Different polymorphisms of the oxytocinergic system explain the prevalence of autistic traits in different populations and the influence of oxytocin on the HPA.

Inaugural Dissertation submitted to the
Faculty of Medicine in partial fulfillment of the
requirements for the PhD-Degree
of the Faculties of Veterinary Medicine and Medicine of
the Justus Liebig University Giessen

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Giessen 2017

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I declare that the present thesis is my original work and that it has not been previously presented at this or any other university for any degree. I have also abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen in carrying out the investigations described in the dissertation.

Abstract

Several recent studies demonstrated that different single nucleotide polymorphisms of the oxytocin receptor gene are associated with impairments of social behavior including the heritability of autism. Another focus of attention has been the CD38 protein, which stimulates the release of oxytocin in the blood stream and probably in the brain. The objective of this study was to investigate if the prevalence and severity of autistic traits in a healthy sample of academic students is influenced by three different SNPs of OXR rs53576 and CD38 rs3796863, and in a second experiment their action in the HPA axis in a social challenge. For the first purpose, 333 subjects, 205 men and 128 women, answered the self-report questionnaires on autistic traits (The Adult Autism Quotient (AQ), Empathy Quotient (EQ), and Systemizing Quotient (SQ)) and were genotyped by cheek swab. For the second, 80 male participants were additionally submitted to the Trier Social Stress Test. Comparisons between men and women confirm reported findings that women have higher scores on the EQ and lower scores on SQ, with no differences on AQ. However, it was

observed that women have less autistic traits on the communication subscale (AQ). In our study a gender-SNP, and in the female sample, a SNP-SNP interaction in prevalence of autistic traits were for the first time observerd. In the female sample, the presence of at least one protective allele (A) on CD38 rs3796863 was associated with a higher score on the EQ and the analysis of the interaction suggested that this effect is partially modulated by the presence of the critical allele (A) on the OXTR rs2254298. In the male, the exclusive effect of OXTR rs53576 on the prevalence of autistic traits and the existence of a dose dependent effect of the critical allele (A), was identified. Our study also confirms the role of oxytocin on the modulation of HPA stress reactivity since higher salivary cortisol levels just before the stress situation and at the endpoint were observed in OXTR rs225429A+. Additionally, this group also showed a higher increase in cortisol concentration during the stress-phase. These results help to explain the difference prevalence of autistic traits and empathy among men and women, since the prevalence of the alleles are different and their effects, gender-specific. Future studies should

also include this and other variables, like ethnicity, which is also associated with different allelic distribution.

Zusammenfassung

Mehrere neuere Studien zeigten, dass verschiedene einzelne Nukleotid-Polymorphismen des Oxytocin-Rezeptor-Gens mit Beeinträchtigungen des sozialen Verhaltens einschließlich der Erblichkeit des Autismus assoziiert sind. Ein weiterer Schwerpunkt der aktuellen Forschung ist das CD38-Protein, das die Freisetzung von Oxytocin im Blutstrom und im Gehirn stimuliert. Das Ziel dieser Studie war es zu untersuchen, ob die Prävalenz und der Schwergrad autistischer Merkmale in einer gesunden Stichprobe von drei verschiedenen SNPs von OXR rs53576 und CD38 rs3796863 beeinflusst werden. In einem zweiten Experiment untersuchten wir deren Einfluss in der HPA-Achse in einer sozialen Herausforderung. Für den ersten Zweck beantworteten 333 Probanden, 205 Männer und 128 Frauen, die Selbstreport-Fragebögen auf autistische Merkmale (The Adult Autism Quotient (AQ), Empathy Quotient (EQ) und Systemizing Quotient (SQ)) und wurden zusätzlich genotypisiert. Zweitens nahmen 80 männliche Probanden an dem Trier Social Stress Test teil. Vergleiche zwischen Männern und Frauen bestätigten, die schon berichteten

Ergebnisse, dass Frauen ein höhere Punktzahl auf dem EQ und niedrigere Punkte auf SQ haben. Allerdings wurde beobachtet, dass Frauen weniger autistische Merkmale auf der Kommunikations-Subskala (AQ) zeigen. In unserer Studie wurde eine Wirkung eines SNP in der männlichen Stichprobe und der SNP-SNP-Interaktion in der weiblichen Stichprobe auf die Prävalenz von autistischen Merkmalen zum ersten Mal beobachtet. In der weiblichen Probe wurde die Anwesenheit von mindestens einem schützenden Allel (A) des CD38 rs3796863 mit einer höheren Punktzahl auf dem EQ assoziiert, und die Analyse der Wechselwirkung schlug vor, dass dieser Effekt vom OXTR rs2254298 teilweise moduliert wird. Bei den männlichen Teilnehmern wurde die exklusive Wirkung des OXTR rs53576 auf die Prävalenz von autistischen Merkmalen und die Existenz einer dosisabhängigen Wirkung des kritischen Allels (A) identifiziert. Unsere Studie bestätigt ebenso die Rolle von Oxytocin in der Modulation der HPA-Stressreaktivität, da höhere Speichelcortisolspiegel unmittelbar vor der Stresssituation und an ihrem Endpunkt in OXTR

