PhD Program in Translational and Molecular Medicine

Role of oxytocin signaling in the development and function of hippocampal neurons

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"Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e conoscenza".

La Divina Commedia-Inferno-Canto XXVI
Dante Alighieri-

TABLE of CONTENTS

Chapter 1

General Introduction

1. The oxytocin system

Substantial progress has been made in recent years in the understanding of how the mammalian brain executes higher functions and how the dysfunction of brain regulatory systems can lead to neurodevelopmental disorders in humans. One system that has generated particular interest due to its therapeutic relevance is operated by two neuropeptides, oxytocin (Oxt) and vasopressin (Avp). Oxytocin and Avp are highly homologous peptides, mostly known for their peripheral physiological effects. The former plays a key role in parturition and lactation, whereas Avp regulates body fluid homeostasis. However, Oxt and Avp and their respective receptors are also expressed in the central nervous system (CNS), where they exert different modulatory effects on complex behaviours. Accordingly, they have been referred to as "social'" neuropeptides as they play a key role as mediators of complex inter-individual recognition and interaction in mammals. In addition, alterations in the Oxt system have been associated with several psychiatric and neurological diseases, such as autistic spectrum disorders (ASDs). Thus, it is a widespread view that a better understanding of the Oxt system could in the future provide potential clues leading to the development of novel therapeutic strategies for these diseases.

1.1 Structure, synthesis and release of oxytocin

Oxytocin is a neurohypophyseal hormone discovered by Sir. Hanry Dale back in 1906. Subsequently, Oxt has been the first peptide hormone to be sequenced and chemically produced in a biologically active form (Du Vigneaud et al., 1953). The name Oxt derives from the Greek word (ωκνξ, τοκοχξ) meaning "quick birth" after its uterotonic (contraction-inducing) activity was discovered and described (Dale, 1990).

Oxytocin is composed of nine amino acids (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2) with a disulphide bond between the two cysteines at positions 1 and 6. The sequence of Oxt is very similar to that of Avp, also a nonapeptide that differs from Oxt by only two amino acids (Phe and Arg at positions 3 and 8, respectively; Lee et al., 2009). Both peptides are extremely well conserved during evolution (Acher et al., 1995; Caldwell and Young, 2006). Accordingly, in all animals the genes for Oxt and Avp are located on the same chromosome (2 in mice and 20 in humans), although their transcription occurs in opposite directions (Mohr et al., 1988), indicating that the two genes derived from the duplication of a common ancestor and an inversion between the two then occurred (Figure 1). Structurally, the two genes are separated by an intragenic region (IGR) varying in length across different animal species (11 kb in rat and human and 3.6 kb in mice). Within the IGR there are regulatory DNA sequences for both Oxt and Avp (Gainer et al., 2001; Young and Gainer, 2003, 2009; Lee et al., 2009).

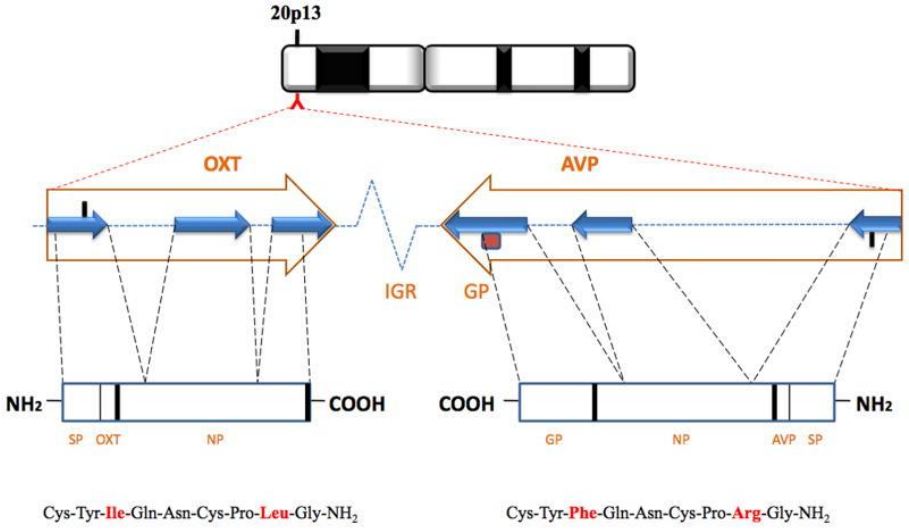

Figure 1. Schematic organization of Oxt and Avp genes in the human genome. The genes for Oxt and Avp are located in the same chromosome in all species separated by an intergenic region (IGR). Both genes contain three exons shown as blue arrows that are separated by two introns (shown as dotted lines). They encode for a pre-prohormone consisting of a signal peptide (SP), a neuropeptide (OXT or AVP), a neurophysin (NP) and, in the case of Avp, a glycopeptide (GP). (Image from Caldwell et al., 2008; Lee et al., 2009).

As shown in Figure 1 Oxt is synthetized as a pre-propeptide sequence that consists of the signal peptide, the nonapeptide and the carrier protein neurophysin (Gimpl and Farenholz, 2001; Lee et al., 2009). The human gene for Oxt is on chromosome 20p13 (Rao et al., 1992) and it is composed of three exons. As depicted in Figure 1, the first exon contains the sequences for the translocator signal, the nonapeptide hormone, the signal peptide, and the first residues of neurophysin; the second exon encodes the middle part of neurophysin; and the third exon contains the sequence for the COOH-terminal region of neurophysin (Gimpl and Farenholz, 2001; Lee et al., 2009). During the

transport down the axons to the neurohypophysis the pre-propetide is cleaved and undergoes several modifications (Brownstein et al., 1980). Once transported to the posterior pituary, the mature peptide and its carrier molecule neurophysin are stored in the axon terminals until they are released upon depolarization (Renaud and Borque, 1991). The main function of neurophysin is to guarantee the proper targeting, packaging and storage of Oxt within the secretory granules before its release into the bloodstream (Gimpl and Farenholz, 2001). In fact, the binding strength of the Oxt-neurophysin complex is pH-dependent. It is high in the neurosecretory granules where the pH is high, but low in the plasma where the pH is more acidic thus facilitating the dissociation of the two peptides (Rose et al., 1996).

Oxytocin and Avp are mainly synthetized in the large (magnocellular) neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus but additional production takes place in the accessory nuclei situated between the PVN and SON (Gimpl and Farenholz, 2001, Stoop, 2012; Benarroch, 2013). These neurons send their projections to the posterior pituitary gland where Oxt and Avp can enter the general circulation hence reaching different peripheral organs where they exert their actions as hormones. Even if the magnocellular neurons represent the main source of Oxt and Avp, these peptides are also synthesized in the small (parvocellular) neurons of the PVN. These neurons project to different brain areas including the median eminence, the brainstem autonomic nuclei and the spinal cord (Benarroch, 2013; Stoop; 2012). As mentioned

previously, in the parvocellular and magnocellular neurons of SON and PVN, Oxt and Avp are stored in secretory vesicles or granules together with their respective neurophysin carriers (Burbach et al., 1995). More specifically, Oxt and Avp are stored in the large dense core vesicles (LDCVs). The neuropeptide-containing LDCVs are present not only at the nerve endings but also in other neuronal compartments, such as the somata, dendrites and axonal varicosities, from where they are released by exocytosis upon elevation of intracellular calcium (Ca^{2+}) (Benarroch, 2013; Stoop, 2012). Unlike the classical neurotransmitter release, which takes place at the presynaptic active zones in response to Ca^{2+} influx via N or P/Q type of voltage dependent Ca^{2+} channels (VDCCs), the release of Oxt and Avp requires high-frequency stimulation and involves Ca^{2+} influx via somato-dendritic N/L type of VDCCs and/or mobilization of Ca^{2+} from intracellular stores (Tobin et al., 2011). Thus, the release of classical neutransmitter requires a low frequency stimulation and a local increase in Ca^{2+} at the pre-synaptic bouton, whereas the peptide release is induced by a more diffuse and long lasting rise in intracellular Ca^{2+} (Hoekfelt, 1991; Stoop 2012). As mentioned before the neuropeptide-containing LDCVs can be stored in all parts of the neuron, including somata and dendrites (Stoop, 2012). For this reason magnocellular neurons of the SON and PVN, which are densely filled with LDCVs both at the somata and dendrites, can give rise to a somato-dendritic release of Oxt and Avp, in addition to the release from nerve endings (Stoop, 2012; Benarroch, 2013). However, it is still unclear how Oxt diffuse after somato-dendritic release and what concentrations are reached in the target areas.

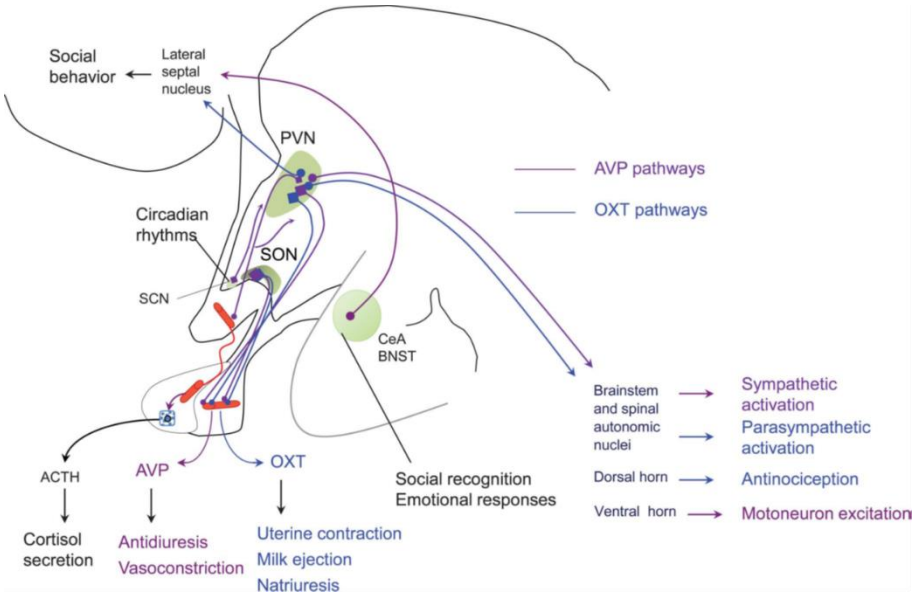

Figure 2 Schematic representation of the main release pathways of Oxt and Avp. Oxt and Avp are synthesized in the parvocellular and magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. Vaopressin (AVP) is also produced in other extra-hypothalamic brain areas such as the suprachiasmatic nucleus (SCN), the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and other regions. Oxytocin and Avp produced in the magnocellular neurons are transported to the posterior pituitary, from where the two peptides can be released into the general circulation. Circulating Avp plays a major role in controlling fluid homeostasis whereas blood Oxt exerts main effects in parturition and lactation. The axons of the parvocellular neurons send their projections to different brain areas, where these neuropeptides regulate higher brain functions, such as social behaviour, cognition and anxiety (Image from Benarroch, 2013).

Due to the different modalities by which Oxt and Avp are released, it is not at all surprising that they participate to very different types of neurochemical signalling processes, i.e. endocrine, autocrine, paracrine, and synaptic, as recently reviewed by Bennarroch (2013). Magnocellular neurons located in the SON and PVN project their axons to the posterior pituitary gland from which these peptides are released into the bloodstream where they exert hormonal functions. In particular, Avp is essential for body fluid homeostasis, whereas Oxt

exerts its main roles in parturition and lactation. However, in both the PVN and SON, Oxt and Avp are also released in a somato-dendritic manner, thus exerting a local autocrine regulation of magnocellular neurons (Benarroch, 2013). Moreover, the axons of parvocellular neurons project to several areas of the CNS, such as the olfactory system, prefrontal cortex, enthorinal cortex, amygdala, bed nucleus of the stria terminalis, hippocampus, lateral septum, autonomic brainstem nuclei and spinal cord (Stoop, 2012; Benarroch; 2013). In all these areas, the release of Oxt and Avp occurs both at pre-synaptic terminals and non-terminal axonal varicosities. In this manner, the two peptides can both participate to the classical synaptic neurotransmission as well as to a volume transmission through the diffusion via the extracellular fluid to different close and distant areas where they act on their respective receptors (de Vries et al., 2012; Stoop, 2012; Neumann and Landgraf, 2012; Benarroch, 2013). The effects of Oxt and Avp in the brain are limited by a half-life of 20 minutes in the cerebrospinal fluid which is determined by the activity of aminopeptidases that cleave the two peptides, thus producing shorter forms that in some instances can exert their own biological effects (Ludwig and Leng, 2006).

The activity of magnocellular and parvocellular neurons and the subsequent release of Oxt and Avp are induced by different stimuli (Benarroch, 2013), such as fast synaptic inputs mediated by classical neurotransmitters, such as L-glutamate and γ-aminobutyric acid (GABA) (Leng et al., 1999), local modulation provided by the

endocannabinoids (De Laurentis et al., 2010), and other signals involving neuronal-glial interactions (Tasker et al., 2012). For example, the magnocellular Avp neurons can change their activity in response to different stimuli. Indeed variations in plasma osmolarity, hypovolemia and hypotension determine a switch in the activity of Avp-secreting neurons from slow irregular firing to phasic burst firing (Benarroch, 2013). This mechanism is triggered by various synaptic inputs from several brain areas such as the neurons of the subfornical organ and the ascending inputs from noradrenergic neurons of the caudal ventrolateral medulla (Leng et al., 1999). Magnocellular Oxt neurons exhibit a tipical burst-like firing activity during parturition and upon the milk ejection reflex (Hirasawa et al., 2004), but can also be activated following alteration in blood hosmolarity (Sladek and Song, 2008). For example, microdialysis studies have shown that social stimuli, both reproduction-dependent and -independent, as well as stresses, trigger Avp and Oxt release within the CNS, not only in the SON and PVN but also in the amygdala, hippocampus and lateral septum (Landgraf and Neumann, 2004). Reproduction-related stimuli include mother-offspring interactions, maternal aggression and socio-sexual interaction like mating both in males and females (Neumann et al., 1993; Lévy et al 1995; Bosch et al., 2005; 2004; Lukas et al., 2011). In these conditions the central release of Oxt is essential to guarantee the individual's emotional stability (Neumann, 2006; Lukas and Neumann 2013). Moreover, Oxt and Avp are also released in response to non-reproductive stimuli, such as the exposure to an unknown adult or juvenile conspecific (Lukas and Neumann, 2013).

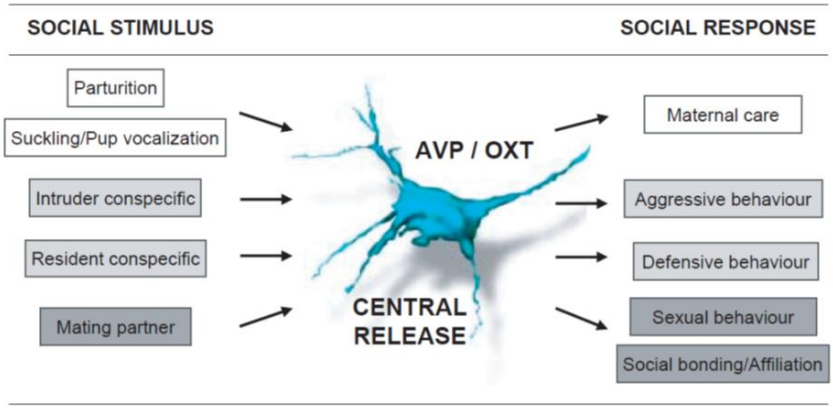

Figure 3 Scheme of the main Oxt- and Avp-mediated effects on social behaviour triggered by different social stimuli (Image from Veenema and Neumann, 2008).

Because Oxt and Avp are so closely related it has been proposed that they evolved with the purpose of interacting between each other to play opposite physiological roles. For instance, both peptides have been shown to affect the control of the autonomic nervous system, with Oxt primarily exerting a parasympathetic activity whereas Avp acting as an important regulatory component of the sympathetic nervous system (Kenkel et al., 2012; Saechenko and Swanson, 1985). For all these reasons, it is important to consider the role of Avp when investigating the Oxt system, even if the present study will mainly focus on the latter.

1.2 The oxytocin receptor: intracellular signalling and distribution

Oxytocin exerts its peripheral and central effects by specifically activating its own receptor (Oxtr). Although there are several

receptors for Avp, i.e. V1a (Avpr1a), V1b (Avpr1b) and V2 (Avpr2), there is only one receptor for Oxt (Caldwell et al., 2008). Similar to their respective peptides, Oxtr and Avprs share a high sequence homology, hence Oxt and Avp are rather unselective ligands being able to interact as partial agonists on the other receptor. Both Oxtr and Avprs belong to the rhodopsin-type (class I) G protein-coupled receptor (GPCR) superfamily (Gimpl and Fahrenholz, 2001), whose members possess seven transmembrane domains, three extracellular and three intracellular loops (Figure 4).

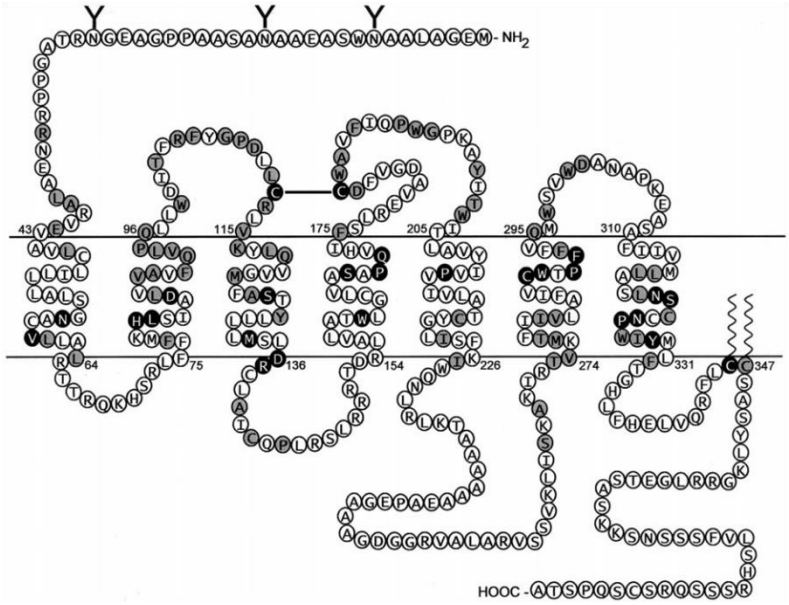

Figure 4. Schematic structure of the human oxytocin receptor [Image adapted from Gimpl and Farenholz, 2001].

It has been hypothesized that there may be different Oxtr subtypes (Page et al., 1990), thus explaining the different pharmacological features of Oxtr in different tissues, such as the uterus (Chan et al., 1993; Chan et al., 1994), kidney (Airpin et al., 1997) and brain (Adan et al., 1995, De Wied et al., 1993). However, there is no evidence to support such a hypothesis, and thus far only one Oxtr has been reported. The *Oxtr* gene, encoding a protein sequence of 389 amino acids, is present as a single copy in the human genome mapping on 3p25-3p26 (Inoue et al., 1994; Michelini et al., 1995; Simmons et al., 1995). The gene is 17 kb long and contains three introns and four exons (Figure 3). Exon 3 and 4 contain the sequence encoding the Oxtr. These two exons are separated by the long intron 3 (12 Kb) following the coding region for the transmembrane domain 6. Exon 4 encodes the 7th transmembrane domain, the COOH-terminus and the entire 3'-non coding region (Figure 5; Gimpl and Fahrenholz, 2001).

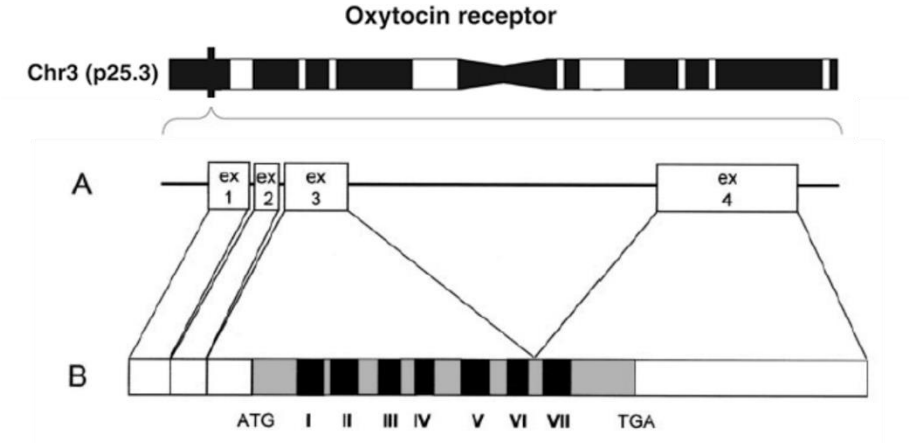

Figure 5 Schematic organization of the human oxytocin receptor gene. [Image modified from Inoue et al., 1994].

A sequence approximately 1000 bp upstream of the coding region is required for Oxtr expression, as shown by deletion experiments (Inoue et al., 1994; Ebstein et al., 2012). The transcription starts at 618 and 621 bp upstream of the methionine initiation codon, in close proximity to a TATA-like motif and a potential SP-1 binding site. In these regions other binding sites for transcriptional regulatory factors, such as AP-1, AP-2, GATA-1, Myb, nucleofactor-interleukin 6 binding consensus sequence and an acute phase reactant-responsive element, are also present (Ebstein et al., 2012; Gimpl and Fahrenholz, 2001). Within the promoter region there is also a three half-palindromic estrogen-responsive element motif (Inoue et al., 1994) that can act synergistically to bind the estrogen receptor (Kato et al., 1992). It has been demonstrated that the Oxtr is up-regulated by estrogens and down-regulated by progesterone (Murata et al., 2000). It is thought that the presence of the estrogen-responsive element motif explains why Oxtr undergoes a time-specific up- and down-regulation in different cell types. For instance, in the uterus the expression of the Oxtr is increased by two-fold during gestation, thus enhancing the local sensitivity to Oxt (Soloff et al., 1979). After birth, the number of uterine Oxtrs decreases whereas their expression in the mammary gland remains elevated during lactation (Soloff et al., 1979; Breton et al., 2001). This tissue-specific regulation of the receptor makes Oxt able to selectively act on different target organs within a defined time frame, thus allowing uterine contraction during birth and milk production soon after birth.

G protein-coupled receptors perceive many extracellular signals and transduce them to the respective cognate heterotrimeric G proteins, which in turn activate the downstream effectors to trigger various signalling cascades. The Oxtr can couple to different G proteins leading to different functional effects (Figure 6).

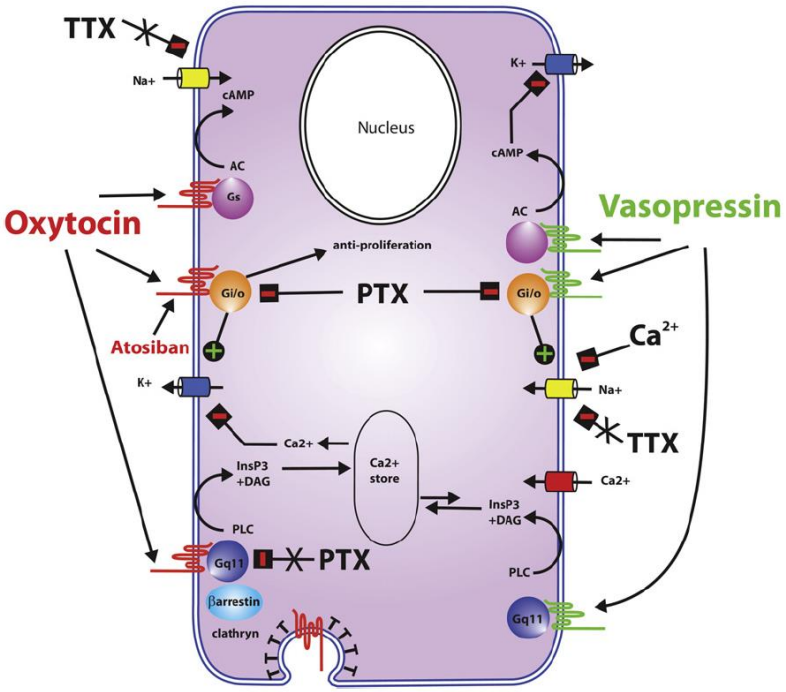

Figure 6 Intracellular pathways of Oxt and Avp singnaling. Summarized in the picture are the main pathways triggered by the activation of Oxtr or Avprs according to the specific cognate G proteins they are coupled with (Image adapted from Stoop 2012).

The Oxtr is mainly coupled to $G_{q/11}$ and triggers phospholipase-Cβ (PLCβ) activation that controls the intracellular production of inositol-trisphosphate (IP3) and diacylglycerol (DAG). In turn, IP3 leads to the

release of Ca^{2+} from intracellular stores hence the activation of protein kinase C (PKC). Peripherally, this pathway is involved in uterine smooth muscle contraction (Alberi et al., 1997), nitric oxide production, and cardio-myogenesis (Danalache et al., 2010) and, in central neurons, leads to the inhibition of inwardly rectifying K^+ channel conductances (Gravati et al, 2010). In many cell types DAG can also interact with other targets, such as protein kinase D (PKD), diacylglycerol kinases (DKG) β and γ, Ras guanyl nucleotide-releasing proteins (RasGPRs), chimerins and mammalian uncoordinated 13 (Munc13s) (Kazanietz et al., 2002; Yang and Kazanietz, 2003; Brose and Rosenmund, 2002). In neurons, Oxt can also induce an inwardly rectifying current through the coupling with a pertussis–sensitive $G_{i/o}$ protein (Gravati et al., 2010). In addition, Oxt can activate adenylyl cyclase via a G_s protein hence increasing cAMP production, that eventually can promote a sodium-dependent TTX-resistant sustained inward current independently from PKA activation (Alberi et al., 1997). Like most other GPCRs, Oxtr undergoes desensitization following a persistent stimulation by agonists. In fact, following Oxt activation, Oxtrs are phosphorylated by G protein-coupled receptor kinase (GRK)-2, and, after binding to β-arrestins, they are endocytosed via clathrin-coated vesicles (Smith et al., 2006). After being internalized, Oxtrs recycle back to the cell surface via the Rab4/Rab5 short cycling pathway (Conti et al., 2009). The intracellular trafficking of the Oxtr is thought to underlie the rapid desensitization that results after receptor activation (Stoop, 2012).

The Avpr1a and Avpr1b subtypes, similarly to Oxtr, are coupled to $G_{q/11}$, whereas Avpr2 couples to G_s. Noteworthy, a significant cross reactivity exists between Oxt and Avp ligands, despite the specific definition proposed by the receptor nomenclature. In fact, Avp can bind the Oxtr with a similar affinity than own Avprs. On the other hand, although Oxt exhibits a higher affinity for Oxtr, it is still capable to bind Avprs with 100-fold lower affinity (Mouilliac et al., 1995; Manning et al., 2012). It has been shown that heterodimeric complexes can form between Avpr1a, Avpr1b, Avpr2 and Oxtr (Cottet et al., 2010; Devost and Zingg, 2003; Terrillon et al., 2003). The formation of heterodimers can have important *in vivo* consequences and eventualy confuse the interpretation of peptide effects. In fact, this introduces a further element of combinatorial variability regarding the identity of the receptor type that is activated either physiologically or experimentally (Ebstein et al., 2011).

