala et al 2011).

Modulating the switch of GABA through an OXTR-mediated pathway?

An association between oxytocin and the switch of GABA polarity had been also previously shown (Tyzio et al 2006). Indeed, Tyzio and coworkers identified a transient excitatory-to-inhibitory switch of GABA action during labor, and demonstrated that it was mediated by the burst of maternal OXT release. Authors evaluated the possibility of an OXT-mediated modulation of KCC2 expression, by giving an OXTR antagonist to a pregnant rat (starting from E18) and subsequently evaluating KCC2 mRNA levels in the hippocampi of newborn pups immediately after birth. However, this transient receptor antagonism was not sufficient to produce any significant change, even though looking at their graph, a decreasing trend could be seen.

Therefore, we decided to check if KCC2 expression could be modulated by OXTR, by treating our *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons with OXT. Interestingly, we found a 50% increase in the KCC2 protein level in neurons exposed to three daily OXT treatment (100nM) starting from DIV3 (the "burst" protocol). Notably, this upregulation was observed, starting from 24 hours after the end of the burst protocol, but it was also maintained later on: at DIV11, five days after the end of treatments, and also in more mature neurons, at DIV14, even if at this time point it did not reach statistic significance.

Quite surprisingly, chronic daily treatment with OXT failed to produce any increase in KCC2 expression in our *Oxtr*$^{+/+}$ neuronal cultures. Actually, this could have a simple explanation in the desensitization and internalization of OXTR due to sustained and prolonged activation (Conti, 2009), as it has been recently described for chronic intranasal OXT administration (Huang et al 2013).

Most importantly, when applied on *Oxtr*$^{-/-}$ neurons, neither burst nor chronic OXT treatment were able to modify KCC2 expression, indicating that OXTR is necessary for mediating this OXT-induced effect.

Finally, our preliminary results indicate that an acute OXT treatment (100 nM) is sufficient to produce the increase in KCC2 level in immature (DIV8) *Oxtr*$^{+/+}$ neurons and that this upregulation needs more than 24 hours to take place. The long latency of this effect suggests that several transcription and/or translation steps may be necessary.

On the contrary, OXT effect on the transient switch of GABA during labor is very rapid, suggesting a post-translational mechanism on CCCs function, possibly mediated by NKCC1, because this effect is occluded by the concomitant administration of Bumetanide, a selective NKCC1 blocker (Tyzio et al 2006). Considering these data together, we cannot exclude a double mechanism underlying OXT effects on this process: a rapid post-translational modulation of either NKCC1 or KCC2 function and a slower, long-lasting modulation of KCC2 expression.

This link between our autism model and the CCCs is particularly relevant in light of positive results obtained by recent clinical trials in which Bumetanide was administered to infants with autism (Lemonnier & Ben-Ari 2010, Lemonnier et al 2012).

Among the various OXTR-mediated signaling molecules that could be involved in KCC2 modulation, we decided to start by analyzing the elongation factor eEF2. eEF2 has been associated with the synthesis of BDNF (Verpelli et al 2010), which is one of most important regulator of KCC2 expression (Aguado et al 2003, Carmona et al 2006, Ludwig et al 2011, Rivera et al 2002, Uvarov et al 2006). eEF2 is an important ubiquitary regulator of protein synthesis, and in neurons it is able to regulate local dendritic protein synthesis in an activity-dependent manner (Chotiner et al 2003, Park et al 2008, Sutton et al 2007). To the best of our knowledge, the effect of OXT treatment on eEF2 activity have never been studied in neurons; conversely, in myometrial cell, OXT was found to induce eEF2 activation by promoting its dephosphorylation (Devost et al 2008, Devost et al 2005).

Our results indicate that in *Oxtr*$^{+/+}$ neuronal cultures, both oxytocin and vasopressin actually inactivate eEF2, inducing its phosphorylation, and that the two peptides display similar dose-response curves. Applying the same treatments on *Oxt*$^{-/-}$ neurons, we also observed increases in eEF2 phosphorylation, suggesting that this effect is OXTR-independent and could possibly rely on V1a/V1b vasopressin receptors activation. This hypothesis was also supported by the

lack of effect that we obtained by treating neurons with a selective OXTR agonist, Thr_4Gly_7-OT (TGOT, 10nM).

It has been reported that some dendritic mRNAs such as CaMKII and Arc, are more efficiently translated when general protein synthesis is slowed down, i.e. when eEF2 is phosphorylated (Belelovsky et al 2005, Chotiner et al 2003, Marin et al 1997, Park et al 2008, Scheetz et al 2000). However, this could not be the case for KCC2. Indeed, in *Oxtr*$^{-/-}$ neurons OXT is still able to elevate p-eEF2, whereas it has no effect on KCC2 expression, suggesting that eEF2 pathway is not involved in OXT-mediated KCC2 upregulation.

Still, its participation in a functional modulation of CCCs cannot be ruled out.

IV. CONCLUSIONS

Taken together our data suggests a possible mechanism underlying the cognitive deficits of our murine model of ASD, the oxytocin receptor knock-out mouse. Since autism has highly heterogeneous etiology and clinical forms, unraveling the pathophysiology of shared behavioral aspects, could provide useful information for designing treatments aimed at controlling specific symptoms. In particular, we highlighted in the *Oxtr*$^{-/-}$ mouse a striatal overuse, possibly arising from hippocampal dysfunction, and proposed it as substrate for habit-like symptoms and cognitive rigidity of *Oxtr*$^{-/-}$ mouse. At the molecular level we identified in *Oxtr*$^{-/-}$ hippocampal neurons aberrations regarding the setting and maintenance of E-I balance, which could be the sources of functional deficits observed in the adult mouse. Particularly interesting is the lack of the physiological upregulation of KCC2 during development, that presumably leads to aberrant GABAergic signaling in mature neurons. Furthermore, we disclosed a long-term effect of OXT treatment on the modulation of KCC2 expression, in developing hippocampal neurons. It will be interesting to test if such OXT effect could rescue E-I balance impairment in other model of neuropsychiatric disease.

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