rs225429A + beobachtet wurden. Darüber hinaus zeigte diese Gruppe auch eine höhere Zunahme der Cortisolkonzentration während der Stressphase. Diese Ergebnisse helfen die unterschiedliche Prävalenz von autistischen Merkmalen und Empathie von Männern und Frauen zu erklären. Da die Prävalenz der Allele unterschiedlich ist und ihre Auswirkungen geschlechtsspezifisch sind. Zukünftige Studien sollten auch diese und andere Variablen, wie Ethnizität, die auch mit verschiedenen Allele-Verteilung verbunden ist, analysieren.

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1. Introduction

Sociability is an important characteristic of several creatures and determines the success in reproduction, breeding and survival in several species. In humans, different pathological conditions like Williams-Beuren syndrome (Martens, Wilson, & Reutens, 2008), borderline personality disorder (Domes, Schulze, & Herpertz, 2009) and Autism (WHO, 2012) are associated with impairments of the capacity to establish satisfactory social interactions and profoundly affect the lives of the people who have these disorders. While some scientists considered social behavior too complex to be understood on the molecular level, several technological and methodological advances, like comparative studies between different species and genetic manipulations, contributed to the development of the current approach that made possible the elucidation of some of the different neurobiological mechanisms associated to it.

Social behavior is a complex and varied behavior, with some animals living in groups with complicated social structures while others are solitary and only engage in

interactions sporadically. Why a specific animal engages in social behavior in certain circumstances and which social or environmental cues are necessary to its occurrence is the object of extensive research in several species that range from *Amoebae sp* to humans (Gregor, Fujimoto, Masaki, & Sawai, 2010). The regulation of social behavior depends on the interaction of genetic and epigenetic factors, physiologic and environmental characteristics that in some situations are species-specific. In some species, the population density, number of stressors (chemical or social), food availability and maturation are factors that determine different social responses. The physiological alterations that influence social behavior have recently also been extensively researched.

The first substances implicated in the regulation of social behavior were the gonadal steroids. This hypothesis arose with the discovery that some hormonal manipulations (castration or replacement) modify social behavior in different species (Berthold AA., 1849). Testosterone is involved in the occurrence of aggressive behavior and dominance in animals and humans (Eisenegger,

Haushofer, & Fehr, 2011) and estrogen seems to be an important regulator of female sexual behavior (Shepard, Michopoulos, Toufexis, & Wilson, 2009). More recently, research in voles pointed out the two similar nonapeptides vasopressin (AVP) and oxytocin (OXT) as important modulators of social behavior in mammalians (Donaldson & Young, 2008) and have been associated with deficits on social cognition that characterize disorders such as autism (Israel et al., 2008).

1.1. The nonapeptide oxytocin

Oxytocin was one of the first hormones to be discovered. At the beginning of the last century, extracts of the posterior pituitary gland were shown to stimulate the contractile activity of the pregnant uterus and, after this discovery, the hormone was named accordingly (Gk: swift (*oxys*) birth (*tokos*)). Its action as the most effective stimulant of milk let-down and ejection in lactating mammals was later identified. The distinction between oxytocin and vasopressin was made by Du Vigneaud

(whom a Nobel Prize was awarded) when both molecules were synthesized for the first time.

Oxytocin is a nine-amino-acid peptide (Cys^{1}-Tyr^{2}-$Ileu^{3}$-Gln^{4}-Asn^{5}-Cys^{6}-Pro^{7}-Leu^{8}-Gly^{9}-NH_2) and differs from vasopressin by having Ileu instead of Phe at position 3 and Leu instead of Arg at position 8. It comprises a six-member ring formed by sulfide bridges between the two cysteines, and a short tail (≈ 1kDa) (Russel & Douglas, 2003). This close chemical similarity to vasopressin indicates that oxytocin arose in evolution later, by a duplication of the vasopressin gene (or its forebear), and then by mutations. The evolutionary precursor of oxytocin and vasopressin probably arose 700 million years ago and underwent only few modifications. Invertebrates have only one oxytocin/vasopressin homolog, whereas vertebrates have two suggesting at least a common origin (Acher, Chauvet, & Chauvet, 1995). The genes of vasopressin and oxytocin remain in close proximity to each other (in humans, on chromosome 20) and in opposite orientation, so that they are transcribed left to right and vice versa and display a marked evolutional conservation in structure and

expression in a variety of species (Donaldson et al., 2008).