The expression of the Oxtr and Avprs vary among different species (reviewed Stoop, 2012; Benarroch, 2013). In the periphery, the expression of Avpr1a has been detected in smooth muscle, liver and platelets, whereas Avpr1b is mainly found in the anterior pituitary, and Avpr2 is more abundant in the kidney. Oxtr is localized in the uterine smooth muscles and in the myo-epithelial cells of the mammary gland (Gimpl and Farenholz, 2001). However, Oxtr, Avpr1a and Avpr1b are also expressed in the CNS (Tribollet, 1999; Stoop, 2012). Due to the lack of specific and reliable antibodies against Oxtr and Avprs, the expression levels have been mainly determined by radioligand binding

studies that depend on the availability of specific labelled agonists and antagonists (Stoop, 2012). Using such technique in rodents it has been shown that Avpr1a is expressed in the olfactory system, neocortex, basal ganglia, dentate gyrus, central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis, ventromedial hypothalamus, lateral septum, thalamus, circumventricular organs, and autonomic and motor nuclei of the brainstem and spinal cord (Raggenbass, 2008). The Avpr1b has only been found in the dorsal one-third of pyramidal cells of the CA2 region, in few cells within the anterior amygdala and in the PVN (Young et al., 2006). The expression of Oxtr is predominant in the accessory olfactory system, CeA, CA1 and CA3 regions of the hippocampus, ventromedial hypothalamus, nucleus accumbens, autonomic nuclei of the brainstem, and dorsal horn. In some of these regions, the receptors are expressed in a complementary way. One example of this complementarity is found in the amygdala, where Avpr1a is expressed in the medial part of the CeA whereas Oxtr is expressed in the lateral part (Stoop, 2012; Goodson and Thompson, 2010; Benarroch, 2013). It has been recently proposed that this complementarity in the amygdala is the basis of the opposite effects exerted by Avp and Oxt on fear (Huber et al., 2005). Different studies, mainly using autoradiography, have shown that the distribution of Oxtr and Avprs is quite different between rodents and humans (Loup et al., 1991). The autoradiographic investigations of human tissues typically use the same radiolabeled probes employed in rodents, although it is clear that human and rodent receptors do not exhibit the same binding properties (Stoop, 2012). In addition, the studies on

human brain are usually performed at much longer post-mortem times as compared to rodent studies and it is unknown whether and how this affects the results (Stoop, 2012). In humans and primates, the espression of Oxtr has been detected in the basal nucleus of Meynert, ventral part of the lateral septal nucleus, subiculum, entorihnal cortex, amygdala and in the globus pallidus and ventral pallidum (Loup et al., 1991; Gimpl and Farenholz, 2001; Stoop; 2012; Benarroch, 2013). Avprs are expressed in the dorsal portion of the lateral septal nucleus, thalamus, hilus of the dentate gyrus, dorsolateral part of the amygdaloid nucleus and brainstem. The current search in this field is for a radioactive ligand that can easily cross the blood brain barrier, thus allowing positrone emission tomography (PET) scanning studies of Oxtr and Avpr distribution in living human subjects (Smith et al., 2012; Stoop, 2012).

2. Neuromodulatory effects of oxytocin on complex behaviour

In the brain, Oxt and Avp play a key role as neurotransmitters or neuromodulators and regulate sexual and social behaviour as well as some higher brain functions, such as stress response, learning and memory (Gimpl and Fahrenholz 2001; Lee et al., 2009). Of note, it is still unclear how the endogenous Oxt and Avp systems are handling and mutually coordinating these regulatory actions. For example, it is still unknown whether there is a massive undistinct release of these

peptides onto various targets or whether a finely tuned activation at the cellular level takes place. Also, it is still undefined whether the neuronal populations that synthesize and release Oxt and Avp coordinate their activities among the brain regions they innervate (Stoop, 2012). As previously mentioned, Avp and Oxt systems are rather unselective and their brain expression is closely superimposed, so that a better understanding of how these peptides modulate the different brain functions would require a discussion of their complementary role. Hence, in the following sections, the central effects of Oxt in animal models will be discussed and the roles of Avp will only be mentioned in relationship with them.

2.1 Oxytocin in social behaviour

Following their central release, Oxt and Avp regulate multiple aspects of social behaviour (reviewed in Lee et al., 2009; Neumann and Landgraf, 2012), such as social preference (Lukas et al., 2011), maternal care and aggression (Neumann 2003; Bosch and Neumann, 2008), sexual behaviour (Melis et al., 2007), pair bonding (Donaldson and Young, 2008; Jarcho et al., 2011), and social recognition (Bielsky and Young, 2004; Gabor et al., 2012).

The main affiliative behaviours regulated by Oxt and Avp are pair bonding and maternal behaviour, together with sexual behaviour. There is a large body of literature related to the positive effects of both Oxt and Avp on these two important aspects of the social sphere using

prairie voles (*Microtus ochrogaster)* as an animal model system (Lukas and Neumann, 2013). Prairie voles have been very useful in these studies since they represent a typical monogamous species that form lifelong bonds between partners after mating. In female prairie voles intracerebroventricular (i.c.v.) administration of Oxt, but not Avp, induces pair bonding without prior mating and this affiliative behaviour is prevented by pre-treatment with a specific Oxtr antagonist (Williams et al., 1994). This Oxt effect has been mapped to the nucleus accumbens (NAc) and the pre-frontal cortex (Young et al., 2001; Lukas and Neumann, 2013). Accordingly, an increased release of Oxt in the NAc during mating (Ross et al., 2009), and an enhanced Oxtr expression in the NAc were found in female prairie voles as compared to non-monogamous female mountain voles, indicating that a different Oxtr expression explains the various social organizations found in different animal species (Insel and Shapiro, 1992). Moreover, the injection of adeno-associated viral vectors encoding the Oxtr gene into the NAc of monogamous prairie voles results in an accelerated formation of partner preference compared to the untreated prairie voles (Ross et al., 2009). On the other hand, central administration of Avp to male prairie voles induces the formation of a partner preference independently from mating (Winslow et al., 1993).

In addition to pair bonding, Oxt and Avp participate in other aspects of rodent affiliation, such as the maternal behaviour (Lee et al., 2009; Likas and Neumann; 2013). Of note, during pregnancy and post-natal nursing, both Oxt and Oxtr levels are significantly increase in the

female rat brain, with Oxtr expression returning to the levels of nulliparous rats by several hours post-partum (Medlle et al., 2007). A possible effect of this phenomenon is the facilitation of the onset and maintenance of maternal behaviour, supported by studies showing that exogenous Oxt can induce maternal behaviour in rodents. Indeed, nulliparous rats typically keep distant and reject stranger pups (Fleming and Anderson, 1987). However, soon after delivery, female rats show a drastic surge in their motivation towards maternal behaviour, presumably as an effect of Oxt release. This hypothesis is supported by the observation that centrally administered Oxt induces the same change in nulliparous female rats (Pederson et al., 1982). It has been shown that Oxt is released into different brain areas, such as the bed nucleus of the stria terminalis (BNST), the ventral tegmental area and the medial preoptic area (MPOA) during nursing and it facilitates maternal care in lactating rats (Bosch and Neumann, 2008; Bosch et al., 2010; Pedersen et al., 1994; Lee et al., 2009). In addition, as a part of maternal behaviour both Oxt and Avp, acting in the PVN and amygdala, promote maternal aggression toward a female intruder carrying potential risks to the offspring (Bosch and Neumann, 2011). On the other hand, aggressive behaviour in male rodents is believed to be strongly under the influence of Avp (Caldwell et al., 2008). The involvement of Oxt in male aggressive behaviour is quite controversial and the different results obtained mainly depend upon the species used and when and how the Oxt system is manipulated (Lee et al., 2009). For instance, Oxt modulates aggressive behaviour in prairie voles only after, and not prior to mating (Winslow et al., 1993).

Additionally, Oxt plays a key role in the regulation of sexual behaviour both in male and female rodents (Lee et al., 2009). In male, Oxt is involved in copulatory activity, erectile functioning and ejaculation (Witt, 1995). In female rodents, Oxt activity has been extensively investigated in different species. In general, it is believed that in female rodents the regulation of copulatory behaviour requires the interaction between estrogens and Oxt (Witt, 1995).

A number of studies have indicated that Oxt and Avp signaling in the lateral septum (LS) is important for social recognition and related social behaviours (Bielsky and Young, 2004; Bielsky et al., 2005; Caffè et al., 1987; Curley et al., 2012). The septal administration of Avp to rats increases short-term social recognition memory (Dantzer et al., 1988) and rescues social memory of Brattleboro rats that are intrinsically deprived of Avp (Engelmann and Landgraf, 1994). Likewise, the overexpression of Avpr1a in the murine LS increases social recognition memory (Bielsky et al., 2005), and virally mediated re-expression of Avpr1a in the LS of knockout mice fully rescues the deficits in short-term social recognition (Bielsky et al., 2005). These studies indicate that LS may play an important role for establishing social and affective bonds. However, despite these important behavioural implications, little is know about how Oxt and Avp act in the septum at cellular levels (Stoop, 2012). In addition to the LS it has been demonstrated that Oxt can influence social recognition by acting in the amydgala (Ferguson et al., 2001). Studies in Oxt and Oxtr knockout mice have improved our understanding of the involvment of

Oxt in social memory and in the modulation of the amygdaloid functions. Indeed, both male and female Oxt and Oxtr knockout mice exhibit deficits in short-term conspecific social recognition (Ferguson et al., 2000). In male Oxt knockout mice these deficits are rescued by the local microinjection of Oxt into the medial amygdala prior to the first exposure to a social stimulus and are mimicked by antisense oligonucleotides targeting the Oxtr (Choleris et al., 2007).

Most research concerning the role of Oxt and Avp in social behaviour have been performed in rodents, where social recognition is highly dependent on the olfactory system (Stoop, 2012). Among the brain areas involved in these effects there are the accessory olfactory system, amygdala, bed nucleus of the stria terminalis, lateral septum and NAc (Tribollet et al., 1997; Ross et al., 2009; Veenema et al., 2008). Pheromones are able to stimulate the accessory olfactory system, which in turn is involved in processing of olfactory signals that are important for short-term conspecific social recognition in males and induction of sexual and maternal behaviour in females (Benarroch, 2013). In this circuit, Oxt and Avp complementary modulate the neuronal activity at various levels (Stoop et al., 2012; Dluzen et al., 1998; Benarroch, 2013).

2.2 Oxytocin and anxiety

Oxytocin is thought to play a role in the control of anxiety both in humans and rodents. While Oxt and Avp act in the same direction in the modulation of social behaviour, they appear to exert opposite effects on the behavioural correlates of anxiety and of the fear associated to social recognition (Lee et al., 2009). Mainly based on studies using murine models, it has been established that Oxt exerts anxiolytic and antidepressant effects, whereas Avp promotes stress responses, anxiety and depression (Stoop, 2012; Neumann and Landgraf, 2012; Meyer-Lindenberg et al., 2011). The evidence that Oxt contributes to the reduction of anxiety (Neumann and Landgraf, 2012; Neumann et al., 2000) comes from different pharmacological and behavioural studies on mice lacking the expression of Oxt or Oxtr (Neumann and Landgraf, 2012; Neumann et al., 2000). On the other hand a variety of gain- and/or loss-of-function studies on Avp and Avpr1a have supported the anxiogenic activity of this peptide (Stoop, 2012). The brain area that appears to be mainly involved in the opposite roles that Oxt and Avp exert in the regulation of anxiety is the CeA, which is important for the autonomic, endocrine and motor responses linked with fear (LeDoux, 2012; Benarroch; 2013). The central amygdala plays an important role as an alert center for potentially dangerous stimuli and its activation typically evokes fear responses (Stoop, 2012). Local injection of Avp into the CeA produces a typical fear response, as reflected by a decreased behavioural motility, whereas Oxt injection does the opposite (Roozentaal et al.,

1993). Moreover, mutant female mice lacking endogenous Oxt are characterized by an increased anxiety-related behaviour (Mantella et al., 2003), which is rescued upon i.c.v. administration of Oxt. Interestingly, other authors have not reported any anxious behaviour in Oxtr knockout mice (Sala et al., 2011) or in a partial forebrain-specific Oxtr knockout line (Lee et al., 2008).

It has been proposed that the opposite effects of Oxt and Avp on anxiety and fear responses are mediated by the different distribution of Oxtr and Avpr1a in the amygdala (Stoop, 2012). In fact, Avpr1a are mainly expressed in the CeA, which provides outputs that control a fear response, whereas Oxtrs are more abundant in the lateral portion, which sends an inhibitory GABAergic output to the central portion (Lee et al., 2009; Benarroch, 2013; Stoop 2012).

2.3 *Oxytocin and stress response*

Oxt and Avp are involved in the regulation of the responses to stress (Amico et al., 2004; Neumann, 2002). Acute or chronic central administration of Oxt exert a potent anxiolytic effects both in female and male rats (Windle et al., 1997; Ring et al., 2006; Blume et al., 2008). In particular, it has been proposed that Oxt can modulate the physiological and behavioural responses to stress both by direct and indirect modulation of the hypothalamic-pituitary-adrenal (HPA) axis (Viero et al., 2010). Animal studies have shown that a variety of physiologically or psychologically stressful stimuli activate Oxt neurons

in the hypothalamus and increase the plasma levels of Oxt (Neumann, 2000). It has been demonstrated that in female rats the Oxt pathway attenuates the responses of HPA axis to psychogenic stress (Mantella et al., 2004) and that the ability of Oxt to inhibit the HPA axis activity is strictly dependent on estrogens levels (Ochedalsky et al., 2007). In particular, Oxt reduces both the neuroendocrine and molecular responses of the HPA axis triggered by stressful stimuli (Windle et al., 2004).

This effect is mainly exerted on the amygdala and PVN (Blume et al., 2008; Neumann, 2008). The latter nucleus contains parvocellular neurons synthesizing corticothrophin-releasing hormone (CRH), which send projections to the median eminence, and Oxt- or Avp-expressing neurons projecting to the autonomic, sensory and motor nuclei of the brainstem (Benarroch, 2013). When these neurons in the PVN are stimulated by different acute and chronic stresses, they release both Oxt and Avp. The effects exerted by the two peptides on endocrine and autonomic responses involved in the control of anxiety are opposite (Benarroch, 2013). In particular, the expression of AVP is up-regulated by parvocellular CRH neurons and this potentiates adrenocorticothrophin (ACTH) release from the adenohypophysis (Neumann, 2002). Moreover, Avp through the interation with Avpr1a receptors in different brain areas increases sympathetic cardiovascular responses (Raggenbass, 2001), whereas Oxt enhances parasympathetic responses (Higa et al., 2002).

2.4 Oxytocin in learning and memory

There are a few lines of evidence supporting the notion that Oxt is involved not only in social memory but also in spatial memory. The first results obtained in rodents about the possible involvement of Oxt and Avp in memory indicated that Avp exerts a positive effect, by antagonizing amnesia and inducing memory consolidation (de Wied et al., 1993). Recently, it has been proposed that Oxt is important in promoting spatial memory especially during motherhood. During this period, long-term spatial memory in female rats is highly enhanced for the necessity to find the best environmental and nutritional conditions for the offspring (Ferguson et al., 2001). Accordingly, it has been shown that the i.c.v. injection of Oxt in nulliparous female mice enhances long-term spatial learning, whereas the administration of an Oxtr antagonist in multiparous mice reduces the improved spatial memory (Tomizawa et al., 2003). Oxt enhances long-term synaptic plasticity and long-term memory thought the activation of the MAPK cascade and CREB phosphorylation. In particular, it has been proposed that Oxt plays a central role in the consolidation processes of spatial memory rather then in spatial learning as recently reviewed by Chini and coll (2013). This hypothesis is supported by the observation that the acquisition phase of spatial memory evaluated using the Morris water maze or the Y maze tests is not impaired in Oxt knockout mice (Ferguson et al., 2000). In addition, Oxtr knockout mice display a normal spatial memory acquisition, as determined by an appetitive-motivated T-maze test (Sala et al., 2011). Accordingly, recurrent

central administration of Oxt does not decrease the short-term memory in virgin female mice but significantly improves long term-memory (Tomizawa et al., 2003) and this supports the notion that Oxt is involved in memory consolidation rather than in the acquisition phase of memory. Another recent study supporting the role of Oxt and Oxt-like peptides on mnemonic stabilization processes has been performed on *Sepia officinalis*, a cephalopod mollusc that exhibits high learning abilities. This study has shown that a peripheral injection of cephalotocin (an Oxt analogue) promotes long-term memory (Bardou et al., 2010; Chini et al., 2013).

Although it is recognised that Oxt is involved in animal learning and memory, the cellular and molecular mechanisms underlying its cognitive actions are less clear. It has been proposed that Oxt influences learning and memory at different times and with different modalities and that the main target of Oxt in this process is the hippocampus, as recently reviewed by Chini and coll. (2013). The first exposure to Oxt in the rodent hippocampus occurs probably peri-natally or post-natally, since there is almost no Oxt immunoreactivity in the developing brain (Alstein and Gainer, 1988). In these two periods Oxt is thought to play both neuroprotective and neurodevelopmental actions (Chini et al., 2013). At birth Oxt is involved into the switch of GABA neurotransmission from excitatory to inhibitory in the rat hippocampus. It has been suggested that the Oxt-induced GABA switch can protect hippocampal neurons from hypoxic insults that can occur during delivery by reducing the neuronal

excitability (Tyzio et al., 2006). Beside its neuroprotective action, Oxt participate in the maturation of neuronal microcircuits. Indeed, in early post-natal period Oxt triggers the appearance of a coherent activity pattern in the hippocampus (Crepel et al., 2007). The abililty of Oxt to form new connections and synaptic contacts can also influence learning and memory in the adult brain (Chini et al., 2013). As mentioned previously, Oxt improves the hippocampus-dependent plasticity in motherhood (Tomizawa et al., 2003) by increasing long term-spatial memory. This is in accordance with the observation that in rats during late pregnancy and lactation maternal brain undergoes changes in hippocampal plasticity in order to provide the optimal conditions for the offprings. Accordingly, a study performed from Kinsley and coll (2006) has demonstrated that lactating rats exhibit an increase hippocampal dendritic spines density as compared to virgin rats. The molecular mechanisms by which Oxt is able to modulate hippocampal function are still not fully understood, hence further investigation is required to understand how Oxt participates in the regulation of memory. For example, it is not clear whether it exerts a direct effect on the synaptic plasticity events (LTP and LTD) involved in memory processes, or whether it directly or indirectly influences other neurotransmitters and/or neuromodulators (Chini et al., 2013).

Behavioral classes	Behaviors	Effects of Oxt in rodents
Social behaviors		
Social memory	Social recognition	– ⇑ odor processing in olfactory bulb – ⇑ social memory – ⇓ social recognition in Oxtr KO mice – abnormal Bruce effect in female Oxt KO mice
Affiliation	Sexual behavior	– ⇑ erections (with T) and ejaculation frequency in males – ⇑ receptivity (with E) in females
	Paternal behavior	– ⇓ parental behavior with concomitant Avp/Oxt antagonism – ⇓ adult paternal behavior with Oxt antagonist on PND1
	Maternal behavior	– ⇑ Oxtr throughout the brain with onset of maternal behavior – necessary for lactation – induces full repertoire of maternal behaviors (in presence of E) – ⇓ pup retrieval and pup survival in Oxtr KO
Aggression	Female aggression	– ⇑ Oxt levels in CeA correlated with aggression
	Male aggression	– may have organizational effect during prenatal period
Non-social behaviors		
Learning and memory	Non-spatial memory	– ⇓ memory in passive avoidance tasks
	Spatial memory	– ⇑ memory when injected into hippocampus – ⇓ memory when injected into NBM
Anxiety and depression	Anxiety	– ⇓ anxiety following Oxt administration – ⇑ anxiety in some Oxtr KO mice; sexually dimorphic
	Depression	– ⇑ active/coping behaviors with i.p. Oxt administration

Table 1. Summary of the neuromodulatory effects of Oxt and Oxtr in rodent. [table adapted from Lee et al., 2009].

2.5 Evidences from oxytocin and oxytocin receptor knockout mice

One useful approach for understanding the effects of neuropeptides such as Oxt is studying animals carrying deletions of the genes coding for the peptide or its receptor(s). Spontaneous mutations in the Oxt gene have not yet been reported in mice, possibly owing to the fact that the lack of Oxt can highly impair reproduction (Carter, 2003). Some information regarding the function of Oxt can be derived from the study of mice that lack the endogenous peptide. Oxytocin knockout mice were firstly generated in 1996 by two independent groups (Nishimori et al 1996; Young et al., 1996). These mice reach the

sexual maturity, display a normal sexual behaviour and delivery, and exhibit a normal care for the offspring. However, the Oxt knockout mice are unable of milk ejection (Nishimori et al., 1996). It has been suggested that the absence of a clear deficit in maternal behaviour shown by the Oxt knockout mice is related to compensatory mechanisms that occur through the activation of Oxtr by Avp (Winslow and Insel, 2002; Lee et al., 2008). Behavioural studies on Oxt knockout mice also demonstrated that Oxt exerts regulatory effects not only on reproduction but also on social behaviour (DeVries et al., 1997; Insel and Young 2001). In fact, Oxt knockout mice show an impaired social memory (Winslow and Insel, 2002; Ferguson et al., 2000) and increased anxiety (Mantella et al., 2003). Moreover, knockout mice lacking CD38, a protein involved in the release of Oxt, are characterized by reduced Oxt release and impaired maternal and social behaviours (Jin et al., 2007).

Subsequently, two groups independently generated Oxtr knockout mice (Lee et al., 2008; Takayanagi et al., 2005). These show no obvious deficits in mating or parturition, although dams exhibit defects in lactation and maternal nurturing (Lee et al., 2008; Takayanagi et al., 2005). Pups demonstrate an increased exploratory activity and decreased ultrasonic vocalizations suggesting a reduced stress or anxiety-like behaviour (Takayanagi et al., 2005). Moreover, Oxtr knockout mice display an increased aggression and impairment of social behaviour (Takayanagi et al., 2005; Sala et al., 2011). Finally, Oxtr knockout mice also show a diminished cognitive flexibility and a

resistance to change of a learned pattern of behaviour (Sala et al., 2011) that are ameliorated by *in vivo* activation of Avpr1a (Sala et al., 2011). Noteworthy, Oxtr heterozygous mice expressing only the 50% of brain Oxtrs display an impaired sociability but normal cognitive flexibility and lack of aggressive behaviour (Sala et al., 2013), thus indicating that the number of Oxtrs is important in determining these phenotypical manifestations. A partial forebrain-specific Oxtr knockout line displays no deficits in maternal behaviour compared to Oxtr knockout mice (Lee et al., 2008). However, the partial forebrain-specific Oxtr knockout line differs from the knockout in the performance of the social recognition task and fails in recognizing individual mice (Lee et al., 2008).

These studies on knockout animals are not only useful for a better understanding of the role of Oxt in the modulation of higher brain function, but also illustrate that in addition to Oxt release, the level of Oxtr expression in the brain may be important in determining Oxt-mediated behaviours (Sala et al., 2013; Chini et al., 2013). Moreover, animal studies on knockout mice for Oxt or Oxtr are beginning to shed some light on the molecular mechanism of Oxt actions, particularly at the neurochemical and electrophysiological levels.

3. The role of the oxytocin system in human brain and pathological conditions

Over the last two decades, animal models have shed some insights on how Oxt contributes to the regulation of social and non-social behaviours. In addition, a growing body of evidence from recent studies has indicated that the effects of Oxt reported in animals can be extrapolated to humans (reviewed in Lee et al., 2009). In particular, it has become clear that Oxt plays a central role in the modulation of complex human behaviours (both social and non-social). Furthermore, it has been proposed that alterations in the Oxt system are involved in different brain disorders and that the modulation of Oxt activity could offer a potential therapeutic target (Meyer-Lindenberg, 2011). The potential of the Oxt system for drug targeting is undoubtedly significant, thus bringing some hope for alleviating serious neurodevelopmental disorders. However, this is hampered by the difficulty to specifically and effectively control with drugs the multiple actions that Oxt performs in the CNS (Manning et al., 2008). Moreover, additional studies are required to increase our understanding of the mechanisms by which Oxt enters the brain following different administration routes, of the relationships between peripheral and central Oxt, and to develop new analogues of Oxt capable to act on the Oxtr in a more specific and long-lasting way (Meyer-Lindenberg, 2011).

3.1 Oxytocin and human complex behaviours

Oxytocin appears to play an important role in the regulation of human complex behaviours as reviewed in Lee et al., 2009. Recently, a number of different researches have begun to investigate the effects of Oxt on social recognition, primarily by testing facial expression recognition in humans following Oxt treatment (Lee et al., 2009). It is generally assumed that Oxt improves the memory of faces even if it is still unclear whether the facial emotional expression is indeed relevant for this process (Meyer-Lindenberg, 2011; Lee et al., 2009). Intranasal Oxt administered to males and females after viewing male faces with different expressions enhances the recognition memory for neutral and angry faces only (Savaskan et al., 2008). Likewise, intranasal Oxt improves the recognition of angry faces in both sex (Guastella et al., 2009). In contrast, a previous paper from the same group (Guastella et al., 2008) demonstrated that in males Oxt acts selectively by increasing the memory for faces with only happy expressions. These discrepancies can be attributed to differences in the proband sample (only male in the first study vs male and female in the second one) and differences in the way the stimuli were presented. Finally, a recent study has shown that male subjects receiving intranasal Oxt administration prior to the presentation of faces have better memory for faces that had already been seen, with no influence on memory for previously seen non-social stimuli (Rimmele et al., 2009), thus further supporting the view that Oxt is indeed involved in the memory of social stimuli. All together, these observations indicate that that Oxt

influences facial recognition at early stages of the emotional processing.

As observed in animal models, the amygdala seems to play an important role for facial recognition in humans and for these reasons a number of studies have investigated Oxt effects on this brain area (Lee et al., 2009). For example, amygdala activation is reduced in male subjects following Oxt administration in response to angry and fearful expressions (Kirsch et al., 2005), but the same response is not seen upon exposure to non-social stimuli. Moreover, intranasal Oxt significantly reduces functional amygdala connections to upper brain regions (Kirsch et al., 2005). Numerous functional magnetic resonance imaging (MRI) studies after intranasal administration of Oxt to males have clarified the mechanisms by which Oxt controls the function and the activity of amygdala. In particular, it has been shown that Oxt differently stimulates various subregions of the amygdala in response to the positive or negative connotation of the social stimuli. For example, Oxt reduces the activation of left amygdala following a negative social stimulus and reduces the connectivity between this region and brainstem areas involved in fear reaction (Kirsch et al., 2005; Domes et al., 2007; Gamer et al., 2010; Meyer-Lindenberg, 2011; Benarroch, 2013). In response to happy faces, Oxt attenuates the reactivity of the right amygdala (Domes et al., 2007) and increases the activity of the left anterior amygdala (Gamer et al., 2010).

