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Università degli Studi di Milano

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Ciclo XXIII

NEW PHARMACOLOGICAL TOOLS FOR AUTISM RESEARCH: OXYTOCIN RECEPTOR MUTANT MICE AND ZEBRAFISH AS NEUROBEHAVIOURAL MODELS

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Abstract

Autism is a neurodevelopmental disorder which is characterized by severe and pervasive impairment in reciprocal socialization, qualitative impairment in communication and repetitive or stereotyped behaviour associated to resistance to change. Oxytocin (OT) is a peptidic hormone best known for its role in lactation and parturition. Recently, it has been also shown by several studies involving different lines of knock-out mice to play an important role in the central nervous system (CNS) by acting on the regulation of social, emotional, aggressive behaviour and on learning and memory. Furthermore, prosocial effects following OT administration in humans have been shown. The link between OT and autism has already been traced in preliminary clinical studies as autism affected patients received a beneficial outcome from treatment with OT. Pharmacokinetic (very short half-life), pharmacodynamic (unspecific binding to vasopressin (AVP) receptors) properties and the presence of peripheral side effects of OT, though, make this peptide an unsuitable target for a future clinical use. It is important, in this perspective, to characterize specific animal models in order to validate the use of OT analogs with more suitable characteristics for preclinical research.

To this end, a characterization of the behavioural phenotype of OTR knock-out mice ($OTR^{-/-}$) and heterozygous littermates ($OTR^{+/-}$) in comparison with their wild type counterparts ($OTR^{+/+}$) has been carried out. General home cage behaviour, sensory, motor abilities and emotional behaviour were not affected by the altered genotype. Interestingly, both and $OTR^{+/-}$ and $OTR^{-/-}$ mice exhibited a significant social impairment as quantified in both the sociability and social novelty tests. Furthermore, $OTR^{-/-}$ mice displayed much higher levels of aggression when facing a stranger mouse as a higher number of attacks and tail rattlings has been registered in the neutral cage paradigm.

Moreover, when tested in the reversal phase of a T-maze task for their cognitive flexibility, $OTR^{-/-}$ showed a profound impairment in responding to the changes applied in their established routine. All in all, the OTR mutant mouse model provides full range autism-related aberrant behaviours, displaying social impairments, altered aggressive behaviour, a strong resistance to change and stereotyped behaviour. Mechanisms underlying the aberrant phenotype revealed by mutant mice were investigated through autoradiographic binding experiments for both OTR and vasopressin 1A receptor (V1aR) distribution. In addition, pharmacological treatments with OT, AVP and V1aR antagonist SR49059 were done. Binding experiments were carried out in specific brain areas known to exert a key role in integrating the processing of olfactory information that is crucial to regulate social and emotional behaviour in rodents. $OTR^{-/-}$ animals displayed an almost undetectable OTR binding in all tested areas. Furthermore, a slight compensatory up-regulation of V1aR in the hippocampus and a significant down-regulation of the V1aR expression in the anterior olfactory nucleus, amygdala, ventral pallidum and lateral septum were found. As for heterozygous mutants their phenotype appeared as halfway between the wild type and knock out counterparts for OTR binding sites and V1aR binding has been subjected to a slight reduction in ventral pallidum and anterior olfactory nucleus only. Hence, the previously mentioned aberrant behavioural phenotype displayed by OTR mutant mice could be due to an altered OT/AVP receptors concentration in crucial brain areas. Interestingly, intracerebroventicular treatment with both OT and AVP (0.5ng/mouse) in mutant mice was able to rescue the impairment shown in all behavioural tasks. Furthermore, pre-treatment with V1aR antagonist SR49059 (0.5 ng/mouse), which *per se* did not exert any effect, in association with OT, blocked the social, aggressive and

cognitive enhancing effects of the neuropeptide. Our results suggest a strong involvement of AVP, alongside OT, and in particular the subtype 1A of the AVP receptor, in the modulation of social abilities and cognitive flexibility of OTR mutant mice.

Finally, as an increasing interest for the use of zebrafish for social behavioural analyses to study the genetic basis of behaviour is rising, we also evaluated zebrafish potential as a screening tool for neuropsychiatric diseases involving deficits in social behaviour. To this end, we analyzed the effect of OT, AVP but most importantly isotocin (ISO) and vasotocin (AVT) (zebrafish homologues of OT and AVP, respectively) using the shoaling preference test as social paradigm. Dose-response parabolic curves were obtained and all neuropeptides showed significant efficacy in enhancing social interaction, suggesting the involvement of the oxytocin/vasopressin systems and their analogs in the modulation of zebrafish social behaviour.

In conclusion, our findings indicate that $OTR^{-/-}$ and in part $OTR^{+/-}$ mice display autistic-like symptoms rescued by administration of AVP and OT to young adult animals. The OTR mutant mouse is thus instrumental to investigate the neurochemical and synaptic abnormalities underlying autistic-like disturbances and to test new strategies of pharmacological intervention. We also suggest the use of zebrafish as an alternative animal model for the study of social behaviour, especially as a screening tool: future studies involving new molecules acting on OT and AVP systems will be carried out, taking advantage of this new promising model.

Introduction

2.1 Autism

Autism is a neuropsychiatric disorder characterized by severe and pervasive impairment in reciprocal socialization, qualitative impairment in communication and repetitive or unusual behaviour (Levy et al., 2009). Clinicians and researchers use autism spectrum disorders (ASD) to include autism, Asperger's syndrome (a disease similar to autism with no cognitive deficit), and "pervasive developmental disorder not otherwise specified" (PDD-NOS).

ASD prevalence seems to have increased greatly since the 1960s. In the 20 years since, in the USA and Europe prevalence rates ranged from 5 to 72 cases per 10 000 children while prevalence of autistic disorder is now between ten and 20 per 10 000 children (Newschaffer et al., 2007). A rise in the number of children identified with autism spectrum disorders in educational systems has resulted in public health concern (Shattuck, 2006): some of the reported increase is attributable to new classifications in education settings and the subsequent reclassification of children from a different category to autism. In fact symptoms of ASD might resemble or arise with intellectual disability, attention deficit-hyperactivity disorder, or obsessive-compulsive disorder (Gillberg et al., 2000), so policy and practice changes rather than true changes in community prevalence might be responsible for recorded increases.

Core symptoms of autism spectrum disorders affect domains of socialization, communication, and behaviour (see panel 1 for details). Clinical signs are usually present by the age of 3 years, but typical language development might delay identification of symptoms (Levy et al., 2009). Results of prospective studies (Volkmar et al., 2008) of infants at risk (i.e. younger siblings of affected children) have shown that deficits in social

responsiveness, communication and play can be present in those as young as age 6–12 months. Diagnoses show heterogeneity of clinical phenotype, severity, and type and frequency of symptoms.

Socialization

- Impaired use of non-verbal behaviours to regulate interactions
- Delayed interactions, few or no friendships
- Absence of seeking to share enjoyment and interests
- Delayed initiation of interactions

Communication

- Delay in verbal language and non-verbal compensation with gestures
- Impairment in expressive language and conversation
- Stereotyped, repetitive language
- Delayed imaginative and social imitative play

Restricted, stereotyped and repetitive patterns of behaviour

- Adherence to routines, rigidity and perseverative behaviour
- Stereotyped and repetitive motor behaviour
- Self-stimulatory behaviour
- Fascination with parts of items and unusual visual exploration

Panel 1: core domains of autism (adapted from Levy et al., 2009)

2.1.1 Neurobiology of Autism

Many studies have attempted to elucidate neurobiological features of autism by examining brain growth, functional neural networks, neuropathology, electrophysiology, and neurochemistry in both patients and animal models of the disease.

Neurobiological findings support different theories. Macrocephaly is noted by age 2–3 years in 20% of children with autism spectrum disorder. Brain growth accelerates at 12 months (Minshew and Williams, 2007). These changes arise in parallel with onset of

core symptoms during the first 2 years of life. Results of neuroimaging studies (Pardo and Eberhart, 2007) have shown overgrowth in cortical white matter and abnormal patterns of growth in the frontal lobe, temporal lobes, and limbic structures such as the amygdala. These brain regions are implicated in development of social, communication, and motor abilities that are impaired in ASD. In post-mortem brain studies (Schultz, 2005), researchers have also noted cytoarchitectural abnormal features, including reduced number and size of purkinje cells and abnormal findings in the cortical minicolumn. Functional MRI has shown differences in patterns of activation and timing of synchronisation across cortical networks, with lowered functional connectivity between language, working memory, social cognition or perception, and problem solving related breain areas. The most replicated functional MRI abnormal finding, though, is the hypoactivation of the fusiform face area, associated with deficits in perception of people compared with objects (DiCicco-Bloom et al., 2006, Schultz, 2005).

2.1.2. Genetics of Autism

Before the 1970s, autism was not widely considered to have a strong biological basis. Instead, various psychodynamic interpretations, including the role of a *cold* style of mothering, were invoked as potential causes. The importance of genetic contributions became clear in the 1980s, when the co-occurrence of chromosomal disorders and rare syndromes with the ASD were noted (Blomquist et al., 1985).

Several lines of evidence support genetic factors as a predominant cause of ASD. First is the growing body of literature demonstrating that mutations or structural variation in any of several genes can dramatically increase disease risk. Second, the relative risk

of a child being diagnosed with autism is increased at least 25-fold over the population prevalence in families in which a sibling is affected (Jorde et al., 1991). Third, siblings and parents of an affected child are more likely than controls to show subtle cognitive or behavioural features that are qualitatively similar to those observed in probands (Bishop et al., 2004).

Different genes have been demonstrated to be involved in the pathogenesis of autism, including neuroligins, post-synaptic scaffold proteins, phosphatases and transcription factors (see tab. 1)

Neuroligins interact with neurexins expressed in presynaptic neurons (Lise and El-Husseini, 2006). The NLGN3 and NLGN4 genes have been found to be mutated in 1/100 individuals with ASD (Lintas and Persico 2009). The clinical phenotype of human NLG mutation carriers is heterogeneous. Mutation carriers typically display no dysmorphic features but, interestingly, they can undergo regression at disease onset, characterized by a loss of initially acquired social and verbal milestones (regressive autism) (Jamain et al., 2003). The neurexin 1 gene (NRXN1) encodes a neurexin1 signal peptide variant. It is a neuronal cell-surface protein that may be involved in cell recognition and cell adhesion by forming intracellular junctions through binding to neuroligins. Neurexin1 gene mutations have been identified in individuals with autism (Yan et al., 2008).

Another autism-related gene is SHANK3. Shank proteins are involved in the assembly of specialized postsynaptic structures and are required for the development of language and social communication. Recent studies confirmed that SHANK3 mutations can cause ASD with phenotype characterized mainly by severe verbal and social deficits (Moessner et al., 2004; Aneja and Tierney, 2008).

Affected gene (OMIM)	Chromosomal locus	Protein name	Gene function	Clinical phenotype	Inheritance	Prevalence among individuals with autism
NLGN3 (300336)	Xq13.1	Neuroligin-3 precursor	Neuroligins function as ligands for the neurexin family of cell-surface receptors	Autism, Asperger syndrome, PDD-NOS	X-linked	<1%
NLGN4 (300427)	Xp22.33	Neuroligin-4, X-linked precursor	Same as *NLGN3*	Autism, Asperger syndrome, X-linked mental retardation, PDD-NOS	X-linked	<1%
SHANK3 (606230)	22q13.3	SH3 and multiple ankyrin repeat domains protein 3	Encodes a scaffolding protein found in the PSD complex of excitatory synapses,where it binds directly to neuroligins	Autism with severe language and social deficits	Unknown	1.1%
NRXN1 (600565)	2p16.3	Neurexin-1α precursor	Neurexins function in the vertebrate nervous system as cell adhesion molecules and receptors	Autism with seizures, facialdysmorphism mild to severe spokenlanguage deficits	Unknown	<1%
MeCP2 (300005)	Xq28	Methyl-CpG-binding protein 2	A transcriptional repressor that binds to methylated CpG dinucleotides generally located at gene promoters and recruits HDAC1 and other proteins involved in chromatin repression	Autism, learning disability, Angelman syndrome phenotype, preserved speech variant of Rett syndrome	X-linked	0.8–1.3% of the female ASD population
HOXA1 (142955)	7p15.3	Homeobox protein Hox-A1	Transcription factor essential to the development of head and neck structures, including hindbrain, ear, and occipital and hyoid bones	Autism spectrum disorder susceptibility	Autosomal recessive	Very rare
PTEN (601728)	10q23.31	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase	A tumour-suppressor gene influencing G1 cell cycle arrest and apoptosis. In the central nervous system, *PTEN* inactivation results in excessive dendritic and axonal growth with increased numbers of synapses	ASD with macrocephaly has been consistently found in approximately 20% of individuals with autism recruited in independent samples	Unknown	4.7%

Tab 1: ASD related genes (taken from Caglayan, 2010)

2.1.3 Autism Treatment

Although many psychosocial interventions have some positive evidence, suggesting that some form of treatment is preferable to no treatment their clinical results are mostly tentative, and there is little evidence for the relative effectiveness of treatment options (Abrahams and Geschwind, 2008). Intensive special education programs and behavioural therapy early in life can help children with ASD acquire self-care, social, and job skills (Sigman et al., 2004), and can often improve functioning, decrease symptom severity and maladaptive behaviours (Arndt et al., 2005). Available approaches include applied behaviour analysis, developmental models, structured teaching, speech and

language therapy, social skills therapy, and occupational therapy (Levy et al., 2009) Educational interventions have some effectiveness in older children: intensive behavioural treatment has demonstrated effectiveness in enhancing global functioning in preschool children and is well-established for improving intellectual performance of young children (Arndt et al, 2005).

Many drugs are prescribed to treat problems associated with ASD. A 2007 study on a U.S. population of children with ASD has shown that 83% had at least one drug claim during the year. Prescribed drugs for these children came from 125 different therapeutic classes. The seven most frequently prescribed classes of psychoactive drugs were antidepressants, stimulants, tranquilizers/antipsychotics, anticonvulsants, hypotensive agents, anxiolytic/sedative/hypnotics and benzodiazepines. Age data indicate that about 70% of 8-year old or older children with autism-spectrum disorders receive some form of psychoactive medication in a given year (Oswald and Sonenklar, 2007). Aside from antipsychotics (Silverman, 2008) there is not much reliable research about the effectiveness or safety of drug treatments for adolescents and adults with ASD (Geschwind, 2008). A person with ASD may respond atypically to medications, the medications can have adverse effects, and still no known medication relieves autism's core symptoms of social and communication impairments (Rogers, 2009).

2.2. Neurohypophysial hormones

2.2.1. Oxytocin (OT)

Oxytocin (OT) is a nonapeptide hormone best known for its role in lactation and parturition. The word *oxytocin* was coined from the Greek words (ωκνε, τοκοχε) meaning "quick birth" after its uterine-contracting properties were discovered by Dale (1906). Shortly thereafter, the milk ejection property of OT was described (Ott and Scott, 1910; Schafer and Mackenzie, 1911).

2.2.1.1. Structure and evolution of oxytocin

OT is composed of nine amino acids (Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly) with a sulfur bridge between the two cysteines. The structure of OT is very similar to another nonapeptide, vasopressin (AVP), which differs from OT by two amino acids only (Ile-Leu → Phe-Arg) (Fig 1).

OT and AVP are neuropeptides that are evolutionarily well conserved across *phyla*. Amino acid substitutions occurred in precise positions. All vertebrate species possess an oxytocin-like and a vasopressin-like peptide so that two evolutionary lineages can be traced. Because a single peptide, vasotocin ([Ile3]-vasopressin or [Arg8]-oxytocin) has been found in the most primitive Cyclostomata, a primordial gene duplication and subsequent mutations are assumed to have given rise to the two lineages. They started with vasotocin ([Arg8]-vasotocin) and isotocin ([Ser4,Ile8]-oxytocin) in bony fishes and culminated with vasopressin and oxytocin in placental mammals. Mesotocin ([Ile3]-

oxytocin), found in lungfishes, amphibians, reptiles, birds and marsupials, appears as an evolutionary intermediate. (Acher et al., 1995)

As a result of gene duplication, the OT gene is located on the same chromosome (chromosome 2 in mice and 20 in humans) as AVP but is oriented in opposite transcriptional direction in mammals. Both OT and AVP contain three exons and two introns and are highly homologous. The two genes are separated by an intergenic region (IGR) that varies in length across species (e.g., 11 kb in rat and human, and 3.6 kb in mouse). The IGR harbors regulatory DNA sequences within conserved portions for both OT and AVP (Gainer et al., 2001). (Fig. 1)

Fig. 1. Schematic diagram of the oxytocin (OT) and vasopressin genes (large arrows), preprohormones (boxes), and neuropeptides (bottom). Their human chromosomal location is shown at the top. Both genes are composed of three exons shown as small blue arrows and separated by two introns (shown as dotted lines between the exons). The genes are located on the same chromosome but are transcribed in opposite directions and separated by an intergenic region (IGR). The preprohormones each contain a signal peptide (SP), a neuropeptide (AVP or OT), and a neurophysin (NP). In the case of AVP, a glycopeptide (GP) as well is present. Protein processing signals are represented by thick lines. Cysteine residues form a disulfide bond to create a cyclic six amino acid ring for both neuropeptides. Seven out of the nine amino acids are identical in the neuropeptides and two are different (red). (Taken from Lee at al., 2009).

Oxytocin is primarily synthesized in magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. The bulk of the peptide is transported to the posterior pituitary where it is released to regulate parturition and lactation. However, some of the OT is transported into the dendrites where regulation of its release is critical for controlling the firing patterns of the OT neurons (Rossoni et al., 2008). Lesser amounts of OT are generated by smaller, parvocellular neurons of the PVN and, depending on species, the bed nucleus of the stria terminalis (BNST), medial preoptic area, and lateral amygdala for release within the brain (Young and Gainer, 2003, 2009).

2.2.1.2. The oxytocin receptor

Oxytocin is currently known to have only one receptor (OTR) (fig. 2), unlike AVP which has at least three different subtypes (V1aR, V1b, V2) (Caldwell et al., 2008). OT acts on OTR as a full agonist capable of promoting the promiscuous coupling of the receptor to different G proteins (G-protein coupled receptor, GPCR), resulting in the activation of multiple downstream signalling pathways (Reversi et al., 2005). Classically, OTR coupling to Gq is responsible of phospholipase C beta (PLCbeta) activation, followed by inositol phosphate (InsP) and diacylglicerol (DG) production, and release of Ca^{++} from intracellular stores.

Centrally, AVP exerts its effects mainly through the vasopressin-1A receptor, which is also coupled to the PLC pathway (Thibonnier et al., 1998; Birnbaumer, 2000). Importantly, both OT and AVP, as well as the OTR and V1aR, display a high degree of

sequence homology, and both peptides can therefore activate both receptors (Chini and Manning, 2007).