Oxytocin is a very abundant peptide and is synthesized in neurons' bodies in the paraventricular and supraoptic nuclei of the hypothalamus, which project their axons to the posterior pituitary gland, where oxytocin is stored. In a study in which the most prevalent hypothalamic-specific RNAs were analyzed, oxytocin was found to be the most abundant of 43 transcripts identified (Gautvik et al., 1996). The gene that encodes for oxytocin, like the genes that encode for other neuropeptides, has several (three) exons and encodes additionally for a much larger protein called oxytocin-neurophysin, which has no hormonal function. The secretion of oxytocin and oxytocin-neurophysin in the circulation occurs through discharges of action potentials from the bodies of the magnocellular cells, which promotes the opening of membrane channels and entrance of calcium ions. The increase in the cytoplasmic calcium concentration promotes the movement of the storage vesicles, their fusion with the membrane and release of their content. Once oxytocin is secreted in the circulation it has a half-

life of approximately 2 minutes, being cleared by tissues on which it acts and by excretion by the kidneys.

Besides the oxytocin secretion from the axons' terminals of magnocellular neurons located in the paraventricular and supraoptic hypothalamic nuclei, oxytocin is also secreted by parvocellular neurons from the paraventricular nucleus, which terminate elsewhere in the brain and exerts distant actions. The hypothalamic magnocellular neuron-posterior pituitary and the paraventricular neuron-central sources of oxytocin are common to all mammals but the distribution of fibers in the brain has species-specific differences. OT fibers and endings have been described in various brain areas in the rat: the dorsomedial hypothalamic nucleus, several thalamic nuclei, the dorsal and ventral hippocampus, subiculum, entorhinal cortex, medial and lateral septal nuclei, amydala, olfactory bulbs, mesencephalic central gray nucleus, substantia nigra, locus coeruleus, raphe nucleus, the nucleus of the solitary tract, and the dorsal motor nucleus of the vagus nerve (Richard, Moos, & Freundmercier, 1991). In the CNS, oxytocin has a much longer half-life (28 min in the CSF) and increases in

blood concentration do not always correspond to increases in the CSF. In monkeys and in humans, a circadian rhythm with peak concentrations in the CSF at midday was identified. The same was not observed in plasma levels (Amico, Tenicela, Johnston, & Robinson, 1983).

There are also peripheral sources of oxytocin that also show some species or class specificity i.e. uterus, corpus luteum, heart and immune system and pancreas (Russel et al., 2003). Regarding the hypothalamic and peripheral production of oxytocin, this nonapetide exerts its function via three different routes. Firstly, oxytocin circulates in the blood and participates as a hormone in the control of uterine contraction during labor and milk ejection and prolactin release during lactation. Secondly, oxytocin reaches, thorough neuronal projections, various targets within the brain and modulates maternal, social and sexual behavior as a peptide (Burbach, Adan, & de Bree, 1992). And finally, oxytocin is produced in the periphery and controls the reproductive function, acts as a natriuretic hormone (in rats), stimulates the production of atrial natriuretic peptide and seems to be involved in

the immune mechanism of self-tolerance in the thymus (Russel et al., 2003). Regarding the fact that oxytocin facilitates reproduction in all vertebrates on several levels and its function in pair-bound formation as a facilitator of species propagation, some authors nicknamed it the “love peptide” or “the great facilitator of life”, suggesting that this nonapeptide is of crucial importance to understand reproductive and social behavior (Lee, Macbeth, Pagani, & Young, 2009; Neumann, 2007b).

As mentioned above, oxytocin and vasopressin genes are in the same chromosomal region but the second is transcribed in the opposite direction. The intergenic distance between these genes ranges from 3 to 13 kb in mice, humans and rats. The human gene for OXT-neurophysin is mapped to chromosome 20p13. This OXT pre-peptide suffers several cleavages and modifications as it is transported to extremities of the magnocelullar neurons. The main function of neurophysin, a small disulfide-rich protein, seems to be related to the proper targeting, packing, and storage of OXT within the granula. The concentration ratio of OXT and neurophysin inside the granules is normally 1:1 and these

molecules are commonly bound via electrostatic and multiple hydrogen bounding interactions and dissociate in the basic medium. The regulation of genetic transcription is complex and not completely understood but some mechanisms like the increase in poly(A) tail length during pregnancy, lactation and dehydration; the interaction with receptors of the nuclear receptor family and the stimulation of promoters by estrogen, thyroid hormone and acid retinoic receptors are described in heterogeneous species (Gimpl & Fahrenholz, 2001).