Even if studies in animal models have demonstrated that Oxt modulates the aggressive behaviour, little is known about the role of

Oxt in human aggression (Lee et al., 2009). As mentioned before, it has been reported that Oxt administration reduces the amygdaloid activity (Kirsch et al., 2005), and anxiety levels seem to be associated with aggression in several animal models (Bosch et al., 2005; Winslow et al., 2000). Although a detailed investigation of the possible consequences of Oxt administration on aggressive behaviour in humans has not yet been performed, a number of studies have focused on the role of Oxt in human anxiety. It has been proposed that Oxt decreases anxiety by promoting human social behaviour, recognition (Savaskan et al., 2008), feelings of affiliation (Kosfeld et al., 2005), and trust (Baumgartner et al., 2008). For example, intranasal administration of Oxt attenuates the stress caused by speaking in public, a situation that has been reported to increase the circulating levels of cortisol (Heinrichs et al., 2003). During breast feeding, owing to the increase of Oxt levels, women usually exhibit reduced levels of ACTH and cortisol (Chiodera et al., 2001; Nissen et al., 1996). Indeed lactation seems to reduce the woman response to stress exposure. In particular, the reduction of HPA axis underlying a lower stress response depend on the type of stressor (Altemus et al., 1995; 2001; Heinrichs et al., 2001), and is mediated by central effects of Oxt in PVN and septum rather than at the peripheral adrenal level (Heinrichs et al., 2002). The role of Oxt in reducing the stress responses in humans has also been investigated after intranasal administration of Oxt before the application of stressful stimuli to healthy subjects receiving a social support from their best friend during the pre-test period versus subjects without any previous social support (Heinrichs et al., 2003).

The results show that the subjects who had received the social support and Oxt exhibit the lowest cortisol levels during the stress exposure, whereas subjects who had received placebo and no social support display the highest anxiety and cortisol levels (Heinrichs et al., 2003). Another study showed that intranasal administration of Oxt promotes the positive communication in both men and women during a couple conflict and it lowers cortisol levels during the conflict, which is in line with the evidence that Oxt facilitates pair bonding behaviour in different species (Ditzen et al., 2009).

However, although numerous studies have demonstrated that Oxt facilitates pair bonding and parental care in animals, the results on humans are less conclusive and mainly agree on the assumption that Oxt facilitates social interaction and feelings of attachment (Lee et al., 2009). Many lines of evidence support the role of Oxt in human maternal care. In particular, it has been proposed that Oxt levels in the first months of pregnancy and of post-partum period are positively related with specific maternal bonding behaviours such as gaze, vocalization toward infants, and affectionate touch (Feldaman et al., 2007). Finally, a number of reports have demonstrated that Oxt enhances the feelings of generosity and trust in a money game test and helps the detection and understanding of others' feelings (empathy) (Zak et al., 2004;2005; Guastella et al., 2008; Domes et al., 2007; Lee et al., 2009).

There is also strong evidence that Oxt is involved in the regulation of human mood (Lee et al., 2009). Subjects affected by major depression

manifest low plasma levels of Oxt (Scantamburlo et al., 2007), whereas increased post-mortem levels of Oxt mRNA in the PVN have been detected in patients with melancholic depression (Meynen et al., 2007). On the other hand, increased post-partum Oxt levels have been associated with positive mental state. In humans, breast-feeding increases Oxt, which is associated with lower negative feelings (Mazzacappa and Katlin, 2002).

Beside the well established role of Oxt in human amygdala, future studies will have to search for alternative neural pathways in other brain regions according to the widespread distribution of Oxtrs in the human brain and the complexity of neuronal networks involved in the processing of social recognition and emotions (Adolphs, 2003). Thus far, it is difficult to have a systematic picture regarding the social effects of Oxt in humans, mainly due to the heterogeneous behavioural tests used in the different studies (Meyer-Lindenberg, 2011). However, the results that have been reported thus far are in agreement with the idea that Oxt enhances social interactions by improving the understanding of emotional stimuli and facilitating trust and cooperative behaviour (Meyer-Lindenberg, 2011).

3.2 Oxytocin and autism

Given the ability of Oxt to modulate various higher brain functions and the positive relationship between Oxt and social bonding in animals and humans, it has been proposed that alterations in the Oxt system

can play a role in the pathogenesis of autism spectrum disorders (ASDs). ASDs are a group of severe neurodevelopmental disorders that are typically defined by four core symptoms, i.e. communication deficit, irritability, repetitive behaviour and restricted interest according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV).

One of the first lines of evidence for a possible involvement of the Oxt system in the development of autism comes from the observations that animal lacking endogenous Oxt and Oxtrs display some social impairments that mimic those observed in ASDs (Insel, 1997; Lee et al., 2002; Takayanagi et al., 2005; Sala et al., 2011; Sala et al., 2013). In addition, early studies in humans documented that subjects with ASDs exhibit altered plasma levels of Oxt. In particular, Modhal and coll. (1998) were the first to correlate lower plasma levels of Oxt with social deficits in ASD children. These findings were extended to demonstrate alterations of Oxt metabolism in ASDs (Green et al., 2001). Indeed, the levels of Oxt precursors are higher in some autistic than in healthy children (Green et al., 2001), indicating that autism can be related to an abnormal synthesis of this neuropeptide. Additional evidence for a possible role of Oxt in ASDs comes from the recent interest towards CD38, a transmembrane protein involved in Oxt secretion in the brain that is thought to influence social behaviour (Jin et al., 2007). Several genetic variants of the CD38 gene were recently identified that show a significant association with a specific type of autism, the so called

“high functioning autism” (Munesue et al., 2010 Meyer-Lindenberg et al., 2011).

Another line of evidence highlights the putative role of Oxtr in ASDs. Different studies have indicated the 3p25 region containing the Oxtr gene as the most promising genetic locus for ASDs (Lauritsen et al., 2006; Ylisaukko-oja et al., 2006). Indeed, different studies have shown that single nucleotide polymorphisms (SNPs) in the Oxt (Yrigollen et al., 2008) and Oxtr (Jacob et al., 2007; Lerer et al., 2008; Wu et al., 2005) genes are linked to ASDs. Particularly, two SNPs in the third intron of the Oxtr gene, rs53576 (G to A) and rs225498 (G to A), have been associated with autism and different social phenotypes. These SNPs have been correlated with variations in the volumes of different brain areas, including the amygdala, anterior cingulate cortex, hypothalamus and posterior brainstem, that influence the effects of intranasal Oxt on the activation of the amygdala in response to social cues (Meyer-Lindenberg et al., 2011). Several studies have reported the familiar transmission of these polymorphisms to the offspring with ASDs (Wu e al., 20005). Interestingly, Gregory and coll. (2009) looking at copy number variations (CNVs) in a group of 119 unrelated probands, observed that the most interesting CNV is a heterozygous deletion of the Oxtr gene in a single individual with autism and his mother with putative obsessive-compulsive disorder (OCD).

Currently, the only available drugs employed for ASDs target certain symptoms, notably aggression, but not the core features of ASDs. The potential usefulness of Oxt in the treatment of the main autistic

symptoms has also been studied. The majority of studies have measured Oxt responses to single doses in ASDs (Hollander et al., 2003; Andari et al., 2010), whereas only few have examined the effects after long-term treatment (Anagnostou et al., 2012). Intravenous Oxt administration facilitates the processing and retention of social information in adults diagnosed with autism or Asperger's syndrome. Compared to subjects who had received placebo, those who assumed Oxt exhibited an increased retention of affective speech comprehension (Hollander et al., 2007). These findings are similar to previous reports in which intravenous Oxt administration reduces repetitiveness in adults with autism or ASDs (Hollander et al., 2003). Intranasal administration of Oxt to a small group of subjects with autism induces a more appropriate social behaviour related to emotional recognition, indicating a potential therapeutic role of Oxt (Guastella et al 2010; Benvenuto et al., 2013; Andari et al., 2010). While single dose studies are important, evaluating long-term effects of Oxt are essential to determine whether Oxt possesses a therapeutic potential in ASDs. Anagnostou and coll. (2012) have investigated the safety and therapeutic effects of intranasal Oxt following a schedule of daily administrations of intranasal Oxt to ASD subjects. This pilot study has indicated a potential therapeutic usefulness of daily administrations of intranasal Oxt to adults with ASD with no serious adverse effects. The results of this six-week study have shown that intranasal Oxt improves social cognition, quality of life, restricted and repetitive behaviours, hence the core domains of ASDs.

3.3 Oxytocin in schizophrenia and obsessive-compulsive disorder

It has been suggested that alterations in the Oxt system are involved in other psychiatric diseases, such as schizophrenia (Beckman et al., 1985) and OCD (Leckman et al., 1994).

The main evidence for a possible involvement of Oxt in schizophrenia has come from the observation that Oxt levels are increased in schizophrenic compared to healthy subjects (Beckman et al., 1985). Additional studies have reported that the basal level of Oxt-neurophysin was three-fold higher in the plasma of schizophrenics (Legros et al., 1992). In contrast, a more recent study has reported a reduced Oxt in a subgroup of schizophrenics who display altered HPA activity (Goldman et al., 2008), and some antipsychotics significantly increase the plasma levels of Oxt (Uvnas-Moberg et al., 1992), indicating that it exerts a natural antipsychotic effect. Interestingly, the amount of circulating Oxt positively predicts the ability to interpretate facial emotions in schizophrenics patients (Goldman et al., 2008), further suggesting the involvement of Oxt in the social symptoms of schizophrenia. Recent studies have also shown positive effects of Oxt administration on schizophrenic symptoms, including cognition, emotional recognition and social perception (Averbeck et al., 2011; Feifel er al., 2013; Fischer-Shofty et al., 2013). Finally, several randomized controlled trials in subjects with schizophrenia kept on a stable dose of antipsychotic medication have shown that the addition of Oxt produces some beneficial effects, although opposite results

have also been reported (Modabbernia et al., 2013; Macdonald et al., 2012).

Alterations of the Oxt system have been also investigated in OCD. Indeed OCD includes cognitive and behavioural symptoms that could be correlated to those behaviours modulated by Oxt (Gimpl and Farenholz, 2001). According to the DSM-V, the main symptoms of OCD are recurrent, intrusive thoughts, fear of danger, and compulsive behaviours or cognition aimed at relieving anxiety. The possibile implication of Oxt in the neurobiology of OCD has been indicated by elevated CSF levels of Oxt and by the correlation between these and the severity of the disorder (Leckman et al., 1994). However, although an early study indicated that intranasal Oxt improves the sympyhoms exhibited by OCD (Ansseau et al., 1987), subsequent studies have not confirmed these therapeutic effects of systemic (Charles et al., 1989) and intranasal administration of Oxt (de Boer and Westenberg, 1992; Salzberg and Swedo, 1992; Epperson et al., 1996).

4. Aims of the work

Beside its role in parturition and lactation, Oxt controls several cognitive, social and neuroendocrine functions. In humans, it is involved in the regulation of learning and memory and in emotional and social behaviours. In rodents, it controls various social and non social behaviours such maternal care, pair bonding, sexual behavior, social memory, anxiety, aggression and memory (Sala et al., 2011; MacDonald and MacDonald, 2010; Lee et al., 2009). The key role played by this neuropeptide in regulating higher brain functions has encouraged the study of the Oxt system in different human pathological conditions, such as ASDs, schizophrenia and OCD. Hovewer, the cellular mechanisms by which the Oxt system controls the development of appropriate synaptic networks and thereby regulates social and cognitive functions are still unclear. The present study is aimed at elucidating the cellular and molecular mechanisms by which Oxt affects the formation and function of central synapses.

In Chapter 2 the effects of Oxt signalling on hippocampal synaptogenesis are addressed. To this end, it has been taken advantage of the autaptic culture system that allows the investigation of pre- and post-synaptic mechanisms triggered by Oxtr activation in individual excitatory or inhibitory neurons using a combination of biochemical, microscopical and electrophysiological techniques.

The Chapter 3 examines whether any synaptic defect(s) are present in hippocampal neuronal primary cultures obtained from embryonic day

18 (E18) Oxtr knockout mice in comparison to naive mice. To achieve this aim, the expression of protein componets of excitatory and inhibitory synapses were evaluated using immunofluorescence and confocal microscopy with high-resolution imaging analysis, and the corresponding data were complmented with electrophysiological recordings of glutamatergic and GABAergic spontaneously evoked ion currents of pyramidal hippocampal neurons using the patch-clamp technique.

In the last Chapter, the main results are discussed highlighting the implications of my findings for potential perspectives in translational medicine.

5. Abbreviations

ACTH: adenocorticothropin

ASDs: autistic spectrum disorders

Avp: vasopressin

Avpr: vasopressin receptor

BNST: bed nucleus of the stria terminalis

CeA: central nucleus of the amygdala

CNS: central nervous system

CRH: corticothrophin-releasing hormone

DAG: diacylglycerol

DKG: diacylglycerol kinases

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

HPA: hypothalamic-pituitary-adrenal axis

i.c.v.: intracerebroventricular

IGR: intragenic region

IP3: inositol trisphosphate

LDCVs: large dense core vesicles

LS: lateral septum

MPOA: medial preoptic area

NAc: nucleus accumbens

Oxt: oxytocin

Oxtr: oxytocin receptor

PET: positrone emission tomography

PKC: protein kinase C

PKD: protein kinase D

PLCβ: phospholipase Cβ

PVN: paraventricular nucleus

RasGPRs: Ras guanyl nucleotide-releasing proteins

SCN: suprachiasmatic nucleus

SON: supraoptic nucleus

VDCCs: voltage dependent calcium channels

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Chapter 2

Oxytocin regulates dendrite growth and synapse density in mouse hippocampal neurons.

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Oxytocin regulates dendrite growth and synapse density in mouse hippocampal neuron

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SUMMARY

Besides its well established roles in parturition and lactation, the peptide hormone oxytocin participates in the regulation of brain development and in multiple brain processes, including cognition and social interactions. However, whether and how oxytocin functions in nerve cell differentiation and synaptogenesis is still poorly understood. To address this issue we examined the morphological and functional consequences of oxytocin exposure in cultured murine hippocampal neurons. We have found that transient oxytocin exposure for 1-3 days decreases dendritic branching, synapse number and synaptic transmission in excitatory glutamatergic neurons. These effects are mediated by a specific activation of Oxt receptors coupled to the downstream activation of phospholipase Cβ (PLCβ). In contrast, GABAergic neurons were found to be refractory to Oxt treatment, which is most likely due to a lack of oxytocin receptor because ectopic lentiviral-mediated expression of oxytocin receptors in the inhibitory neurons of the striatum makes

these fully responsive to oxytocin. Taken together our data show that oxytocin exerts an early priming effect on developing neurons containing oxytocin receptor to regulate their differentiation and function.

INTRODUCTION

The nonapeptide oxytocin (Oxt) is primarily synthesized in the magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, and is released from the axon terminals of these cells in the posterior pituary gland into the general circulation, where it exerts its hormonal roles in the regulation of parturition and lactation (Russel et al., 2003). Interestingly, Oxt is also released into the brain, both from the axonal projections of the parvocellular neurons of PVN and SON and from the dendrites of the magnocellular neurons. Released Oxt regulates Oxt receptor (Oxtr)-containing neurons in a variety of brain areas, including the hippocampus, medial amygdala, lateral septum, bed nucleus of the stria terminalis, medial preoptic area, anterior and ventromedial hypothalamus, tegmentum and periaqueductal gray matter. There, Oxt acts as a neurotransmitter or neuromodulator to control many important aspects of behaviour, such as social recognition and interaction (Ferguson et al., 2000; 2001), anxiety (Bale et al., 2001; Yoshida et al., 2009), sexual behaviour (Bale et al., 2001), and spatial memory (Tomizawa et al., 2003).

The many peripheral and central effects of Oxt are exerted upon interaction with a unique Oxtr that belongs to the seven transmembrane G protein-coupled receptor (GPCR) superfamily. Its activation leads to pleiotropic cellular responses, the most common being the stimulation of phospholipase Cβ (PLCβ) activity via $G_{q/11}$ heterotrimeric G-protein (Stoop 2012). Additional Oxtr-mediated responses have been reported in certain cell types, including the inhibition or stimulation of inwardly rectifying K^+ channels through a pertussis toxin (PTX)-sensitive $G_{i/o}$ protein (Gravati et al., 2010), and the G_s-mediated activation of adenylyl cyclase, leading to an increased cAMP production as well as to a cAMP-independent, sodium-dependent tetrodotoxin (TTX)-resistant sustained inward current (Alberi et al., 1997).

Maternally-released hormonal Oxt is known to induce a switch of GABAergic transmission from excitatory to inhibitory in fetal brain neurons at the time of delivery to prevent neuronal damage during labor (Tyzio et al., 2006). Intriguingly, the switch was not observed in rodent models of autism showing impairments of social behaviour (Tyzio et al., 2014). Moreover, autism-like features were observed in the offspring of naïve mothers treated with an Oxtr antagonist on the day before delivery (Tyzio et al., 2014). This raises the possibility that Oxt acts as an early priming factor to shape the brain so that “social” animals, including humans, are capable of living with their conspecifics (Carter, 2013). Consequently, impaired Oxt signaling has been implicated in pathological defects of social behaviour, such as those occurring in autism spectrum disorders (ASDs), and Oxtr knockout

mice exhibit some core and accessory autism-like symptoms, such as social deficits, enhanced aggressiveness and brain excitability, and lowered cognitive flexibility as well as a reduced ratio between inhibitory and excitatory synapses in the hippocampus (Sala et al., 2011).

Currently, the cellular effects of Oxt on nerve cell development and function are still poorly understood, particularly with respect to their relevance for pathophysiology. In the present study, we investigated the consequences of Oxt exposure for the formation and function of murine hippocampal synapses in culture. Our results show that the exposure to Oxt for 1-3 days in culture (DIV) significantly reduces the dendritic branching and the number of synapses of glutamatergic neurons in an Oxtr-dependent manner. No effects of Oxt were observed with hippocampal and striatal inhibitory GABAergic neurons, likely owing to their lack of Oxtr because the lentiviral-induced expression of Oxtr in inhibitory neurons make these susceptible to the regulatory effects of Oxt. Taken together, our findings support the notion that Oxt regulates dendrite growth and synapse formation in brain neurons that express Oxtr, independently of their excitatory or inhibitory nature. Thus, a dysfunctional Oxt transmission could determine a maladaptive synaptic organization that may contribute to the development of different pathological conditions that is has been implicated in like ASDs and other neurodevelopmental disorders, such as anxiety, depression and schizophrenia (Meyer-Lindenberg et al., 2011).

EXPERIMENTAL PROCEDURES

Animals

Mice used in this study were bred in our animal facilities from adult C57BL/6J mice, which were obtained from Charles River Laboratories. For neuronal cultures, newborn postnatal day 0 (P0) pups from pregnant female C57BL/6J mice were used. All animal experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the State Government of Lower Saxony, Germany (comparable to NIH guidelines).

Cell culture

Microisland cultures of hippocampal and striatal neurons were prepared and cultured as described previously (Jockusch et al., 2007; Burgalossi et al., 2012). In brief, astrocytes for microisland cultures were obtained from mouse cortices dissected from P0 wild type animals and enzymatically digested for 15 min at 37°C with 0.05% (w/v) trypsin-EDTA (Gibco). The cells were plated onto T75 culture flasks in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS; Gibco) and penicillin (100 U/ml) /streptomycin (100 µg/ml) (Gibco), and grown for 7-10 days. Upon reaching the 60-70% confluence, astrocytes were trypsinized and plated at a density of ~30,000 cells/well onto 32 mm-diameter glass coverslips. The coverslips used for microisland cultures had been previously coated with 0.15% (w/v) agarose (Sigma-Aldrich) and then with a coating solution containing 0.5mg/ml poly-D-lysine (Sigma-

Aldrich), 17mM acetic acid and 1mg/ml collagen (DB) using a custom made stamp to generate 200x200 μm microislands. Hippocampi and striata from P0 animals were dissected in cold Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco) and incubated for 60 min at 37°C in DMEM containing 2.5U/ml papain (Worthington Biomedical Corporation), 0.2 mg/ml cysteine (Sigma), 1mM $CaCl_2$ and 0.5mM EDTA. Following inactivation of the papain enzyme activity with a solution containing 25 mg albumin, 25 mg trypsin inhibitor, 1 ml FCS and 9 ml DMEM, hippocampi and striata were mechanically triturated and the resulting cell suspension was plated onto the astrocyte microisland plates in pre-warmed Neurobasal medium (Gibco) supplemented with B27 (Gibco), Glutamax (Gibco) and penicillin (100 U/ml) /streptomycin (100 μg/ml) at a density of ~4,000 cell/well. Neuronal cultures were exposed to Oxt (100nM; Bachem), atosiban (100nM; Sigma-Aldrich) or both during the first three DIV by adding fresh drug solution every 24 h. The other compounds used in the present study were 1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U-73122, 3μM; Calbiochem) and pertussis toxin (PTX, 100ng/μl; Calbiochem). Pertussis toxin was added to the cell media from DIV 1 through 3, and following an 8 h incubation, Oxt (100 nM) or vehicle treatments were carried out. In the case of U-73122, cells were exposed to the compound alone or in combination with Oxt (100nM) only at DIV 1 due to the high neuronal death observed after three-days exposure. Electrophysiological recordings were performed between 9 and 14 DIV whereas immunofluorescence assays were carried out at 14 DIV.

DNA constructs

Lentiviral expression vectors encoding full length human Oxtr with a Myc epitope tag sequence at 5', in frame with an N-terminal EGFP tag were engineered in a modified FUGW vector carrying a synapsin promoter instead of a ubiquitin promoter (Lois et al., 2002). The lentiviruses were generated as described previously (Naldini et al., 1996). Striatal autaptic cultures were infected 6h after plating and treated with Oxt starting from 1 to 3 DIV.

Electrophysiology

Autaptic neuronal cultures (9-14 DIV) were subjected to whole-cell voltage clamp recordings at -70 mV with an amplifier EPSC10 (HEKA) under the control of the Patchmaster 2 program (HEKA). All traces were analyzed using AxoGraph (AxoGraph Scientific). EPSCs and IPSCs were evoked by depolarizing the cell from -70 to 0mV for 2 ms. The readily releasable vesicle pool (RRP) size was measured after the application of a hypertonic solution containing 0.5M sucrose. The vesicular release probability (Pvr) was calculated by dividing the charge transfer occurring during a single EPSC or IPSC by that occurring during the response to hypertonic sucrose. Short-term plasticity was assessed by measuring the evoked EPSC depression during 10Hz stimulation train. mEPSCs and mIPSCs were recorded in the presence of 300nM tetrodotoxin (TTX, Tocris Biosciences). Cell surface glutamate and GABA receptors were activated by the focal application of 100 µM glutamic acid or 3 µM GABA, respectively. The extracellular solution used for all recordings contained (mM): 140 NaCl, 2.4 KCl, 10

Hepes, 10 glucose, 4 $CaCl_2$ and 4 $MgCl_2$ (320mOsml/liter), pH, 7.3. The patch pipettes were filled with a solution containing (mM): 136 KCl, 17.8 Hepes, 1 EGTA, 4.6 $MgCl_2$, 4 NaATP, 0.3 Na_2GTP, 15 creatine phoshatase and 5U/ml phosphocreatine kinase (315-320 mOsml/liter), pH 7.4. All extracellular solutions were applied by means of a custom-built flo array of flow pipettes controlled by a stepper that permits the complete rapid replacement of solution within 30 ms.

Immunocytochemistry

Neuronal cultures were fixed by incubation in a solution containing 4% paraformaldehyde (w/v)/4% sucrose (w/v) in phosphate-buffered saline, pH 7.4, for 20 min. Subsequently, cells were incubated for 20 min in PBS containing 0.3% (v/v) Triton, 10% (v/v) normal goat serum (GIBCO) and 0.1% (w/v) fish skin gelatin (Sigma) to permeabilize membranes and block nonspecific binding sites. The same solution was used for diluting both primary and secondary antibodies. Neurons were incubated with primary antibodies for 2 h at room temperature or overnight at 4°C, washed three times with PBS, and then incubated in secondary antibody for 2 h. After three washes, coverslips were mounted using the Vectashield® medium with DAPI (Vector Laboratories). The same protocol was followed for the staining of all antigens with the exception of the staining for PSD95, for which the cells were fixed in absolute methanol for 7 min at -20°C. The following primary antibodies were used: rabbit polyclonal directed against vGAT (1:1000; Synaptic Systems), vGLUT1 (1:1000; Synaptic System), Map2 (1:500; Chemicon); GFP

(1:1000; Synaptic Systems); chicken polyclonal against Map2 (1:500; Novus Biologicals); mouse monoclonals against gephyrin (3B11, 1:500; Synaptic Systems), and PSD95 (6G6-1C9, 1:200; Abcam). For the identification of inhibitory and excitatory synapses, triple stainings for gephyrin, vGAT, and Map2 or for PSD95, vGLUT1 and Map2, were performed, respectively. All images were captured using a confocal microscope (TCS SP2 Leica equipped with AOBS [acousto-optical beam splitter] at high magnification (40X; numerical aperture=1.0; resolution=1024x1024 pixels). Image analysis was carried out using the ImageJ software (NIH). Synapses were counted as described previously (Varoqueaux et al., 2002). Briefly, the signals of pre- and post-synaptic markers were first thresholded to omit any background contribution from the analysis. Subsequently, the number of puncta in each cell were measured after the application of a specific filter capable to distinguish larger puncta. The same images were also analyzed to determine the co-localization between immunostained pre- and post-synaptic proteins. In this particular case, the background was substracted using the Subtract Background Plugin, and the Intensity Correlation Analysis plugin was used to calculate the Manders' overlap coefficient (Manders et al., 1993). The dendritic arborizations were assessed using the advanced Sholl analysis plugin of ImageJ, in which concentric circles are drawn at 5 µm intervals around a common center in the cell body and the numbers of crossing dendrites are counted in each of these circles (Sholl, 1953). The images used for the Sholl analysis were acquired using an upright

epifluorescence microscope (Zeiss Axio Imager Z1) with a 25X objective lens.

Data analysis

All data are shown as mean values ± SEM, resulting from at least three independent experiments. Statistical analyses were performed using a one-way ANOVA followed by a post-hoc Bonferroni test. Results were considered statistically significant at $p<0.05$. All statistical analyses were performed using the Prism® version 5 software (GraphPad, San Diego, CA).

RESULTS

Oxt exposure reduces synaptic transmission in glutamatergic but not in GABAergic hippocampal neurons

To investigate the potential role of Oxt on synaptic transmission in neurons of the central nervous system, we investigated the effects of the exposure of GABAergic and glutamatergic hippocampal neurons to Oxt. We incubated autaptic hippocampal cultures from C57BL/6J mice to 100nM Oxt for 1 or 3 DIV starting on the first day after plating and then carried out patch-clamp recordings between 9 and 14 DIV. We first determined the evoked excitatory and inhibitory post-synaptic currents (EPSCs and IPSCs, respectively) upon depolarizing the cells from -70 to 0 mV for 2 ms. The EPSC amplitudes in glutamatergic neurons were significantly reduced to about 40% and 30% of control

values, respectively, upon exposure to Oxt for 1 or 3 DIV (Figure 1, panels A and C; $p<0.001$ Oxt 1 DIV vs control; $p<0.05$ Oxt 3 DIV vs control).