In freshly dissociated rat supraoptic neurons, OT and AVP induce a significant increase in the intracellular Ca^{++} concentration which persists in the absence of external Ca^{++}, suggesting that it mainly involves Ca^{++} release from intracellular stores (Sabatier et al., 2004). Similar results have been also obtained in primary cultures of cerebral cortical astrocytes as well as in brain neurons derived from hippocampus, lamina terminalis of the subfornical organ and area postrema (Consolim-Colombo et al., 1996; Jurzak et al., 1995; Strosser et al., 2001). However, in vagal motor neurons of brainstem slices, the OT-evoked current is not mediated by an increase in Ca^{++} concentration, indicating that in these cells OTR is not functionally coupled to PLCbeta (Alberi et al., 1997). Thus, the peptide-activated second messengers pathways may be different in different neuronal populations and/or at different stages of neuronal development (Raggenbass, 2001).

In general, OTR is widely distributed throughout the brain. In rodents, it is often especially prominent in the olfactory bulb and tubercle, neocortex, endopiriform cortex, hippocampal formation (especially subiculum), central and lateral amygdala, BNST, nucleus accumbens (NAc), and ventromedial hypothalamus (VMH) (Insel et al., 1991; Veinante and Freund-Mercier, 1997). In humans, expression is prominent in the basal nucleus of Meynert, the nucleus of the vertical limb of the diagonal band of Broca, the ventral part of the lateral septal nucleus, the preoptic/anterior hypothalamic area, the posterior hypothalamic area, the substantia nigra pars compacta, the substantia gelatinosae of the caudal spinal trigeminal nucleus and of the dorsal horn of the upper

spinal cord, as well as in the medio-dorsal region of the nucleus of the solitary tract (Loup et al., 1989, 1991).

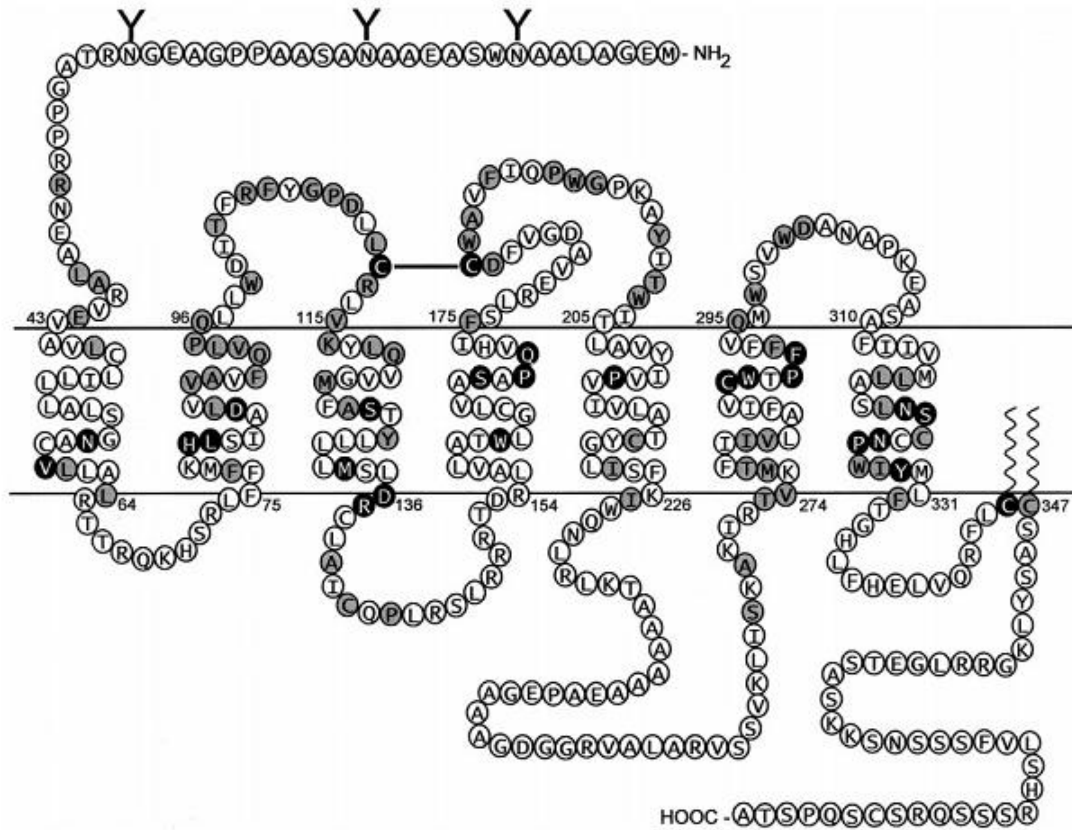

Fig 2: Schematic structure of the human OT receptor with amino-acids residues shown in one-letter code. Residues conservative within the OT/AVP receptor subfamily are outlined in grey, and residues conservative for the whole GPCR superfamily are outlined in black (taken from Gimpl and Fahrenholz 2001).

2. 2.2. Vasopressin (AVP)

2.2.2.1. Properties and receptors

AVP is mainly synthesized in the magnocellular cells of the hypothalamic supraoptic (SON) and paraventricular nuclei (PVN) whose axons project to the posterior pituitary (Young and Gainer, 2003). AVP is then released into the blood stream upon appropriate stimulation (for example, hemorrhage or dehydration) to act at the kidneys and blood vessels (Nishimura and Fan, 2003). The brain also contains several populations of smaller, parvocellular neurons whose projections remain within the brain. These populations are located within the PVN, bed nucleus of the stria terminalis (BNST), medial amygdala (MeA) and suprachiasmatic nucleus (SCN) (De Vries et al., 1985). There are three major receptor types for AVP: V1aR, V1bR, and V2R. The AVP receptors have seven transmembrane domains: V1aR and V1b couple to Gaq/11 GTP binding proteins which activate PLC activity whereas V2R couples to Gs and acts through the cyclic AMP system (Michell et al., 1979; Jard et al., 1987). Although not confined to these tissues in the periphery, the V1aR is prominent in the liver, kidney and vasculature. The V1bR is prominent in the anterior pituitary in cells that make adrenocorticotropin hormone (Antoni, 1984) but is detected in many other tissues (Lolait et al., 1995). V2R is mainly expressed in the kidney and the antidiuretic function of AVP is mostly transduced via this receptor in the renal collecting duct (Bankir, 2001). The V1aR and V1bR are expressed throughout the brain, but evidence for the presence of the V2R there is scarce (Foletta et al., 2002).

As for receptors localization within the brain, major V1aR binding is present in the rat lateral septum (LS), neocortical layer IV, hippocampal formation, amygdalostriatal

area, BNST, various hypothalamic areas, ventral tegmental area, substantia nigra, superior colliculus, dorsal raphe, nucleus of the solitary tract and inferior olive (Johnson et al., 1993). V1aR binding is moderate throughout the spinal cord, but binding is higher in the dorsolateral motoneurons in general and all motoneurons in the lumbar 5/6 levels, where innervation to the perineal muscles originates (Tribollet et al., 1997). It should be noted, however, that V1bR distribution has not been mapped by receptor autoradiography due to the lack of a specific radiolabeled ligand.

2.2.3. OT, AVP and Autism

Recently, researchers have begun to examine OT effects on human social recognition, primarily by testing recognition of faces and expressions. OT administered intranasally and intravenously to males and females after viewing male faces (with happy, angry, or neutral expressions) significantly improves recognition memory 30 min and 24 h later for neutral and angry faces only (Savaskan et al., 2008). In contrast, an earlier study by the same group reports that OT (administered to males only) increases memory for previously seen faces with happy expressions only, not angry or neutral (Guastella et al., 2008b, 2009). Why this discrepancy exists remains unclear, but may be due to presentation of different stimulus faces (male, male and female, drawings). In a more recent study, intranasal OT was given to male subjects 40 min prior to presentation of faces. Twenty-four hours later, subjects treated with OT had better memory for faces seen the previous day, with no influence on memory for previously seen non-social stimuli (Rimmele et al., 2009): this is a further indication that OT is specifically involved in

memory of social stimuli. OT in the medial amygdala underlies social recognition in rodents, as discussed previously. Similarly, the human amygdala seems important for facial recognition in humans. Lower blood oxygenation level dependent functional magnetic resonance imaging (fMRI) activity is seen in the right amygdala after intranasal OT, as compared with placebo, when viewing emotional (happy, fearful, or angry expressions) faces, regardless of type of emotion displayed (Domes et al., 2007). Similarly, male subjects have reduced amygdala activation when shown angry or fearful expressions following OT administration. The same reduction is not seen when presented with non-social stimuli, such as fearful or threatening scenes. Furthermore, functional amygdala connections to upper brainstem regions (such as the periaqueductal gray) are significantly reduced with intranasal OT (Kirsch et al., 2005), indicating that OT can affect a circuit for social recognition and response. However, it should be noted that although intranasal application of large proteins may reach the olfactory bulbs and small peptides the cerebrospinal fluid (Born et al., 2002), no studies have shown that the intranasal OT is reaching the central nervous system areas involved in facial recognition.

Animal studies of social cognition and the human research suggesting prosocial effects of OT have let researchers trace a link between this neuropeptide and autism. A few autism cases have been identified with deletions in chromosome 3 that essentially knock out the human oxytocin receptor. In particular, Gregory et al. reported on a deletion of the OTR in an autistic boy and his mother, who had obsessive-compulsive disorder. An affected brother did not have the OTR deletion but exhibited epigenetic silencing of the OTR due to hypermethylation of the OTR promoter. In an independent sample, they not only found additional autism cases with hypermethylation of the OTR gene but reported

reductions in OTR mRNA in temporal cortex associated with hypermethylation, demonstrating likely epigenetic silencing of the OTR even in the absence of a genetic mutation (Gregory et al., 2009). Many studies support the hypothesis that also early environmental experience, especially social experience, can have enduring effects on the OTR system (Carter et al., 2009). While there are many reports for associations between variations in OTR and AVP receptor genes and risk for autism, data are still not entirely consistent, as there is still little evidence that these variants are functional.

Modahl and colleagues reported a marked reduction in OT in children with autism relative to age-matched controls (Modahl et al., 1998). There have been few attempts to replicate these findings, although profoundly reduced OT plasma levels have been reported in high-functioning autism patients (Andari et al., 2010). Cerebrospinal fluid measures of OT could be informative but are not currently available from individuals with autism. It is notheworthy that cerebrospinal fluid OT has been reported to be selectively reduced in both women subjected to childhood abuse and monkeys raised with social deprivation (Heim et al., 2009; Winslow, 2005).

As for the possible treatment for ASD affected people, different studies have examined the effects of OT administered intranasally to high-functioning autistic patients (Andari et al., 2010; Guastella et al., 2009; Hollander et al., 2007). While not a cure, the results are promising. These initial trials report that, relative to placebo, OT improves eye contact, social memory, and use of social information. These reports should be considered a proof of principle. With the advent of nonpeptide agonists (Ring et al., 2010) and expanded clinical trial infrastructure allowing research with a broader range of

children and adults with autism, there may soon be an opportunity to develop new pharmacological agents specifically directed to social deficits.

Many of the defining characteristics of autism spectrum disorders such as atypical social behaviour, repetitive and stereotypic behavioural patterns, and increased anxiety and emotionality are, as expected, affected by AVP. Interestingly, ASD is more prevalent in males (Chakrabarti and Fombonne, 2005), likewise the effects of AVP on these behaviours are most pronounced in male animals (Bielsky et al., 2005). There have been a few studies of human sibling pairs (1 out of 2 affected) suggesting that the V1aR is an autism susceptibility gene (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006). A more powerful approach using more extended families found a significant linkage to chromosome 112q14 where, among other genes, the V1aR gene is located (Ma et al., 2007). While the 5' flanking region of the V1aR is not thought to cause autism, because ASD likely has diverse etiologies (Muller, 2007), it has been proposed that its variation could contribute, along with other genetic and environmental factors, to affect the onset and severity of the disease (Carter, 2007).

The microsatellite sequences of the 5' flanking region of the V1aR have been examined for their influences on various behaviours. In addition, a very strong linkage to creativity in dance was found by Bachner-Melman et al. (2005). These linkage studies, taken together, suggest a possible role for these polymorphisms influencing V1aR's role in social communication and the interpretation of social context. Also a particular polymorphism in the V1bR is associated with reduced susceptibility to recurrent major depression (van West et al., 2004). These genetic approaches, coupled with the

development of better behavioural animal models, suggest that the in the next future the answer to many of the ongoing questions regarding AVP's role in behaviour could be found.

2.3. Neurohormones and behaviour

2.3.1 Rodent models

2.3.1.1 Social memory and social recognition

A great body of evidence indicates that both neuropeptides AVP and OT influence social memory. Early work by Dluzen and colleagues indicates that OT influences social recognition responses in males by affecting the ability to process odors. Infusion of OT into the ofactory bulb facilitates social recognition at both a 30- and 120-min delay compared to vehicle-treated animals, but infusion of the OT antagonist OVTA into the same region of the olfactory bulb fails to block social recognition at either time delay (Dluzen et al., 1998). OT also facilitates social recognition when administered in other regions. Infusion into the lateral ventricles significantly enhances social recognition 120 min later at doses of 1 fg–10 ng/rat when injected after the first encounter (Benelli et al., 1995), indicating a role for OT in the acquisition phase of social memory. Similarly, infusion of the OT antagonist d(CH2)5[Tyr(Me) 2–Orn8] vasotocin (CPOVT) 5 min before OT injection abolishes the social memory-enhancing effect. Social recognition is facilitated by OT injection into the medial preoptic area of the hypothalamus (mPOA) with

a wide range of doses (0.3–1000 pg), but not when injected into the septum (Popik and van Ree, 1991). Interestingly, AVP facilitates social recognition when injected into the septum (Engelmann and Landgraf, 1994), but not in the medial pre-optic area (Popik and vanRee, 1991), indicating that these two neuropeptides influence social recognition in different brain regions. Finally, peripheral subcutaneous administration of OT and related peptides containing the C-terminal glycinamide has been shown to facilitate social recognition at low doses (Popik et al., 1996).

The development of OT and OT receptor KO mice has led to the further characterization of OT's role in social recognition responses. Male OT KO mice fail to develop social memory on both the habituation–dishabituation test (Lee et al., 2008) and the social recognition test (Ferguson et al., 2001). OT in the medial amygdala is necessary to facilitate social recognition, as demonstrated by *c-fos* activation in the medial amygdala of wildtype (WT) but not OT KO mice during the initial exposure (Ferguson et al., 2001). Interestingly, two independently derived lines of OT KO mice fail to show any deficits in general sociability (as measured by the social approach task (Crawley et al., 2007), indicating that OT seems to be primarily involved in the memory component of social recognition. Recently, though, a similar impairment in social recognition in these two lines of OTR KO mice was described. Specifically, unlike WT controls, OTR KO mice continue to investigate a 'familiar' female as if she were 'novel' (Takayanagi et al., 2005). Furthermore, a line of conditional OTR KO mice to reduce OTR expression in parts of the forebrain (OTR FB/FB) was generated. Compared to WT littermate controls, OTR FB/FB mice also show social recognition impairment but in a

different manner, with decreased investigation of both ‘familiar’ and ‘novel’ females on the second trial (Lee et al., 2008).

Generation of KO mice has allowed for investigation into the importance of proteins that regulate OT secretion. OT release is controlled, in part, by intracellular calcium stores (Ludwig and Leng, 2006). CD38 is a transmembrane glycoprotein that, through formation of cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate, mobilizes Ca^{++} and affects OT secretion. CD38 KO mice show normal synthesis and storage of OT in axon terminals, but abnormally low plasma and high hypothalamic OT concentrations, indicating abnormal release of OT in these mice, from both axon terminals and soma and/or dendrites (Jin et al., 2007). Interestingly, these mice show deficits in social recognition similar to those of the OT and OTR KO described above.

There is compelling evidence that AVP is important for social memory formation and social recognition. Furthermore, the use of V1aR and V1b KO mice has further elucidated the role of AVP in the formation of social memory. In the NIMH line of V1aR knockout mice the effects on social recognition have been mixed. Bielsky et al. (2004) demonstrated that male $V1aR^{-/-}$ mice have impaired social recognition that can be rescued by over-expression of V1aR in the LS. On the other hand, Wersinger et al. (2007) found no deficits in social recognition but rather a deficit in olfaction in $V1aR^{-/-}$ mice. Whether this discrepancy is due to slightly different strain backgrounds or testing procedures is unknown, but it is clear from previous works that AVP within the LS is important for social recognition. Interestingly, V1b mice also show mild impairments in social recognition (Wersinger et al., 2002).

2.3.1.2. Aggression

Aggressive behaviour occurs in situations of competition (for food, mates or space) to establish hierarchy in a social group. Generally, aggression in rodents is believed to be heavily under the control of AVP (Caldwell et al., 2008). The role of OT in controlling aggressive behaviour in males is ambiguous and depends upon the species used, the test animals' sexual status and the age at which OT levels are manipulated. In prairie voles, ventricular delivery of OT reduces sexual behaviour but has no effect on aggression (Mahalati et al., 1991) and it has been shown to affect aggressive behaviour after, but not prior to, mating (Winslow et al., 1993b). Interestingly, male Wistar rats introduced as an intruder to the cage of a singly housed male rat have between two- and five-fold increases in OT levels in the SON and anterior ventrolateral portion of the hypothalamus (Engelmann et al., 1999). This suggests that OT's role in aggressive encounters may have more to do with the stress response to this type of social interaction than aggression *per se*.

In male squirrel monkeys pair-housed for a sufficient length of time in which to form a stable dominant-subordinate relationship, OT significantly increases sexual and aggressive behaviours in the dominant, but not subordinate male, during interaction with a female. Similarly, the increase in aggression is blocked following concomitant administration of OT and the OT antagonist OVTA (Winslow and Insel, 1991). A clear picture has yet to emerge from studies using transgenic mice. One line with inactivation of the OT gene is mildly less aggressive than WT or heterozygous controls, and shows no difference in anxiety behaviour in an open field (DeVries et al., 1997). In a different line of OT KO mice, other researchers have reported increased aggressive behaviour in the

resident-intruder paradigm and decreased anxiety in the elevated plus maze (EPM) (Winslow et al., 2000). These effects were noted only in KO mice born from KO–KO matings; KO mice, and their WT controls, were cross-fostered to WT mothers. KO's produced from HET-HET matings show no reduction in anxiety and a small increase in aggression only on the third aggressive encounter. This suggests that the effects on aggression and anxiety are due to the lack of OT in the prenatal environment, or an interaction of genotype and the stress of cross-fostering. Elevated levels of aggression are also reported in OTR knockouts generated from non-obligates, consistent with the idea that a lack of prenatal activation of the OT system results in increased adult aggression (Takayanagi et al., 2005).

Studies of the role of AVP in the regulation of aggression have mostly focused on offensive aggression, which in rodents is examined using a resident-intruder test.

Two important concepts have emerged so far: AVP primarily modulates intermale aggression and AVP-facilitated aggression appears to be dependent upon prior experience, suggesting the presence of synaptic learning and-or epigenetic regulation of gene expression. Also, while key anatomical sites have been identified, their interactions with one other, as well as the interactions of AVP with other neurotransmitter systems are still poorly understood. The vasopressinergic projections from the BNST and MeA to the caudal LS are important for aggression and are androgen-dependent (De Vries and Miller, 1998). The anterior hypothalamus (AH) is also consistently implicated in the regulation of aggression and V1aR in this area demonstrates considerable plasticity (Askew et al., 2006).