1.2. Oxytocin receptor and its distribution in the CNS

The isolated and identified cDNA encoding the human OXT receptor is a 389-amino acid polypeptide with 7 transmembrane domains and belongs to the class I G protein-coupled receptor family (Kimura, Tanizawa, Mori, Brownstein, & Okayama, 1992). The OXT receptor gene is present in a single copy in the human genome, was mapped to the gene locus 3p25-3p26.2, spans 14 kDa and has 3 introns and 4 exons (Inoue et al., 1994).

Exons 1 and 2 correspond to the 5'-prime noncoding region while exons 3 and 4 encode the amino acids of the OXT receptor. The intron 3 is the largest and separates the coding region immediately after the putative transmembrane domain 6. Exon 4 contains the sequence encoding the seventh transmembrane domain, the COOH terminus, and the entire 3'-noncoding region.

The expression and the binding capacity of the OXT receptor are probably controlled by several pathways. Among them, the influence of estrogen, cytokines, cAMP and methylation seem to play an important role but species-specific differences have been described. Although several findings suggest the existence of OXT receptor subtypes in the rat uterus, kidney, and brain, the applied techniques failed to indentify another gene. When oxytocin binds to its receptor, which is functionally coupled to $G_{q/11}\alpha$ class GTP binding proteins that stimulate together with $G\beta\gamma$ the activity of phospholipase C-β isoforms, the generation of inositol trisphosphate and 1,2-diacylglicerol occurs. Inositol trisphosphate triggers Ca^{2+} release from intracellular stores and diacylglicerol stimulates protein kinase C, which phosphorilate

unidentified target proteins. Finally, in response to an increase in the intracellular concentration of Ca^{2+}, a series of cellular events occur. The forming Ca^{2+}-calmodulin complexes trigger the activation of neuronal and endothelial isoforms of nitric oxide synthase, which stimulates the production of cGMP. In the uterus and mammary gland, the Ca^{2+}-calmodulin system promotes activation of smooth muscle cells and consequently their contraction whereas in the neurosecretory cells, the elevation Ca^{2+} levels control cellular excitability and modulate their firing patterns, which leads to transmitter release. Furthermore, oxytocin seems to exert its activity through voltage-gated or receptor-coupled channels, inhibition of Ca^{2+}/Mg^{2+}-ATPase and PGE_2 synthesis (Gimpl et al., 2001).

Experiments with cell cultures showed that OXT receptors are localized both on hypothalamic neurons and astrocytes (Di Scala-Guenot & Strosser, 1995) and pronounced species-specific differences concerning the regional distribution of OXT binding sites have been reported. In rats, OXT receptors are abundant in some cortical areas, the olfactory system, the basal ganglia, the

limbic system, the thalamus, the hypothalamus, the brain stem and the spinal cord. In human brains, dense OXT binding sites were found in the pars compacta of the substantia nigra unlike several other species examined so far. This finding indicates that in humans the nigrostriatal dopamine neurons could be a target for OXT, and this neuropeptide is probably involved in motor and other basal ganglia related functions. Another area that shows intense binding in humans is the basal nucleus of Meynert but areas with demonstrated binding in other species like hippocampus, amygdale, entorhinal cortex, and olfactory bulb are not evident in our species.

The number and the distribution of OXT receptors undergo important changes during development and only a fraction is constant throughout the life-cycle whereas others are expressed only during infancy or maturation. It was observed in rats that during aging the number of OXT receptor binding sites in some areas decreases under the influence of decreasing testosterone exposure. The expression of OXT receptors in the olfactory tubercle and in the ventralmedial hypothalamic nucleus was shown in rats to be depended on gonadal steroids,

and testosterone replacement in aged rats could restore the levels of OXT receptors in these areas to levels observed in adults (Tribollet, Duboisdauphin, Dreifuss, Barberis, & Jard, 1992; Arsenijevic, Dreifuss, Vallet, Marguerat, & Tribollet, 1995). Castration and inhibition of aromatase activity reduce, whereas estradiol and testosterone increase the number of receptors in regions of the brain involved in reproductive functions, such as the ventrolateral part of the hypothalamic ventromedial nucleus (VMN) and the islands of Calleja and neighboring cell groups (Tribollet, Charpak, Schmidt, Duboisdauphin, & Dreifuss, 1989). Additionally to estrogen, it was also shown that the treatment with progesterone or glucocorticoids can also alter the density of OXT receptors and this effect, like that observed with estrogen, seems to be different in different brain areas and also species-specific (Caldwell, Walker, Pedersen, Barakat, & Mason, 1994; Coirini, Schumacher, Flanagan, & Mcewen, 1991; Patchev, Schlosser, Hassan, & Almeida, 1993; Schumacher, Coirini, Pfaff, & Mcewen, 1990; Insel, Young, Witt, & Crews, 1993). Some evidence that the specific distribution of OXT receptors