The size of the post-synaptic current (PSC) to a single action potential (AP) depends on many factors, such as the quantal response to a single synaptic vesicle (SV), the number of synapses, and the release probability (for review, see Stevens, 2003). In order to investigate the causes of the altered EPSC amplitude following Oxt exposure we determined the size of the readily releasable pool (RRP) of SVs, that corresponds to the vesicles that are docked and primed for membrane fusion. Similar changes to those detected for the evoked EPSC amplitudes were observed in the PSC triggered by exposure to a hypertonic sucrose (0.5 M) solution (Figure 1, panels B and D), which is known to cause the release of the RRP (Rosenmund and Stevens, 1996; Jockusch et al., 2007). In particular, we observed that glutamatergic neurons exposed to Oxt for 1 or 3 DIV display a reduction in the apparent RRP size of 60 and 55%, respectively (Figure 1, panels A and D; $p<0.05$). However, neither IPSC amplitudes (Figure 1, B and C) nor RRP sizes (Figure 1, panels B and D) of GABAergic neurons were altered by Oxt exposure for 1 and 3 DIV, indicating a specific action of the peptide on excitatory neurons in the hippocampus. Then, in order to investigate whether the reduction of the EPSC amplitude was caused by a decrease in release sites or by a change in the release machinery, we compared the vesicular release probability (Pvr) across the different treatment conditions. The Pvr, calculated by dividing the charge transferred during an action

potential-evoked PSC by the RRP charge, was equally unchanged in Oxt-treated glutamatergic and GABAeric neurons compared to control ones (Figure 1E), indicating that Oxt exposure does not alter the probability by which an individual release-competent vesicles will fuse in response to an AP.

We next examined the change in synaptic responses induced by repetitive stimulation at 10Hz. Indeed, depending upon the release probability, synapses show a depression or facilitation of PSCs during trains of stimuli, most likely because of the balance between fusion and resupplying of synaptic vesicles (SVs). Corroborating our analysis of the Pvr, the analysis of short-term plasticity showed that EPSC and IPSC amplitudes in Oxt-treated hippocampal glutamatergic and GABAergic neurons progressively depressed during 10-Hz stimulation trains to the same steady-state depression levels as control cells (Figure 1, panels J and M). Taken together, these data indicate that Oxt exposure does not alter the release probability in response to single or repetitive APs.

The changes in synaptic transmission observed in glutamatergic neurons after Oxt exposure can be caused by a reduction in the number of synapses present in neurons, or by functional defects at the level of individual synapses. To distinguish between these two possibilities, we analyzed miniature EPSCs (mEPSCs) and IPSCs (mIPSCs) which occurr from spontaneous vesicle fusion events. We found that mIPSC amplitudes and frequencies were not altered by Oxt treatment of GABAergic neurons (Figure 1, panels G-I). However, mEPSC amplitudes were unchanged between control and Oxt-treated

glutamatergic neurons (Figure 1, panels F and H), whereas the frequencies of mEPSCs in Oxt-treated neurons were reduced to 50% of control values (Figure 1, panels F and I; $p<0.05$). The observation that mEPSC amplitudes are not affected by Oxt treatment indicates that the quantal size of neurotransmitter at a single synapse is similar between Oxt-treated and control neurons and that the sensitivity to glutamate in each functional synapse is identical under the two conditions analysed. However, the reduction of mEPSC frequencies upon Oxt-treatment indicates a reduced number of functional glutamatergic synapses.

To further assess the effect of Oxt in shaping hippocampal synaptic transmission and to further test the hypothesis that Oxt exposure reduces the number of functional synapses, we applied exogenous glutamate (100 µM) or GABA (3 µM) to autaptic cultured neurons to stimulate all functional glutamate and GABA receptors on cell surface. Similarly to what we observed before, GABA-induced currents were the same in treated and control groups (Figure 1, panels N and O), while Oxt-treated glutamatergic neurons at DIV 10-14 exhibited a significant reduction of their responses to 100µM glutamate as compared to control cells (Figure 1, panels K and L; $p<0.05$), indicating a reduction in the number of post-synaptic glutamate receptors. These findings support the hypothesis that Oxt induces a decrease in the number of functional synapses.

Taken together, the data described above indicate that Oxt exposure selectively influences glutamatergic synaptic transmission in the mouse hippocampal neurons. In particular, the fact that the current

induced by the application of glutamate is reduced in Oxt-treated glutamatergic neurons, together with the lower evoked EPSC and mEPSC frequencies, suggest that Oxt induces an alteration in synaptic transmission, which is most likely due to a reduction in the number of normally functioning synapses.

Oxt exposure reduces dendrite branching and synapse number in glutamatergic hippocampal neurons

The architecture of the dendritic arborization determines the amount and the integration of the synaptic inputs that a neuron receives (Koester and O'Leary, 1994). To examine whether Oxt affects neuronal maturation, we analysed the morphology of individual hippocampal neurons treated with 100nM Oxt in autaptic cultures (Bekkers and Stevens, 1991) by seeding an identical number of cells (~4000 cells per 35 mm^2) on astrocytic islets. The numbers of neurons at 10 DIV were not affected by Oxt (data not shown). However Oxt treated-neurons exhibited a reduced morphological complexity as compared to untreated neurons. To examine whether this structural feature was related to an impaired dendritic development induced by Oxt, we stained neurons fixed at 14 DIV with an antibody directed against the microtubule-associated protein 2 (Map2), a specific dendritic marker. Following Oxt exposure for either 1 or 3 DIV, neurons were found to show less dendritic branching than untreated cells (Figure 2A). We quantified this effect using the Sholl analysis, which calculates the number of dendrites crossing concentric circles drawn at 5 µm

intervals around the neurons (Sholl, 1953). The number of crossing dendrites was highest at 30 µm distance from the cell body in both treated and untreated neurons. However, between 20 and 85µm from the cell body, all-Oxt treated neurons showed less crossing dendrites than untreated neurons (Figure 2B). The number of dendrites at 30µm distance from cell bodies was significantly reduced in neurons exposed to Oxt for 1 or 3 DIV as compared to control cells (Figure 2C, $p<0.05$). These data indicate that the early application of Oxt influences dendrite development and arborization.

The defective dendritogenesis upon Oxt treatment could be associated with alterations in synapse development, which in turn could explain the phenotypic change in synaptic transmission observed in glutamatergic neurons exposed to Oxt. To study the effects of Oxt on synapse formation, we made use of antibodies directed against vGLUT1 (Wojcik et al., 2004), to specifically label glutamatergic pre-synapses and against PSD95 to stain excitatory post-synapses (Schlüter et al., 2006; Figure 2D). Using laser scanning confocal microscopy coupled to computer-assisted image analysis, the number of vGLUT1- and PSD95-positive fluorescent puncta were counted in each glutamatergic autaptic neuron, whose morphology was identified through Map2 labeling. The quantitative analysis revealed that the number of glutamatergic nerve terminals (Figure 2E) and the number of juxtaposed post-synapses (Figure 2G) were reduced following Oxt exposure, in agreement with the reduced evoked EPSC amplitudes and mEPSC frequencies and the impaired responses to exogenous glutamate observed in Oxt-treated cells.

Hovewer, Oxt exposure did not influence the spatial matching of pre- and post-synapses. In fact, the determination of the Mander's overlap coefficient between vGLUT1 and PSD95 signals revealed no significant difference between control neurons and neurons treated with Oxt for 1 or 3 DIV (Figure 2G), indicating that the gross structure and association of pre- and post-synapses in Oxt-treated neurons remains intact.

Finally, to test whether the effects induced by Oxt are exerted at early stages of neuronal development and then affect subsequent steps of the synaptogenetic process or whether Oxt treatment affects later stages of synapse consolidation, we started the exposure at 7 DIV to Oxt of autaptic hippocampal cultures, i.e. at a time when the process of synaptogenesis is already under way (Fletcher et al., 1994). The postponed application of 100 nM Oxt to excitatory hippocampal neurons did not cause those effects on evoked EPSC amplitudes, mEPSC frequencies, RRP sizes and glutamate-induced currents that were observed when Oxt was administered to cultures starting one day after seeding (Supplementary Figure 1)

Taken together, these results show that Oxt exposure causes an impaired dendritic development, which in turn leads to a reduction in the number of synapses and that these effects are exerted exclusively at an early phase of synaptic development.

Oxt exerts its effect on neuronal differentiation through an interaction with Gq-coupled receptors

The effects of Oxt are mediated through a high affinity interaction with a unique Oxtr GPCR. However, due to the high sequence similarity with arginine vasopressin (Avp), Oxt can also bind to Avp receptor subtypes with lower affinity, and thereby act as a partial agonist (Chini et al., 1996). To investigate whether the effects elicited by Oxt on glutamatergic neurons were caused via an interaction with Oxtrs, we exposed hippocampal autaptic neuronal cultures to the selective Oxtr antagonist atosiban (100nM) for 1 or 3 DIV in combination with 100nM Oxt. The analysis of synaptic transmission performed on glutamatergic neurons between 9 and 14 DIV showed that the presence of the antagonist abolished all the Oxt-induced effects (Figure 3, A-K) whereas atosiban alone had no effects ineffective. In addition, the antagonist inhibited the ability of Oxt to reduce dendritic arborisation when applied at early stages (Figure 4A). Both the Sholl analysis and the quantitative analysis of the number of dendrites crossing at 30µm distance from cell bodies revealed no significant differences between control neurons and atosiban-treated neurons (Figure 4, panels A and C). Taken together, these data show that Oxt exerts its action on hippocampal glutamatergic neurons through a specific interaction with Oxtrs, and not via Avprs.

The Oxtr can promiscuously couple to different heterotrimeric G proteins in several cell types (Zhou et al., 2007; Gravati et al., 2010; Rimoldi et al., 2003). In response to agonist binding, Oxtr activation leads to the stimulation of PLCβ via an interaction with $G_{q/11}$. Hovewer,

inhibition and stimulation of adenylyl cyclase via $G_{i/o}$ and G_s, respectively, have been demonstrated. To investigate which intracellular pathway is activated by Oxt in hippocampal glutamatergic neurons, we treated hippocampal cultures with Oxt together with 3 µM U73122, an aminosteroid that is reported to act as a specific inhibitor of phospholipase C. In particular, this compound inhibits the PLCβ-mediated hydrolysis of phosphoinositides to inositol tris-phosphate, while leaving the synthesis of cAMP unaffected. Neuronal cultures were exposed to U73122 alone or in combination with Oxt for 1 DIV since 3-DIV incubation led to profound morphological abnormalities and excessive neuronal death (Supplementary Figure 2). Co-incubation with U73122 blocked all electrophysiological effects induced by Oxt (Figure 5, panels A-K) and the Oxt-induced impairment of dendrite development (Figure 5, panels L-N). In contrast, the exposure to U73122 per se did not significantly alter any of the analysed parameters (Figure 5, panels A-N).

To further investigate whether the observed effects of Oxt also involve Oxtrs coupled to heterotrimeric G proteins of the Gi/Go subfamily we took advantage of pertussis toxin (PTX), which selectively catalyzes the irreversible ADP-ribosylation of the $G\alpha_{i/o}$-subunits, thus preventing the signalling through G_i/G_o-coupled Oxtrs. Hippocampal neurons were thus exposed to 100ng/µl PTX from 1 through 3 DIV, and 8 h after PTX addition 100nM Oxt was also added to the cultures. Oxt was still able to exert its actions on glutamatergic neurons, causing a reduction of evoked EPSC amplitudes (Suppl. Figure 3, panels A and C), mEPSCs frequencies (Suppl. Figure 3, panels F and H), RRP sizes (Suppl. Figure

3, panels B and D) and responses to exogenous glutamate application (Suppl. Figure 3, panels J and K).

Intriguingly, PTX did not prevent Oxt-induced effects after 1 DIV treatment and it was only partially effective after 3 DIV treatment without reaching statistical significance. The latter finding may result from the activity of PTX on other pathways than the G_i/G_o-mediated signaling pathways.

Taken together, these results suggest that Oxt affects synaptic transmission and neuronal morphology of hippocampal glutamatergic neurons through the activation of Oxtrs coupled to $G_{q/11}$.

Inhibitory neurons are resistant to Oxt-induced effects due to the lack of Oxtrs

Our experiments so far showed that Oxt only affects excitatory neurons in the hippocampus. This is in conflict with other reports (Zaninetti and Raggenbass 2000) indicating that Oxt influences inhibitory synaptic currents. To determine the reasons for this discrepancy we investigated the effects of Oxt on the inhibitory transmission in striatal autaptic cultures, which mostly contains inhibitory neurons.

Oxt was added the striatal cultures for 1 or 3 DIV starting on the day after seeding and synaptic transmission was monitored between 9 and 14 DIV. Similar to what was observed in hippocampal inhibitory neurons, Oxt exposure did not influence the evoked IPSC amplitudes, the size of the RRP, the vesicular release probability, the response to

exogenous application of GABA, short-term plasticity during 10Hz-stimulation trains, or the mIPSC amplitude and frequency of striatal cells (Figure 6 A-K). In agreement with this, the analysis of striatal neuronal morphology at 14 DIV did not reveal any obvious differences in dendritic branching between control and Oxt-treated neurons (Figure 7, panels A and B). Furthermore, we quantified GABAergic pre- and post-synapses of striatal neurons at 14 DIV by immunostaining for vGAT and gephyrin, respectively (Wojcik et al., 2006; Fritschy et al., 2008) and found that the treatment with Oxt did not significantly alter the number of GABAergic synapses in striatal cells (Figure 7, panels D-F).

To verify whether the absence of an Oxt response in striatal neurons is due to an intrinsic resistance of inhibitory neurons or to the lack of Otrs, we induced the overexpression of Oxtrs in striatal neurons by means of infection with a lentivirus encoding the full length human Oxtr carrying an N-terminal Myc tag (Suppl. Figure 4). The treatment with Oxt for 1 or 3 DIV caused a reduction of evoked IPSC amplitude in Oxtr-positive GABAeric striatal neurons (Figure 8, panels A and D) as compared to cells expressing EGFP (1d=$p<0.05$; 3d=$p<0.01$). Similar differences were observed in the post-synaptic responses triggered by a hypertonic sucrose solution (Figure 8, panels B and E; 1d=$p<0.05$; 3d=$p<0.01$) or by the exogenous application of 3 µM GABA (panels C and G; 1d=$p<0.05$; 3d=$p<0.01$). These Otx-induced alterations in synaptic transmission were associated with an impaired dendritic development. Following Oxtr expression and subsequent Oxt exposure for either 1 or 3 DIV, striatal neurons were characterized by

less dendritic branching than EGFP-expressing cells (Figure 8 H) as assessed by Sholl analysis (Figure 8 I). Moreover, comparison of the number of dendrites at 30µm distance from cell bodies showed significantly lower values in Oxtr-expressing treated cells than in cells expressing EGFP (panel J, 1d=$p<0.05$; 3d=$p<0.01$).

Taken together, these data indicate that striatal GABAergic neurons lack sensitivity to Oxt due to the absence of Oxtrs. However, sensitivity to Oxt-induced effects on synaptic transmission and dendritic arborization can be induced in these neurons upon induction of Oxtr receptor expression.

DISCUSSION

Oxytocin is believed to play a key role in brain development and to modulate higher brain functions, such as cognition and social behaviour. In particular, Oxt acts during brain development at perinatal and early postnatal stages. Prior to delivery, a surge of Oxt in the maternal circulation occurs, which serves a dual physiological role. It promotes parturition and lactation in the mother, and it protects the fetal brain from hypoxic insults, which can occur during labour, by promoting the switch of GABAergic effects from excitatory to inhibitory (Tyzio et al., 2006). In addition to providing neuroprotection to the fetal brain, Oxt has been proposed to influence brain development and maturation of central circuitries (Crepel et al., 2007).

Our results are coherent with the view that Oxt is of crucial importance during neurodevelopment, by showing that Oxt signalling affects the development and functions of murine hippocampal synapses. Hereby we show that early and transient treatment of excitatory glutamatergic neurons with Oxt causes a reduction of dendrite arborisation and a concomitant decrease in the number of synapses. These changes are specifically driven by the activation of Oxtrs and the downstream activation of phospholipase Cβ (PLCβ). Interestingly, cultured GABAergic neurons did not exhibit such effects in response to Oxt treatment. However, the ectopic viral-mediated expression of Oxtrs in GABAergic neurons lowered the dendrite growth, thus suggesting that the lack of sensitivity to Oxt of naïve GABAergic neurons is due to a lack of Oxtrs.

Our results are in accordance with previous reports showing that Oxt regulates the plasticity of SON neurons, where Oxt is synthesized, and acts in an autocrine manner after being released from the neuronal somata and/or dendrites (Theodosis 2002; Veenema and Neumann, 2008). This self-regulation, which is maximal during the second post-natal week, is correlated with a transient increase of dendritic branching (Chevaleyre et al., 2001) and results from a coordinated action of Oxt (and Avp) together with glutamate presumably released from incoming afferents (Chevaleyre et al., 2002). Hereby we show that Oxt exposure of cultured murine hippocampal neurons affects nerve cell differentiation by reducing dendrite branching through $G_{q/11}$-mediated intracellular signalling pathways. The exact molecular mechanisms by which Oxt controls neuronal development is still

unclear, but it is possibile that it is achieved via the modulation of cytoskeletal component(s), as was reported in the case of the α7 nicotinic receptor and the α1 adrenergic receptor, whose activation triggers the $G_{q/11}$ intracellular signalling cascade. Indeed, a recent study has shown that the activation of the α7 nicotinic receptor by its ligand acetylcholine in differentiating neuronal cells inhibits microtubule dynamics at growth cones, via the activation of $G_{q/11}$, the production of inositol trisphosphate (IP3) and the subsequent release of Ca^{2+} from intracellular stores (Nordman and Kabbani, 2014). Likewise, the increased noradrenergic signalling that follows repetitive stress exposures induces changes in neuronal cytoarchitecture, such as dendrite retraction and reduced spine density in pyramidal neurons (Radley et al, 2006, 2008; Cook and Wellman 2004, Wellmann 2001) through the activation of $G_{q/11}$-coupled α1 adrenergic receptors and PKC-mediated downstream cascade. *In vitro* studies showed that a sustained activation of PKC induces changes of spine density in hippocampal cultured neurons through a disruption of actin cytoskeleton cross linking (Calabrese and Halpain, 2005). Recently, these findings were validated *in vivo* (Hains et al., 2009), indicating that $G_{q/11}$-mediated PKC activation plays a prominent role in shaping the dendritic morphology.

Our data show that Oxt does not only shape the dendritic arborisation but also it reduces the synapse number in glutamatergic hippocampal neurons, and that these effects are exerted in an early phase of synaptic development. Recently, it has been reported that the maternal treatment of mice and rats with a specific Oxtr antagonist

one day before delivery enhances the electrophysiological activity of glutamatergic neurons in hippocampal slices of juvenile rodents (Tyzio et al., 2014). The Authors interpreted their results as an indication that the Oxt-induced switch of GABAergic signalling from excitatory to inhibitory had not occurred (Tyzio et al., 2006). Our results offer an alternative interpretation, according to which Oxt negatively affects the development of excitatory glutamatergic synapses by acting on Oxtrs coupled to PLCβ. The finding that Oxt acts only on excitatory neurons, contrarily to what was previously reported about the inhibitory activity of Oxt on GABAergic interneurons (Muhlethaler et al., 1983; 1984; Zaninetti and Raggenbass, 2000; Owen et al., 2013) is certainly intriguing. Indeed, intracellular recordings carried out in hippocampal slices had previously revealed that Oxt powerfully excites a class of non pyramidal neurons and that this in turn may indirectly depress the activity of CA1 pyramidal neurons (Muhlethaler et al., 1983; 1984). More recently, it has been proposed that Oxt enhances the inhibitory synaptic transmission in the rat hippocampus by activating interneurons located in the stratum pyramidale (Zaninetti and Raggenbass, 2000) by modulating fast-spiking interneurons (Owen et al., 2013). The different animal species used by us and others (mouse versus rat) can possibly explain these discrepancies. In fact, it has been proposed that the activity of Oxt on hippocampal neurons is species-specific. For instance, in the guinea pig hippocampus no Oxt binding was detected and CA1 interneurons were not responding to Oxt exposure (Reggenbass et al., 1989). In addition, the different experimental setups used for analysing the neuronal effects of Oxt

could account for these discrepancies. We analysed the effects of 1-3 DIV Oxt exposure on individual neurons grown as autaptic cultures, whereas previous studies mainly examined the effects of a shorter Oxt application to hippocampal slices. Using autaptic cultures, we studied the consequences of Oxt exposure in isolated neurons hence we assessed cell autonomous responses to Oxt. This can be considered a more direct approach as compared to what has been previously performed using hippocampal slices, where indirect effects due to the presence of a neuronal network or to homeostatic changes cannot be ruled out. Finally, the high concentrations of Oxt used in previous studies (50-100-fold over the affinity constant) could limit the specificity of the effects, as it is known that Oxt can bind Avp receptors at a lower affinity (for a recent review see Manning et al., 2012). In contrast, the concentrations of Oxt and the Oxtr antagonist used in the present study guarantee the selective targeting of Oxtrs (Busnelli et al., 2013). This allowed us to avoid the parallel activation of Avp receptors by an excessive concentration of Oxt and corresponding complications in the analysis and interpretation of Oxt-induced effects.

Hereby, we propose that the lack of effects of Oxt on hippocampal inhibitory neurons is simply due to the fact that Oxtrs are not expressed in these neurons. In situ hybridization studies using radiolabeled probes or radioligand binding assays demonstrated that Oxtrs are indeed expressed in the rodent hippocampus, but both techniques did not provide any clues on the excitatory or inhibitory identities of the Oxtr-expressing neurons. The exact neuronal

localization of Oxtrs has also been compromised by the lack of sensitive and specific antibodies. However, our data, together with the observation that Oxtr-expressing cells do not contain GAD67 in the striatum and hippocampus of knockin mice expressing tagged Oxtrs (unpublished personal communication from Katsuhiko Nishimori), indicate that Oxt cannot influence inhibitory neurons due to the lack of Oxtrs. The finding that the sensitivity to Oxt can be established in inhibitory neurons following ectopic expression of Oxtrs supports this notion and indicates that the signalling cascade triggered by Oxt to regulate neuronal differentiation and function is the same in excitatory and inhibitory neurons despite the obvious differences of the pre- and post-synaptic protein repertoires. Since the neuronal effects of Oxt are induced upon activation of Oxtrs coupled to the activation of PLCβ, it is possible that the stimulation of other GPCRs coupled to PLCβ can compensate Oxt signalling defects in Oxtr-null neurons. In this regards, it is worth mentioning that the behavioural alterations reported in adult Oxtr knockout mice were rescued by the administration of a selective agonist of Avp1a receptors that are also coupled to PLCβ (Sala et al., 2011)

In conclusion, our findings support the notion that Oxt exerts an early regulatory role *in vitro* on synapse formation and function of the murine hippocampal neurons that express Oxtrs, i.e. the excitatory glutamatergic neurons. As a consequence, less excitatory synapses are formed. This could also happen *in vivo,* when fetal brain is exposed to the perinatal surge of maternal Oxt. Thus dysfunctional Oxt transmission could alter the excitation/inhibition balance in the brain

resulting in neurobehavioural disturbances. Interestingly, an increasing number of studies indicate that the impairment of Oxt neurotransmission is related to ASDs (Neuman, 2008; Donaldson and Young, 2008; Carter, 2003, 2007; Modhal et al., 1998; Hollander et al., 2003; 2007; Guastella et al., 2009). The data from animal models indicate that a lack of a proper supply of exogenous Oxt from the mother to the fetus or to the breast fed newborns affects the neuronal development in genetically susceptible mice, thus increasing the risk of developing autistic-like behaviours (Higashida et al., 2010). Moreover, studies performed on Oxt and Oxtr knockout animals have shown that the lack of Oxt signalling causes alterations in social behaviours similar to those observed in autistic patients (Takayanagi et al., 2005; Sala et al., 2011). A possible involvement of Oxt in ASDs is further supported by the observation that plasma Oxt levels in autistic children are lower than in age-matched normal controls (Modhal et al., 1998), and that the infusion of Oxt reduced some of the core symptoms in adults with autism or Asperger's syndrome (Hollander et al., 2003; 2007). It has been reported (Tanoue and Oda, 1989) that autistic children were breast-fed significantly shorter than infants in control groups, and that Oxt is critical for regulating social behaviour during early experience in infants (Fries et al., 2005). More importantly, it has been proposed that ASDs are associated with an imbalance between excitatory and inhibitory synaptic transmission, whose correct balance is of fundamental importance for the development of a proper neuronal network (Rubestein and Merzenich, 2003). Indeed, more than 30% of autistic patients also

exhibit epileptic seizures (Gillberg and Billstedt 2000), thus supporting the notion of an E/I imbalance in ASDs. Moreover, many of the genes that have been directly or indirectly implicated in autism, encode for proteins that are important for the establishment and/or the maintenance of a proper E/I balance, such as cell adhesion molecules (LeBlanc and Fagiolini, 2011). Our observation that Oxt can control the balance between inhibitory and excitatory activities in cultured hippocampal neurons fits with what has been reported for ASDs. Hence, alterations of Oxt/Oxtr signalling could determine a maladaptive synaptic organization that in turn may contribute to the development of different neurodevelopmental disorders, such as ASDs.

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FIGURE LEGENDS

Figure 1. *Exposure to* oxytocin *reduces evoked and spontaneous synaptic transmission in glutamatergic but not in GABAergic hippocampal neurons.* (**A,B**) Representative traces (left) of depolarization-evoked EPSCs (**A**) and IPSCs (**B**) and responses after application of hypertonic (0.5 M) sucrose (right) in hippocampal glutamatergic and GABAergic neurons unexposed (Ctrl; black) or exposed to oxytocin (Oxt; 100 nM) for 1 (Oxt 1d; light grey) or 3 DIV (Oxt 3d; dark grey. (**C**) Mean evoked PSC amplitudes in naive and Oxt-treated hippocampal glutamatergic and GABAergic neurons. (**D**) Mean charge transfer in hippocampal glutamatergic and GABAergic neurons exposed to hypertonic sucrose (apparent readily releasable pool (RRP) size estimates). (**E**) Average vesicular release probability (Pvr) in hippocampal glutamatergic and GABAergic neurons following Oxt exposure. The Pvr values, expressed as percentages of RRP, were calculated by dividing the charge transfer during an action potential evoked response by the charge transfer measured during a response to hypertonic sucrose (reflecting the apparent RRP). (**F,G**) Representative mEPSC traces recorded at -70 mV holding potential in the presence of 300 nM tetrodotoxin (TTX) in naïve and Oxt-treated hippocampal glutamatergic and GABAergic neurons. (**H,I**) Mean mPSC amplitudes (**H**) and frequencies (**I**) in hippocampal glutamatergic and GABAergic neurons. (**J**) Evoked EPSC depression during 10 Hz (control, n=49; Oxt 1d, n=40; Oxt 3d, n=44) stimulation train. Data were normalized to the first response in the train. (**K**) Representative traces of responses to the application of 100 µM glutamate in glutamatergic control and Oxt-treated cells. (**L)** Mean amplitudes of responses to 100 µM glutamate in hippocampal glutamatergic neurons. (**N**) Representative traces following application of 3 µM GABA in GABAergic Oxt-treated and untreated hippocampal neurons. (**N**) Mean amplitudes of responses to exogenous 3 µM GABA in hippocampal

GABAergic neurons. (**M**) Evoked IPSC depression during 10 Hz (control, n=39; Oxt 1d, n=35; Oxt 3d n=37) stimulation train. Data were normalized to the first response in the train. Error bars indicate S.E.M. The number of cells analysed are given in the histogram bars (*P<0.05; **P<0.01 using one-way ANOVA followed by post-hoc Bonferroni test).