2.3.1.3. Learning and memory

Memory processes are highly influenced by neuropeptides. Generally, AVP seems to enhance both non-spatial and spatial memory, likely through connections between the hippocampus and septum (Caldwell et al. 2008). In contrast, OT seems to attenuate memory processes. Recently, de Oliveria and colleagues also showed that i.p. OT administered prior to testing impairs inhibitory avoidance measuring 'step-down latency without causing increases in anxiety alone (tested on EPM) with an accompanying decrease in corticosterone levels (de Oliveira et al., 2007). Stress hormones and effects on the hypothalamic–pituitary–adrenal (HPA) axis may therefore mediate the amnesic effects of OT. The site of injection can also affect OT-induced changes in memory. Passive avoidance behaviour is impaired with post-learning injections of OT in either the dentate gyrus or dorsal raphe nucleus; however, memory is facilitated with OT injection into the dorsal septal nucleus (Kovacs et al., 1979). Furthermore, OT antiserum injected into dorsal hippocampus, dorsal raphe nucleus, or lateral habenula does not impair passive avoidance (Greidanus and Baars, 1993), contradicting the idea that OT is generally an amnesic peptide. Whether OT influences spatial memory is unclear. OTR are highly expressed in the hippocampus of mice (Insel et al., 1991). Hippocampal slices treated with OT in vitro maintain long-term potentiation (LTP) significantly longer than untreated slices, and have higher levels of phosphorylated CREB (Tomizawa et al., 2003). In other brain regions, OT also appears to inhibit spatial memory. Injections of OT into the nucleus basalis of Meynert (NBM), a region that provides primary cholinergic pathways to the cortex and is involved attention and memory (Wenk, 1997), significantly

increases latency to escape onto the hidden platform in the Morris water maze. Furthermore, injection of the OT antagonist atosiban into the NBM blocks the OT induced impairment, indicating that action at the OTR in the NBM is responsible for inhibition of spatial memory (Wu and Yu, 2004). Interestingly, mice lacking the OT gene throughout the brain and body do not show deficits on the Morris or Y-maze, indicating that OT is not necessary for spatial memory (Ferguson et al., 2000).

In addition to its effects on social memory and interactions, de Wied in the 1960s firmly established a modulatory effect of hypothalamic AVP on non-social learning and memory: he found that removal of the posterior pituitary impaired active avoidance shuttle box performance in rats (de Wied, 1965) that could be improved with AVP administration (Bohus et al., 1972). Since then, AVP has repeatedly been shown to affect non-spatial and spatial memory, with most studies finding an enhancing effect on memory, most likely through influences on the hippocampal-septal interactions (Croiset et al., 2000;). AVP can enhance consolidation of memories, but seems more important in memory retrieval (Gulpinar and Yegen, 2004). In passive avoidance, both administration of AVP immediately after the last learning trial or just prior to the retention trial improves learning (Kovacs et al., 1986).

More recently, subcutaneous administration of NC-1900, an AVP analog with a longer half-life, was shown to ameliorate deficits in passive avoidance memory produced by inhibition of the COX-2 pathway (Sato et al., 2007), indicating that AVP may affect memory through this pathway. As for spatial memory, performance in the radial arm maze is enhanced by AVP, as administration significantly enhances learned extinction

behaviour, that is learning that food was no longer present in the arms (Packard and Ettenberg, 1985). Scopolamine, an anticholinergic, produces deficits in reference and working memory in the radial arm maze. These memory deficits are reversed with administration of AVP (Taga et al., 2001) and NC-1900 (Mishima et al., 2003). Moreover, V1aR KO mice have impaired working memory in the radial arm maze as compared to controls (Egashira et al., 2006), indicating a role for the V1aR receptor in spatial task performance. Conversely, V1b KO mice do not have impaired spatial memory on the Morris water maze (Wersinger et al., 2002; Egashira et al., 2004).

2.3.1.4. Emotional behaviour

Oxytocin is thought to work as an anxiolytic as it decreases release of stress hormones in both humans (Legros, 2001) and rats (Stachowiak et al.1995). Endogenous release of OT in males during mating can reduce anxiety-like behaviours (Waldherr and Neumann, 2007). In male rats, OT administration reduces anxiety-like behaviours in a number of behavioural tests; in mice, this effect is blocked in some tests by the OT antagonist WAY-162720 (Ring et al., 2006). The anxiolytic properties of OT may be mediated, at least in part, via action at the OTR in the amygdala which is well known for its role in the acquisition, modulation and storage of emotional memory (LeDoux, 2007). OT infusion into the amygdala has an anxiolytic effect on ovariectomized female rats in an open field (Bale et al., 2001). Projections from the septum and the amygdala may modulate the hypothalamic-pituitary-adrenal axis via connections to OT neurons in the PVN and SON (Oldfield et al., 1985). Within the extended amygdala there are many regions with binding sites for OT (Veinante and Freund- Mercier, 1997). In one line of OT

KO mice, male offspring of KO–KO parents show reduced anxiety on the elevated plus maze, though this effect may be due to lack of exposure to OT in utero (Winslow et al., 2000). It has also been shown that there is no effect on anxiety-like behaviour in OTR KO mice or in a partial forebrain-specific OTR knockout line (Lee et al., 2008).

High densities of vasopressinergic fibers (De Vries and Buijs, 1983) and V1aR (Ostrowski et al., 1992) are found within the septum. Anxiety-related behaviour, as measured on the elevated plus maze (EPM), is significantly reduced with septal infusion of antisense oligodeoxynucleotide to the V1aR mRNA (Landgraf et al., 1995), as well as with the V1aR antagonist d(Ch2)5Tyr(Me)AVP (Engelmann et al., 2000), in the absence of altered locomotor activity. However, Appenrodt et al. (1998) found both intraseptal and intraperitoneal (i.p.) application of AVP is also anxiolytic, indicating possible actions outside of the septum. Moreover, some transgenic mice studies indicate a role for V1aR in modulating anxiety. V1aR KO males display significantly less anxiety-related behaviour than wildtype males on a variety of tasks (Egashira et al., 2007). Additionally, over-expression of the V1aR gene in the LS significantly increases anxiety-related behaviour (Bielsky et al., 2005), further implicating V1aR in controlling the anxiety response. However, in another lab, no anxiolytic phenotype has been found in this line (Caldwell et al., 2006). Ample evidence indicates that, in addition to affecting anxiety-related behaviour, AVP affects depression-related behaviour. In rodents, the forced swim task produces immobility that can be reversed with antidepressants (Porsolt et al., 1977). Acute forced swimming induces a significant increase in AVP release in both the SON and PVN that increases further in the PVN with repeated swimming (Wotjak et al., 1998).

Applications of the V1aR antagonist d(Ch2)5Tyr(Me)AVP into both the mediolateral septum and the amygdala have antidepressant-like effects (Ebner et al., 1999, 2002). Antidepressant-like effects are also observed with oral and intraperitoneal (i.p.) administration of the V1b antagonist SSR149415, even after hypophysectomy (Griebel et al., 2002). Infusion of SSR149415 into the LS and into three nuclei of the amygdala also decreases immobility time in the forced swim task (Salome et al., 2006). However, wildtype, V1aR $^{-/-}$ and V1bR $^{-/-}$ mice do not differ in this swimming behaviour (Egashira et al., 2007).

2.3.2. The Zebrafish model

2.3.2.1. General description and behavioral models

Zebrafish, *Danio rerio*, is one of the most important vertebrate model organisms in genetics, developmental biology, neurophysiology and biomedicine (Amsterdam and Hopkins, 2006). It has a number of attributes that make it particularly useful for experimental manipulation. It is a small, robust fish and large numbers of individuals can be kept easily and cheaply in the lab, where it breeds all year round. Females can spawn every 2-3 days and a single clutch may contain several hundred eggs. Generation time is short, typically 3-4 months, making it suitable for selection experiments. Furthermore, fertilisation is external so live embryos are accessible to manipulation and can be monitored through all developmental stages under a dissecting microscope (Kimmel et al., 1995). Zebrafish embryos and early adults are optically transparent, a characteristic that facilitates direct observation of internal organs by light microscopy; during organogenesis, embryos are permeable to small molecules and drugs, providing easy access for drug administration and vital dye staining. Expression of specific proteins can be suppressed throughout the developing embryo using of morpholino antisense oligodeoxynucleotides; this enables monitoring the roles of specific proteins in development and/or drug response. Conversely, embryos can be microinjected with either capped mRNA or plasmids to express specific proteins and variants (including splice variants, point mutants, etc.), either throughout the embryo or in a tissue-specific manner. In combination with morpholino antisense oligodeoxynucleotides, this approach resembles ‘‘knock-out/knock-in’’ approaches widely used in mice. Zebrafish genome has

been sequenced and can be accessed at UCSC genome browser (Kari et al., 2007). In fact the greatest advantages of the zebrafish as a model system come from its well-characterised genetics, genetic and developmental techniques and tools and the availability of well-characterised mutants. Its nervous system is more comparable to that of humans than Drosophila. Zebrafish are also tractable species for behavioural experiments, readily acclimatising to new environments, being constantly active and little disturbed by the presence of observers. In order for the zebrafish to be more widely adopted as a model by the behavioural ecology community there is a need for more behavioural and field-based studies in order to catalogue natural variation in morphological, physiological and behavioural traits.

Zebrafish appears ideally suited to studies of social and cognitive behaviour and it is surprising that it has been so little utilised for this purpose. There is increasing interest in employing social and cognitive tests with zebrafish to study the genetic basis of behaviour and there is a need for more comprehensive and better controlled studies in this area (Spence et al., 2008). What it is known is that in zebrafish the preference for associating with other fish is innate while the preference for particular colour patterns is based on learned behaviour. Individuals raised in isolation do not display colour pattern preferences whereas cross-reared individuals prefer to associate with the colour pattern with which they were raised (Engeszer et al., 2004; Spence et al, 2008). Learned preferences are mediated by olfactory as well as visual cues; zebrafish can differentiate between familiar and unfamiliar conspecifics on the basis of odour and thus appear capable of individual recognition (Gerlach and Lysiak, 2006). The response shown by

zebrafish to alarm substance is also innate but appears to function as a means of learned predator recognition. Alarm substance can initiate a conditioned response to an innocuous odour, such as morpholine, when the two are presented simultaneously (Suboski et al., 1993). Hall and Suboski (1995) further elicited a learned response to a visual cue by pairing alarm substance with a red light as well as with morpholine. Thus, conditioning can operate across different sensory modalities. The same authors also demonstrated second-order conditioning whereby fish conditioned with alarm substance to respond to either light or morpholine then learned to react to the second neutral stimulus when presented in combination with the first conditioned stimulus in the absence of alarm substance. An alternative approach to studying learning is to use an operant conditioning paradigm, whereby fish are trained to swim in a specific direction for a food reward paired with a visual cue. This approach has been used to study spatial memory, landmark use and orientation in other species (Braithwaite et al., 1998), and the few studies available indicate that zebrafish are potentially a useful model for research in this area. In a study to investigate spatial learning and memory, Williams, White and Messer (2002) trained adult zebrafish to swim alternately to one or other side of a divided aquarium to receive a food reward. Once trained, the fish could remember the task after a 10-day period during which they were fed ad libitum in another aquarium. Zebrafish was also able to learn to swim into one of three compartments when the one containing the reward was cued by a white light (Bilotta et al., 2005). A three-choice design provides better evidence of learning than a two-choice design as the level of a chance response is reduced to one-third. Different tasks have been shown to elicit different preferences. Colwill et al. (2005) used a T maze with different-coloured arms (green versus purple or

red versus blue) to assess visual discrimination learning in zebrafish. They found that while fish could be trained to swim down whichever coloured arm was associated with a food reward, they learned faster and retained the response longer when the colour associated with the reward was purple or blue than when it was green or red. Thus, not only were the stimuli not perceived as equal, but the colour preferences shown in this context differed from those in the foraging study by Spence and Smith (2007).

Furthermore zebrafish model has been validated for studying the process of addiction in vertebrates, for example using the CPP test with cocaine (Darland and Dowling, 2001) and amphetamine (Ninkovic et al., 2006). Moreover, ethanol-induced hyperlocomotion can be blocked by the addition of a dopamine antagonist, suggesting the involvement of the brain dopamine system (Lockwood et al., 2004).

2.3.2.2. The social zebrafish

Zebrafish is a highly social species that prefers swimming in groups, an aggregation behaviour termed shoaling (Pitcher, 1983) and described in a number of fish species (Wright et al., 2006). Other traditional laboratory organisms including the mouse and the rat, or simpler organisms such as Drosophila or the nematode, do not exhibit the degree of social cohesion and group preference displayed by zebrafish. Thus, in addition to the genetic tools developed for zebrafish, its behavioural characteristics make this species particularly appropriate for this kind of studies. It has to be noted that the mechanisms of social behaviour of vertebrates including our own species are not clearly understood and as a result diseases associated with abnormal social behaviours in humans (including social phobias and autism spectrum disorders) have been difficult to

treat. Given the high nucleotide and amino acid sequence homologies found at the DNA and protein levels between zebrafish and mammals including humans (Barut and Zon, 2000) and the similarities of the basic layout of the brain of these species, it is not unlikely that the analysis of genetic mechanisms of social behaviour of zebrafish will lead to results that generalize and translate to other vertebrate species including our own (Tropepe and Sive, 2003). Preference of zebrafish for certain stimulus fish was investigated in choice paradigms (Engeszer et al., 2004). Results demonstrated that zebrafish were sensitive to certain visual characteristics of the stimulus fish and the preference was strongly influenced by early experience during development.

2.3.2.3. Zebrafish and autism

Studies regarding autism spectrum disorders have recently taken advantage of the zebrafish model. Aberrant social behaviour, the most typical characteristic of the disorder, can be easily modelled in zebrafish by evaluating different parameters such as the tendency to join a shoal, social isolation, the distance from a predator (Dadda et al., 2010), inhibitory avoidance (Blank et al., 2009), social interaction (Delaney et al., 2002), dominance-subordination relationships (Larson et al., 2006), repetitive and stereotyped movements (López-Patiño et al., 2008), aggressive behaviour (Colman et al., 2009).

In humans, about 1% of ASD cases is associated to deletions involving chromosome 16, which contains 25 genes that are homologous to zebrafish (Eichler & Zimmerman, 2008). The extensive use of morpholino anti-sense oligos to inhibit mRNA maturation of specific genes (Nasevicius & Ekker, 2000) made it possible to identify genes involved in neuronal and brain development in zebrafish. Very recently, the use of morpholino knock out

zebrafish has helped in demonstrating an important role of the so-called *Sushi domain,* consisting in 4 SUDS4 proteins, which results deleted in a group of affected ASD patients. In fact, SUDS4 is highly expressed in CNS of different species, such as humans, mice and zebrafish in particular, where it plays an essential role in its development. (Tu et al., 2010).

These and other pioneering studies suggest how zebrafish can also be used as a model for future sperimentations in order to characterize cellular and molecular mechanisms of ASD.

In conclusion, in the near future, zebrafish behavioural quantification methods will most probably be a great tool for developing automated and high throughput paradigms with which the biological mechanisms of different kind of zebrafish behaviour will be investigated, a goal that will ultimately lead to better understanding of the genetics of complex behaviours in other vertebrates including humans.

Aim of the study

Autism is a neurodevelopmental disorder which is characterized by severe and pervasive impairment in reciprocal socialization, qualitative impairment in communication and repetitive or stereotyped behaviour associated to resistance to change. Oxytocin (OT) is a peptidic hormone best known for its role in lactation and parturition. Recently, it has been also shown by several studies involving different lines of KO mice to play an important role in the CNS by acting on the regulation of social, emotional, aggressive behaviour and on learning and memory. Furthermore, prosocial effects following OT administration in humans have been shown. The link between OT and autism has already been traced in preliminary clinical studies as autism affected patients have received a beneficial outcome from treatment with OT. Pharmacokinetic (very short half-life), pharmacodynamic properties (unspecific binding to vasopressin receptors) and the presence of peripheral side effects of OT, though, make this peptide an unsuitable target for a future clinical use. It is important, in this perspective, to characterize specific animal models in order to validate the use of OT analogs with more suitable characteristics in preclinical research.

On this purpose our aim will be to characterize more specifically a pre-existing mice model lacking the OT receptor in both its heterozygous and homozygous form. General health (fur, pilo-erection, body tone, skin colour), reflexes (righting, corneal, pinna, tail pinch) and sensory abilities (vision, hearing, smell and pain) will be firstly investigated. Motor function will be analyzed through a motor activity cage for spontaneous motor activity, rotarod for motor coordination, hanging wire for muscle strength. Emotional reactivity will be evaluated in the elevated plus maze for anxiety-like behaviour and in the forced swim and tail suspension test for depressive symptoms.

Finally, mice will be tested for the core symptoms of autistic disorders: aberrant social behaviour in the sociability and social novelty test, aggression in the neutral cage test and resistance to change in the T maze task. The effect of OT, AVP and V1aR antagonist SR49059 treatment (0.5 ng/mouse i.c.v) will also be investigated in these tasks in order to analyze the mechanism through which these altered behaviours take place.

For an additional characterization of the model, OTR and V1AR receptor binding will be evaluated in different brain areas already known to be associated with social and cognitive impairments such as anterior olfactory nucleus, vertical limb of the diagonal band, pyriform cortex, lateral septum, ventral pallidum, hypothalamus, amygdala, hippocampus and supraoptic nucleus.

Finally, as an increasing interest in the use of zebrafish for social and cognitive behavioural analyses to study the genetic basis of behaviour is rising, we will evaluate its potential as a screening tool for neuropsychiatric diseases involving deficits in social behaviour. In order to do so, we will perform dose-response studies to analyze the effect of OT, AVP but most importantly isotocin (ISO) and vasotocin (AVT) (zebrafish homologues of OT and AVP, respectively) using the shoaling preference test as social paradigm.

Materials & Methods

4.1. OTR mutant mice model

4.1.1. Animals

The $OTR^{+/+}$, $OTR^{+/-}$ and $OTR^{-/-}$ mice were obtained from L. Young (USA). All mice used were littermates from mated heterozygous and were genotyped by PCR. Mice were initially tested for general health, followed by reflexes, sensory, motor and emotional-like reactivity and then for behavioural tasks relevant to autism (sociability and social novelty, aggression, T maze and seizure susceptibility). For sociability and social novelty and neutral cage tests, *stranger* adult male mice were used, belonging to the DBA/2J strain (Charles River, Calco, Italy) chosen for their very low scores of native aggression (Crawley, 1997).