is associated with different social organization was found in monogamous vs. polygamous voles (Insel & Shapiro, 1992) and the demonstration of OXT receptor binding sites and OXT innervations indicate that oxytocin is also involved in autonomic and sensory functions (Reiter et al., 1994). Recently, a study suggested that a polymorphic variation of the OXT receptor gene (OXTR) explains part of the variance observed in reproductive behavior in humans. Caucasian women who carry the long version of the OXTR used contraceptives less often and have children at an earlier age compared to women possessing the short variant (Prichard, Mackinnon, Jorm, & Easteal, 2007).

1.3. Effects of Oxytocin

1.3.1. The peripheral oxytocinergic system

The pregnant uterus was the first described target of oxytocin. This peptide is the most potent uterotonic substance and has established clinical use in labor induction. Furthermore, the development of OXT

antagonists seems to be of therapeutic value to prevent preterm birth, to the regulation of dysmenorrhea and to improve the efficacy of in vitro fertilizations (Chou, Wu, Pan, Hung, & Chang, 2011; Manning et al., 1995; Williams, Beck, Evans, Freidinger, & Pettibone, 1998). During pregnancy and labor, oxytocin may act primarily as a local mediator and not only as a circulating hormone. In this autocrine/paracrine system within the uterus, significant change of OXT, prostaglandins, and sex steroids concentrations are associated with prolonged or pre-term labor and occur without reflecting an increase in OXT levels in the maternal circulation (Mitchell, Fang, & Wong, 1998). Equally important are studies in rats and in humans that failed to demonstrate significant alterations in the blood and intra-uterine concentration of oxytocin around the onset of labor (Hirst, Haluska, Cook, & Novy, 1993). Complementarily an increased uterine sensitivity to OXT associated with both an increase in the density of myometrial OXT receptors and an up-regulation of their mRNA, which reaches its peak during early labor and represents a level 200 times greater than that in the non-pregnant state, is described by many authors in this

period of the reproductive cycle (Fuchs, Fields, Freidman, Shemesh, & Ivell, 1995; Kimura et al., 1992; Fuchs & Fuchs, 1984). This enhancement in its response is transitory and the levels of expression and the density of receptors drop significantly just after the parturition. The uterus hyperreactivity to OXT is regulated by a not well understood hormonal regulation that involves steroids, progesterone, fetal and placentary CRH, and cortisol. An important phenomenon seems to be the increase of the ratio of estrogen to progesterone, which occurs in the days preceding birth concomitantly with the formations of gap-junctions, increased production of prostaglandins and the up-regulation of OXT receptors (Fuchs et al., 1995; Mitchell & Chibbar, 1995). In fact, it is well known that estrogen and progesterone act in different directions in the regulation of oxytocin response in the pregnant uterus. Progesterone can inhibit the action of oxytocin through genomic and non-genomic mechanisms while estrogen is associated with the increase in expression of the OXT receptor gene (Soloff et al., 1983; Larcher et al., 1995).

The increase in OXT receptors before labor is also found in the decidua. During the course of parturition, their gene expression in human chorio-decidual tissue is increased fivefold (Takemura et al., 1994). In this tissue, OXT stimulates the release of prostaglandin $PGF_{2\alpha}$, which in higher levels drives luteolysis and thus leads to progesterone withdrawal and labor initiation in rodents. Notwithstanding, the parturition of OXT-deficient mice is not delayed (Nishimori et al., 1996). That could be explained by the fact that OXT has a luteothropic function and helps maintain the production of progesterone by the corpus luteum. In other words, in mice lacking OXT, the absence of oxytocin has a luteolytic activity that promotes labor at the proper time (Gross et al., 1998). Thus, the oxytocin system is considered to have an important role in the regulation of parturition, which includes the induction of OXT receptors and regulation of progesterone activity as determining events.

Outside of pregnancy, the number of OXT receptors in the endometrium also influences the length of the luteal phase and the administration of OXT antagonists or the

continuous administration of OXT, which downregulates the OXT receptor, lengthens the cycle (Flint, Riley, Kaluz, Stewart, & Abayasekara, 1995; Silvia et al., 1994). In the nonpregnant human uterus, the OXT receptor mRNA is mainly expressed in the glandular epithelial cells of the endometrium and the highest expression occurs during ovulation. However, its physiological significance is not known (Takemura et al., 1993).