Figure 2. *Oxytocin exposure impairs dendrite branching and reduces synapse numbers in hippocampal glutamatergic neurons*. (**A**) Representative binary images of autaptic hippocampal neurons exposed to Oxt (100 nM) for 1 (Oxt 1d) or 3 DIV (Oxt 3d) and processed for immunofluorescence at 14 DIV using an anti-Map2 antibody to label dendrites. (**B**) Sholl analysis of control and Oxt-exposed neurons. For each condition, 40 cells from three independent experiments were analysed. (**C**) Number of crossing dendrites at a distance of 30 µm from cell bodies in Ctrl and Oxt-treated neurons (n=40). The numbers of analysed cells is reported in each histogram bar. Data are expressed as means ± S.E.M. (*P< 0.05 using one-way ANOVA followed by post-hoc Bonferroni test). (**D**) Immunofluorescence staining of 14 DIV autaptic hippocampal neurons (labelled in blue by Map2 staining) following Oxt exposure for 1 or 3 DIV using vGLUT1 (red) and PSD95 (green) antibodies as markers for glutamatergic pre- and post-synapses, respectively. (**E,F**) Quantitative analysis of the average numbers of vGLUT1-positive pre-synaptic (**E**) and PDS95-positive post-synaptic puncta (**F**) in individual autaptic hippocampal neurons unexposed or exposed to Oxt for 1 or 3 DIV. The numbers of analysed cells are given in the histogram bars. Error bars represent the means ± S.E.M. (*P< 0.05 using one-way ANOVA followed by post-hoc Bonferroni test). (**G**) Quantification of the colocalization between vGLUT1 and PSD95 immunofluorescences using the Manders' overlap coefficient. Error bars indicate S.E.M.

Figure 3. *Oxytocin receptor antagonist atosiban blocks the oxytocin-induced effects in glutamatergic hippocampal neurons.* Autaptic hippocampal cultures were exposed for 1 or 3 DIV to 100 nM atosiban alone (ATO 1d and 3d, respectively) or atosiban + Oxt (ATO+Oxt 1d and 3d; 100 nM) by adding fresh drug solution every 24 h, and synaptic transmission was monitored using the whole-cell voltage clamp technique between 9 and 14 DIV. Only the data of control (Ctrl, black), ATO 1d (dark blue), and ATO-Oxt 1d (light blue) treated neurons are shown. Representative traces (**A**) and average amplitudes (**C**) of evoked EPSCs in excitatory hippocampal neurons. Representative traces (**B**) and averaged apparent RRP sizes released upon application of hypertonic sucrose (0.5 M) for 6 s (**D**) and average Pvr (**E**). Representative traces (**F**) and average mean amplitudes (**G**) and frequencies (**H**) of mEPSCs recorded in the presence of 300 nM TTX in hippocampal gutamatergic neurons for the different condition analysed. (**I**) Analysis of short-term plasticity during 10 Hz train stimulation (Ctrl, n=42; ATO 1d, n=30; ATO+Oxt 1d, n=38; ATO 3d,n=37; ATO+Oxt 3d, n=34). Representative traces (**J**) and mean amplitudes (**K**) of responses to the application of 100 µM glutamate. In all graphs, n values are indicated at the bottom of each bar. Results are expressed as means ± S.E.M. of at least three independent experiments.

Figure 4. *Atosiban application antagonizes the effects of oxytocin on dendrite branching*. (**A**) Representative 14 DIV hippocampal autaptic neurons labelled with Map2 untreated (Ctrl) and treated with atosiban (100nM; ATO) or ATO + Oxt (100 nM). (**B**) Sholl analysis of control (n=50), ATO-treated (1d=44; 3d=49), and ATO+Oxt-treated (1d=50; 3d=49) hippocampal neurons . (**C**) Quantitative analysis of the number of dendrite crossings at 30 µm distance from cell bodies. The number of neurons analysed for each condition is indicated within each bar. Data are expressed as means ± S.E.M. of three independent neuronal preparation.

Figure 5. *The effects of Oxt on hippocampal glutamatergic neurons are mediated by the interaction with a Gq-coupled receptor*. Autaptic hippocampal neurons were exposed to the phospholipase C inhibitor (PLC) U-73122 (3 μM), alone or in combination with Oxt (100nM) for 1 DIV. Electrophysiological recordings were performed between 9 and 14 DIV whereas immunofluorescence assays were carried out at 14 DIV. (**A**) Sample traces of evoked EPSCs from control (Ctrl, black) and U-73211 (U73211, red)-treated cultures in the absence or presence of Oxt (U73211+Oxt, orange). (**B**) Representative traces of post-synaptic currents evoked by the application of hypertonic sucrose in glutamatergic neurons for each analysed condition. (**C**) Sample traces of glutamate-induced currents in the presence of U73211 alone or in combination with Oxt in control glutamatergic autaptic neurons. Quantitative evaluations of average evoked EPSC amplitudes (**D**), mean readily releasable vesicle pool size (**E**), average Pvr (**F**) and mean amplitudes (**M**) of responses to exogenous application of 100 μM glutamate in glutamatergic hippocampal cells for all the analysed experimental conditions. (**H**) Evoked EPSC depression during 10 Hz stimulation train (Ctrl, n=45; U73122, n=31; U73122+Oxt, n=32). Data were normalized to the amplitudes of the first response in the train. (**I**) Sample traces of mEPSCs and quantitative analysis of averaged mEPSC amplitudes (**J**) and frequencies (**K**) in glutamatergic hippocampal neurons untreated or exposed to U73211 alone or in combination with Oxt. (**L**) Example of representative autaptic neurons labelled for Map2 at 14 DIV and converted to binary images for each analysed condition. (**M**) Sholl analysis of control (n=36) and cells treated with U73211 alone (n=34) or combined with Oxt (n=34). (**N**) Quantitative analysis of the number of crossing dendrites at 30 μm distance from the neuronal somata in each experimental condition. In all bar graphs, n values are indicated at the bottom of the bars. Bars represent the means ± S.E.M.

Figure 6. *Oxt exposure does not influence synaptic transmission of striatal GABAergic neurons*. Autaptic striatal cultures were exposed to Oxt (100 nM) for 1 (Oxt 1d) or 3 DIV (Oxt 3d) and synaptic transmission was recorded in GABAergic neurons using the whole-cell voltage clamp technique between 9 and 14 DIV. Representative traces of depolarization-evoked IPSCs (**A**), responses after application of hypertonic sucrose (**B**) or 3 µM GABA (**C**) in GABAergic striatal neurons untreated (Ctrl, black) or exposed to Oxt (1d, light grey; 3d, dark grey). Quantitative analysis of average evoked IPSC amplitudes (**D**), mean RRP sizes (**E**), average Pvr (**F**) and mean amplitudes of responses to application of 3 µM GABA for all the analysed experimental conditions G). (**H**) Analysis of short- term plasticity during 10 Hz train stimulation (Ctrl, n=34; Oxt 1d, n=37; Oxt 3d, n=36). Data were normalized to the first response in the train. (**I**) Representative traces of mIPSCs and quantitative analysis of averaged mEPSC amplitudes (**J**) and frequencies (**K**) in GABAergic striatal neurons of control and Oxt-treated cultures. The numbers of analysed neurons in each data set is depicted within each histogram bar. Error bars indicate S.E.M.

Figure 7. *Early Oxt exposure does not influence the morphology and the synapse number of striatal GABAergic neurons*. (**A**) Example of binary images of autaptic striatal neurons exposed to Oxt (100 nM) for 1 (Oxt 1d) or 3 DIV (Oxt 3d) and processed for immunofluorescence at 14 DIV using an anti-Map2 antibody to label the dendrites. (**B**) Sholl analysis of control and Oxt-exposed neurons (Ctrl, n=36; Oxt 1d, n=30; Oxt 3d=31). (**C**) Number of crossing dendrites at 30 µm distance from cell bodies in control and Oxt-treated neurons. The number of analysed cells is indicated within each histogram bar. Data are expressed as means ± S.E.M. (**D**) Staining of striatal autaptic neuronal cultures at 14 DIV following Oxt exposure for 1 or 3 DIV using antibodies directed against vGAT and gephyrin to label GABAergic pre- and post-synapses, respectively, and against Map2 to label dendrites. (**E**) Quantitative analysis of the average numbers of pre-synaptic

puncta (vGAT-positive) and (**F**) post-synaptic puncta (gephyrin-positive) in individual Oxt-treated or control autaptic striatal neurons. The number of analysed cells are given within the histrogram bars. Error bars represent the means ± S.E.M. of four independent neuronal preparations.

Figure 8. *Overexpression of oxytocin receptor in striatal cells impairs synaptic transmission and dendrite branching following Oxt exposure.* Striatal autaptic cultures were infected 6 h after plating with lentiviruses coding for EGFP or Oxtr-EGFP and subsequently treated with Oxt (100 nM) from 1 to 3 DIV. Representative traces of depolarization-evoked IPSCs (**A**), responses to hypertonic sucrose (**B**) and 3 μM GABA (**C**) in striatal GABAergic neurons expressing EGFP or Oxtr-EGFP and exposed to Oxt for 1 (EGFP+Oxt 1d, Oxtr+Oxt 1d, respectively) or 3 DIV (EGFP+Oxt 3d, Oxtr+Oxt 3d, respectively). Quantitative analysis of average evoked IPSC amplitudes (**D**), mean RRP size (**E**), average Pvr (**F**) and mean amplitudes (**G**) of responses to exogenous application of 3 μM GABA for all the analysed experimental conditions. (**H**) Representative binary images of autaptic striatal neurons exposed to Oxt for 1 or 3 DIV following infection with EGFP or Oxtr-EGFP encoding lentiviruses and processed for immunofluorescence at 14 DIV using an antibody against Map2 to label dendrites. (**I**) Sholl analysis (EGFP+Oxt 1d, n=43; Oxtr+Oxt 1d, n=21; EGFP+Oxt 3d, n=44; Oxtr+Oxt 3d, n=20) and (**J**) number of crossing dendrites at 30 μm distance from cell bodies. The number of analysed cells is reported within each histogram bar. Data are expressed as means ± S.E.M.

Figure1

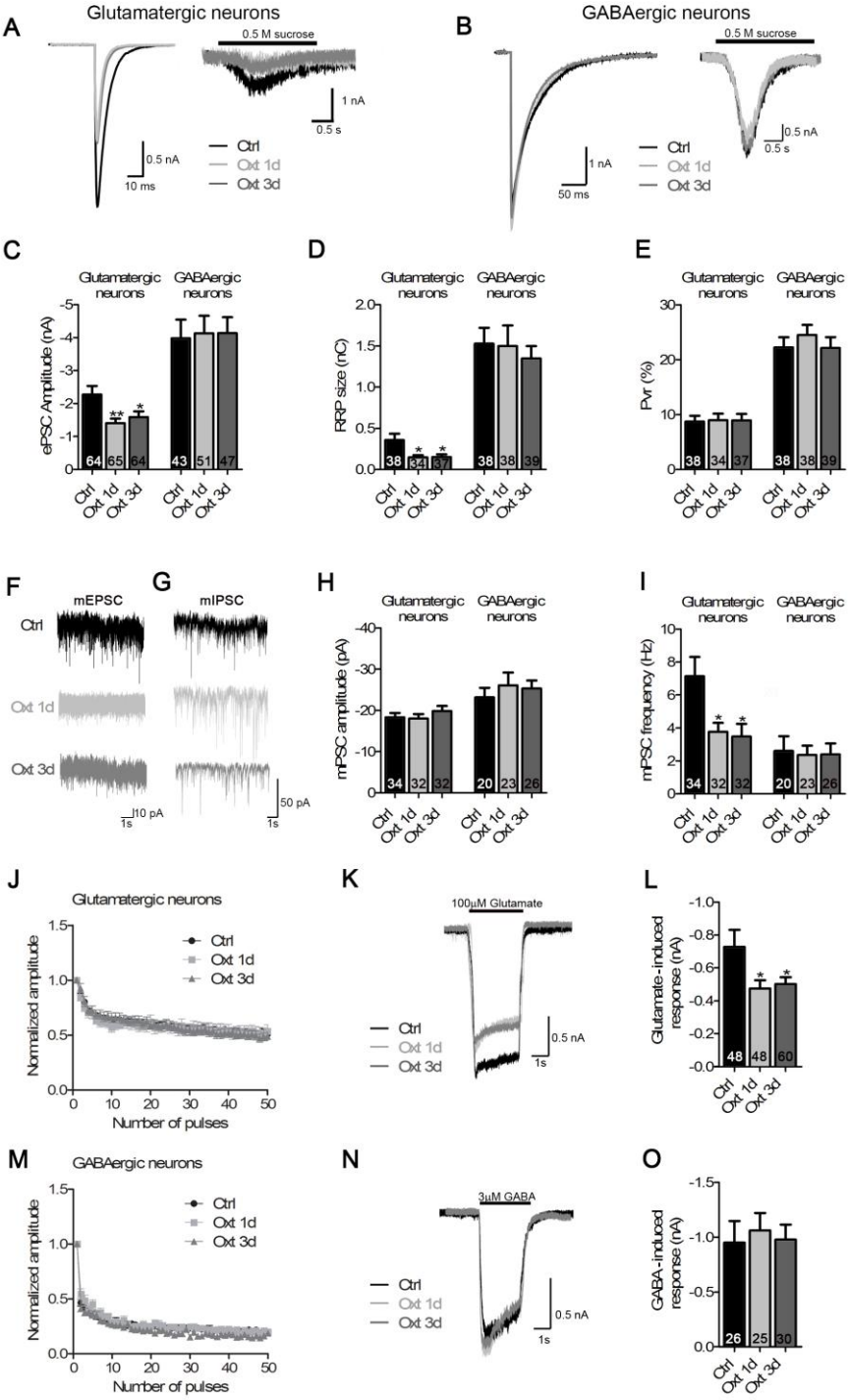

A
Glutamatergic neurons
0.5 M sucrose
1 nA
0.5 s
0.5 nA
10 ms
Ctrl
Oxt 1d
Oxt 3d
B
GABAergic neurons
0.5 M sucrose
0.5 nA
0.5 s
1 nA
50 ms
Ctrl
Oxt 1d
Oxt 3d
C
Glutamatergic neurons
GABAergic neurons
ePSC Amplitude (nA)
64 65 64 43 51 47
D
RRP size (nC)
38 34 37 38 38 39
E
Pvr (%)
38 34 37 38 38 39
F
mEPSC
Ctrl
Oxt 1d
Oxt 3d
10 pA
1s
G
mIPSC
50 pA
1s
H
mPSC amplitude (pA)
34 32 32 20 23 26
I
mPSC frequency (Hz)
34 32 32 20 23 26
J
Glutamatergic neurons
Normalized amplitude
Number of pulses
K
100μM Glutamate
0.5 nA
1s
L
Glutamate-induced response (nA)
48 48 60
M
GABAergic neurons
Normalized amplitude
Number of pulses
N
3μM GABA
0.5 nA
1s
O
GABA-induced response (nA)
26 25 30

Figure 2

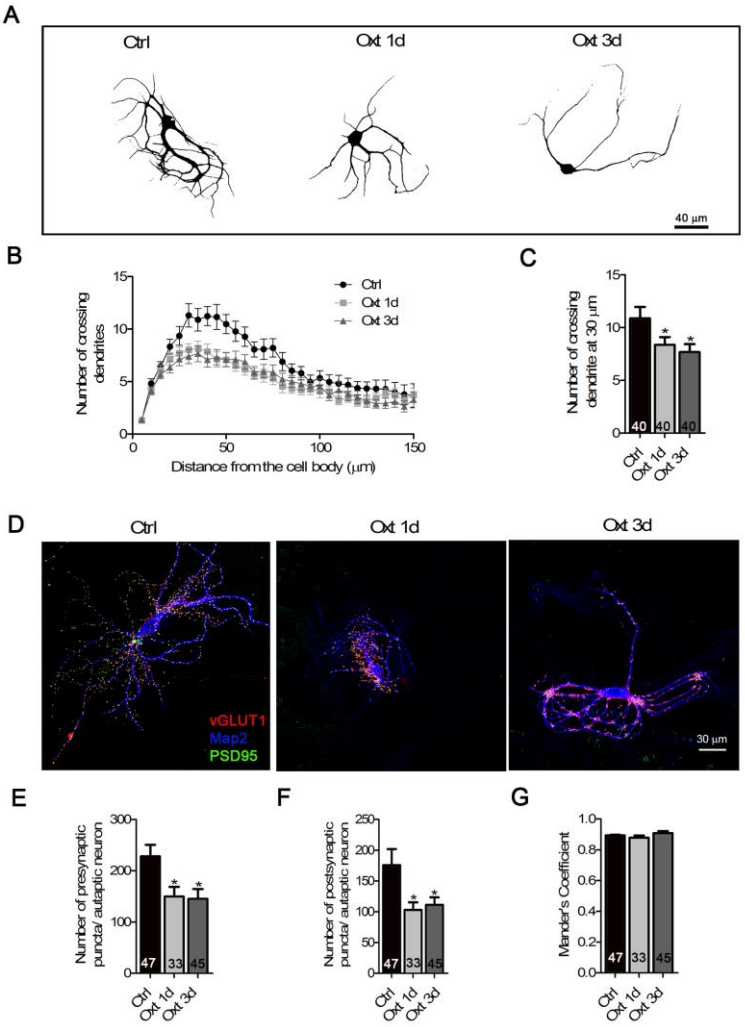

A
Ctrl
Oxt 1d
Oxt 3d
40 µm
B
Number of crossing dendrites
Distance from the cell body (µm)
Ctrl
Oxt 1d
Oxt 3d
C
Number of crossing dendrite at 30 µm
40
40
40
D
Ctrl
Oxt 1d
Oxt 3d
vGLUT1
Map2
PSD95
30 µm
E
Number of presynaptic puncta/ autaptic neuron
47
33
45
F
Number of postsynaptic puncta/ autaptic neuron
47
33
45
G
Mander's Coefficient
47
33
45

Figure 3

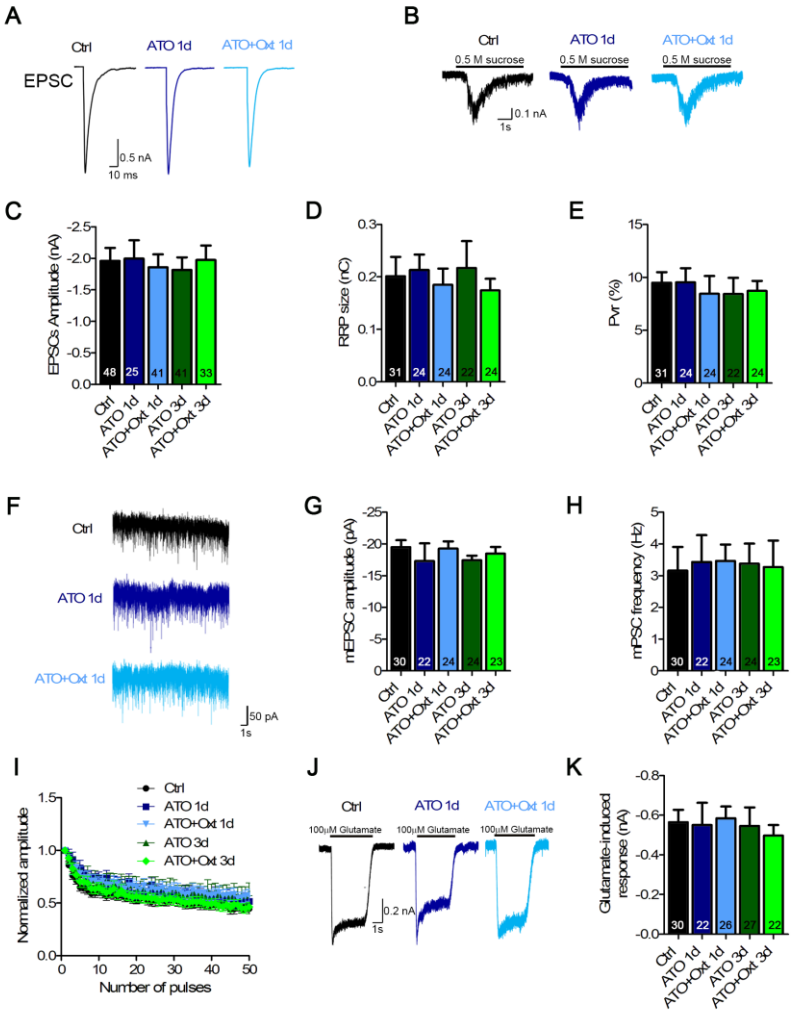

A
Ctrl
ATO 1d
ATO+Oxt 1d
EPSC
0.5 nA
10 ms
B
Ctrl
ATO 1d
ATO+Oxt 1d
0.5 M sucrose
0.5 M sucrose
0.5 M sucrose
0.1 nA
1s
C
EPSCs Amplitude (nA)
48
25
41
41
33
Ctrl
ATO 1d
ATO+Oxt 1d
ATO 3d
ATO+Oxt 3d
D
RRP size (nC)
31
24
24
22
24
E
Pvr (%)
31
24
24
22
24
F
Ctrl
ATO 1d
ATO+Oxt 1d
50 pA
1s
G
mEPSC amplitude (pA)
30
22
24
23
H
mPSC frequency (Hz)
30
22
24
23
I
Ctrl
ATO 1d
ATO+Oxt 1d
ATO 3d
ATO+Oxt 3d
Normalized amplitude
Number of pulses
J
Ctrl
ATO 1d
ATO+Oxt 1d
100μM Glutamate
100μM Glutamate
100μM Glutamate
0.2 nA
1s
K
Glutamate-induced response (nA)
30
22
26
22

Figure 4

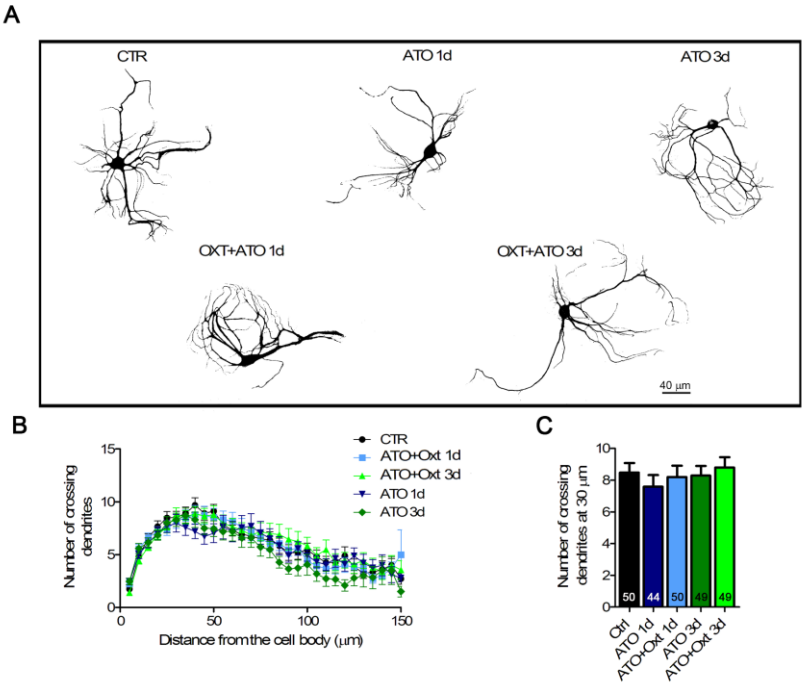

A
CTR
ATO 1d
ATO 3d
OXT+ATO 1d
OXT+ATO 3d
40 µm
B
CTR
ATO+Oxt 1d
ATO+Oxt 3d
ATO 1d
ATO 3d
Number of crossing dendrites
Distance from the cell body (µm)
C
Number of crossing dendrites at 30 µm
50
44
50
49
Ctrl
ATO 1d
ATO+Oxt 1d
ATO 3d
ATO+Oxt 3d

Figure 5

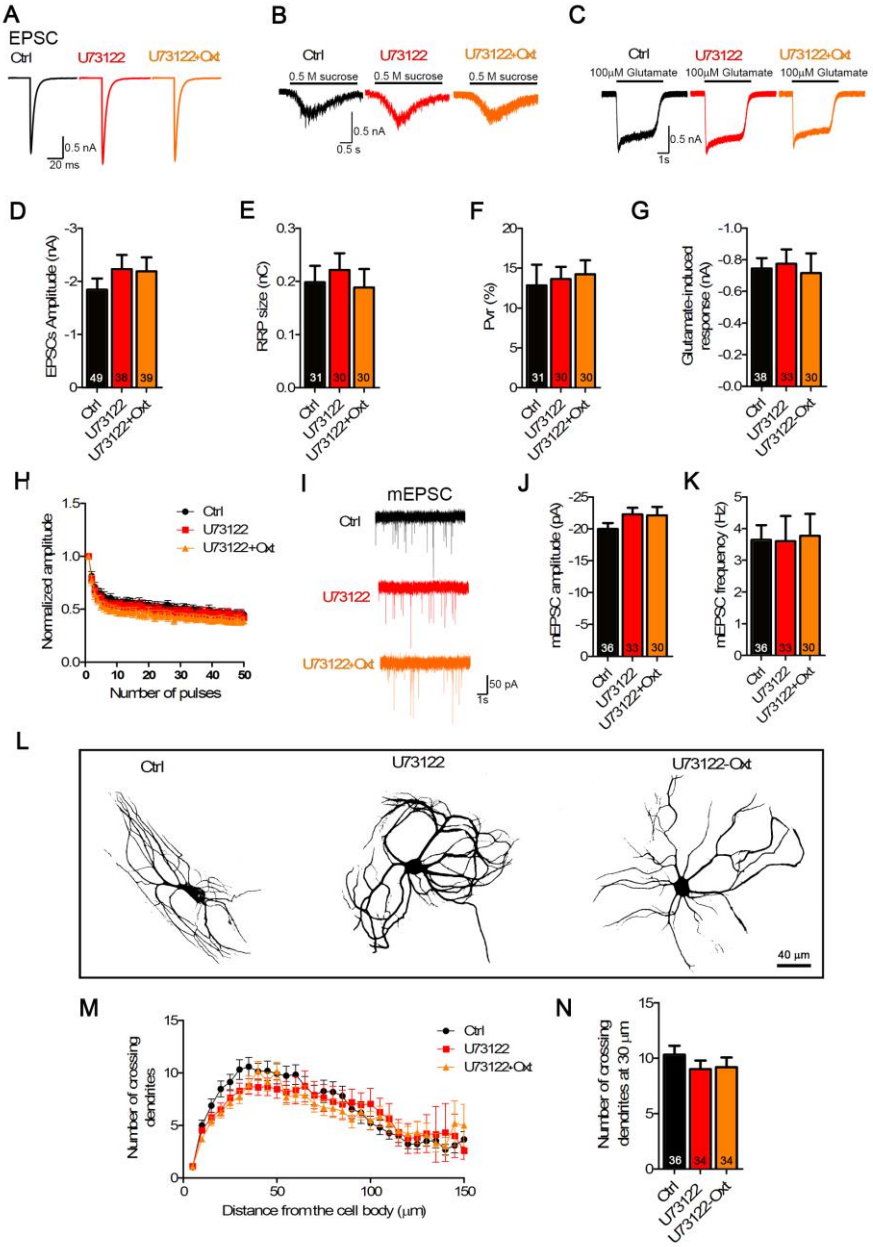

A
EPSC
Ctrl
U73122
U73122+Oxt
0.5 nA
20 ms
B
Ctrl
U73122
U73122+Oxt
0.5 M sucrose
0.5 M sucrose
0.5 M sucrose
0.5 nA
0.5 s
C
Ctrl
U73122
U73122+Oxt
100μM Glutamate
100μM Glutamate
100μM Glutamate
0.5 nA
1s
D
EPSCs Amplitude (nA)
49
38
39
E
RRP size (nC)
31
30
30
F
Pvr (%)
31
30
30
G
Glutamate-induced response (nA)
38
33
30
H
Normalized amplitude
Number of pulses
Ctrl
U73122
U73122+Oxt
I
mEPSC
Ctrl
U73122
U73122+Oxt
50 pA
1s
J
mEPSC amplitude (pA)
36
33
30
K
mEPSC frequency (Hz)
36
33
30
L
Ctrl
U73122
U73122-Oxt
40 μm
M
Number of crossing dendrites
Distance from the cell body (μm)
Ctrl
U73122
U73122+Oxt
N
Number of crossing dendrites at 30 μm
36
34
34