4.1.1.1. Maintenance of mice

Male mice were individually housed in an air-conditioned room (22 ± 1 °C) with a 12-h day-dark cycle (lights on: 08:00 hours) and had free access to food and water. Then, they were randomly assigned to each experimental group (10 naive animals per group for behavioural tasks, 3 animals per group in the autoradiographic binding assay) and tested at 3 month of age. All testing took place during the first half of the light period (between 09:00 and 13:00 hours). Water was always available while food was provided *ad libitum* except during T maze experiments which were conducted under dietary restriction. All the experimental procedures followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government decree No. 37/2007. All efforts were made to minimize the number of subjects used and their suffering.

4.1.1.2. Genotyping

Mice were genotyped by PCR using specific primers on DNA extracted from tail snips. A single forward primer (N248-5'CTGGGGCTCAGTCTTGGAAG), and two reverse primers (N2475'GTTGGGAACAGCGGTGATTA;WT25'CCTCGATACTCCAGTTGGCTG) were used for amplification. The PCR was carried out for 35 cycles with denaturation at 98 °C for 30 sec, annealing at 55 °C for 45 sec, and extension at 72 °C for 1 min. $OTR^{+/+}$ and $OTR^{-/-}$ mice were identified by the presence of either a 700bp or a 450bp specific product; $OTR^{+/-}$ mice were identified by the presence of the two products.

4.1.1.4. Surgery

Mice were anaesthetized with chloral hydrate (Sigma-Aldrich, Chemicals, Co, MO, USA), (450 mg/kg, i.p.). A steel guide cannula (24 gauge) anchored to a acrylic pedestal (Greto, Italia) was stereotaxically implanted as per the coordinates referred from Franklin and Paxinos (2007)(AP -0.82mm; ML +1.5 mm; and DV +3.0 mm; related to bregma). The guide cannula was secured to skull using a mounting screw (Plastic One Inc.,USA) and dental cement (Palavit, New Galetti Rossi, Milan, Italy). Each mice was allowed to recover for 1 week under antimicrobial cover (Ceftriaxone, Sigma-Aldrich, Chemicals, Co, MO,USA, 50 mg/kg i.p.). To ascertain the accuracy of the ICV injections, at the end of the experiments, mice were injected by the same route with 2 μl of a saturated solution of Evans blue (Merck, Whitehouse Station New Jersey) using a Hamilton syringe (Hamilton, Nevada, USA) and killed immediately. Macroscopic examination of the brain confirmed that only the area around the lateral ventricles was stained.

4.1.2. Behavioural Assays

4.1.2.1. General health and reflexes

Each mouse was observed and any abnormalities in the general appearance of the fur (3-point scale), pilo-erection, body tone (3-point scale), skin colour were recorded according to Lee et al. (2008). Furthermore, mice were observed for the empty cage behaviour (nest-building behaviour) according to Deacon (2006) using a 5-point scale.

Neurological reflexes were assessed, including righting reflex (4-point-scale) corneal (8-point scale), pinna (8-point scale) and tail pinch (8-point scale) according to Irwin (1968).

4.1.2.2. Sensory abilities

- *Hearing* was evaluated using Preyer's reflex testing through a handclap sound. The reflex was considered positive (and scored 1 in 0-1 point scale) when a rapid movement of the whole body of the animal was clearly noticed as previously described (Jero et al., 2001).
- *Visual acuity* was performed according to Brandewiede et al. (2005) using the visual cliff test. The apparatus consisted of a plexiglass box (60x40x61 cm). A platform was installed adjacent to a wall 32 cm above the floor of the box. The platform and the inner surface of the box were covered with black and white checkboarded contact paper that emphasised the ledge drop-off. A piece of clear Plexiglas covered the platform and spanned the ledge so that there was no actual drop-off but only the visual appearance of the cliff. Mice were placed on the

platform close to the wall. Mice that spent a significantly higher amount of time on the checkboarded platform rather than in the transparent one were considered to have good sight.

- *Olfactory test* was carried out according to Moy et al. (2004). Two days before the test, an unfamiliar palatable food (Kellogg's chocolate cereal) was placed overnight in the home cage of the subject mice. On the day of the test, each mouse was placed in a large cage containing 3 cm deep sawdust and allowed to explore for 5 minutes. The animal was removed from the cage, and one cereal was buried in the cage bedding. The animal was then returned to the cage and given 15 minutes to locate the buried food. Latency to find the cereal was recorded.

- *Pain sensitivity.* The hot-plate assay was performed at 52°C. The time (s) elapsing to the first pain response (licking or jumping) was recorded. A maximal latency of 40 s was used (Crawley, 2000).

4.1.2.3. Motor functions

- *Spontaneous motor activity* was evaluated in an activity cage (41 X 41 X 32 cm) (Ugo Basile, Varese, Italy), placed in a sound-attenuating room (Braida and Sala, 2000). The cage was fitted with two parallel horizontal and vertical infrared beams located 0 cm from the floor. Cumulative horizontal and vertical movement counts were recorded for 30 min.

- *Motor coordination* and balance were evaluated during four consecutive 120 s trials by quantifying the ability to maintain balance on a rotating

cylinder using the rotarod test as described by Daugé et al. (2001). The latency to fall from the rotarod was recorded

- *Muscle strength* was measured using the hanging wire cage test. Mice were placed on the underside of a standard wire rat cage top approximately 20 cm above a large cage containing soft sawdust bedding. The latency to release was recorded, with a maximum latency of 300s (Crawley, 2000).

4.1.2.4. Emotional like reactivity

- *Anxiety* was measured according to Lister (1987) in an Elevated Plus Maze. Each animal was placed onto the centre of the apparatus and the time spent on and entries onto each arm were registered for 5 min. The percentage of time spent in the open arms and the percentage of open-arm entries id used as measure of anxiety.
- Forced swim (FST) and tail suspension (TST) tests were used to assess the depressive phenotype. FST (Porsolt et al., 1977) consisted in forcing mice to swim for 6 min under conditions in which escape is not possible. Time spent immobile (sec) was measured in the last 4 minutes of the test only, considering the first 2 minutes as an habituation phase. TST is based on the observation that a mouse suspended by the tail alternates periods of complete immobility and agitation. The test was conducted according to the procedure of Steru et al., (1985). Mice were held by the tail suspended dangling in the air at 35 cm from the floor for 5 min. The duration of immobility (sec) was measured.

4.1.2.5. Core symptoms of autism

- *Sociability and preference for social novelty test:* the sociability and preference for social novelty test was used as described by Moy et al. (2007), with slight modifications. The apparatus consisted in a three equal and communicating transparent Plexiglas chambers (41 X 41 X 32 cm). The test procedure consisted in three separated phases. *Habituation*: the test mouse was placed in the middle chamber and allowed to explore the whole apparatus for 10 minutes. Each of the two outermost sides contained an empty wire cage. *Sociability*: after the habituation period, the test mouse was enclosed in the centre compartment of the box, and an unfamiliar male mouse (stranger 1, a never-seen-before adult male DBA/2J mouse) was enclosed in one of the wire cages and placed in one of the side chambers. The location of stranger 1 alternated between the left and right sides of the box across subjects. Following placement of stranger 1, the doors were re-opened and the subject was allowed to explore the apparatus for 10 min. The time spent in each chamber and the number of entries made in each chamber was recorded. The *difference score* for sociability was calculated as difference between the time spent in the compartments containing the familiar littermate and the time spent in the empty compartment according to DeVito et al., (2009). *Preference for social novelty:* at the end of the sociability test, each mouse was tested in a further 10-min session to quantify preference to spend time with a new stranger. A new unfamiliar mouse (adult male DBA/2J mouse) was placed in the wire cage that had been empty in the previous phases. The test mouse had to choose between spending time in the proximity of the first, already-

investigated, familiar mouse (stranger 1) and the novel unfamiliar mouse (stranger 2). The time spent in each chamber and the number of entries made in each chamber was recorded, as described above. The *difference score* for social novelty was calculated as difference between the investigation times for the compartments containing the novel animal and that for the familiar littermate according to DeVito et al., (2009).

- *Aggression:* the test was performed according to Tordjman et al. (2003) in a neutral area (a transparent cage with dust-free clean sawdust). The tested male was placed in the neutral cage and followed 10 min later by the opponent, a never-seen-before DBA/2J male adult mouse. The test lasted 6 min during which we measured attack latency (the time elapsing between the first sniffing until the first attack) and the number of attacks.

- *T maze:* the task was conducted according to Moy et al. (2007). Before starting, animals were food deprived to 85-90% of their free-feeding body weight. Mice were then habituated to a black T- maze and shaped to obtain food across the maze for 5 days. In the first *acquisition* phase, for each mouse one arm was designated as the correct arm where one palatable food reward (Kellogg's chocolate rice cereal) was available for each trial. The reinforced arm was on the left side for half of the mice, and on the right side for the other half. At the beginning of each session, each mouse was placed at the start of the maze and was then given a choice between entering either arm. If the mouse made the correct choice, it was given time to consume the pellet and then guided back to the start for the next 9 daily trials. Incorrect choices were not rewarded or punished.

For each successive trial the reward was always placed in the same arm. The criterion was reached when the mouse made 80% of correct choices for three consecutive days. The number of days taken to reach the criterion was recorded. Each mouse that met criterion for acquisition was the tested using a *reversal* procedure, in which the reinforce location was switched to the arm opposite to its previous location. Ten trials per day were administered for reversal learning, using the same methods and criterion as described above.

4.1.3. Autoradiography binding studies

(in collaboration with prof. Daniela Parolaro, Università dell'Insubria, Italy)

Brains were rapidly removed, frozen in liquid nitrogen and stored at -80°C until processing. Twenty-micron coronal sections were cut with a cryostat, thaw-mounted on gelatin-coated slides and processed following Francis et al., 2002. Specific and non specific binding were determined by incubating adjacent sections with the specific ^{125}I-OVTA (Elands et al., 1988) (NEX254, 2200 Ci/mmol, Perkin Elmer, Boston, MA, USA) and [^{125}I] Linear AVPA (NEX310, 2200 Ci/mmol, Perkin Elmer, Boston, MA, USA) ligands at final concentrations of 50 pM with or without 50 pM unlabelled Thr4Gly7OT, a selective OT ligand (Peninsula Laboratories, Belmont, CA, USA), or 50 pM of unlabelled Linear AVPA, a selective V1aRR antagonist (Schmidt et al., 1991). Autoradiographic ^{125}I receptor binding was quantified as in Rubino et al., 2005. The intensity of the autoradiographic film was assessed by measuring the grey levels with an image analysis system provided by

Immagini & Computer (Milan, Italy) consisting of a dual scanner Artixscan 1800F connected to a personal computer (PC). The software IMAGE-PRO PLUS version 5.0 (Media Cybernetics Inc., Silver Spring, MD, USA) was used. Each cerebral area was traced with the mouse cursor control and the light transmittance was determined as the grey level. The grey level of densitometric measurements calculated after subtracting the film background density was established within the linear range determined using tritium standards (3H Microscales, Amersham Pharmacia Biotech, Milan, Italy) for receptor binding studies made in the laboratory for [35S]GTPγS binding studies.

3.1.4. Drugs and Treatments

Oxytocin (OT) and vasopressin (AVP) (Bachem, Germany, EU) were dissolved in artificial cephalorachidian liquid (aCSF) and given intracerebroventricularly (ICV) 10 min before each test. In particular for the T maze task treatments were performed before each daily trial in the reversal phase only. The selective V1aR antagonist, SR49059 (Sanofi-Aventis, Paris, France) was dissolved in aCSF and given ICV at a dose of 0.5 ng/2μl/mouse 20 minutes before each test. OT and AVP were given at a dose of 0.5 ng/2μl/mouse since a previous study (Meisenberg and Simmons, 1987) demonstrated grooming behaviour, starting from 1 ng/mouse.

4.1.5. Statistical Analysis

All measurements were compared between genotypes using a one- or two-way repeated measures ANOVA test where appropriate, followed by Tukey's, Bonferroni or Dunn's tests. Data expressed as percentage were analyzed with Fisher exact probability test. The accepted level of significance was $p<0.05$. All statistical analyses were done with software Prism, version 5 (GraphPad, San Diego, California).

4.2. Zebrafish model

4.2.1. Subjects

Adult AB wild type (WT) zebrafish (*D. rerio*) (50% male and 50% female) and were purchased from a local pet shop (Aquarium Center, Milan, Italy). *Nacre* mutant zebrafish were a kind gift of Dr Mione (IFOM-IEO, Milan, Italy). Both strains were kept at approximately 28.5°C on a 14-h light/10-h dark cycle. All fish were raised with their wild-type siblings in visual isolation from other phenotypes. Behavioural testing took place during the light phase between 9.00 and 14.00 hours. Tank water consisted of deionized H_2O and sea salts (0.6 g/10 l of water; Instant Ocean, Aquarium Systems, Sarrebourg, France). The home tanks with groups of approximately 30 adult fish were maintained with constant filtration and aeration. Fish were fed daily with brine shrimp and flake fish food (Tropical fish food, Consorzio G5, Italy). All the fish were drug naive and each fish was used only once. 10 fish per group were used. The experimental protocol was approved by

the Italian Governmental Decree No. 14/07. All efforts were made to minimize the number of animals used and their discomfort.

4.2.2. Behavioural assays

4.2.2.1. Shoaling preference test

Shoaling preference test was carried out according to Engeszer et al. (2008*). Nacre* mutants and WT counterparts were used as stimulus fish shoals. All stimulus shoals comprised two males and two females randomly chosen from each group tank. Shoals of as few as four zebrafish exhibit shoaling behaviours indistinguishable from those of larger groups (Breder, 1959). A glass test tank *(*122 cm long x 55 cm tall x 32 cm wide) divided into five equal compartments, was used. Outermost compartments, hereafter referred to as stimulus areas, were separated from inner compartments by glass walls, which were sealed with silicon aquarium sealant to isolate water in the stimulus areas from the inner compartment. We further subdivided the inner compartment, marking off three zones of equal volume comprising a left preference area, a central no-preference area, and a right preference area. The tank was lit by two 250-W halogen lamps placed above and on either side of the test tank. Light from these lamps then reflected off two sheets of Teflon hung at a 45° angle from the top of the tank. Thin sheets of opaque plastic, as temporary visual barriers to separate the exterior compartments from the interior compartment, were used. The water level in the tank was kept at 25 cm depth. Opaque barriers were placed in the central

compartment, to visually isolate the subject fish from the stimulus areas, one containing the stimulus (*nacre*) shoal, the other the WT shoal. Immediately after treatment, the subject fish was placed in the central compartment. The fish were allowed 5 min to acclimate to the test tank. Then the opaque barriers were removed and started recording shoaling behaviour by using Canon Digital MV900 compact camera. Fish were allowed up to 15 min to recognize both stimuli. We started recording time spent in association with each stimulus after the first 5-min period in which the subject recognized both stimuli. If the subject did not recognize both stimuli in 15 min, the test was cancelled and redone at the beginning of the following week. When the fish subject swam parallel to one of the shoal members, it was considered to recognize the stimulus shoal. (Engeszer et al., 2004). Shoaling preference was quantified by recording the total time spent by the WT test fish in proximity of each stimulus shoal.

4.2.3 Drugs and treatment

Body weight was measured as previously described (Braida et al., 2007). Briefly, fish were removed from their tank using a net and placed in a container containing tank water, positioned on a digital balance. Fish weight was determined as the weight of the container plus the fish minus the weight of the container before the fish was added. The mean of three measurements was recorded. Fish were injected intramuscularly (i.m.) in the caudal musculature using a volume depending on the weight of the fish (2 μl/g) using a Hamilton syringe (Hamilton Bonaduz AG,

Bonaduz, Switzerland). Oxytocin, vasopressin ((Arg^{8})-Vasopressin), isotocin ((Ser^{4},Ile^{8})-Oxytocin) and vasotocin ((Arg^{8})-Vasotocin) (Bachem, Germany) were disolved in saline (0.9%). All drugs were prepared fresh daily.

4.2.4 Statistical analysis

All data are expressed as mean±sem. For the dose-response analysis of the different peptides, data were analyzed against the log of the administered doses; the values of the social parameter considered were calculated by the statistical analysis adapted to curvilinear regressions according to Steel and Torrie (1960). All statistical analyses were done using software Prism, version 5 (GraphPad, San Diego, CA, USA). The accepted level of significance was $P<0.05$.

Results

5.1 OTR mutant mice

5.1.1. OTR mutant mice phenotypical characterization

5. 1.1.1 General phenotypic and sensory characteristics

Table 1 reports the general phenotypical characteristics of OTR $^{+/+}$, OTR $^{+/-}$, OTR $^{-/-}$ mutant mice. No significant differences were found in weight, fur condition, piloerection body tone and skin colour. Nest building abilities did not differ among different genotypes nor did general reflexes (righting, corneal, pinna and tail pinch). No difference was found in visual, olfactory, hearing and pain sensitivity (fig. 1) as tested respectively in the visual cliff test (fig. 1A), buried pellet test (fig 1B), preyer reflex (fig. C) and hot plate test (fig. 1D). In particular for the visual cliff test a significant difference between the time spent on the plain and check boarded floor side was found by 2way repeated measure ANOVA (with side as between-subject factor and genotype as within-subject factor, side x genotype interaction: not significant; effect of side: $F(1, 28) = 526.3$; effect of genotype: not significant) followed by Bonferroni post hoc analysis.

5.1.1.2. Motor abilities

Motor abilities of OTR$^{+/+}$, OTR $^{+/-}$, OTR $^{-/-}$ mutant mice are shown in fig. 2. The mean number of horizontal (fig. 2A) and vertical counts (fig. 2B) was not different among genotypes. Fig 2C reports results of the rotarod test. In the first of the four trials of the test a slight not significant difference is shown as a shorter latency to fall from the rotarod is

displayed by heterozygous and KO mice cpmpared to WT mice. Recovery from this small deficit is reached across the remaining trials as a significant difference from the first trial is found (2way repeated measure ANOVA, with trial number as between-subject factor and genotype as within-subject factor, treatment x genotype interaction: $F(7, 17) = 0.59$; effect of trial: $F(3, 26) = 13.38$; effect of genotype: $F(2,27) = 3.09$) followed by Bonferroni post hoc analysis.

No differences among groups were found in the hanging wire test performance as measured by the latency to fall from the overturned grid (fig. 2D).

5.1.1.3. Emotional behaviour

No changes in anxiety or depressive-like behaviour emerged in any of the groups tested in our studies: in the elevated plus maze the number (fig. 3A) and time spent (fig. 3B) in the open arms did not differ among genotypes and time spent immobile was very similar also in both the forced swim test (fig. 3C) and tail suspension test (fig. 3D).

5.1.1.4. Social Behaviour

Social behaviour was evaluated in the sociability and social novelty test (respectively fig. 4A-C and fig. 4B-D). One way ANOVA found a significant difference in the difference score time in both sociability ($F(2, 27) = 9.600$, $p<0.001$) and social novelty ($F(2, 27) = 23.99$, $p<0.0001$). Tukey's post hoc test revealed significant decrease in the difference scores for both heterozygous and knock out mice compared to wild type mice in both tasks. The number of entries in the different compartments was similar among groups (fig. 4C-D).