In several species, the ovary has been shown to contain OXT and may be a site of local oxytocin production (Ivell & Richter, 1984) and in the marmoset monkey OXT seems to be a follicular luteinization factor. Other studies suggest that OT may be an autocrine factor during follicular grow (Okuda et al., 1997) and play a role in the early stage of development of fertilized oocytes (Furuya et al., 1995b). In humans, it was demonstrated that both OXT and OXT receptors are expressed in cumulus cells surrounding the oocytes and, in consequence, may participate in fertilization and early embriogenic development (Furuya et al., 1995a). In addition, as cited above, in the corpus luteum OXT participates with

estrogen, progesterone and $PGF_{2\alpha}$ in a system that balances luteolytic and luetotrophic processes outside the gestation (Gimpl et al., 2001).

OXT has been identified in the testes of various mammalian species and a mesotocin-like pepitide, a correspondent peptide in several species, was found in the testes of birds and marsupials (Pickering et al., 1989; Bathgate & Sernia, 1995). There is also evidence that OXT is produced in the testes and maybe in the epididymis and prostate and a different distribution between species is observed (Ivell, Balvers, Rust, Bathgate, & Einspanier, 1997). In humans, a complete oxytocin system appears to be present in the gonads and prostate (Frayne & Nicholson, 1998). Primarily, it has been postulated that oxytocin in the testes has two functions, i.e. the regulation of seminiferous tubule contractility and the modulation of steroidogenesis. In fact, OXT may promote the spermiation and the subsequent transport of the immobile spermatozoa to the epididymis, increases the activity of the enzyme 5α-redutase in the testes and epididymis and therefore may have an autocrine/paracrine role modulating steroid

metabolism in these tissues (Nicholson & Jenkin, 1995). In the prostate it enhances the resting tone of the gland and promotes contractions, which result in the expulsion of prostatic secretions during ejaculation (Nicholson et al., 1995; Bodanszky, Sharaf, Roy, & Said, 1992). It also suggests that OXT activity could be a component in the development of prostate benign hyperplasia. Besides glandular hyperplasia, an enhanced muscular tonus and contractibility are also postulated as possible pathophisiological mechanisms and treatment with OXT antagonists have been tested (Nicholson et al., 1995). Another indirect mechanism of cell-growth under influence of 5α-redutase and OXT has been also complementarily suggested (Nicholson, 1996).

Among all the effects of oxytocin in the reproductive system, one of the first described is the promotion of milk-ejection, which is triggered when the baby sucks the nipple. The stimulation of tactile receptors at that site generates sensory impulses that are transmitted from the nipples to the spinal cord and then to the secretary oxytocinergic neurons in the hypothalamus, which display a synchronized high-frequency bursting activity.

This bursting consists of a brief (3-4s) discharge of action potentials recurring every 5-15 min and promotes OXT release in the blood stream. Reaching the lactating breasts, it causes a contraction of myoepithelial cells in the walls of lactiferous ducts, sinuses, and breast tissues' alveoli. In humans, milk ejection occurs within 30 s to 1 min after the baby begins to suck, the so-called milk let-down reflex (Gimpl, Reitz, Brauer, & Trossen, 2008). The activation of oxytocinergic neurons can also be stimulated by the baby crying and occurs before the baby begins to suck. The important role of OT in the lactation was demonstrated in knockout mice. Despite the occurrence of normal labor, these mice are completely unable to nurse their offspring (Nishimori et al., 1996). In addition to the well known milk let-down reflex, oxytocin has an important role in the necessary proliferation of the alveoli and differentiation of epithelial cells that are indispensable for nursing.

Oxytocin not only has reproductive functions and is present in a variety of tissues where it exerts diverse functions. In the rat, neurons in the hypothalamus are also stimulated by hypovolemia or hyperosmorality and

OXT has a natriuretic effect mainly due do to a reduction in tubular Na^+ reabsorption, probably in the terminal distal tube or in the collecting duct. When the plasma sodium concentration increases up to 130 mM, the OXT and vasopressin levels increase as an exponential function of sodium serum concentration (Verbalis & Dohanics, 1991). Moreover, injections of oxytocin cause proportional increases in urinary osmolality, natriuresis, and kaliuresis and evoke concomitant release of atrial natriuretic peptide (Haanwinckel et al., 1995). Although the natriuretic response to OXT has also been described for conscious dogs, it probably does not occur in humans and nonhuman primates. Therefore, the contribution of OXT to renal physiology in these species is not elucidated (Conrad, Gellai, North, & Valtin, 1993).