Figure 6

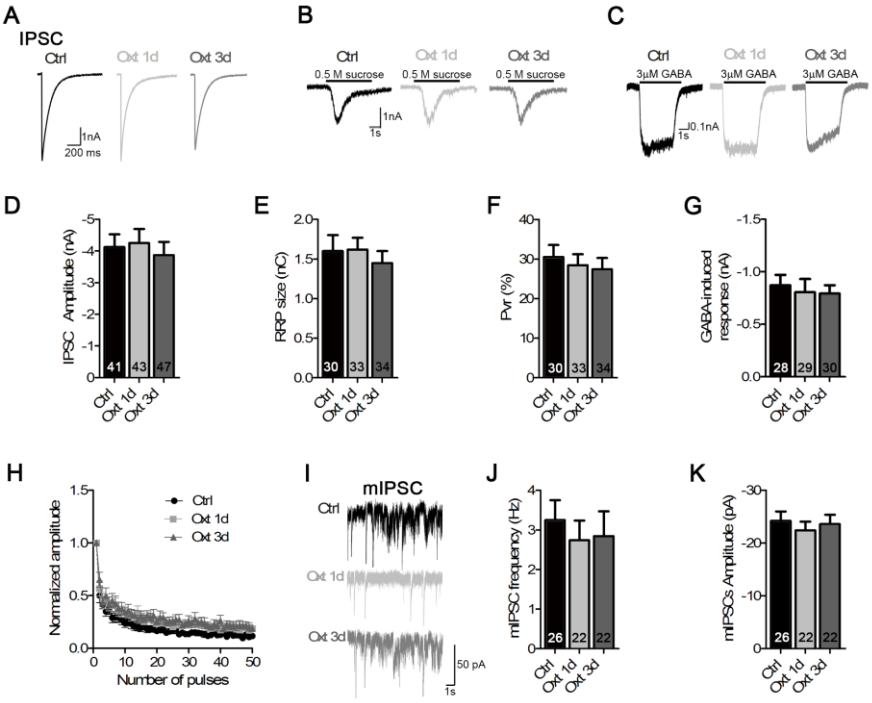

A
IPSC
Ctrl
Oxt 1d
Oxt 3d
1nA
200 ms
B
Ctrl
Oxt 1d
Oxt 3d
0.5 M sucrose
0.5 M sucrose
0.5 M sucrose
1nA
1s
C
Ctrl
Oxt 1d
Oxt 3d
3μM GABA
3μM GABA
3μM GABA
0.1nA
1s
D
IPSC Amplitude (nA)
41
43
47
Ctrl
Oxt 1d
Oxt 3d
E
RRP size (nC)
30
33
34
F
Pvr (%)
30
33
34
G
GABA-induced response (nA)
28
29
30
H
Normalized amplitude
Ctrl
Oxt 1d
Oxt 3d
Number of pulses
I
mIPSC
Ctrl
Oxt 1d
Oxt 3d
50 pA
1s
J
mIPSC frequency (Hz)
26
22
22
K
mIPSCs Amplitude (pA)
26
22
22

Figure 7

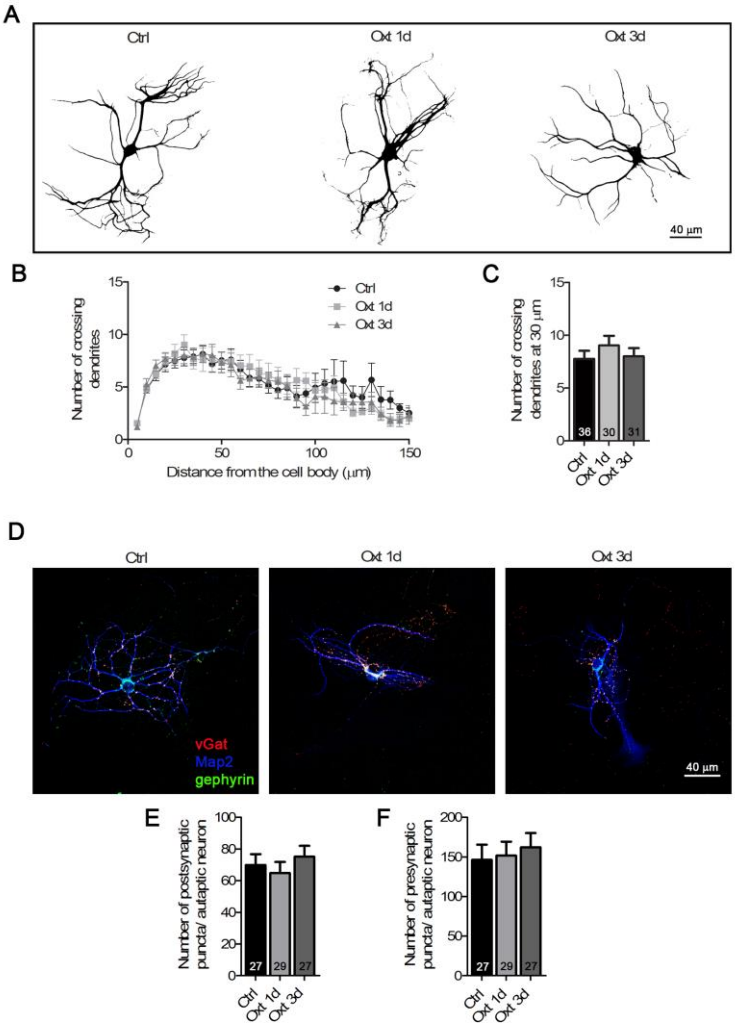
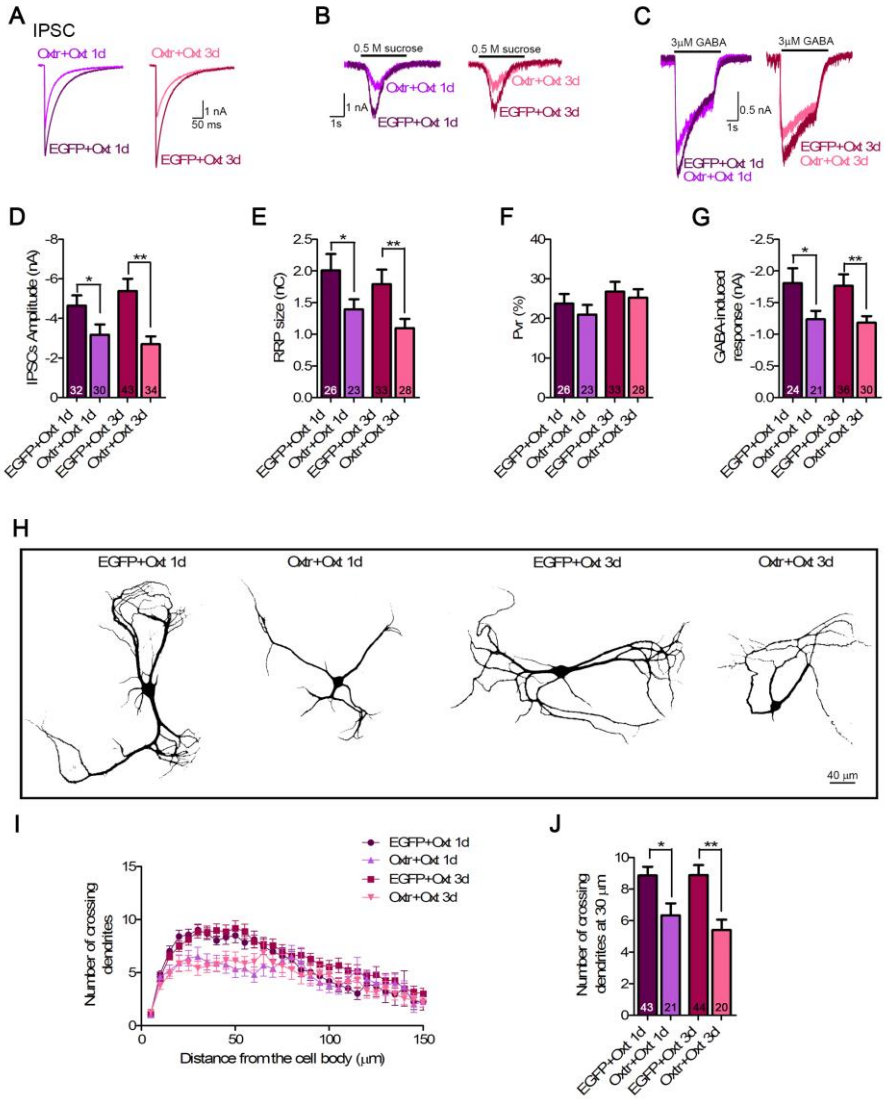

A
Ctrl
Oxt 1d
Oxt 3d
40 µm
B
Number of crossing dendrites
Ctrl
Oxt 1d
Oxt 3d
Distance from the cell body (µm)
C
Number of crossing dendrites at 30 µm
36
30
31
D
Ctrl
Oxt 1d
Oxt 3d
vGat
Map2
gephyrin
40 µm
E
Number of postsynaptic puncta/ autaptic neuron
27
29
27
F
Number of presynaptic puncta/ autaptic neuron
27
29
27

Figure 8


A
IPSC
Oxtr+Oxt 1d
Oxtr+Oxt 3d
1 nA
50 ms
EGFP+Oxt 1d
EGFP+Oxt 3d
B
0.5 M sucrose
0.5 M sucrose
Oxtr+Oxt 1d
Oxtr+Oxt 3d
1 nA
1s
EGFP+Oxt 1d
EGFP+Oxt 3d
C
3μM GABA
3μM GABA
0.5 nA
1s
EGFP+Oxt 1d
Oxtr+Oxt 1d
EGFP+Oxt 3d
Oxtr+Oxt 3d
D
IPSCs Amplitude (nA)
32
30
43
34
E
RRP size (nC)
28
23
33
28
F
Pvr (%)
26
23
33
28
G
GABA-induced response (nA)
24
21
36
30
EGFP+Oxt 1d
Oxtr+Oxt 1d
EGFP+Oxt 3d
Oxtr+Oxt 3d
H
EGFP+Oxt 1d
Oxtr+Oxt 1d
EGFP+Oxt 3d
Oxtr+Oxt 3d
40 μm
I
EGFP+Oxt 1d
Oxtr+Oxt 1d
EGFP+Oxt 3d
Oxtr+Oxt 3d
Number of crossing dendrites
Distance from the cell body (μm)
J
Number of crossing dendrites at 30 μm
43
21
44
20
EGFP+Oxt 1d
Oxtr+Oxt 1d
EGFP+Oxt 3d
Oxtr+Oxt 3d

Supplementary Figures

Figure S1

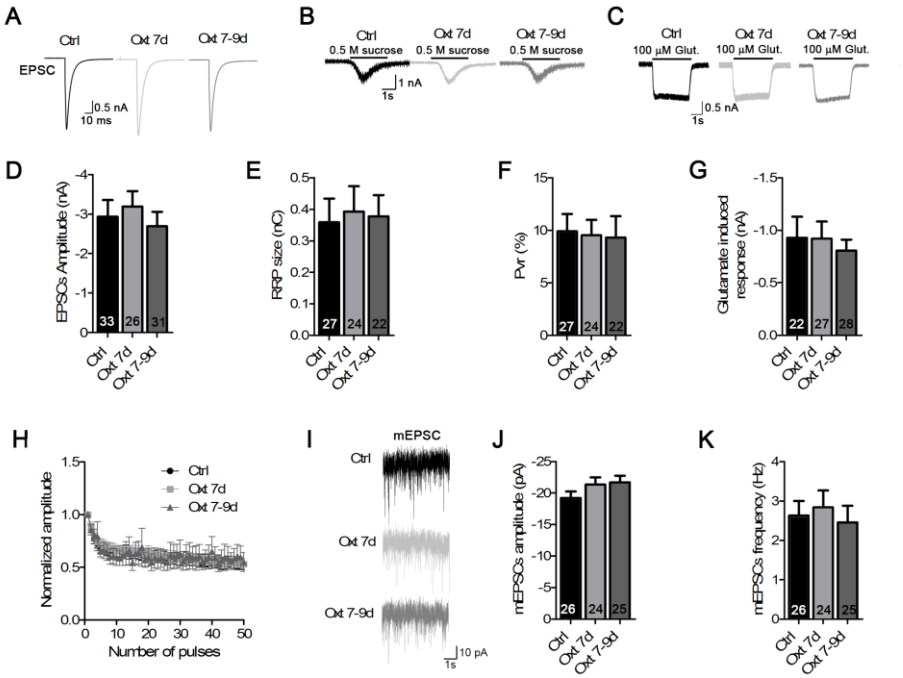


Figure S1. *Oxt exposure at later stages of neuronal development does not influence synaptic transmission of hippocampal glutamatergic neurons.* (**A**) Sample traces of depolarization-evoked EPSCs in hippocampal glutamatergic neurons exposed to Oxt (100 nM) at 7 DIV (Oxt 7d, light grey) or from 7 to 9 DIV (Oxt 7-9d, dark grey) and controls (Ctrl, black). (**B**) Representative traces of responses induced by the application of hypertonic sucrose (0.5 M) in control (Ctrl) and Oxt-treated (Oxt 7d and Oxt 7-9d). Black bars above traces correspond to the 6 s application of sucrose. (**C**) Representative traces of glutamate-induced currents in control (Ctrl) and Oxt-treated (Oxt 7d Oxt 7-9d) autaptic glutamatergic neurons. The black bars above the traces indicate the application time of exogenous 100 µM glutamate. Quantitative analysis of average evoked EPSC amplitudes (**D**), mean

readily releasable vesicle pool sizes (**E**), average Pvr (**F**) and mean amplitudes of responses to exogenous application of 100 µM glutamate (**G**) in hippocampal glutamatergic neurons for all the analysed experimental conditions. (**H**) Evoked EPSC depression during 10 Hz stimulation train (Ctrl, n=28; Oxt 7d, n= 27; Oxt 7-9d, n=30). (**I**) Representative mEPSC traces recorded at -70 mV holding potential in the presence of 300 nM TTX in control and Oxt-treated (Oxt 7d; Oxt 7-9d) glutamatergic neurons. Quantitative analysis of mean mEPSC amplitudes (**J**) and frequencies (**K**) in hippocampal glutamatergic neurons for all the analysed experimental conditions. The numbers of cells are given within the histogram bars. Error bars indicate S.E.M.

Figure S2

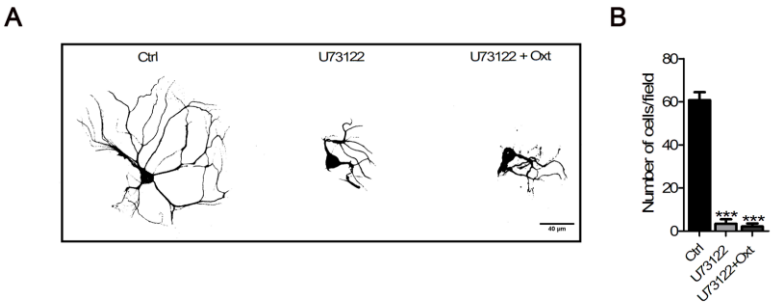


Figure S2. *The 3-day exposure to the phospholipase C inhibitor U73122 induces neuronal death***.** (**A**) Representative binary images of autaptic hippocampal neurons exposed to 3 µM U73122 for 3 DIV alone (U73122) or in combination with 100 nM Oxt (U73122+Oxt) and control neurons. Cultures were processed for immunofluorescence at 14 DIV using an anti-Map2 antibody to label dendrites. (**B**) Quantitative analysis of the number of cells counted in each field using a bright field microscope, used as an index of neuronal survival. The long-term exposure to the U74122 inhibitor led to profound morphological abnormalities and substantial cell death. Data are expressed as mean ± S.E.M. (***$p<0.0001$ using one-way ANOVA followed by post-hoc Bonferroni test).

Figure S3

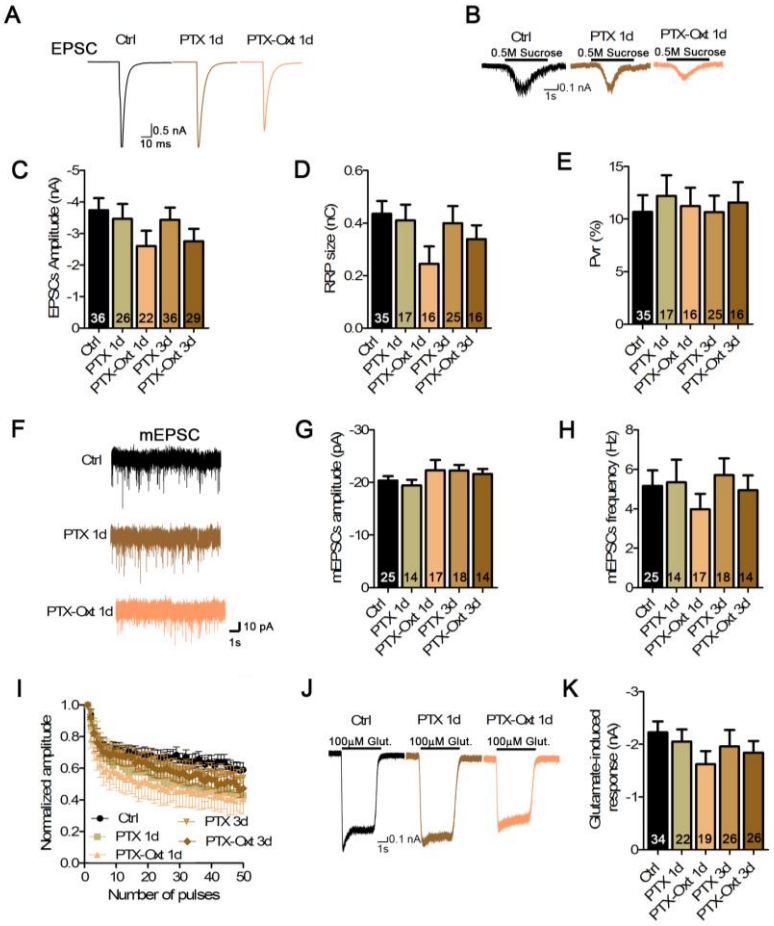


Figure S3. *Pertussis toxin does not completely block the effects induced by Oxt in glutamatergic hippocampal neurons.* Pertussis toxin (PTX, 100 ng/µl) was added to the cell media for 1 (PTX 1d) or 3 DIV (PTX 3d), and after 8 h incubation Oxt (100 nM; PTX+Oxt 1d; PTX+Oxt 3d) or vehicle (Ctrl) treatments were carried out. Synaptic transmission was monitored between 9 and 14 DIV. For brevity only the traces related to controls (Ctrl) and 1day treatments with PTX 1 day or PTX-Oxt are shown. Sample traces (**A**) and average amplitudes (**C**) of evoked EPSCs in excitatory hippocampal neurons. Representative traces (**B**) and averaged RRP sizes released upon application of hypertonic sucrose (0.5 M) for 6 s (**D**) and average Pvr (**E**). Representative traces (**F**) and

average mean amplitudes (**G**) and frequencies (**H**) of mEPSCs recorded in the presence of 300 nM TTX in hippocampal gutamatergic neurons for the different analysed conditions. (**I**) Analysis of the short-term plasticity during 10 Hz train stimulation (Ctrl, n=36; PTX 1d, n=24; PTX+Oxt 1d, n=19; PTX 3d, n=27; PTX+Oxt 3d=25). Representative traces (**J**) and mean amplitudes (**K**) of responses to the application of 100 µM glutamate. In all bar graphs, n values are indicated at the bottom of each bar. Results are expressed as means ± S.E.M. of at least three independent experiments.

Figure S4

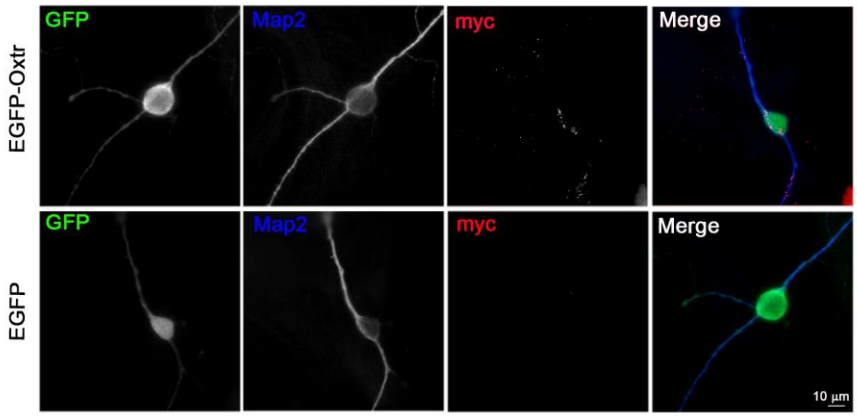


Figure S4. *Overexpression of oxytocin receptor in striatal autaptic cultures.* Striatal autaptic cultures were infected 6 h after plating with lentiviruses coding for EGFP or Oxytocin receptor (Oxtr)-EGFP carrying a Myc epitope tag at the N-terminus and immunolabeled at 10 DIV using antibodies against GFP (green), Map2 (blue) and Myc (red) to test for the expression level of Oxtr after infection. Striatal neurons overexpressing Oxtr exhibit a detectable Myc staining, that is absent in EGFP-expressing cells.

Chapter 3

Hippocampal Excitatory/Inhibitory Synaptic Imbalance In The Oxytocin Receptor Null Mouse Model Of Autism

Silvia Ripamonti, Francesca Guzzi, Marta Gravati, Gerardo Biella, Marta Busnelli, Katsuhiko Nishimori, Bice Chini, Mauro Toselli and Marco Parenti

Manuscript in preparation

Hippocampal Excitatory/Inhibitory Synaptic Imbalance In The Oxytocin Receptor Null Mouse Model Of Autism*

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*Running title: *Oxytocin and hippocampal synaptogenesis*

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Keywords: Oxytocin; synaptogenesis; hippocampus; autism

Background: Oxytocin receptor gene knockout mice exhibit autism-like symptoms, including increased seizure susceptibility and impaired cognitive flexibility.

Result: Lack of oxytocin receptor function increases while activation decreases excitatory synaptic proteins and current in hippocampal neuronal cultures.

Conclusion: Oxytocin affects the excitatory/inhibitory synaptic balance in hippocampus.

Significance: Learning how synapses are formed during development can unveil the pathogenesis of neurobehavioral disorders, such as autism.

SUMMARY

Our previous work (Sala et al., 2011) validated the oxytocin receptor null mouse (*Oxtr*$^{-/-}$) as a model for autism as it recapitulates some autistic-like symptoms, i.e. decreased social behavior and cognitive flexibility, increased seizure susceptibility and aggression. To search for the underlying synaptic mechanism(s) of impaired cognitive flexibility, we compared primary cultures of hippocampal neurons from 18-day *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ murine embryos using confocal microscopy and patch-clamp recordings. *Oxtr*$^{-/-}$ neurons showed a decreased immunofluorescence for pre-synaptic vesicular GABA transporter (vGAT) and post-synaptic inhibitory neuroligin 2 (NL2) adhesion and gephyrin scaffolding proteins in *Oxtr*$^{-/-}$. Conversely, the expression of presynaptic vesicular glutamate transporter 1 (vGLUT-1), post-synaptic excitatory NL1 adhesion and PSD95 scaffolding proteins, and GluR1-subunit of AMPA receptor were significantly enhanced in *Oxtr*$^{-/-}$ cultures. Moreover, both the

frequency and amplitude of spontaneous glutamatergic post-synaptic currents (EPSCs) were enhanced in *Oxtr*$^{-/-}$ neurons. Subsequently, the effects of the selective Oxtr agonist [Thr4-Gly7]-Oxt (TGOT) exposure on hippocampal neurons from C57BL/6J mice were evaluated to understand how the Oxt system could influence hippocampal synaptogenesis. We observed that TGOT treatment increased inhibitory vGAT and NL2, and decreased excitatory vGLUT-1 and NL1 immunofluorescences, together with a reduction in both frequency and amplitudes of sEPSCs. We conclude that Oxt regulates the balance between excitatory (E) and inhibitory (I) hippocampal synapses during development and suggest that the lack of Oxt may lead to neurobehavioural disturbances with an altered E/I ratio, such as autism and schizophrenia.

INTRODUCTION

Oxytocin (Oxt) is a nonapeptide primarily synthesized in the magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. The peptide secreted from the nerve endings in the posterior pituitary gland exerts key regulatory roles during parturition and lactation (1). In addition, Oxt acts in the brain to affect social recognition and interaction (2, 3), anxiety (4, 5), sexual behavior (4), and spatial memory (6) in different animal species.

We have recently proposed (7) that the oxytocin receptor gene knockout (*Oxtr*$^{-/-}$) mice originally developed by Takayanagi and coll. (8)

represent a valid experimental model of autism. Their behavioural phenotype recapitulates some primary and accessory autistic symptoms, i.e. an impairment of social behavior, increased aggression, increased susceptibility to pentilentetrazol-induced myoclonic seizures, and reduced cognitive flexibility in the T-maze spatial memory task.

Cognitive flexibility or reversal learning, that is the ability to flexibly adapt acquired information to changed contingencies, depends on hippocampal functioning (9-11). Thus, the decreased spatial memory skills observed in the *Oxtr*$^{-/-}$ mice can suggest the existence of some alterations of the hippocampal neuronal circuitries, resulting from the constitutive lack of Oxt function.

Oxytocin receptors are expressed in the rodent hippocampus (6, 12) and parvocellular Oxt neurons in the paraventricular nucleus (PVN) of the hypothalamus send projections to the hippocampus (13, 14). In addition Oxt released from the dendrites of magnocellular neurons of the hypothalamic supraoptic nucleus (SON) can reach by diffusion distant brain targets including the hippocampus (15). Based on these premises, here we have investigated whether any synaptic defect(s) are present in hippocampal neuronal primary cultures obtained from embryonic day 18 (E18) *Oxtr*$^{-/-}$ mice in comparison to *Oxtr*$^{+/+}$ cultures. To this aim the expression of some excitatory and inhibitory pre- and post-synaptic proteins was evaluated using immunofluorescence and confocal microscopy high-resolution imaging analysis and glutamatergic and GABAergic spontaneously evoked ion currents were recorded from pyramidal hippocampal neurons using the patch-clamp

technique. Our results show that the balance between excitation and inhibition (E/I) is shifted in favor of the former in the *Oxtr*$^{-/-}$ hippocampus, both in terms of synaptic protein machinery and ion currents. In addition, the exposure of wild type hippocampal cultures to the selective Oxtr agonist [Thr^{4}Gly^7]-Oxt (TGOT) causes a decreased expression of excitatory pre- and post-synaptic and glutamatergic current. We put forward the hypothesis that Oxt function is needed during embryonic development to ensure a balanced E/I hippocampal circuitry. This is discussed in terms of psychiatric and neurological human pathologies of altered neuronal excitation.