5.1.1.5. Aggressive behaviour

Alterations of OTR system result in changes in aggressive behaviour of mice (fig. 5). In the neutral cage test we registered the number of attacks (A) and tail rattlings (C) and the latency to the first attack (B) and tail rattling (D). Significant difference was revealed by one way ANOVA in the latency to first attack ($F(2, 27) = 9.200$, $p<0.0003$), number of attacks ($F(2, 27) = 10.51$, $p<0.0001$), latency to first tail rattling ($F(2, 27) = 7.2$, $p<0.003$) and number of tail rattlings ($F(2, 27) = 4.3$, $p<0.01$). In particular Tukey's test revealed a significant increase in aggressive behaviour in the OTR $^{-/-}$ group in comparison with both OTR $^{+/+}$ and OTR $^{+/-}$ mice in all considered parameters.

5.1.1.6. T maze

OTR$^{+/+}$, OTR$^{+/-}$, OTR$^{-/-}$ mutant mice performance on both the acquisition and reversal phase of the T maze task is shown in fig. 5. The above panels show the general trend of the % of animals reaching the criterion throughout the testing days in both acquisition (A) and reversal (B) phases. The number of days needed to reach the criterion is displayed in panel C (acquisition phase) and D (reversal phase) for all genotypes. No significant difference is found in the acquisition phase among groups; in the reversal phase, ANOVA revealed a significant genotype effect ($F(2, 27) = 13.34$, $p<0.0001$) and post hoc comparison detected a significant increase in the number of days needed by the OTR$^{-/-}$ mice group to reach the criterion compared to both OTR$^{+/+}$ and OTR$^{+/-}$ group.

5.1.1.7. Autoradiography binding assay

Figure 6 shows the different autoradiographic binding densities of OTR (A) and V1aR (B) in different social/emotional-related brain areas of OTR mutant mice. In figure 6A, statistical analysis performed as two-way ANOVA with brain areas as between-subject factor and genotype as within-subject factor (brain areas x genotype interaction: $F_{(12, 54)} = 17.5$; effect of areas $F_{(8, 73)} = 402.1$, effect of genotype $F_{(2, 78)} = 62.13$) found a significant difference. Bonferroni's post-hoc analysis revealed a significant decrease in OTR density in both heterozygous (in AON, Pyr Ctx, LS, VP, Amy, Hippo and SON) and knock out mice (in all tested areas exluding Hyp) compared to the wild type group. Furthermore, OTR binding was found to be significantly different between OTR $^{+/-}$ and OTR $^{-/-}$ in AON, Pyr Ctx, LS, VP, Amy and Hippo and SON.

As for V1aR (fig. 6B), two-way ANOVA with brain areas as between-subject factor and genotype as within-subject factor (brain areas x genotype interaction: $F_{(12, 54)} = 2.31$; effect of brain areas $F_{(8,73)} = 127.6$, effect of genotype $F_{(2,78)} = 7.96$) revealed significant differences among genoypes. In particular, Bonferroni's post-hoc analysis revealed a significant decrease in V1aR density in both heterozygous (in AON and VP) and knock out mice (in AON, LS, VP and Amy) compared to the wild type group. Furthermore, V1aR binding was found to be significantly different between OTR $^{+/-}$ and OTR $^{-/-}$ in Amy.

5.1.2 Effects of treatments on OTR mice aberrant behaviours

5.1.2.1. Motor abilities

No difference was found in spontaneous motor activity following i.c.v. treatment of OTR $^{+/+}$, OTR $^{+/-}$, OTR $^{-/-}$ mutant mice with OT, AVP, SR and SR+OT 30 minutes for both horizontal and vertical counts (fig. 8A-B).

5.1.2.2. Social behaviour

The effect of the different treatments on sociability and social novelty tests is reported in fig. 9. In the sociability task (A), two-way repeated measure ANOVA with genotype as between subjects factor and treatment as within-subject factor, found a significant difference between groups (genotype x treatment interaction: $F(8, 135) = 3.07$; effect of treatment: $F(4, 145) = 18.7$; effect of genotype: $F(2,147) = 16.65$) Bonferroni's post-hoc analysis revealed a significant effect of treatment with both OT and AVP (0.5 ng/2µl) in reversing both OTR$^{+/-}$ and OTR$^{-/-}$ mice social impairment. Pre-treatment with SR49059 (0.5 ng/2µl) was able to antagonize OT's beneficial effect. After ICV injection of OT or AVP, OTR-/- mice showed a preference for the stranger mouse in the social recognition task (fig. 9B) and statistical analysis found a signicant difference between groups following different treatments: two-way repeated measure ANOVA, with treatment as between-subject factor and genotype as within-subject factor, treatment x genotype interaction: $F(8, 135) = 4.33$; effect of treatment: $F(4, 145) = 43.46$; effect of genotype: $F(2,147) = 16.36$. Bonferroni's post-hoc analysis revealed a significant effect of treatment with both OT and AVP in reversing KO and heterozygous mice social impairment.

Moreover pre-treatment with SR49059 (0.5 ng/2μl) significantly reversed the OT-induced improvement in social skills.

5.1.2.3. Aggressive behaviour

Significant treatment effects were revealed in the number of attacks (fig. 10A) (treatment x genotype interaction: $F(8, 135) = 3.63$; effect of treatment: $F(4, 145) = 5.32$; effect of genotype: $F(2,147) = 22.40$), in the latency to first attack (fig. 10B) ($F8, 135) = 2.98$; effect of treatment: $F(4, 145) = 6.50$; effect of genotype: $F(2,147) = 12.19$), in the number of tail rattlings (fig. 10C) ($F(8, 135) = 4.77$; effect of treatment: $F(4, 145) = 14.89$; effect of genotype: $F(2,147) = 5.14$) and in the latency to first tail rattling (fig. 10D) ($F(8, 135) = 7.57$; effect of treatment: $F(4, 145) = 10.81$; effect of genotype: $F(2,147) = 3.65$) by two way repeated measures ANOVA with treatment as between-subject factor and genotype as within-subject factor in aggressive behaviour. Bonferroni's post hoc analysis shows a significant decrease in the number of attacks and tail rattlings displayed by knock out mutant mice and a significant increase in the latency to the first attack and tail rattling following i.c.v. treatment with both OT and AVP. SR49059 had no per se effect but pre-treatment was able to antagonize OT's effect in all considered parameters.

5.1.2.4. T maze

The general trend of the performance of $OTR^{+/+}$, $OTR^{+/-}$, $OTR^{-/-}$ mutant mice following i.c.v. injection of vehicle, OT, AVP, SR49059 and SR49059 in association with OT is shown in panels A-E of figure 11 expressed as the % of animals reaching the criterion throughout the first 12 days of the reversal phase of the T maze task. Poor performance

of $OTR^{-/-}$ mice following vehicle treatment improved following both OT and AVP treatment. SR alone did not seem to induce any change in animals' behaviour *per se* while when administered in association with oxytocin it was able to reverse OT's beneficial effects. Statistically (two way repeated measures ANOVA with treatment as between-subject factor and genotype as within-subject factor), significance was revealed in panel F, which shows the number of days needed to reach the criterion in the different groups (treatment x genotype interaction: $F(8, 135) = 5.40$; effect of treatment: $F(4, 145) = 2.93$; effect of genotype: $F(2,147) = 5.70$). In particular, Bonferroni's test found a significant decrease in the number of days needed to reach the criterion following OT and AVP treatment in KO mice, and a significant difference between OT treated KO mice and SR+OT treated KO mice, with SR49059, that had no per se effect, antagonizing OT-induced performance improvement. Finally, OT and AVP treatment on wild type animals induced a significant increase in the number of days to reach the criterion, when compared to the vehicle-treated group.

5.2. Zebrafish

5.2.1. Effects of neurohypophysial hormones on zebrafish social behaviour

5.2.1.1. *Shoaling preference test*

Figure 12 shows behavioural performance of WT zebrafish in the shoaling test expressed as difference between time spent in proximity of the nacre shoal compartment and time spent near the WT shoal compartment following i.m. treatment with vehicle, oxytocin, isotocin (A) and vasopressin and vasotocin (B). Vehicle treated animals spend the majority of the time near the WT-associated compartment showing an innate aversion for the *nacre* shoal group. Isotocin (0.1-10 ng/kg) and OT (5-30 ng/kg) treatments increase preference for nacre: in particular a non-linear regression on the log of administered doses was found to be statistically symmetrical parabolas (R^2 (27)= 0.55, $p<0.001$ for isotocin, R^2 (48)= 0.42, $p<0.001$ for OT). Similarly, as shown in panel B, AVT (0.1-30 ng/kg) and AVP (10-40 ng/kg) treatments resulted in significanlty fitted parabolic curves (R^2 (60)= 0.52, $p<0.001$ for AVT, R^2 (35)= 0.28, $p<0.05$ for AVP).

Discussion

6.1. The OTR mutant mouse model

The OTR$^{-/-}$ mutant mouse has been reported to display specific deficits in social behaviour and communication, two core symptoms of ASD, and increased aggression in a previous work (Takayanagi et al., 2005).

In order to confirm and complete the behavioural characterization of both OTR$^{-/-}$ and OTR$^{+/-}$ mice, we first investigated their general health, reflex and sensory abilities, motor function and emotional-like reactivity. Our results show that both mutant genotypes displayed normal motor activity, anxiety-like behaviour, and no correlates of depression. When tested for sociability, OTR$^{+/+}$ mice behaved normally spending longer time to explore the compartment occupied by the stranger mouse than the empty cage. This behavioural response was altered in OTR$^{-/-}$ and OTR$^{+/-}$ mice that did not discriminate between the two compartments. Moreover, when subjected to a social recognition test, both OTR$^{-/-}$ and OTR$^{+/-}$ mice spent the same time with the stranger and the familiar mouse, exhibiting a diminished attitude for social novelty and a decrease in social memory in comparison with the wild type littermates. Other factors that could influence the transition between chambers, such as locomotor activity and olfactory sensitivity were not different between the three genotypes. In the same way, any influence by aberrant emotional behaviour such as increased anxiety can be also ruled out by experiments that confirmed no difference between genotypes.

The original trademark of autism (Kanner, 1943) characterized autistic children by a dramatic lack of interest in others. Current DSM-IV guidelines recognize the variable severity of deficits in social interaction, unusual and inappropriate social approach

behaviours and the changes in these symptoms across development (American Psychiatric Association, 1994).

Mice are a highly social species, displaying social investigation of an unfamiliar conspecific (an individual of the same species), communal nesting, sleeping in group huddles, aggression directed towards intruders, sexual approach and mating behaviour patterns, parental care of the pups and juvenile play (Blanchard et al., 2003; Grant and MacIntosh, 1963; Ricceri at al., 2007). Thus, a critical component in a mouse model of autism is a quantitative measure of appropriate social interaction: the simple social approach task we used is able to let us measure quantitatively the tendency of the subject mouse to approach another mouse and engage in social investigation.

OT's involvement in regulation social behaviour is well-known. Male OT KO mice fail to develop social memory on both the habituation–dishabituation test (Lee et al., 2008) and the social recognition test (Ferguson et al., 2001). OTR knock out mutant line had already been demonstrated to have impaired social recognition (Takayanagi et al., 2005). However it is the first time that such impairment is demonstrated in the heterozygous line of OTR mutants, indicating that even a minor loss in the presence of the OT receptor can lead to a strong deficit in their behavioural phenotype.

In male mice, social aggressive behaviour is characterized by overt attacks with biting on the back or neck of the intruder and by the presence of tail rattlings, i.e. very quick tail movements that mice display when a threat is perceived (Krsiak, 1975). Such behaviour was only observed in $OTR^{-/-}$ mice. Generally, aggression in rodents is believed to be mainly under the control of AVP (Caldwell et al., 2008). The role of OT in controlling aggressive behaviour in males is ambiguous. In male squirrel monkeys in a stable

dominant-subordinate relationship, OT increases sexual and aggressive behaviours in the dominant during interaction with a female and this is blocked following administration of OT and the OT antagonist OVTA (Winslow and Insel, 1991). Behavioural studies on transgenic mice have not yet given any steady statement about this aspect. $OT^{-/-}$ mice are mildly less aggressive than their WT counterparts or heterozygous controls (DeVries et al., 1997). However, in a different line of $OT^{-/-}$ mice, another group has reported increased aggressive behaviour in the resident-intruder paradigm (Winslow et al., 2000). Accordingly to our results, higher levels of aggression have already been reported in OTR knockouts generated from non-obligates, which suggests the idea that a lack of prenatal activation of the OT system could result in increased aggression later on in adult life (Takayanagi et al., 2005).

Autistic individuals often maintain rigid habits, similar to individuals with obsessive–compulsive disorders, and frequently show a strong insistence on sameness and difficulty in changing a previously established routine (Hollander et al., 2003). We tried to replicate this complex pattern of behaviour using the T maze task in which mice were trained to establish a habit, and then asked to make a change in the established routine. T maze acquisition learning involves finding a food reward in one of two available locations at opposite ends of a T-shaped apparatus. Reversal learning requires the mouse to extinguish the location of the reinforce in one arm, and learn a new location of the reinforce in the opposite arm. Reversal learning requires different skills than the original acquisition learning. The latter serves as a control for general procedural and spatial-cognitive abilities. Ability of the subjects to switch quickly to the new location is quantified by the number of trials required to consistently choose the opposite T maze arm to obtain

the food reward. Thus, ability to change, resistance to change and responses to the change in routine are measured. We compared the cognitive flexibility of $OTR^{-/-}$, $OTR^{+/-}$ and $OTR^{+/+}$ mice. Although all three genotypes learned to correctly locate the baited arm at comparable rates during acquisition, indicating the absence of alteration in spatial memory components, switching the reward position to the opposite arm in the reversal learning phase induced a significantly slower learning rate in $OTR^{-/-}$ mice suggesting the presence of an impaired cognitive flexibility, strong resistance to change and stereotyped behaviour. Our results agree with the recent findings by Blundell et al. (2010) and Silverman et al. (2010) who have shown respectively that NL1-deficient mice and BTBR mice, both promising models for ASD, exhibit a dramatic increase in repetitive, stereotyped grooming behaviour. Furthermore, in a recently published paper (Moy et al., 2009) NRCAM (Neuronal Cell Adhesion Molecule) null mice are described as an interesting ASD model as they display both impaired sociability and altered cognitive abilities in the T maze reversal learning phase.

Oxytocin has been shown to promote the rapid formation of inhibitory synapses in hypothalamic GABAergic neurons, thus supporting the view that this neuropeptide can contribute to the functional remodelling of synapses (Theodosis et al., 2006). In a similar way, the neuronal cell adhesion molecule (NRCAM) has been demonstrated to have an important role in axonal guidance and the organization of neural circuitry during brain development. Autism has in fact been suggested to reflect an alteration of synaptic plasticity and/or neuronal connectivity occurring at the time when higher-order neuronal networks are established (Zoghbi 2003; Geschwind and Levitt, 2007). Even if mild mental retardation is a common characteristic in autistic patients, autism is not characterized by

a global impairment of cognition, whose functions are often spared, or even enhanced, as in the Savant syndrome, where ‘islands of geniality’ stand out, indicating that only certain brain circuits are impaired (Treffert, 2009). Thus, a crucial issue will be to understand what are the neural pathways selectively ‘disconnected’ in ASD and what common features make them so vulnerable.

Neurochemical mechanisms underlying the cognitive flexibility impairment of $OTR^{-/-}$ mice are starting to be elucidated. In fact, it has been shown in very recent studies (Sala et al., 2010, *accepted*) that primary cultures of hippocampal neurons from $OTR^{-/-}$ display significantly less GABAergic synapses. As just mentioned, it is well known that OT can play an important role in modulating synaptic plasticity in several brain areas. OT triggers a switch from excitatory to inhibitory GABA activity in the hippocampus immediately before birth (Tyzio et al., 2004) to promote the rapid formation of functional GABA synapses in the adult supraoptic nucleus (Theodosis et al., 2006) and to remodel neuronal-glial interactions in the hypothalamus (Oliet et al., 2010). As it was found that hippocampal neurons obtained from $OTR^{-/-}$ mice express a lower percentage of GABAergic synapses, it is possible that an imbalance in glutamate/GABA transmission could be responsible for a hippocampal, working memory-based, cognitive flexibility deficit. Interestingly, Morellini et al. (2010) have recently shown that more efficient reversal learning correlates with enhanced GABAergic innervation in the dentate gyrus of hippocampus. This report fits nicely with our results showing defective reversal learning and decreased hippocampal GABAergic synapses in $OTR^{-/-}$. In rodents, a circuitry between the hippocampus, the prefrontal cortex and the striatum is involved in cognitive flexibility (Floresco et al., 2009) and we can also hypothesize that similar

GABA/glutamate imbalance in prefrontal and/or striatal areas contributes to the specific impairment in reversal learning observed in these animals.

To assess the effects of OT and AVP administration on ASD-like symptoms, OT and AVP were administered i.c.v. to all genotypes. Our results point out how both peptides rescued a normal level of social exploration and recognition and that the pretreatment with the selective V1aR antagonist SR49059 significantly reversed the OT-induced improvement. Horizontal locomotor activity, as measured in the spontaneous motor activity test, was not different among treatments ruling out any of its possible influence on more complex behavioural responses. OT and AVP also reversed the aggressive behaviour exhibited by $OTR^{-/-}$ mice, whereas pre-treatment with SR49059 significantly blocked such effect. Finally, treatment with both peptides reduced reversal learning impairment in $OTR^{-/-}$ mice in comparison with the vehicle-treated counterparts, rescuing the altered cognitive flexibility of these mice.

In the brain, OT and AVP bind to and activate three related receptors, all belonging to the GPCR superfamily, OTR, V1aR and V1bR. Due to the similarities between OT and AVP peptides, that differ for only two out of eight aminoacids, and the high homology in their receptor sequences, this peptide/receptor system is not sufficiently selective. In particular, OT has been shown to also bind with very good affinity the V1aR subtype, acting as a partial agonist (Chini et al., 1996). Thus, since the rescue effects of OT and AVP could be blocked by a V1aR antagonist, SR49059, we suggest that OT acted on V1aR in our KO animals. The distribution of OTRs and V1aRs seems to occur in different, mainly not overlapping regions (Tribollet, 1992; Johnson et al., 1993), indicating that the two peptides cooperate in the regulation of complex behaviours by acting at different

sites. In order to more deeply investigate this aspect, we checked whether in mutant mice an altered expression and/or distribution of V1aR could account for the phenotypical rescue that we showed. OTR$^{-/-}$ animals displayed an almost undetectable OTR binding in all tested areas. Furthermore, a slight compensatory up-regulation of V1aR in the hippocampus and a significant down-regulation of the V1aR expression in AON, Amy, and VP and LS were found. As for heterozygous mutants their phenotype appears as expected halfway between the wild type and knock out counterparts for OTR binding sites and V1aRs binding has been subjected to a slight reduction in ventral pallidum and anterior olfactory nucleus only. Affected areas exert a key role in integrating the processing of olfactory information that is crucial to regulate social recognition in rodents (Choleris et al., 2009). Moreover, in these regions, the OTR/V1aR system seems to be both necessary and sufficient to modulate social recognition (Bielsky et al, 2005; Ferguson et al., 2001; Landgraf et al., 1995).