In the heart and cardiovascular system, oxytocin is able to reduce the heart rate and the force of atrial contractions and the OXT antagonist reverses the bradycardia caused by OXT (Favaretto et al., 1997). Higher intracardial concentrations also stimulate the production of atrial natriuretic peptide and contribute to the regulation of blood osmolality (Gutkowska et al.,

1997). The effect of oxytocin in the heart is mediated by OXT receptors in the atria and ventricle, which are known to be present in the rat at levels 10 times lower than in the nonpregnant uterus. As described in the reproductive system, oxytocin exerts in the heart a paracrine/autocrine function and OXT was detected in heart perfusates and in the medium of cultured atrial myocytes, which confirms that in the rat a cardiac synthesis of OXT exists (Jankowski et al., 1998). The diuretic action that involved OXT and atrial natriuretic peptide is not completely elucidated but presumably involves the release of OXT by the stimulation of baroreceptors, activation of OXT receptors with subsequent elevation of intracellular [Ca^{2+}] and finally, stimulation of atrial natriuretic peptide secretion. In addition, the plasma concentration of OXT and atrial natriuretic peptide after parturition were found to be increased, suggesting that OXT is involved in the massive diuresis observed postpartum. It is also postulated that OXT contributes to the closure of umbilical vessels of the newborn since the evidence of its vasoconstrictive effect in the placenta and in the

umbilical cord is well established (Maigaard, Forman, & Andersson, 1986). In the vasculature of the body outside the reproductive system, OXT is involved in vasodilatation and vasoconstriction and, in rats, chronic and acute SC injections have opposite effects under the regulation of sex hormones (Petersson, Lundeberg, & UvnasMoberg, 1997; Petersson, Lundeberg, & Uvnas-Moberg, 1999). Although the majority of studies were conducted in rats, several studies confirm an OXT-induced cardiovascular activity in other species and, as mentioned above on the distribution of OXT receptors in the body, some species-specific differences were found.

Despite the fact that OXT receptors and OXT production were also found in the thymus and that their functions were not completely elucidated, there is some evidence that OXT actively mediates T-cell differentiation both as a neuroendocrine self-antigen and as a promoter of T-cell focal adhesion. Additionally, it is also suggested that some immune pathologies in humans could be explained by the involvement of OXT in T-cell-positive selection and activation (Gimpl et al., 2001). Likewise, the OXT function in adipocytes has only been recently

investigated. In these cells, OXT has a so-called insulin-like activity and stimulates glucose oxidation and lipogenesis and increases pyruvate dehydrogenase activity (Hanif, Lederis, Hollenberg, & Goren, 1982). In humans, OXT is involved in the regulation of fat cell differentiation and/or maintenance of the differentiate state through the production of H_2O_2. Studies on rats and some on humans pointed out that the administration of OXT was also associated with the release of glucagon and, in lower levels, insulin, which are regulated by the serum glucose concentration. Altered glucose metabolism is commonly associated with obesity and obese patients have a fourfold higher OXT plasma level (Stock, Granstrom, Backman, Matthiesen, & UvnasMoberg, 1989) and a reduced insulin-induced increase in OXT plasmatic levels when compared with healthy subjects. Taking in account the known effects of OXT in adipocytes and pancreas, some authors have postulated a role for OXT in the pathophysiology of obesity under modulation of endogenous opioids (Coiro et al., 1990). Finally, OXT and vasopressin were also identified in the adrenal glands of humans and rats (Ang & Jenkins, 1984)

and both occur in concentration far greater than in plasma. Also, in other species OXT has been identified in the cortex and in the medulla of this gland. Besides the postulation that, in humans, OXT decreases cortisol release and/or synthesis, a proliferative effect of chromaffin-cells was suggested in rats (Legros, Chiodera, & Geenen, 1988; Popovic, Plecas, Ugresic, & Glavaski, 1996).

The multitude of tissues where OXT exerts its functions is not identified completely, but these studies suggest that OXT participates in several physiological processes including immune and haemodynamic response and hydroelectrolytic and glucose homeostasis. OXT receptors were also found in osteoblasts and in the human epithelial osteosarcoma cell-line (Copland, Ives, Simmons, & Soloff, 1999), which leads some authors to suspect that OXT also has an additional anabolic effect.