EXPERIMENTAL PROCEDURES

Animals

Oxtr$^{+/+}$ and *Oxtr*$^{-/-}$ mice (8) were obtained from L. Young (Emory University, Atlanta, GA) and maintained and genotyped as previously reported (7). C57BL/6J mice were purchased from Harlan Laboratories (Udine, Italy).

DNA Constructs

The expression vectors for neuroligin 1 (NL1) fused to the HA epitope (YPYDVPDYA, NL1-HA, Plasmid 15261: pCAG-NL1B) and NL2 (Plasmid 15259: pNICE-NL2A) were purchased from Addgene (www.addgene.org).

Cell Cultures

Primary hippocampal neuronal cultures were generated from the hippocampi of 18-day old mouse embryos (E18) as described (16). For each preparation two pregnant mice of each type carrying 6-8 embryos each were used. Neurons were maintained at 37°C and 5% CO_2 up to 14 days *in vitro* (DIV) with the addition of B27 supplement (Invitrogen-Gibco, San Giuliano Milanese, Italy) to ensure a nearly pure neuronal population with negligible (<0.5%) glial contamination. Culturing is performed for 14DIV to allow the clear detection of pre- and post-synaptic marker proteins.

COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (SigmaAldrich, Milan, Italy) supplemented with 10% (v/v) fetal bovine serum (Euroclone, Pero, Italy) and transfected with FuGENE® 6 (Roche, Mannheim, Germany). Transfected COS-7 cells were trypsinized 24 h after transfection, washed and plated at ≈80,000 cells/coverslips onto hippocampal neuron monolayers pre-grown for 7 DIV. At this stage NL1 and NL2 expressed in COS-7 cells can effectively drive the formation of synaptic-like contacts with co-cultured neurons. Cytosine arabinoside (10 µM; AraC, Sigma-Aldrich) was added to the culture medium at a final concentration of 0.5 µM to inhibit overgrowth of COS-7 cells. After 72 h of co-culture, cells were fixed for 20 min with 4% paraformaldehyde (w/v) /4% sucrose (w/v) in phosphate-buffered saline.

Cultures from C57BL/6J mouse hippocampi were exposed to the selective Oxtr agonist TGOT (10 nM; Bachem, Weil am Rhein,

Germany) during the first three days after plating by adding fresh solution every 24 h and processed for immunofluorescence at 14 DIV.

Immunocytochemistry

Immunofluorescence staining was carried out as described (Lentini et al., 2008). The following mouse monoclonal antibodies were used: vescicular GABA transporter (vGAT; 10 µg/mL), gephyrin (hybridoma supernatant 1:200), vesicular glutamate transporter 1 (vGLUT1; 13 µg/mL), NR1subunit of NMDA receptor (6 µg/mL), and synaptophysin (6 µg/mL) from Synaptic Systems (Göttingen, Germany); post-synaptic density protein 95 (PSD-95; 22 µg/mL) from Pierce Biotechnology (Rockfort, IL). Rabbit polyclonal antibodies against vGLUT1 (7 µg/mL), NL2 (20 µg/mL), γ_2-subunit of $GABA_A$ receptors (2 µg/mL), synaptophysin (10 µg/mL), and parvalbumin (5 µg/mL) were purchased from Synaptic Systems; GluR1-subunit of AMPA receptor (6 µg/mL) from Abcam (Cambridge, UK); microtubule-associated protein 2 (Map2; 2µg/mL) and glutamic acid decarboxylase 65/67 (GAD65/67; 5µg/mL) from Chemicon (Hampshire, UK); NL1 (2.5 µg/mL) and goat polyclonal antibody against HA epitope (1 µg/mL) from Santa Cruz Biotechnology. Alexa 488, 568 and 633 conjugated anti-mouse, anti-rabbit, and anti-goat secondary antibodies were from Molecular Probes (Leiden, Netherlands).

Imaging Analysis

Stained cells on glass coverslips were subjected to imaging analysis using a Zeiss LSM 710 laser scanning confocal microscope with a 63x objective while keeping constant all instrumental settings to allow direct comparisons between different fluorescence intensity scans. For each coverslip (two for each experimental condition) from 3-4 independent cell preparations eight random fields were captured and analyzed. In vGAT and vGLUT1 experiments z-axis stacks of 1 µm-thick images were taken and only puncta juxtaposed to Map2-positive processes with different lengths were counted and normalized to 10 µm dendrite using the NIH ImageJ software. In each field, 7/8 dendritic tracts with different lengths were analyzed up to a total of 105/120 dendrites for each of the three preparations. In the artificial synapse formation assay z-stack images of 30 randomly chosen COS-7 cells from 5 different coverslips for each co-culture were taken and the transfected COS-7 cell contours were chosen as regions of interest for quantitation. Fluorescence optical densities of vGAT- and vGLUT1-positive puncta normalized to each corresponding COS-7 cell areas were quantified using the MetaMorph® Software (Molecular Devices, Sunnyvale, CA). Finally, to determine the expression of post-synaptic markers (NL1, NL2, PSD-95, gephyrin, NR1, GluR1, and GABAγ_2) three distinct parameters were calculated using the NIH ImageJ software, i.e. the total number of merged fluorescent clusters, the intensities and areas of fluorescence in dendrites with different lengths. The data were then normalized to 10 µm dendritic length. In each field, 7/8

dendritic tracts with different lengths were analyzed up to a total of 105/120 dendrites for each of the three preparations.

Electrophysiological Recordings

To record spontaneously evoked postsynaptic currents (sPSCs) patch pipettes were filled with an internal solution containing: 135 mM CsCl, 3 mM NaCl, 10 mM EGTA, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM ATP, 0.3 mM GTP, and 10 mM Hepes, pH 7.4, with CsOH. Seals were formed and whole-cell configurations were obtained in a bath solution containing: 140 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, with NaOH. During spontaneous PSC recording 5 mM N-ethyllidocaine (QX-314) was added to the internal solution to block action potentials in the patch-clamped neuron. Moreover, during glutamatergic PSC (sEPSC) recording, 10 µM bicuculline was added to block $GABA_A$ receptors whereas during GABAergic PSC (sIPSC) recording 10 µM 2,3-dihydroxy-6-nitro7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) and 30 µM 4-(3phosphonopropyl)piperazine-2-carboxylic acid (CPP) were added to block AMPA and NMDA receptors, respectively. All drugs were stored at -20°C as concentrated aliquots and diluted into the recording saline on day of experiment. To detect spontaneous synaptic events, NeuroMatic, a collection of Igor Pro (WaveMetrics Inc., Oswego, OR) functions (http://www.neuromatic.thinkrandom.com/) was used. Detection threshold was set at 10 pA. The amplitudes of spontaneous synaptic events obeyed a lognormal distribution in all neurons tested. Accordingly, the amplitude averages and standard deviations were

computed using the lognormal function of the Origin 7.5 software (OriginLab Corp., Northampton, MA). Interevent intervals for spontaneous synaptic events were distributed exponentially and mean intervals were obtained from the best monoexponential fit of the inter-event interval distributions.

Statistical Analysis

In immunocytochemical analyses the paired Student's t-test was applied to calculate significant differences of results expressed as the means ± S.E.M. Results were considered statistically significant at $p < 0.05$. All statistical analyses were performed using the Prism® version 5 software (GraphPad, San Diego, CA).

RESULTS

***Hippocampal Neurons from Oxtr$^{-/-}$ Mice Exhibit Significantly Less GABAergic and More Glutamatergic Pre-synaptic Terminals*.** Fourteen-DIV hippocampal cultures from *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice were subjected to double staining immunofluorescence with antibodies against vGAT or vGLUT1 to stain pre-synaptic terminals together with Map2 antibody to label neuronal processes. Using laser-scanning confocal microscopy coupled to computer-assisted image analysis, the vGAT- or vGLUT1-positive fluorescent areas juxtaposed to Map2 fluorescent processes were quantified (Figure 1, panels A and B). The results show a significant increase of vGLUT1 fluorescence (*Oxtr*$^{+/+}$ =

112.6 ± 7.43 vs *Oxtr*$^{-/-}$ = 165.5 ± 9.74; P < 0.0001; n = 3 independent cell preparations) as well as a significantly reduced vGAT fluorescence (*Oxtr*$^{+/+}$ = 104.1 ± 9.32 vs *Oxtr*$^{-/-}$: 81.8 ± 7.34; P < 0.05; n = 3 independent cell preparations) in *Oxtr*$^{-/-}$ versus *Oxtr*$^{+/+}$ monolayers (Figure 1, panels C and D), suggesting an enhanced balance of excitatory over inhibitory pre-synaptic terminals in *Oxtr*$^{-/-}$ hippocampi. To check whether the diminished vGAT fluorescence was not due to a loss of GABAergic neurons but indeed to a reduced number of nerve terminals, we evaluated the GABA synthetic GAD65/67 fluorescence expressed as percent of total nuclear fluorescence from DAPI staining. The analysis did not reveal any significant difference of GAD65/67 fluorescence between *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ cultures (*Oxtr*$^{+/+}$ = 11.5 ± 1.4%; *Oxtr*$^{-/-}$ = 12.1 ± 0.3%; P > 0.05; n = 3 independent cell preparations)(Supplementary Figure 1). Likewise, the fluorescence from parvalbumin, that labels a sub-population of inhibitory neurons, over DAPI fluorescence is identical in *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ cultures (*Oxtr*$^{+/+}$ = 8 ± 2.5%; *Oxtr*$^{-/-}$ = 9.7 ± 1.8%; P > 0.05; n = 3 independent cell preparations) (Supplementary Figure 1). The different vGAT and vGLUT1 staining in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons could be due to a different ability to recruit the synaptic vesicles to respectively the inhibitory and excitatory pre-synaptic terminals. To verify this, the *in vitro* synapse-formation assay developed by Scheiffele and coll. (17) was employed. *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ neurons were co-cultured with COS-7 cells, serving as post-synaptic-like neurons upon transient expression of HA-tagged NL1 or NL2 proteins, that specifically label excitatory and inhibitory post-synaptic hemiportions, respectively. Following double

immunostaining with anti-HA tag and vGAT or vGLUT1 antibodies the merged fluorescence was taken as an evidence for the formation of artificial synaptic-like contacts between neurons and COS-7 cells. The imaging results (Figure 2, panels C and D) clearly demonstrate that *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ neurons do not differ in their ability to *in vitro* form synaptic-like contacts with COS7 cells overexpressing either excitatory or inhibitory NLs. Thus, it appears that *Oxtr*$^{-/-}$ neurons possess an intact molecular machinery required for the organization of both GABAergic and glutamatergic pre-synaptic compartments following trans-synaptic stimulation by the respective post-synaptic NLs. Intriguingly, *Oxtr*$^{-/-}$ neurons performed even better than *Oxtr*$^{+/+}$ ones forming significantly more vGAT-positive inhibitory contacts with co-cultured NL2 expressing COS-7 cells (*Oxtr*$^{+/+}$ = 11.4 ± 1.37; *Oxtr*$^{-/-}$ = 19.8 ± 1.63; $P < 0.01$; 30 COS-7 cells from 3 independent cell preparations) (Supplementary Figure 2, panel C). No significant difference was observed between *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ neurons when excitatory vGLUT1/NL1 merged fluorescence was quantified (*Oxtr*$^{+/+}$ = 13.8 ± 5.49 vs *Oxtr*$^{-/-}$ = 14.1 ± 4.16; $P > 0.05$; 30 COS-7 cells from 3 independent cell preparations) (Supplementary Figure 2, panel D).

Oxtr$^{-/-}$ Hippocampal Neurons Display a Reduced Expression of Inhibitory and an Increased Expression of Excitatory PostSynaptic Markers.

The genesis of mature, functionally active synapses depends on the coordinated expression of pre- and post-synaptic hemiportions. Thus, if any alteration(s) occur(s) in the development of pre-synaptic compartment it is not unreasonable to expect that also the postsynaptic compartment is affected. To verify this, the expression of some post-synaptic excitatory proteins, i.e. NL1 adhesion and PSD-95 scaffolding proteins, AMPA receptor GluR1, and NMDA receptor NR1 subunits, and of inhibitory proteins, such as NL2 adhesion and gephyrin scaffolding proteins, and GABA receptor γ2-subunit, was evaluated in *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ neurons. The analysis of protein expression was performed using immunofluorescence and confocal microscopy whose resulting images were analyzed evaluating three different parameters (see Experimental Procedures). Only the total number of fluorescent clusters is shown for brevity as other two parameters give identical results.

Neuroligin 1 (18) and 2 (19) are postsynaptic proteins that stabilize excitatory and inhibitory synapses, respectively, by forming trans-synaptic bridges with pre-synaptic neurexins (20). As shown in Figure 2, panel B and D, we detected a significantly increased NL1 (*Oxtr*$^{+/+}$ = 176.2 ± 10.42 vs *Oxtr*$^{-/-}$ = 222.7 ± 13.13; $P < 0.001$; n = 3 independent cell preparations) and a reduced NL2 immunofluorescence (*Oxtr*$^{+/+}$= 235.6 ± 19.03 vs *Oxtr*$^{-/-}$ = 174.4 ± 16.12; $P < 0.001$; n = 3 independent cell preparations) in 14 DIV *Oxtr*$^{-/-}$ than *Oxtr*$^{+/+}$ neurons. These results

indicate that similarly to what had been observed for the presynaptic expression of vGLUT1 and vGAT, *Oxtr*$^{-/-}$ neurons exhibit a higher expression of post-synaptic excitatory NL1 and a lower expression of inhibitory NL2. Post-synaptically, NLs bind to several PDZ domain-containing scaffolding proteins such as the excitatory PSD-95 and the inhibitory gephyrin, guiding the assembly of neurotransmitter receptors, ion channels and signaling proteins at the neuronal surface (21-23). Thus, 14 DIV *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ cultures were stained for PSD-95 and gephyrin in conjunction with synaptophysin labeling of presynapses. The imaging analysis (Figure 2, panel B and D) showed that *Oxtr*$^{-/-}$ neurons display a significant increase of PSD-95 (*Oxtr*$^{+/+}$ = 207.5 ± 13.27 vs *Oxtr*$^{-/-}$ = 290.6 ± 15.00; $P < 0.0001$; n = 3 independent cell preparations) and a decreased gephyrin (*Oxtr*$^{+/+}$ = 258.4 ± 14.36 vs *Oxtr*$^{-/-}$ = 185.4 ± 10.51; $P < 0.001$; n = 3 independent cell preparations) as compared to *Oxtr*$^{+/+}$ neurons. Finally, the AMPA receptor GluR1 subunit, the $GABA_A$ receptor γ2 subunit and the NMDA receptor NR1 subunit were analyzed. The total number of clusters for GluR1 subunit was significantly increased (*Oxtr*$^{+/+}$ = 282.5 ± 16.95 vs *Oxtr*$^{-/-}$ = 334.4 ± 18.50; $P < 0.05$; n = 3 independent cell preparations), whereas no significant differences were detected for NR1 (*Oxtr*$^{+/+}$ = 97.8 ± 13.54 vs *Oxtr*$^{-/-}$ = 78.59 ± 9.81; $P > 0.05$; n = 3 independent cell preparations) and GABAγ2 subunits (*Oxtr*$^{+/+}$ = 205.0 ± 12.28 vs *Oxtr*$^{-/-}$ = 218.9 ± 13.10; $P > 0.05$; n = 3 independent cell preparations) (Figure 3, panels B and D). Taken together these results suggest that the balance ratio between excitatory and inhibitory pre-and post-synaptic markers is

shifted to a higher set-point in the hippocampal neurons whereby Oxt function is constitutively impaired following *Oxtr* gene deletion.

Amplitudes and Frequences of sEPSCs are Higher in Oxtr$^{-/-}$ Hippocampal Neurons.

To assess whether the observed increments of excitatory pre- and post-synaptic markers in *Oxtr*$^{-/-}$ neurons had any functional impact on glutamatergic transmission, a comparative analysis of sEPSCs in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons was undertaken. Whole-cell recordings were obtained from visually identified 12 DIV hippocampal pyramidal neurons in culture. In a sample of 85 *Oxtr*$^{+/+}$ neurons and 73 *Oxtr*$^{-/-}$ neurons, voltage-clamped at −70 mV, the input resistance (R_{in}) ranged between 26 and 141 MΩ, without any significant difference between *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons. Recordings of sEPSCs were performed in the presence of 10 µM bicuculline in the extracellular saline to block the $GABA_A$ receptors. Under these conditions, sEPSCs appeared as inward currents blocked by NBQX (10 µM) and CPP (30 µM), as shown in the representative traces of Figure 4, panels A and B, from *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons, respectively. Both the frequency and the amplitude of sEPSCs were significantly higher in *Oxtr*$^{-/-}$ than *Oxtr*$^{+/+}$ neuron (Figure 3, panels C and D). A quantitative comparison of the excitatory synaptic transmission between the two cultures was obtained by frequency and amplitude analyses of sEPSCs. The details

on how sEPSC inter-event intervals and amplitudes were analysed are reported in Supplementary Figure 3.

The panels C and D of Figure 3 summarize the data collected from recordings of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$neurons. A significant decrease in the inter-event interval (Figure 3C) and a significant increase in sEPSCs amplitude (Figure 3D) in *Oxtr*$^{-/-}$ neurons compared to *Oxtr*$^{+/+}$ neurons were observed (mean inter-event interval for *Oxtr*$^{+/+}$ neurons = 266 ± 49 ms, n = 11; for *Oxtr*$^{-/-}$ neurons = 149 ± 31 ms, n = 7, $P < 0.05$; mean sEPSC amplitude for *Oxtr*$^{+/+}$ neurons = 19.7 ± 2.1 pA, n = 13; for *Oxtr*$^{-/-}$ neurons = 30.9 ± 1.9 pA, n=15, $P < 0.001$).

To investigate the inhibitory profile of neuronal activity, in terms of sIPSCs, an electrophysiological analysis was performed in the presence of NBQX (10 µM) and CPP (30 µM) in the bath solution to block glutamatergic transmission respectively mediated by AMPA and NMDA receptors. The panels G and H of Figure 3 show that no significant differences of sIPSC intervals and amplitudes were detected between *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ neurons.

Our results indicate that both the amplitude and the frequency of sEPSCs were significantly higher in *Oxtr*$^{-/-}$ than *Oxtr*$^{+/+}$ neurons, suggesting that the observed increased expression of excitatory pre- and post-synaptic proteins correlate with an up-regulated glutamatergic electrical activity. Intriguingly, no significant change was detected in GABAergic electrical activity between *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ neurons, despite the significantly lower expression of inhibitory pre- and post-synaptic proteins.

Exposure of Wild Type Hippocampal Cultures to a Selective Oxtr Agonist Increases the Inhibitory and Decreases the Excitatory Pre- and Post-Synaptic Proteins

The increased expression of excitatory pre- and post-synaptic proteins and of sEPSCs in hippocampal neurons constitutively deprived of Oxt neurotransmission suggests that Oxt exerts a regulatory role on the development of excitatory synapses. To verify this, we exposed hippocampal cultures from C57BL/6J mice to 10 nM TGOT, a selective agonist of Oxtr (24) for 3 DIV starting right after cell adhesion to coverslips. Then, we quantified in 14 DIV *Oxtr*$^{+/+}$ neurons the vGAT- or vGLUT1-positive immunofluorescence clusters juxtaposed to Map2-positive processes as an indication of the numbers of GABAergic and glutamatergic synaptic terminals (Figure 4). As shown in Figure 5 panel B, the treatment with TGOT significantly increased the vGAT-positive clusters (no treatment = 94.37 ± 6.93 vs TGOT treatment = 131.2 ± 16.55; n=3 independent cell preparations; $P < 0.05$) and decreased the vGLUT1-positive ones (no treatment = 154.3 ± 17.81 vs TGOT treatment = 97.43 ± 6.27; n = 3 independent cell preparations; $P < 0.001$) (Figure 4 panel B) .

Subsequently, we evaluated the effect of the three-day TGOT exposure on NL1 and NL2 (Figure 4). TGOT treatment increased the number of NL2 clusters (no treatment = 214.5 ± 14.58 vs TGOT treatment = 262.5 ±17.44; $P < 0.05$; n = 3 independent cell preparation). Conversely, TGOT significantly reduced the total number of NL1 clusters (no treatment = 170.8 ± 7.78 vs TGOT treatment = 145.1

± 7.99; P < 0.05; n=3 independent cell preparations; P > 0.05) (Figure 4, panel D).

These observations suggest that the selective activation of Oxtr in hippocampal neurons up-regulates the expression of pre- and post-synaptic inhibitory proteins and downregulates the excitatory counterparts.

Exposure of Wild Type Hippocampal Cultures to a Selective Oxtr Agonist Decreases the sEPSCs

To assess whether the observed decrements of vGLUT1- and NL1-positive stainings in cultures pre-exposed to TGOT was correlated with a decreased glutamatergic transmission, sEPSCs were recorded in 14 DIV C57BL/6J neurons (representative traces are shown in Figure 5, panels A and B). As summarized by the data shown in Figure 5, panels C and D, TGOT-treated neurons displayed significantly increased sEPSC interevent intervals and amplitudes versus untreated neurons (inter-event interval of control neurons = 108 ± 9 ms, n = 16; TGOT-treated neurons = 471 ± 60 ms, n = 11, P < 0.001; amplitudes of control neurons = 40.5 ± 2.2 pA, n = 16; TGOTtreated neurons = 29.5 ± 4.6 pA, n = 11, P < 0.05). Our results suggest that the early transient selective activation of *Oxtr* in normal hippocampal neurons led to a lower expression of the post-synaptic glutamatergic electrical activity in mature neurons, and this fits with the observed decreases of pre-synaptic vGLUT and post-synaptic PSD95 immunoreactivities.

DISCUSSION

The present work shows that in the hippocampus of the *Oxtr*$^{-/-}$ mouse model of autism (7) the constitutive deprivation of Oxt activity caused a higher excitatory and a lower inhibitory expression of pre- and post-synaptic proteins versus *Oxtr*$^{+/+}$ primary cultures. In addition, both amplitude and frequency of spontaneously evoked glutamatergic current increased in *Oxtr*$^{-/-}$ pyramidal neurons whereas no significant changes were observed in GABAergic current between *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ neurons. This suggests that the E/I balance is offset toward an increased excitation in the hippocampal neuronal circuits of *Oxtr*$^{-/-}$ mice. In addition, the early transient exposure of immature *Oxtr*$^{+/+}$ hippocampal neurons to the selective Oxtr agonist TGOT led to a decreased developmental expression of excitatory synaptic proteins and glutamatergic current as well as an increased expression of inhibitory synaptic proteins without a corresponding change of GABAergic current. The latter finding suggests that Oxt input exerts a priming role in the developing hippocampus to regulate the E/I synaptic balance.

It is well recognized that Oxt affects the functioning of mammalian brain both at fetal and adult ages. Embryonic brains are *in utero* exposed to Oxt released into the bloodstream of the dam to regulate the rythmic contractions of the uterus during parturition (1). The lack of *in utero* exposure to the peptide affects the brain development resulting in permanent alterations of adult behaviour. Thus, adult Oxt$^{-/-}$ male mice born from Oxt$^{-/-}$ dams exhibit a highly aggressive

phenotype that is rescued upon perinatal systemic treatment of the dam with Oxt (8). Likewise, the *Oxtr*$^{-/-}$ male adult mice employed in the present study showed increased aggression (8, 7) and their phenotype was reverted to normal upon intracerebroventricular administration of Oxt and AVP acting on V1a receptors (7). Beside affecting the development of the embryonic brain, Oxt centrally released from the terminals of hypothalamic parvocellular neurons and/or the dendrites of the PVN/SON magnocellular neurons acts perinatally to influence the dam's brain activity to make her ready to satisfy the upcoming demands from the offspring. In this respect the hippocampal regulation of spatial learning function, needed to better localize food sources, seems to be particularly sensitive to Oxt. Tomizawa and coll. (6) reported that the intracerebroventricular administration of Oxt to mice that had never been pregnant improved their behavioural performances in the spatial learning eight-armed radial maze test. In addition, Oxt prolonged the long-term potentiation and the phosphorylation of cAMP-responsive element binding protein (CREB) in hippocampal slices, both indications of an increased synaptic plasticity, hence of improved memory skills. Conversely, the central administration of an Oxt antagonist to multiparous mice reduced the improved spatial learning developed prior delivery (6). We have previously shown (7) that male *Oxtr*$^{-/-}$ mice exhibit a defective spatial learning flexibility, that is the reduced ability to localize food once its learned location in one arm of a T-maze is shifted to the other. Cognitive flexibility has been associated to the hippocampal function (9-11), thus suggesting the presence of alterations in neuronal

circuitries of *Oxtr*$^{-/-}$ mouse hippocampi. Accordingly, we show here that cultured hippocampal neurons from *Oxtr*$^{-/-}$ mice display an altered expression of pre- and post-synaptic proteins. The vGAT staining was found to be significantly reduced and the vGLUT1 staining enhanced, thus suggesting that inhibitory and excitatory pre-synaptic terminals *in vitro* developed abnormally in the hippocampal neurons constitutively deprived of Oxt input (Figure 1). The lower vGAT staining was not simply due to a decreased number of GABA neurons since the immunoreactivities for GAD65/67 and parvalbumin in *Oxtr*$^{-/-}$ neurons were not detected significantly different from *Oxtr*$^{+/+}$ neurons. We can only hypothesize that the decreased vGAT could either reflect a reduced number of GABA terminals or lower quantities of vesicles clustered in the pre-synapses. Scheiffele and coll. (17) demonstrated that vesicle clustering is triggered in cultured neurons by the ectopic expression of NLs in co-cultured nonneuronal cells. Neuroligins are a family of neuronal adhesion proteins that jut from post-synaptic membranes into the cleft and, through the binding with pre-synaptic neurexins, specifically dictate the maturation and function of inhibitory and excitatory synapses (25, 26). Thus, it is of interest that we detected a decreased staining for the inhibitory NL2 (19) and an increased excitatory NL1 (18) staining in *Oxtr*$^{-/-}$ versus *Oxtr*$^{+/+}$ neurons (Figure 3), to suggest that NL changes caused the alterations in the corresponding pre-synaptic organizations. This is supported by the observation that the ectopic overexpression of NL1 in nonneuronal COS-7 cells promoted the *in vitro* formation of vGLUT1-positive synaptic-like contacts as efficiently with co-cultured hippocampal *Oxtr*$^{-}$

$^{-/-}$ and *Oxtr*$^{+/+}$ neurons and NL2 overexpression was even more efficient in triggering vGATpositive *Oxtr*$^{-/-}$ contacts (Figure 2) Thus, *Oxtr*$^{-/-}$ hippocampal neurons are capable to assemble both inhibitory and excitatory vesicle clusters as well as *Oxtr*$^{+/+}$ neurons provided that corresponding NLs are adequately expressed in recipient cells. Beside signaling to the pre-synapses, NLs guide the assembly of proteins at the postsynapses, including the scaffolding PSD-95 and gephyrin proteins and the receptors (27, 28). Correspondingly with changes in NLs we have detected a decreased staining for inhibitory gephyrin and an increased staining for excitatory PSD-95 and GluR1-subunit of AMPA receptor in the *Oxtr*$^{-/-}$ neurons, respectively (Figure 3). At difference, no changes were observed for the γ2subunit of the GABA and the NR1-subunit of the NMDA receptors. The latter finding is in agreement with the observation that PSD-95 overexpression in cultured neurons promoted the synaptic clustering of AMPA receptors as well as the AMPA-mediated, not the NMDAmediated, EPSCs (29, 30), whereas the RNAi-induced PSD-95 knockdown lowered the AMPA-mediated, not the NMDA-mediated, synaptic current in brain slices (31). Interestingly, it has been shown that the excitatory and inhibitory systems influence each other. The overexpression of PSD-95 in hippocampal cultures reduced the number of GABA synapses (32, 25) whereas the downregulation of gephyrin expression beside lowering the GABA synaptic components, enhanced the size of PSD-95 clusters and the number of vGLUT-1-positive pre-synaptic terminals (33). The enhanced expression of excitatory pre- and post-synaptic proteins detected in *Oxtr*$^{-/-}$ neurons is physiologically relevant as an

increase in the spontaneously evoked glutamatergic current was recorded. Intriguingly, the GABA current was not found to be altered in *Oxtr*$^{-/-}$ neurons despite the decreased inhibitory pre- and post-synaptic protein expression (Figure 4). At present, we do not have any explanation for this discrepancy and can only speculate that the inhibitory system has a higher redundancy thus resulting more refractory to changes in synaptic protein machinery. Maternal Oxt exerts a specific protective role on fetal neurons subjected to the extremely stressful conditions occurring during parturition. It has been shown that in the rat hippocampus Oxt promotes the switch of GABA function from being the primary excitatory neurotransmitter in immature neurons to becoming inhibitory shortly before delivery (34). Here we show that the transient exposure of *Oxtr*$^{+/+}$ immature neurons from E18 embryos (i.e. shortly prior parturition as the gestation period of mice is usually 19-21 days) to TGOT led to a lower expression of excitatory pre- and post-synaptic proteins (Figure 5), decreased glutamatergic sEPSCs (Figure 6), and a higher expression of inhibitory synaptic proteins in mature neurons (Figure 5). The increased E/I balance here observed in the hippocampus of *Oxtr*$^{-/-}$ mice could occur in other brain areas. An increased *Oxtr*$^{-/-}$ brain excitability is indicated by the higher spike activity and shorter time latency to pentilentetrazole-induced seizures previously observed by us upon EEG recording (7). Intriguingly, epilepsy is often observed in autistic subjects (35). If this is due to an absent or defective perinatal exposure to Oxt it may be relevant our present observation that the short-term activation of *Oxtr* in immature neurons led to a decreased E/I balance

in mature neurons. This suggests that Oxt input exerts an early primimng role for neuronal development implying a role in the pathogenesis of neuropsychiatric disorders of altered E/I balance, such as epilepsia, autism and schizophrenia.