Our data reveal that infusion of OT or AVP to OTR mutant mice completely normalizes their social deficits. OT was already reported to rescue social deficits in OT$^{-/-}$ and CD38$^{-/-}$ animals in which defective OT production or secretion, respectively, are responsible for social impairment, but, in these models, AVP was found to be ineffective (Ferguson et al., 2000; Jin et al., 2007). This discrepancy suggests that subtle differences exist between OT$^{-/-}$ and OTR$^{-/-}$ mice, one possibly residing in compensatory up- and/or down-regulation of V1Rs. OT and AVP cooperate in regulating social behaviour, but it is still unknown whether they act independently from each other on neuronal circuits that control distinct aspect of social recognition, or they exert their role at different steps along common pathways. Our current data showing that OT and AVP acting on V1aR

effectively rescue the social deficits of $OTR^{-/-}$ mice suggest that the two peptides act cooperatively on distinct sites along the same pathways (Young et al., 2005), as it has been shown within the central nucleus of the amygdala, where the stimulation of neuronal OTRs in the lateral part inhibits, through a GABA-dependent mechanism, the activity of ventral neurons under direct excitatory control by V1aR (Huber et al., 2005).

OT and AVP administration was also very effective in normalizing the increased aggressive behaviour observed in $OTR^{-/-}$ mice. This effect was unexpected because AVP is generally considered a promoter of social aggression (de Wied et al., 1993). In fact, when injected in the anterior hypothalamus, AVP facilitates aggression (Stribley and Carter, 1999), whereas a V1aR antagonist injected in the ventral and lateral hypothalamus results in reduced aggression (Winslow 1993). However, the role of AVP and V1aR in aggression is far than clear as a $V1aR^{-/-}$ mice line displays normal aggression (Wersinger et al., 2997), and, in a rodent line genetically selected for high aggressive behaviour, a reduced AVPergic fibre network and a reduced neuronal activation were observed in the lateral septum (Veenema et al., 2010), a region very enriched in V1aR (Johnson et al., 1993) that is strongly activated during fighting (Siegel et al., 2007). As OTRs have been recently found on GABA-positive cells in the lateral septum and on serotoninergic neurons of the raphe nuclei (Yoshida et al., 2009), an interplay between OT/AVP and serotonin can be hypothesized to differentially regulate male versus male aggression in different brain regions.

Finally, OT and AVP infusion in three-month old $OTR^{-/-}$ mice induce a complete rescue of the cognitive flexibility, implying that anatomical substrates and neurochemical modulators are entirely intact hence it can be restored by pharmacological treatment.

Interestingly, we observed a reduction in the performance of the $OTR^{+/+}$ animals after OT or AVP infusions, indicating that the hyper-stimulation of 'normal' circuitry have a detrimental effect. These results suggest that the selection of patients will play a key role in evaluating the result of the treatment with these or similar neuropeptides, the use of which should be restricted to those individuals who could really benefit from it. Similarly to our results, in a mouse model of syndromic autism, the $tsc2^{+/-}$ mouse model of sclerosis tuberosis, learning deficits of adult animals could be rescued by pharmacological treatment with rapamycin (Ehninger et al., 2008). However, $tsc2^{+/-}$ and $OTR^{-/-}$ mouse models share the potential for pharmacological rescue of cognitive functions well after birth. For how long in life the rescue is possible and what may happen with chronic treatment are the next issues to address.

Different groups of researchers are currently working trying to investigate the complete behavioural phenotype of OTR mutant mice. Very recently (2010) Schorscher-Petcu et al., have characterized the phenotype of both OTR and V1aR null mutant mice in a battery of pain assays. OTR knock-out mice displayed a pain phenotype identical to their wild-type littermates, accordingly to our results. Moreover, systemic administration of OT dose-dependently produced analgesia in both wild-type and OTR knock-out mice in three different assays, the radiant-heat paw withdrawal test, the von Frey test of mechanical sensitivity, and the formalin test of inflammatory nociception. Interestingly, OT-induced analgesia was completely absent in V1aR knock-out mice and could be fully prevented by pretreatment with a V1AR but not an OTR antagonist suggesting the interaction between these two strictly connected systems in the modulation of pain sensitivity, too.

While no animal model can be expected to replicate the full complexity of the human behavioural autistic phenotype, a model that selectively exhibit features of all core symptoms of autism may be instrumental to understand the neurochemical/neuroanatomical basis for the co-occurrence of these symptoms as a syndrome. Moreover, the presence of a social deficit in OTR heterozygous mutant mice could lead to the use of this particular model as a tool in future drug discovery studies meant to find new OTR-directed molecules that could rescue this aberrant phenotype.

6.2 The zebrafish model

Our results suggest that the OT/AVP system seems to be also involved in social-related behaviours in non-mammalian species. In the shoaling preference task, WT zebrafish increased the time spent in proximity of the usually neglected *Nacre* mutant following treatment with OT and AVP and with their respective non-mammalian homologues Isotocin (ISO) and Vasotocin (AVT). At the tested dosed we did not observe any effect of the peptides in swimming activity (data not shown) so any non-specific effect on arousal or motor functioning can be ruled out. Our data are the first to demonstrate a specific pro-social effect of these peptides on the zebrafish model. As expected ISO and AVT acted as far more specific peptides than OT and AVP, reaching their peak effect respectively at a 20 and 30 times lower concentration than their mammalian homologues. Interestingly, all four peptides showed a parabolic trend in their dose-effect curves, with lowest and highest doses exerting no effect at all: the reason for that could be that the lowest drug concentrations might not be sufficient to engage an adequate number of

receptors in order to induce a pro-social response; highest doses, instead, might be responsible for the presence of non-specific side effects such as anxiety-related responses. Of course this hypotheses must be validated by specific future experiments on emotional responses induced by these peptides.

It has been shown that as with oxytocinergic neurons of mammals, isotocinergic neurons project widely in the brain of trout (Saito et al., 2004) and zebrafish (Khan et al, 2010). Moreover, isotocin and vasotocin influence social and reproductive behaviours in fish (Goodson and Bass, 2000, 2001) much like the equivalent hormones in mammals. Previous studies involving fish behaviour and OT/AVP/ISO/AVT systems have revealed their crucial role in influencing social-related behavioural responses. Thompson and Walton in 2004 measured the effects of centrally infused peptides on social approach in the social teleost goldfish (*Carassus auratus*). Vasotocin inhibited approach responses toward the visual stimuli of conspecifics and a V1 receptor antagonist stimulated such responses in animals that were not highly social in baseline conditions, as did isotocin. These experiments indicate that AVT and ISO induce opposite effects on social approach responses in male goldfish. Discrepancies may be found as these studies are far from being completely exhaustive: still no isotocin or vasotocin receptors have been located in zebrafish so it is not clear which is the mechanism behind the effect of these peptides. Very recently Khan and colleagues (2010) have revealed that the novel and brain specific cyclic nucleotide gated channel alpha subunit, CNGA5, is enriched in synaptic terminals of isotocinergic neurons in zebrafish brain and in particular in the neurohypophysis. Cyclic nucleotide gate channels have been reported to modulate transmitter release at synaptic

terminals and CNGA5 channels seem thus to be important presynaptic modulators of neuroendocrine systems that influence reproductive and social behavior in zebrafish.

Of course our study does not aim at elucidating the mechanisms that regulate zebrafish social behaviour. Here, our aim was instead to show how this behaviour can easily be modulated through pharmacological treatments and how this small teleost can be used as a model to investigate potential pro-social effects of novel drugs especially as a large-scale screening tool prior to engage expensive and time-consuming researches using the classical rodent models.

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Figures

General Health and Characteristics

	$OTR^{+/+}$	$OTR^{+/-}$	$OTR^{-/-}$
General Health			
Weight (g)	31.0 0.5	29.2 0.4	31.1 0.4
Fur condition (3-point scale)	3.0 0.0	3.0 0.0	3.0 0.0
Piloerection (%)	0	0	0
Body Tone (3-point scale)	2.0 0.0	2.0 0.0	2.0 0.0
Skin colour (%)	100	100	100
Empty cage behaviour			
Nest building (5-point scale)	3.8 0.4	4.0 0.3	4.1 0.3
Reflexes			
Righting reflexes (4-point scale)	0.05 0.05	0.02 0.02	0.04 0.04
Corneal (8-point scale)	3.9 0.2	3.8 0.2	4.0 0.0
Pinna (8-point scale)	3.9 0.1	3.8 0.2	3.9 0.04
Tail pinch (8-point scale)	3.9 0.1	3.9 0.04	3.98 0.2

Table 1: General phenotypic characteristics, empty cage behaviour and reflexes of $OTR^{+/+}$, $OTR^{+/-}$ and $OTR^{-/-}$ mutant mice

Sensory Abilities

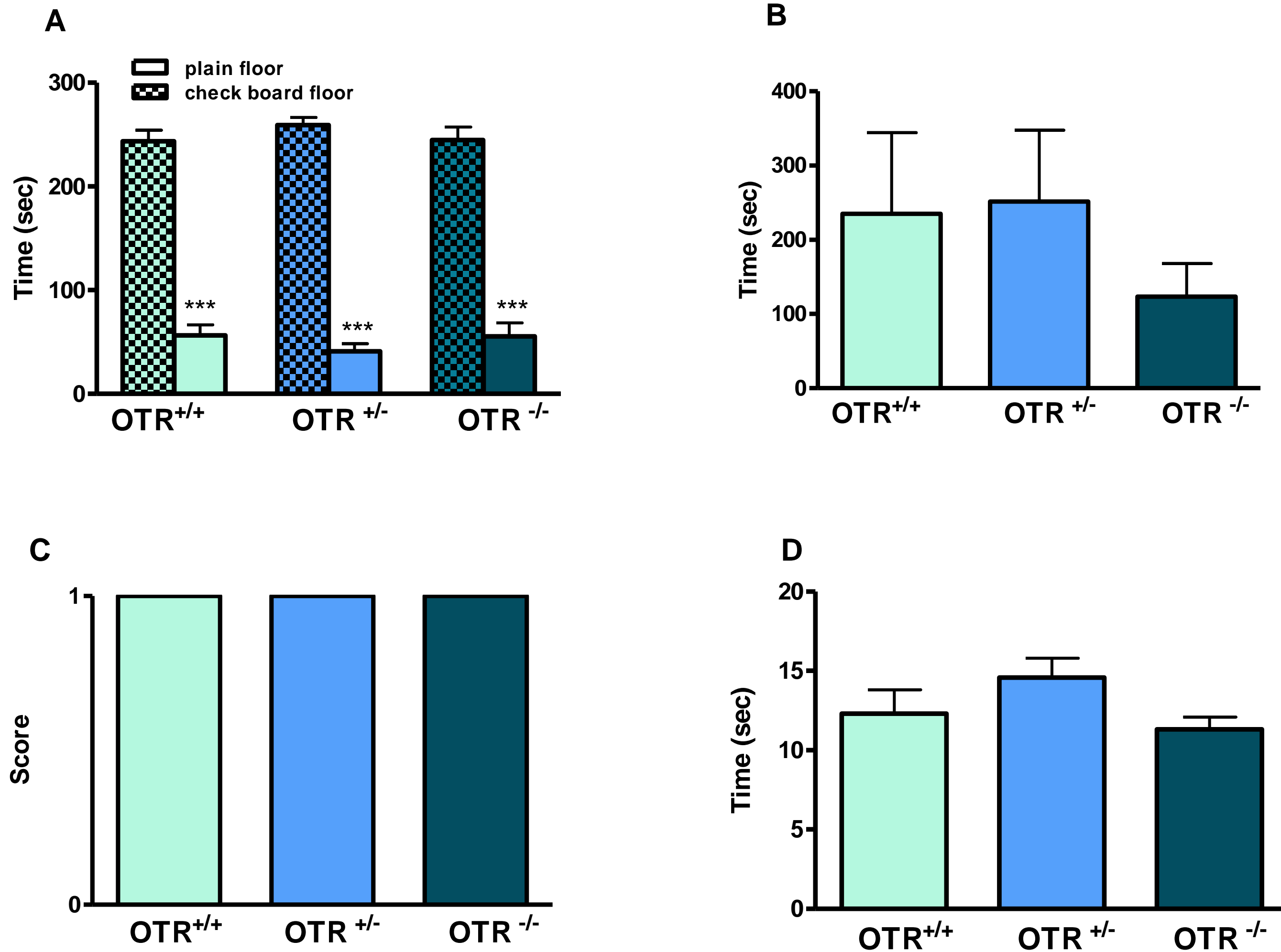


Fig 1: sensory abilities of OTR wild type, heterozygous and knock out mice as evaluated in the visual cliff test (A), buried pellet test (B), preyer reflex test (C) and hot plate test (D) (mean SEM). *** $p<0.001$ vs time spent on the check boarded side (2way ANOVA, Bonferroni's test).

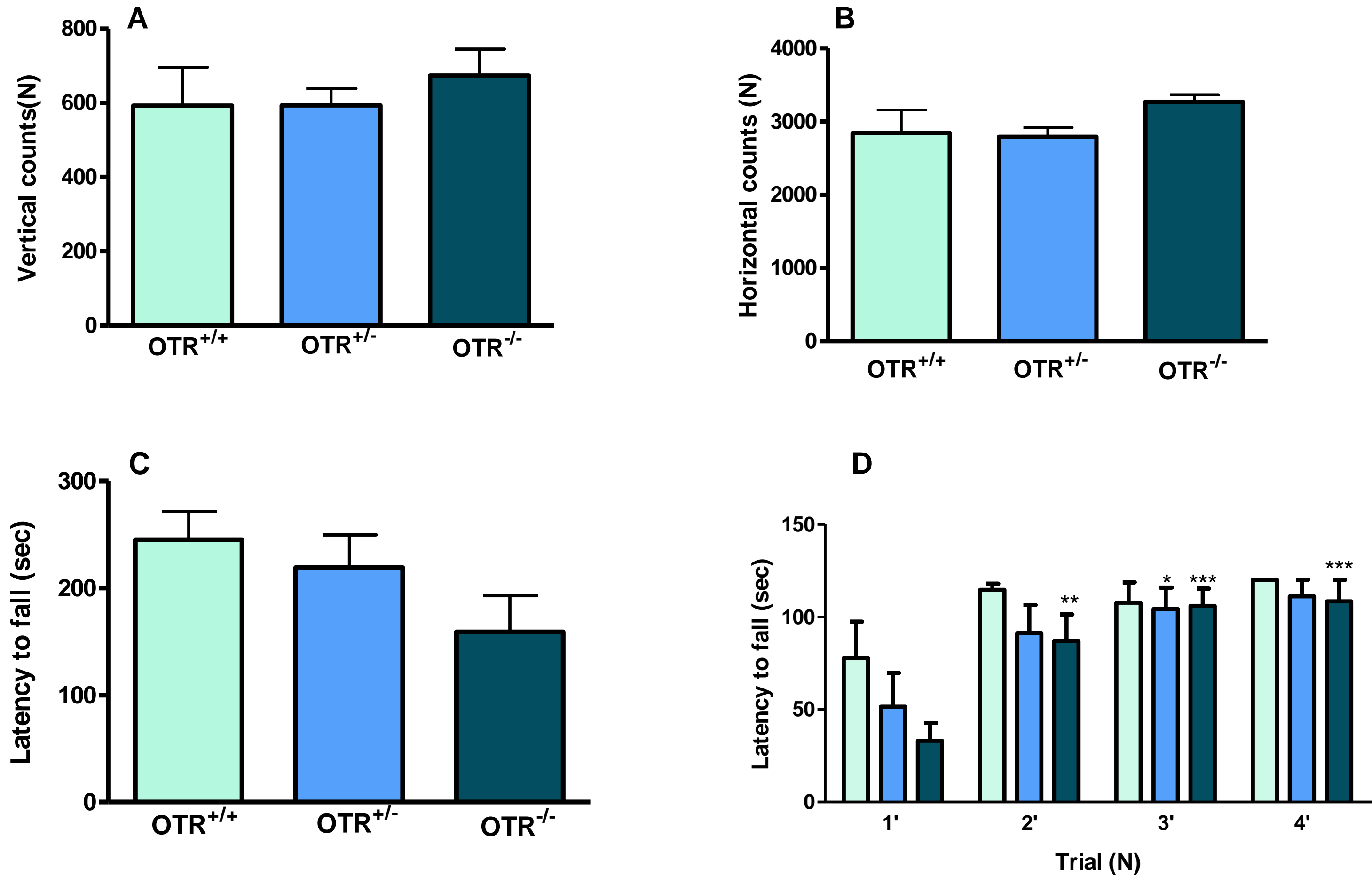


Fig 2: motor abilities (mean SEM) of OTR$^{+/+}$, OTR$^{+/-}$, OTR$^{-/-}$ mice as evaluated in an activity cage measuring horizontal (A) and vertical (B) counts, in the rotarod test (C) and hanging wire test (D). *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs 1st trial (2way ANOVA, Bonferroni's test)

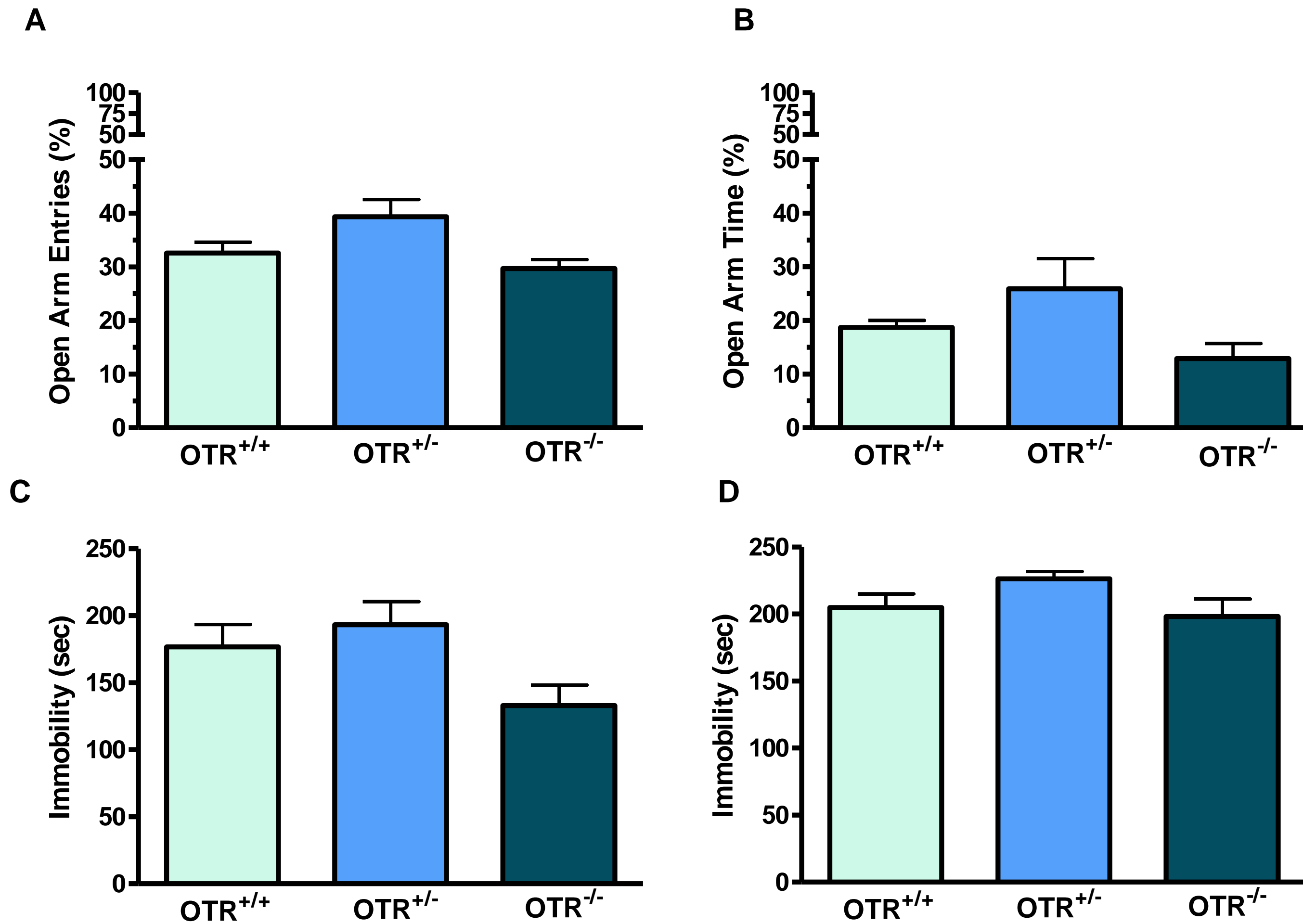


Fig 3: genotype effect (mean SEM) in $OTR^{+/+}$, $OTR^{+/-}$, $OTR^{-/-}$ mice on emotional behaviour evaluated in the elevated plus maze as open arm entries (A) and time (B), in the forced swim test (C) and tail suspension test (D) as time spent immobile.