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FIGURE LEGENDS

Figure 1. *Lower GABAergic and higher glutamatergic nerve terminals in Oxtr$^{-/-}$ than Oxtr$^{+/+}$ hippocampal cultures.* Fourteen DIV hippocampal cultures were doubly immunostained for (**A)** the vesicular GABA transporter (vGAT; red) or (**B**) the vesicular glutamate transporter (vGLUT1; red) to label respectively the inhibitory and excitatory pre-synaptic terminals together with Map2 staining (green, all panels) to visualize processes. Scale bar: 10 µm. Histograms show the quantitative analysis of vGAT- (**C**) and vGLUT1-positive (**D**) fluorescent puncta juxtaposed to Map2-positive processes measured in a series of 1 µm-thick z-stack confocal sections and normalized for 10 µm dendritic length. Results are expressed as means ± S.E.M. of three independent experiments (*P < 0.05; ***P < 0.0001; data are expressed as arbitrary units).

Figure 2. *Reduced expression of inhibitory and increased expression of excitatory post-synaptic proteins in Oxtr$^{-/-}$ versus Oxtr$^{+/+}$ neurons.* Fourteen DIV *Oxtr$^{-/-}$* and *Oxtr$^{+/+}$* hippocampal cultures were doubly immunostained for (**A**) excitatory NL1 (green) or (**C**) inhibitory NL2 (green) adhesion proteins to label the post synapses, and for vGAT or vGLUT1 (red) to stain the GABA or glutamatergic pre-synapses, respectively. Post-synapses were doubly stained for: **(A)** the inhibitory gephyrin or (**C**) the excitatory PSD-95 scaffolding proteins, (**C**) the AMPA receptor GluR1-subunit, (**B**) the $GABA_A$ receptor γ2-subunit or (**C**) the NMDA receptor NR1-subunit (green) together with synaptophysin (red) to label pre-synapses. Scale bar: 10µm. (**D**) Histogram show the total number of fluorescent clusters normalized for 10 µm neurite lengths in *Oxtr$^{-/-}$* and *Oxtr$^{+/+}$* neurons. Error bars represent the S.E.M. of three independent experiments (*P < 0.05; **P < 0.001; ***P < 0.0001; data are expressed as arbitrary units)**.**

Figure 3. *$Oxtr^{-/-}$ neurons display higher spontaneously evoked excitatory currents (sEPSCs) than $Oxtr^{+/+}$ neurons and the same spontaneously evoked inhibitory currents (sIPSCs).* Representative traces of sEPSC patch-clamp recording (V_{hold} = -70 mV) in a *$Oxtr^{+/+}$* (**A**) and *$Oxtr^{-/-}$* (**B**) 11DIV neurons. Bottom traces in (**A**) and (**B**): effects of the application of AMPA antagonist NBQX (10 µM) and NMDA antagonist CPP (30µM). (**C**) Average inter-event intervals and (**D**) average amplitudes of sEPSCs in *$Oxtr^{+/+}$* and *$Oxtr^{-/-}$* neurons. Representative recording (V_{hold} = -70 mV) of sIPSCs in *$Oxtr^{+/+}$* (**E**) and *$Oxtr^{-/-}$* (**F**) 11DIV neurons. Bottom traces in (**E**) and (**F**): effects of GABA antagonist bicuculline (10 µM) application. Average inter-event intervals (**G**) and average amplitudes (**H)** of sIPSCs in *$Oxtr^{+/+}$* and *$Oxtr^{-/-}$* neurons. Histograms in panels (**C**) and (**G**) show the averages of the mean intervals calculated by fitting the interval exponential distribution of each data set with an exponential probability density function. Histograms in panels (**D**) and (**H**) show the averages of the mean amplitudes calculated by fitting the amplitude distribution of each data set with a lognormal probability density function (see Supplementary Figure 3 for details). Bars represent the means ± S.E.M. (*P < 0.05)**.**

Figure 4. *In vitro exposure to the selective Oxtr agonist TGOT increases the expression of both pre- and post-synaptic inhibitory proteins and decreases that of excitatory proteins in C57BL/6J mouse neurons.* Cultures were exposed to 10 nM TGOT for 3 DIV following plating on coverslips and processed for immunofluorescence at 14 DIV using antibodies against (**A**) vGAT (red) or vGLUT1 (red) combined with Map2 antibody (green). Scale bar: 10µm. (**B**) Histogram show the quantitative analysis of vGAT and vGLUT1 fluorescences juxtaposed to Map2-positive processes in a series of 1 µm z-stack confocal sections and normalized for 10 µm dendritic length. In (**C**) cultures were respectively doubly immunostained for NL2 (green) and vGAT or NL1 (green) and vGLUT1 (red). Scale bar: 10µm. (**D**) Quantification of the

total number of fluorescent clusters normalized for 10 µm neurite length of *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ neurons. Error bars represent the S.E.M. of three independent experiments (*P < 0.05; **P< 0.001; data are expressed as arbitrary units).

Figure 5. *In vitro treatment with TGOT decreases the sEPSCs in C57BL/6J mouse neurons.* Representative traces of sEPSCs patch-clamp recorded (V_{hold} = -70mV) from a 14 DIV control neuron (**A**) and a neuron exposed TGOT application (10 nM) for 3 days after cell plating. (**C**) Average intervals of sEPSCs in control and TGOT-treated neurons quantified by averaging the mean intervals calculated by fitting the interval exponential distribution of each data set with an exponential probability density function. (**D**) Average amplitudes in control and TGOT-treated neurons obtained by averaging the mean amplitudes calculated by fitting the amplitude distribution of each data set with a lognormal probability density function. Bars represent the means ± S.E.M. (*P < 0.05).

FIGURES

Figure 1

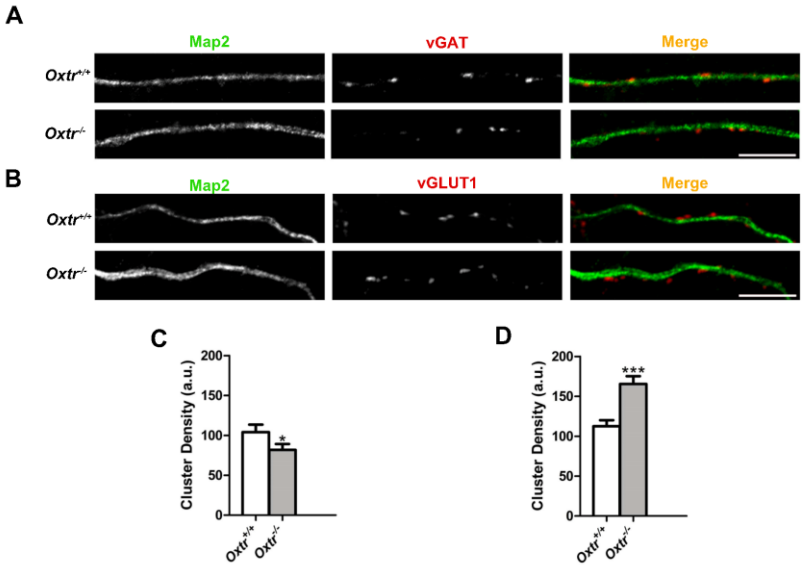
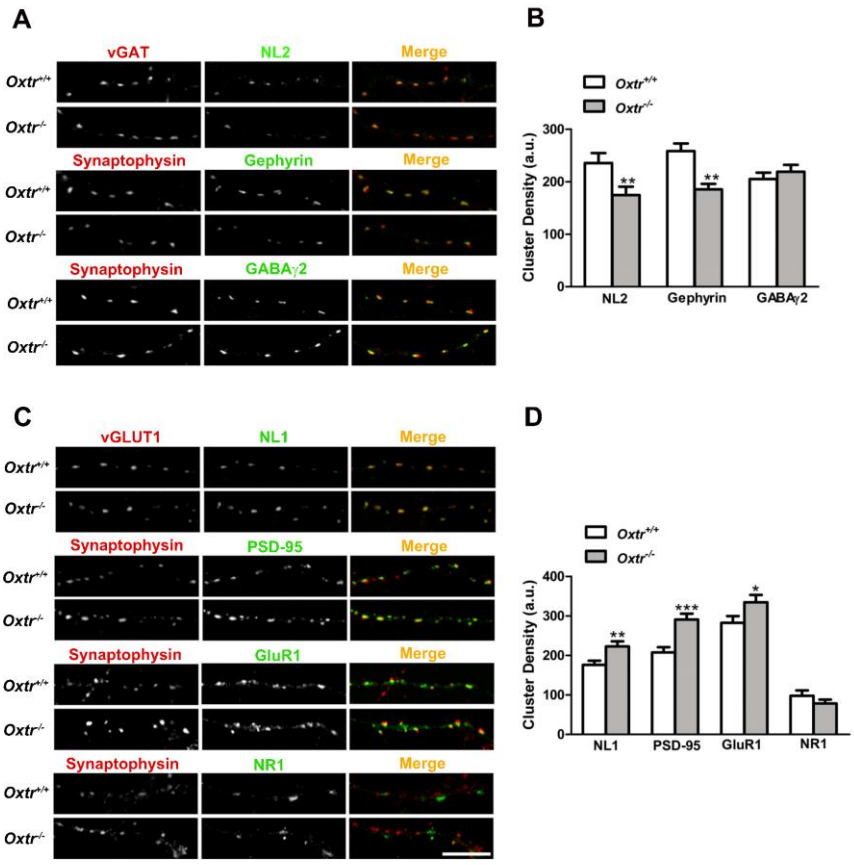


Figure 2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Gephyrin
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vGLUT1
NL1
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Synaptophysin
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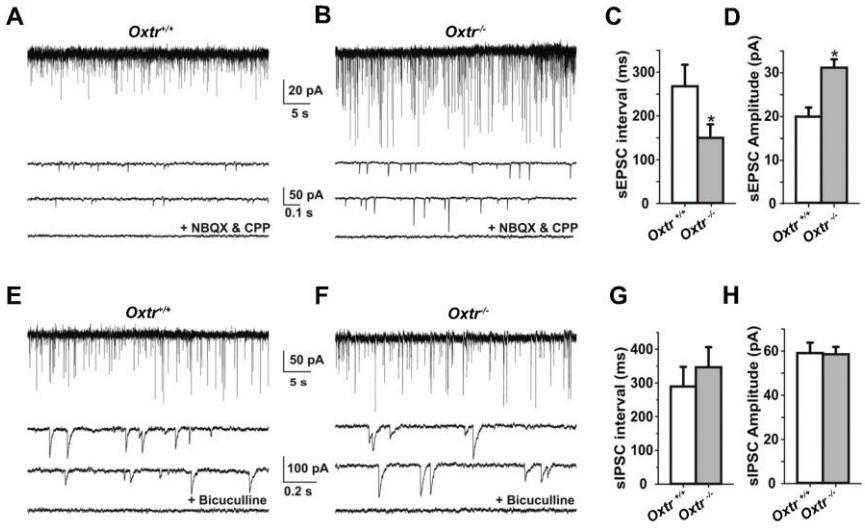
Figure 3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Oxtr +/+
Oxtr -/-
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D
sEPSC Amplitude (pA)
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50 pA
5 s
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+ Bicuculline
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Figure 4

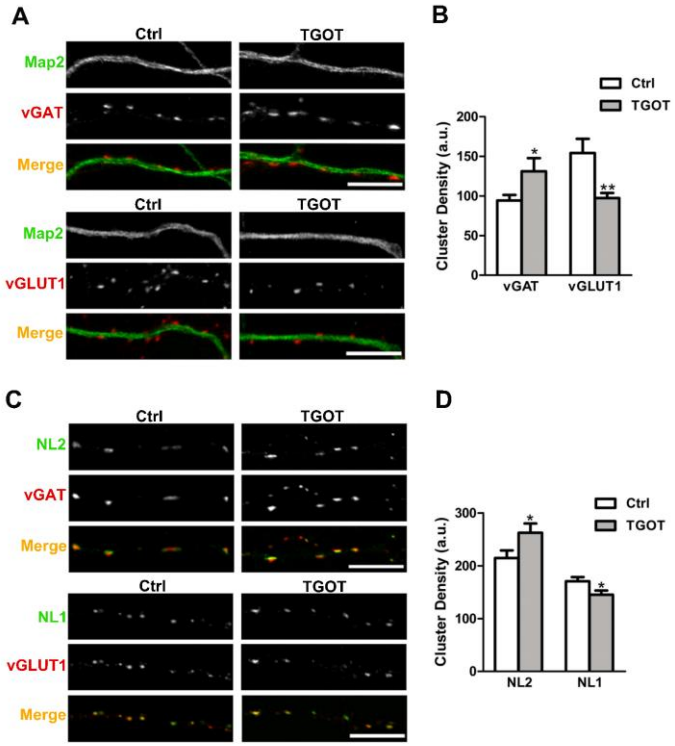
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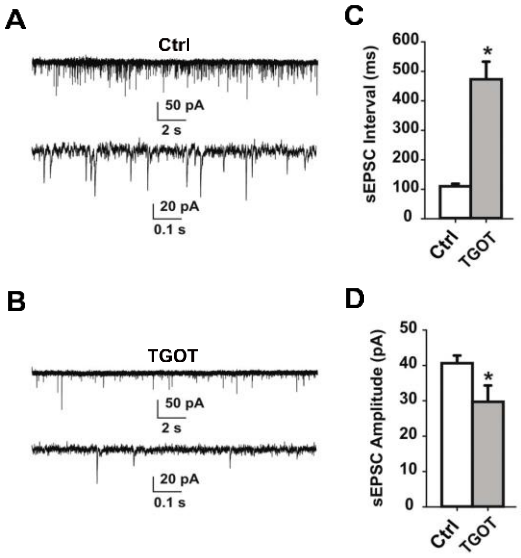
Figure 5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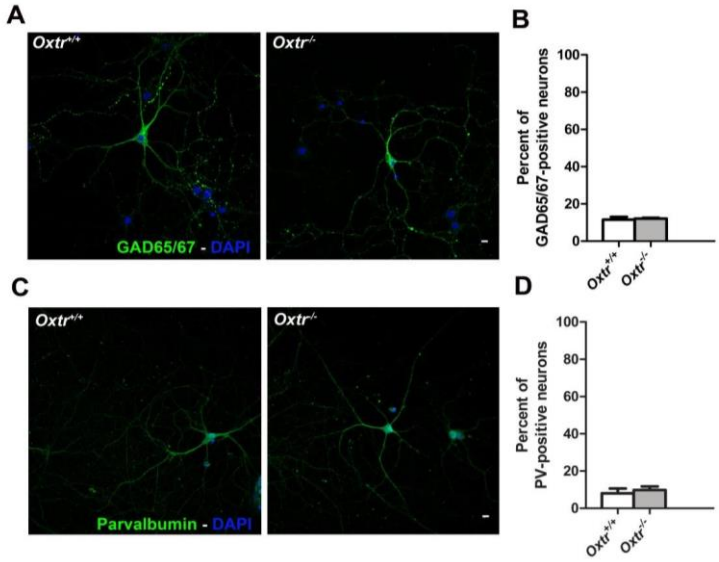
50 pA
2 s
20 pA
0.1 s
C
sEPSC Interval (ms)
600
500
400
300
200
100
0
*
Ctrl
TGOT
D
sEPSC Amplitude (pA)
50
40
30
20
10
0
*
Ctrl
TGOT

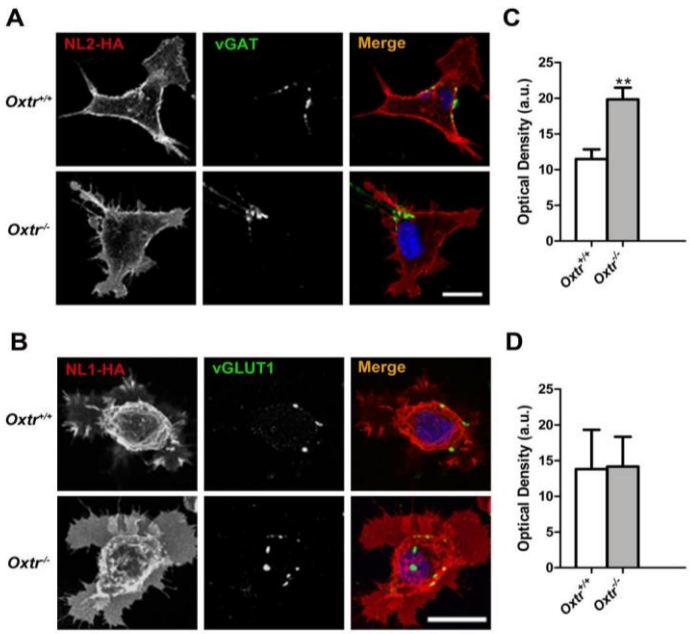
SUPPLEMENTARY FIGURES:

Supplemetary Figure 1



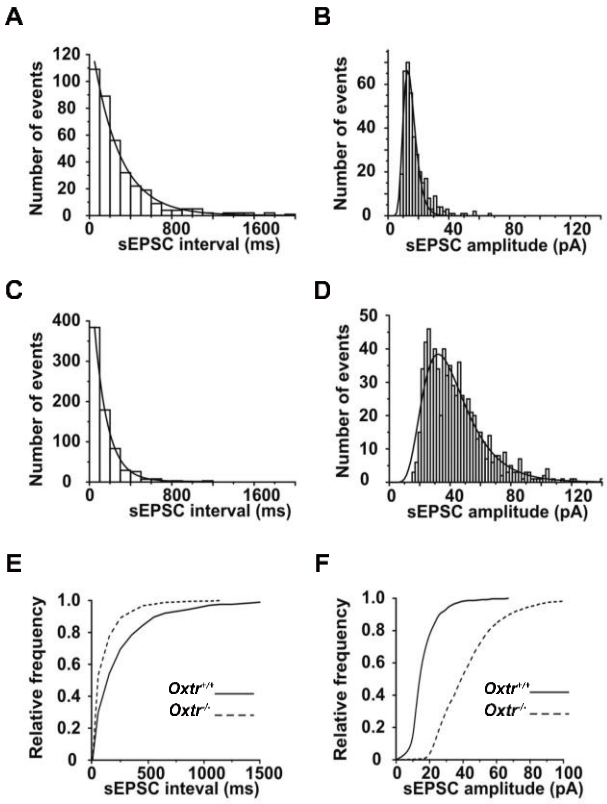
Supplementary Figure 1. *$Oxtr^{-/-}$ and $Oxtr^{+/+}$ cultures show equal number of GAD65/67- and PV-positive GABAergic neurons.* Hippocampal cultures were immunodecorated with antibodies against (**A**) the glutamic acid decarboxylase 65 and 67 enzymes (GAD65/67; green) to label GABAergic neurons and (**C**) parvalbumin (PV; green), staining a specific subpopulation of GABAergic neurons. Scale bar, 10 µm. Bar graphs show that the percentage of GAD65/67- (**B**) or PV-positive (**D**) neurons with respect to total nuclei labeled with DAPI is the same in *$Oxtr^{+/+}$* and *$Oxtr^{-/-}$* cultures. Data are shown as means ± S.E.M.

Supplemetary Figure 2:



Supplementary Figure 2. *Oxtr$^{-/-}$ and Oxtr$^{+/+}$ neurons in vitro form synaptic-like contacts with co-cultured NL-overexpressing COS-7 cells.* Transfected COS cell expressing either NL1-HA or NL2-HA were seeded onto a low density culture of hippocampal Oxtr$^{-/-}$ and Oxtr$^{+/+}$ neurons at 7 DIV. Co-cultures were examined by double immunofluorescence confocal microscopy using antibodies to HA-tag to identify the transfected COS-7 cell (red in all panels) and against vGAT (green in panel **A**) or vGLUT1 (green in panel **C**) to label pre-synaptic terminals. Scale bar: 10µm. Quantitative analysis show the integrated vGAT (**B**) or vGLUT1 (**D**) immunofluorescence from at least 30 different transfected COS-7 cell for each of three independent experiments normalized for the corresponding cell area. Error bars represent the S.E.M. from three independent experiments.

Supplemetary Figure 3



Supplementary Figure 3. *Representative analysis of sEPSC inter-event intervasl and amplitudes from the same Oxtr$^{+/+}$ and Oxtr$^{-/-}$ neurons whose activity is shown in Figures 3A and 3B, respectively.* (**A** and **B**) Histogram plots of sEPSC intervals from *Oxtr$^{+/+}$* neuron (bin width=100 ms, 365 events) and Oxtr$^{-/-}$ neuron (bin width = 100 ms, 725 events), respectively. The solid lines are the best fitting curves obtained using an exponential probability density function for *Oxtr$^{+/+}$* neuron (τ = 263 ms) and Oxtr$^{-/-}$ neuron (τ = 128 ms). (**C** and **D**) Histogram plots of sEPSC amplitudes from Oxtr$^{+/+}$ neuron (bin width = 2 pA, 367 events) and *Oxtr$^{-/-}$* neuron (bin width = 2 pA, 726 events),

respectively. The solid lines represent the best fitting curves obtained using a log-norm function for *Oxtr*$^{+/+}$ (μ= 14.2 pA) and Oxtr$^{-/-}$ (μ= 38.8 pA) neurons. (**E** and **F**) Cumulative plots of sEPSC interval (**E**) and amplitude distributions (**F**) from *Oxtr*$^{+/+}$ (solid line) and *Oxtr*$^{-/-}$ neuron (dashed-line).

Chapter 4

Conclusions

Since its discovery by Sir Henry Dale in 1906 the nonapeptide Oxt has been the object of extensive investigation owing to its essential physiological roles in parturition and breast feeding. In the last decade Oxt has gained further attention as a key player of the team of neurotransmitter molecules that shape human psychology. In fact, this peptide plays a key role in the modulation of complex cognitive and behavioural aspects linked to the social interaction, such as maternal nurture, pair bonding, social exploration, recognition and aggression, as well as anxiety and fear-mediated responses. Accordingly, different lines of evidence have suggested that the function of Oxt is altered in various brain disorders associated with social deficits, including autism, anxiety, depression and schizophrenia (Meyer-Lindenberg, 2011). Hence, the exogenous modulation of Oxt system in combination with psychotherapy has been proposed as a possible novel strategy for the treatment of neurological disorders characterized by social abnormalities (Meyer-Lindeberg, 2011). The highly distinctive features of the dysfunctions affecting the human social sphere have been effectively defined by Thomas R. Insel as: *"We are, by nature a highly affiliative species craving social contact. When social experience become a source of anxiety rather than a source of comfort, we have lost something fundamental-whatever we call it"* (Insel, 2002).

Based on these issues, it clearly emerges the need of devoting a great research effort to understand the molecular mechanisms by which Oxt contributes to the shaping of human complex behaviours. These

studies will contribute to clarify how alterations in the Oxt system are involved in human diseases and eventually will allow the development of novel efficaceous pharmacological interventions. Unfortunately, thus far the relationship(s) linking the Oxt system to the regulation of the mechanisms that orchestrate the formation of the appropriate synaptic brain networks needed to set up the social and cognitive abilities are not yet clear.

This study is a contribution to a better knowledge of the role of Oxt signalling in the development and function of murine hippocampal neurons. The results described in Chapter 2 show the effects of Oxt signalling on hippocampal synaptogenesis. In particular, we have demonstrated that Oxt exposure during the early stages of neuronal development exerts a priming role in the developing hippocampus by regulating the dendrite branching and the synapse formation of excitatory glutamatergic neurons. Upon learning how Oxt can influence the central mechanisms of synaptogenesis we further proceeded to search for the possible consequences that its impairment could have on the pathogenesis of neurobehavioural disorders. Accordingly, in Chapter 3 we report data showing how the lack of Oxt signaling leads to hippocampal synaptic defects that may underlie the impaired social and cognitive functions exhibited by the Oxtr null mouse model of autism. In particular, our work has highlighted how the constitutive deprivation of Oxt function makes the E/I synaptic balance to be offset toward an increased excitation in the hippocampal neuronal circuits of the Oxtr knockout mice.

In conclusion, the results reported in this dissertation suggest that Oxt input exerts an early priming role for the hippocampal development *in vitro*. This could also happen *in vivo* when the fetal brain is exposed to the perinatal surge of maternal Oxt. The lack of exposure to Oxt could thus result in permanent alterations of E/I balance in the hippocampus leading to behavioural disturbances, such as the spatial memory deficits observed in autistic children (Steele et al., 2007).

These observations highlight the key role that Oxt system can play in translational medicine (Meyer-Lindenber, 2011). One of the most exciting challenges of neuroscience is the ability to translate the results of social behavioural studies in animals to preclinical and clinical research in humans. In particular, establishing a bridge from animal to human Oxt neurobiology will be important for the development of more efficient treatments of those pathologies affecting the human social behaviour.

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