Social Behaviour

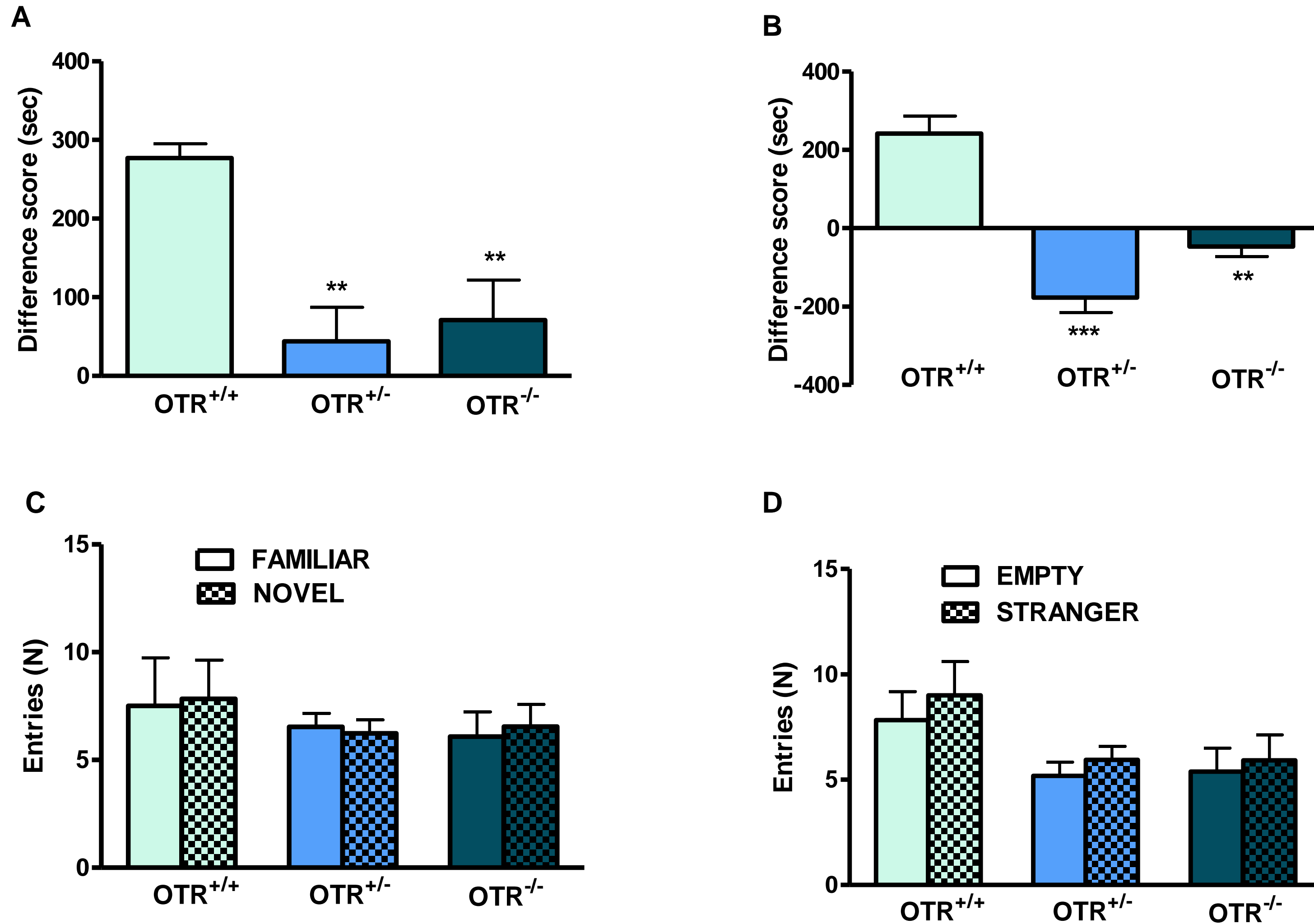


Fig 4: social behaviour of OTR$^{+/+}$, OTR$^{+/-}$, OTR$^{-/-}$ mice displayed in the sociability (A-C) and social novelty test (B-D) expressed as mean SEM of the difference score and number of entries made in the different compartments. ** $p<0.01$, *** $p<0.001$ vs OTR $^{+/+}$ (ANOVA, Tukey's test).

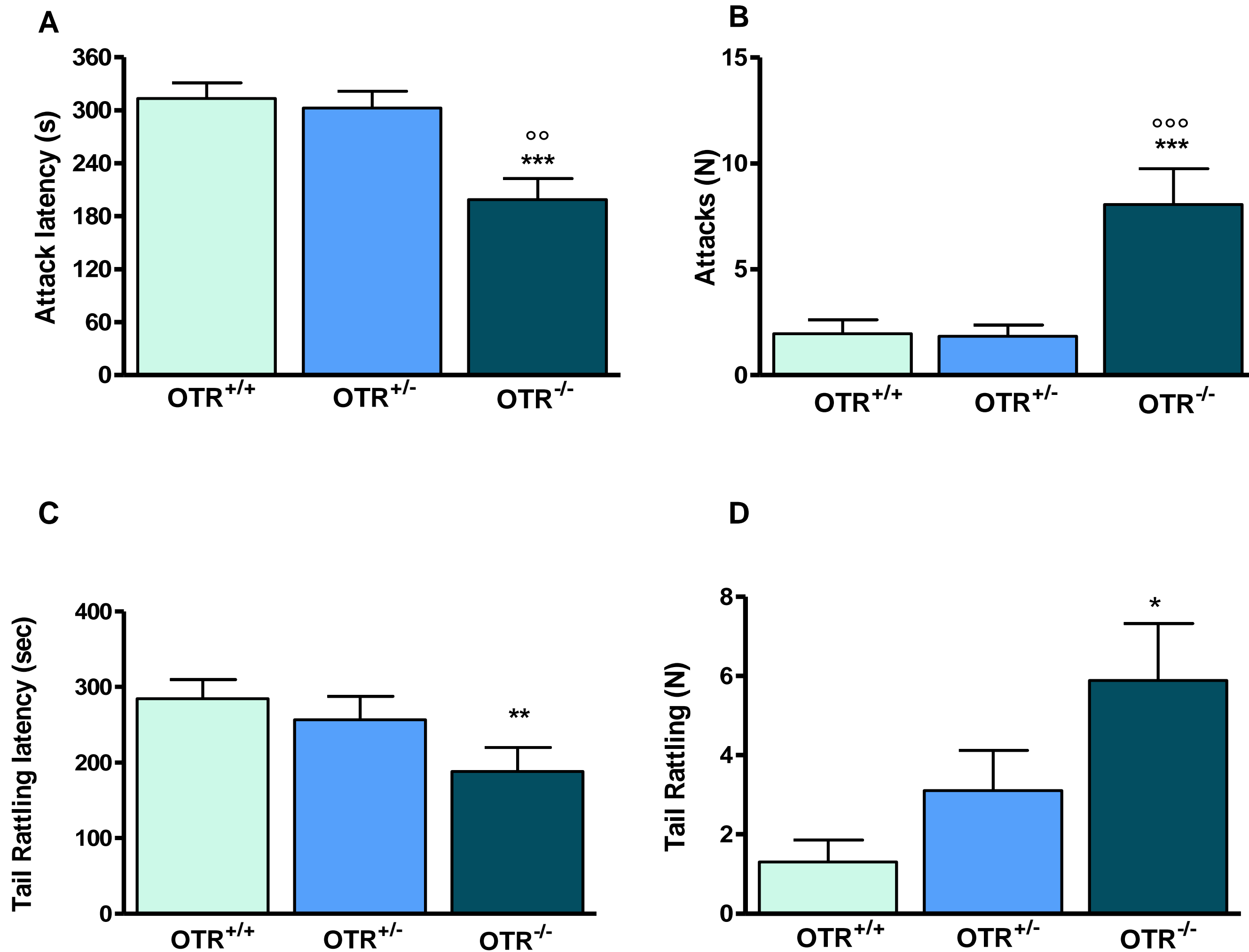


Fig 5: aggressive behaviour of $OTR^{+/+}$, $OTR^{+/-}$, $OTR^{-/-}$ mice in the neutral cage test. Mean SEM of the latency to first attack and tail rattling is shown in panels A and C, the number (mean ± SEM) of attacks and tail rattling in panels B and D.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs $OTR^{+/+}$, °° $p<0.01$, °°° $p<0.001$ vs $OTR^{+/-}$ (ANOVA, Tukey's test).

T-maze

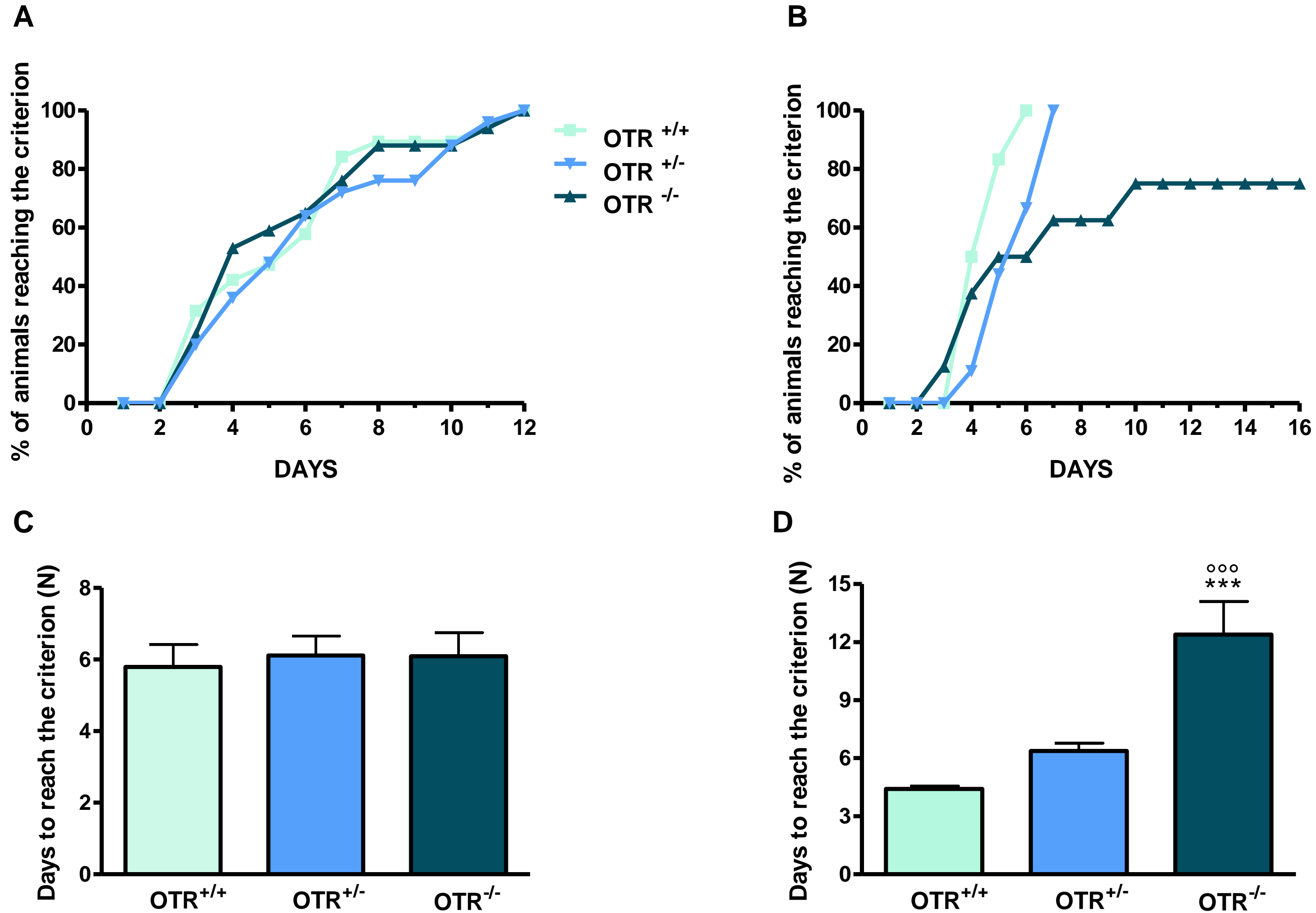


Fig 6: OTR$^{+/+}$, OTR$^{+/-}$, OTR$^{-/-}$ mice performance in both the acquisition (A-C) and reversal phase (B-D) of the T maze task. Data are expressed as % of animals reaching the criterion (A-B) and number of days (mean SEM) needed to reach the criterion (C-D). *** $p<0.001$ vs OTR $^{+/+}$, °°° $p<0.001$ vs OTR $^{+/-}$ (ANOVA, Tukey's test).

OT and V1a autoradiographic receptor binding assay

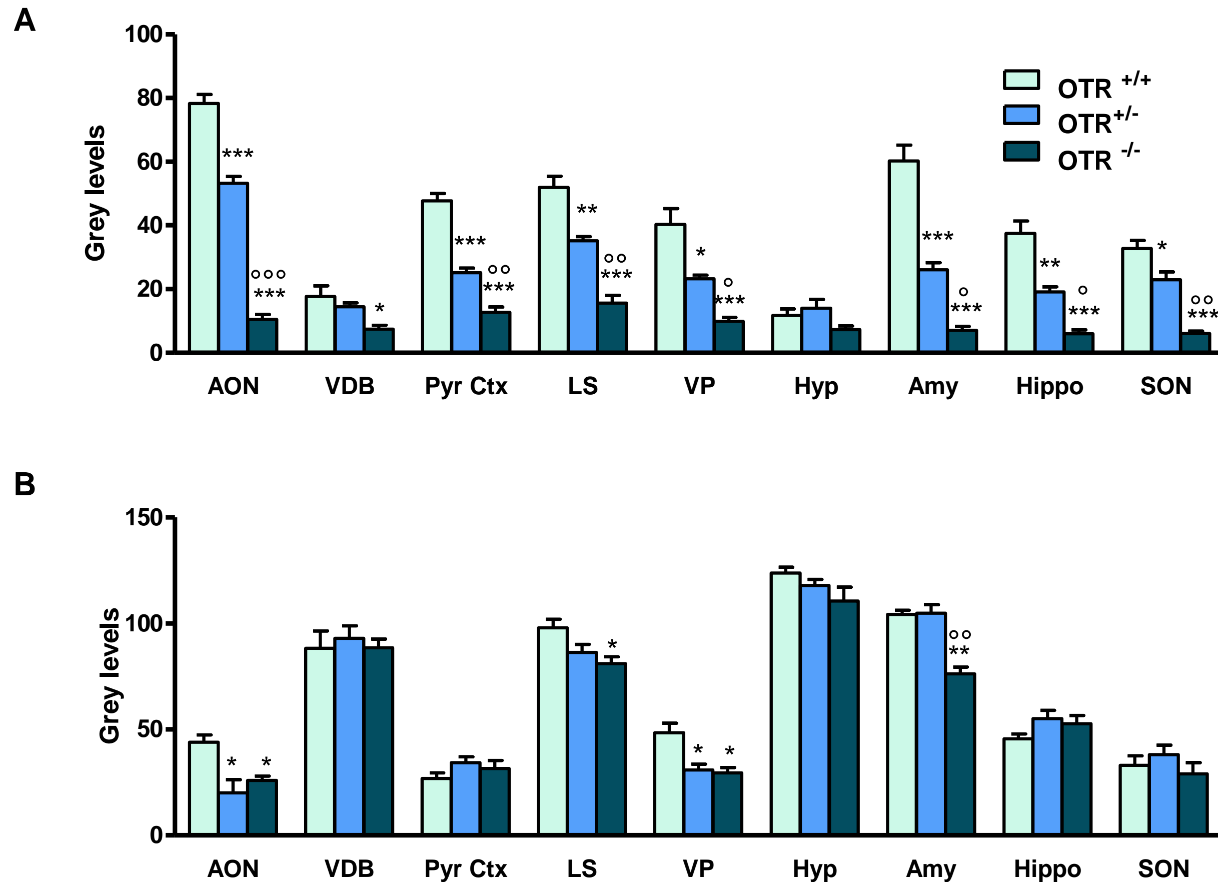
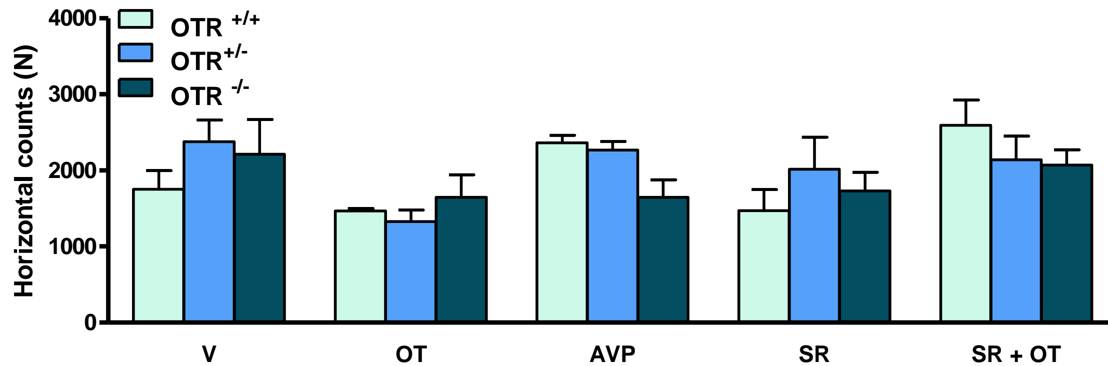


Fig 7: brain expression of OTR (A) and V1aR (B) as results by the quantification of the autoradiographic receptor binding in 9 different brain areas of OTR $^{+/+}$, OTR $^{+/-}$, OTR $^{-/-}$ mice. *P<0.05, **P<0.01, ***P<0.001 vs OTR $^{+/+}$, ° p<0.05, °° p<0.01, °°° p<0.001 vs OTR $^{+/-}$ (2way ANOVA, Bonferroni's test)

AON, Anterior Olfactory Nucleus, VDB, vertical limb of the diagonal band, PyrCtx, Pyriform Cortex, LS, Lateral Septum, VP, ventral Pallidum, Hyp, Hypothalamus, Amy, Amygdala, Hippo, Hippocampus, SON, Supraoptic Nucleus.

Motor Abilities: treatments

A



B

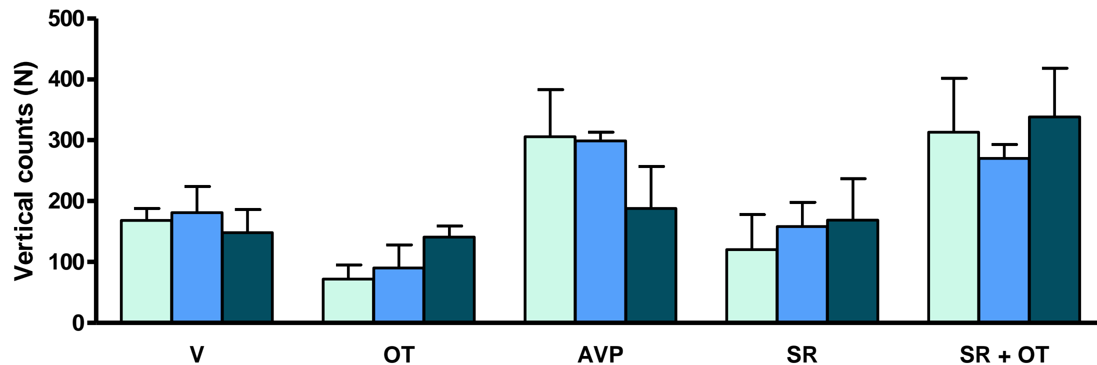


Fig 8: motor abilities of $OTR^{+/+}$, $OTR^{+/-}$, $OTR^{-/-}$ mice expressed as horizontal (A) and vertical (B) counts following i.c.v. administration of vehicle (V), oxytocin (OT), vasopressin (AVP), SR49059 alone (SR) or in association with oxytocin (SR + OT).

Social behaviour: treatments

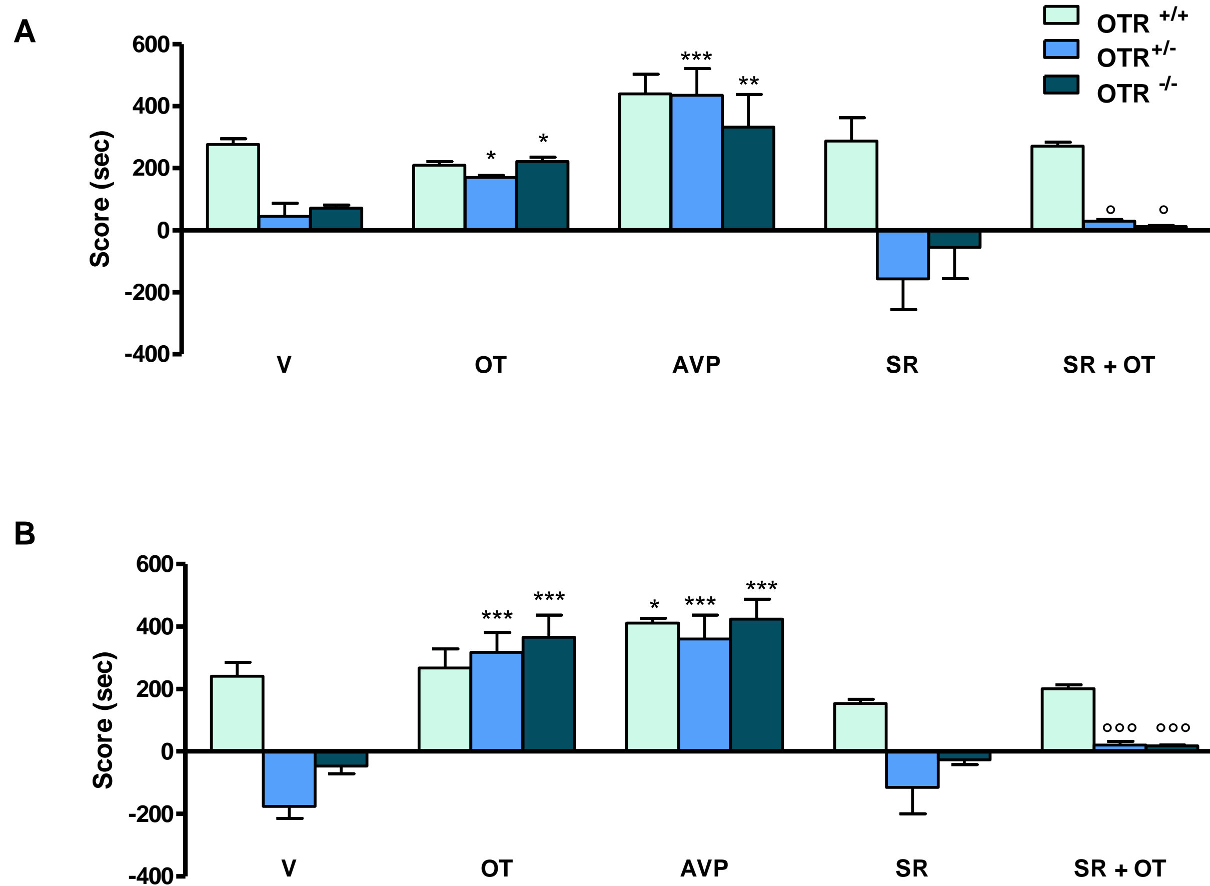


Fig 9: performance by OTR$^{+/+}$, OTR$^{+/-}$, OTR$^{-/-}$ mice on the sociability (A) and social novelty (B) tests following i.c.v. administration of vehicle (V), oxytocin (OT), vasopressin (AVP), SR49059 alone (SR) or in association with oxytocin (SR + OT). * $p< 0.05$, ** $p< 0.01$, *** $p<0.001$ vs V; ° $p<0.05$, °°° $p<0.001$ vs OT (2way ANOVA followed by Bonferroni's test).

Aggressive behaviour: treatments

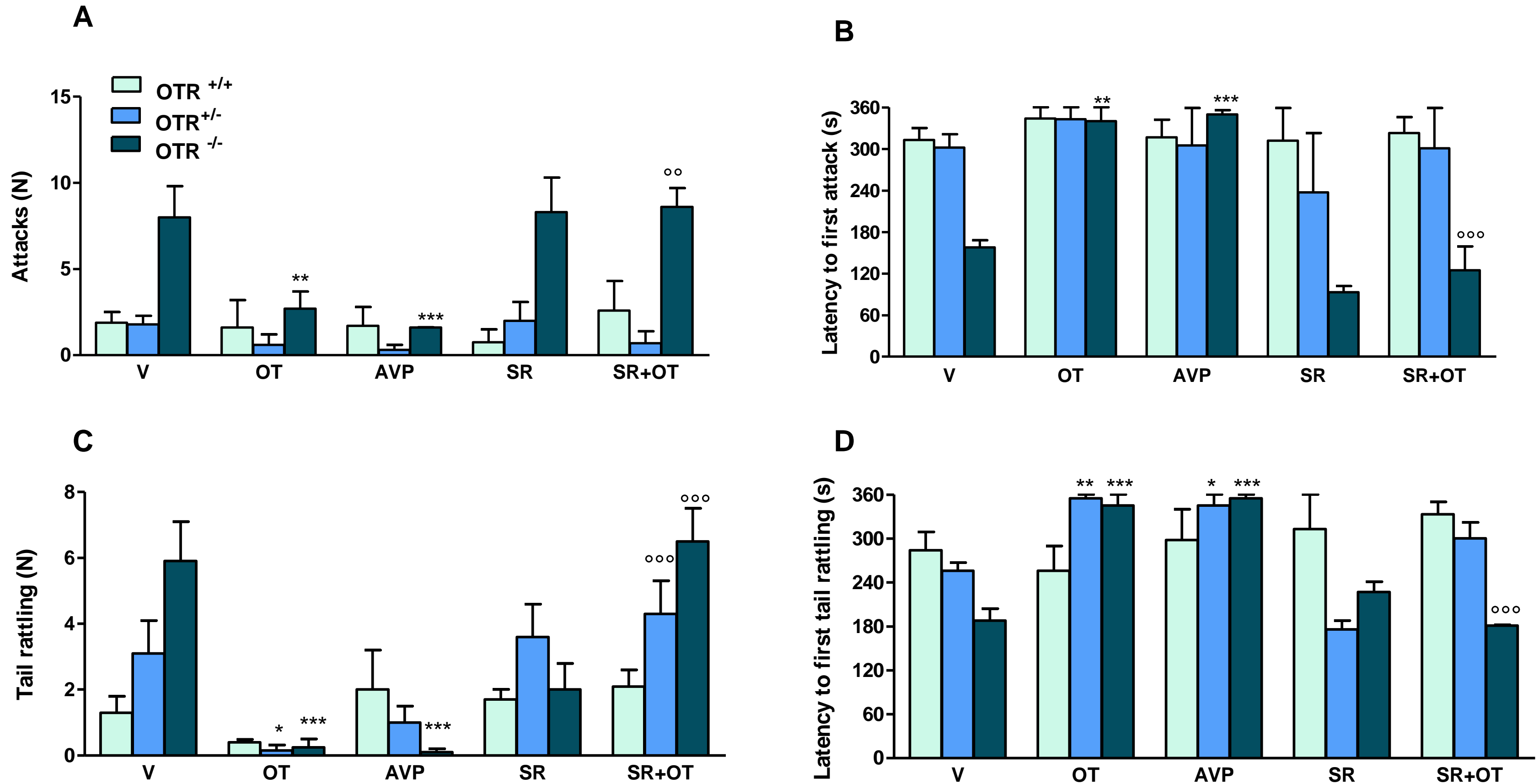


Fig 10: aggressive behaviour evaluated in the neutral cage test as number of attack and tail rattlings (A-C) and latency to first attack (B) and tail rattling (D) following i.c.v. administration of vehicle (V), oxytocin (OT), vasopressin (AVP), SR49059 alone (SR) or in association with oxytocin (SR + OT). * $p< 0.05$, ** $p< 0.01$, *** $p<0.001$ vs V; °° $p<0.01$, °°° $p<0.001$ vs OT (2way ANOVA followed by Bonferroni's test).

T-maze: treatments

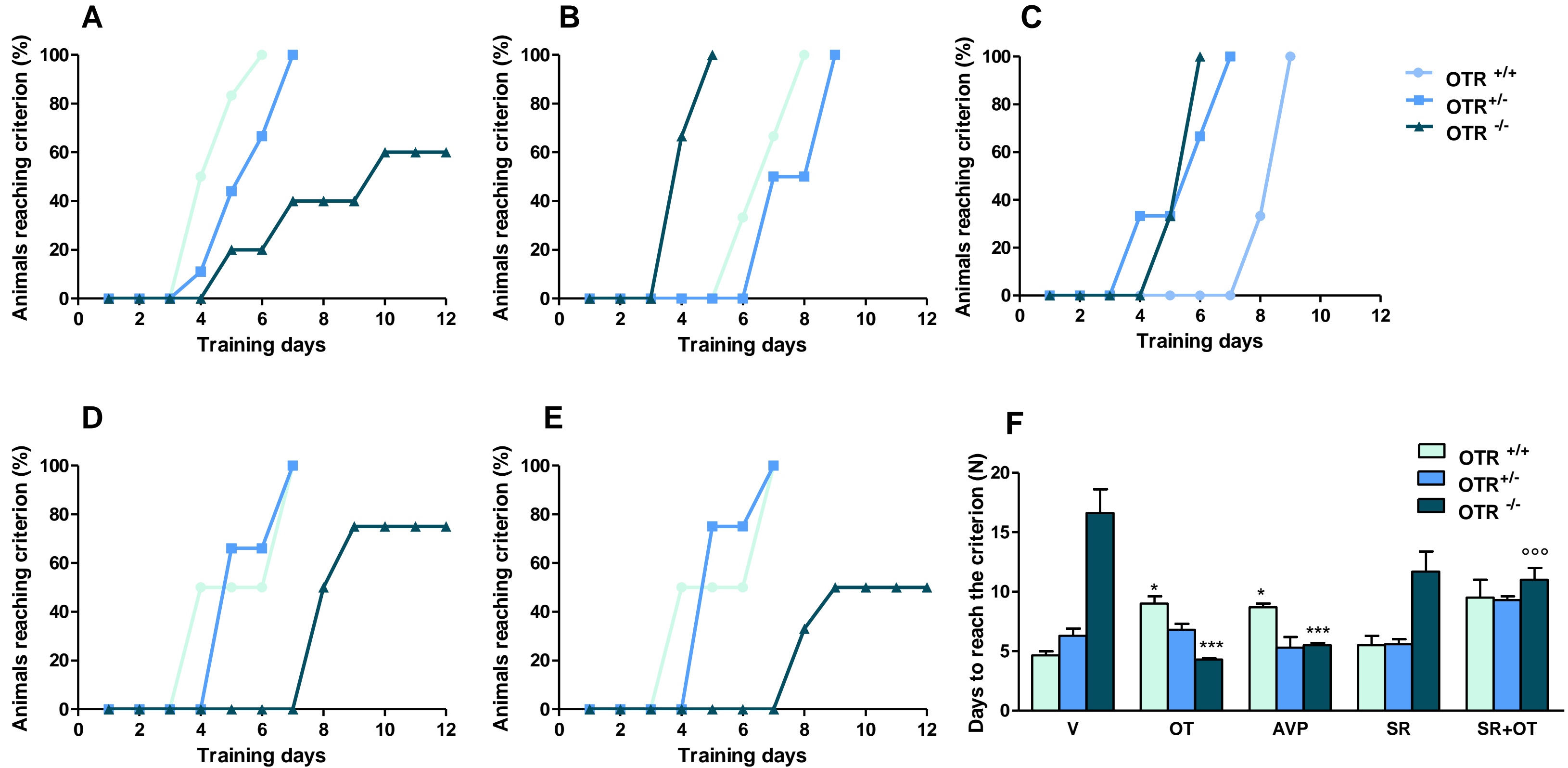


Fig 11: OTR+/+, OTR+/-, OTR-/- mice performance in the reversal phase of the T maze task following i.c.v. administration of vehicle (V) (A), oxytocin (OT) (B), vasopressin (AVP) (C), SR49059 alone (SR) (D) or in association with oxytocin (SR + OT) (E) expressed as % of animals reaching the criterion. The number of days taken to reach the criterion is shown in panel F. * $p<0.05$, *** $p<0.001$ vs V , $p<0.001$ vs OT (2 way ANOVA, Bonferroni's test).

Zebrafish: social behaviour

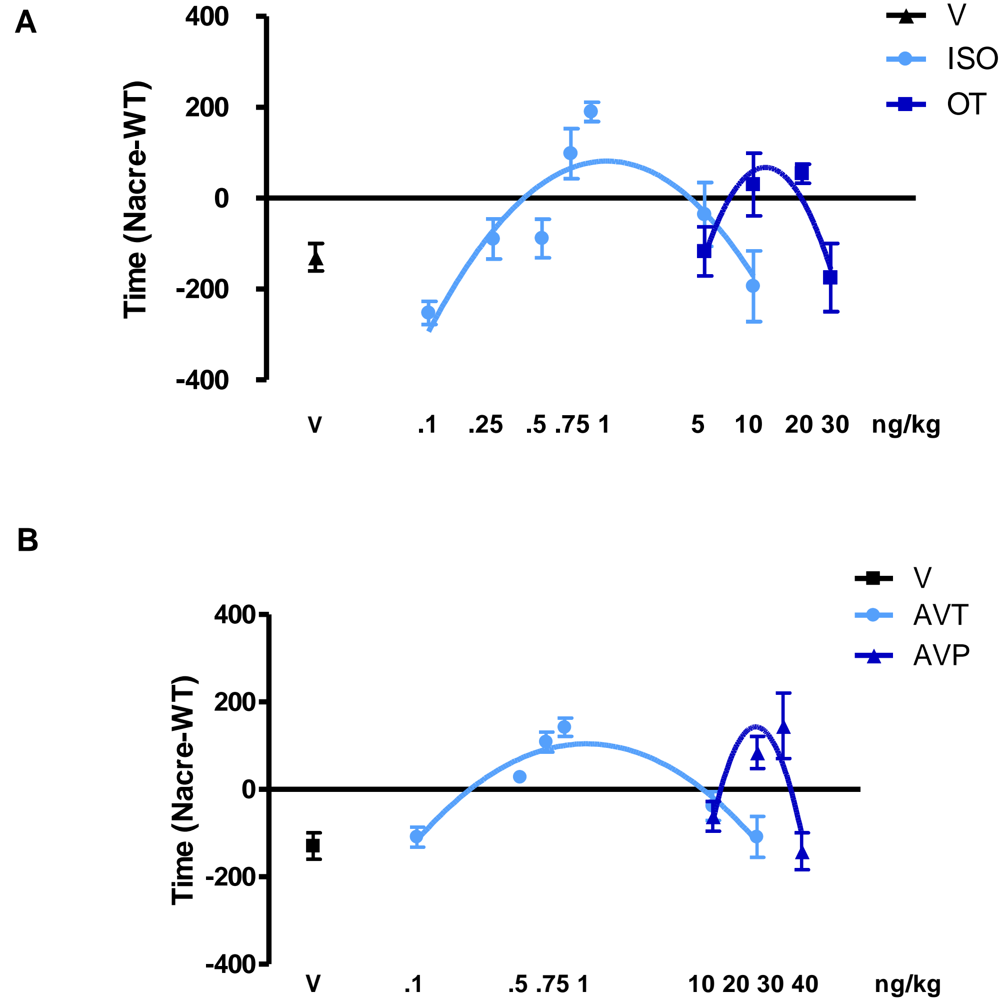


Fig 12: social behaviour of WT zebrafish 10 minutes following i.m. administration of vehicle (V) and different doses of oxytocin (OT), isotocin (ISO) (A), vasopressin (AVP), vasotocin (AVT) (B).