1.3.2. Oxytocin effects in the central nervous system

1.3.2.1. Behavioral studies in animals

1.3.2.1.1. Memory and social recognition

The first described effects of oxytocin on the CNS were the effects on memory, i.e. on avoidance paradigms in rats. It was observed that the administration of different doses, or of different sub-products have opposing effects. A small dose of oxytocin attenuates retention whereas higher doses facilitate it. In addition to these findings, a dependence on the task was also observed, since low doses of oxytocin can attenuate retention in active-avoidance and had no effect on passive-avoidance paradigms. In another study, where high doses of OXT were tested, it was demonstrated that the same dose provoked different responses. In the pole-jumping active avoidance, oxytocin provoked facilitation of retention, i.e. the occurrence of expected avoidant behavior, but, in step-through passive avoidance, the rats that received oxytocin showed a lower rate of avoidance suggesting impaired retention. Based on these findings, some authors suggested that different OXT metabolites have

opposing actions on memory consolidation (McEwen, 2004).

In comparison to vasopressin, oxytocin effects on memory consolidation are not well understood but there is sufficient evidence pointing out that oxytocin interacts with central catecholaminergic and cholinergic transmitter systems that project and modulate activity in limbic, striatal and cortical areas involved in retrieval. Oxytocin may also exert its action through enhancing neural responsivity to glutamate, which facilitates retention. On the other hand, OXT also may exhibit amnestic effects due to interaction with GABA-ergic interneurons in the hippocampus, which causes inhibition of CA1 pyramidal neurons (McEwen, 2004).

The influence of vasopressin and oxytocin on social memory has also been investigated. While studies with vasopressin have been showing more conclusive data, studies with oxytocin are less numerous and more inconsistent. Protocols examining the effects of peripherically and centrally administrated oxytocin and analogs indicate both facilitative and attenuating effects

on social recognition memory and lead to the speculation about the existence of two functional oxytocinergic systems. The type-I OXT-ergic system would be activated in situations of high emotional arousal that are associated with certain reproductive functions (lactation, reproduction and parturition). In this type of circumstances, levels of OXT in the CFS and plasma high above the basal levels are found. On the contrary, the type-II OXT-ergic system would rather be activated in social contacts with lower levels of arousal and with negligible stress and promotes social memory that is involved in affiliation and reproductive success. Several studies confirm this hypothesis, demonstrating that small doses of OXT provoke facilitating effects in social memory acquisition whereas higher doses impair retrieval. Several authors assume that the attenuation of acquisition appears to be protective in situations of higher stress as oxytocin diminishes the frequency of anxious behavior in several species. In rats, oxytocin reduces ultrasonic distress calling in 6-8 day-old pups elicited by removing them from their dams or siblings (Insel & Winslow, 1991). In this species, OXT also

reduces anxiety behavior triggered by noise stress (Windle, Shanks, Lightman, & Ingram, 1997) and the anxiety behavior observed in the elevated plus maze (Windle et al., 1997). The activation of the type II OXT-ergic system associated with the release of small amounts of oxytocin would explain the parent-offspringbound formation in sheep (Kendrick, Keverne, & Baldwin, 1987), mate selection and bonding, occurrence of parental behavior in prairie voles (Carter, Williams, Witt, & Insel, 1992; Carter, 1992), and social affiliative behaviors in rats and mice (Nelson & Panksepp, 1998).

1.3.2.1.2. Social behavior

1.3.2.1.2.1. Social approach and motivation

The neurobiology of social approach has been studied in three different animal models, i.e. parental behavior, infant-mother attachment and adult affiliation. In these three models, rats and mice have been preferably investigated, while fewer studies with non-human primates and sheep have also been conducted.

Despite the significant discrepancies between rat and human social behavior, rat and mice models enable us to understand the underpinnings of human social behavior in a suitable way (Numan, 1988). Female rats, unlike other mammals, avoid contact with infants of their own species until the day shortly before parturition. At this moment, they display an abrupt behavioral change and start to show a non-selective interest in infants, which is demonstrated by the appearance of vigorous nest building, retrieval, grooming and defense of the young (Winslow & Insel, 2002). Since these behaviors are easily observed and are not present in virgin rats, this is an interesting, predictable and useful model for the study of maternal care and bonding. Oxytocin neurons derived from PVN and SON are important to the development of the above mentioned behaviors. Lesions in the PVN, which are associated with the disruption of the oxytocinergic system, delay maternal care (Insel & Harbaugh, 1989) and intracerebroventricular injections of OXT promote it in virgin, otherwise avoidant rats (Pedersen, Ascher, Monroe, & Prange, 1982). In contrast, OXT antagonists injected intracerebroventriculary, in the

medial preoptic area (MPA), and in the ventral tegmental area inhibit or delay maternal behavior arousal (Pedersen, Caldwell, Walker, Ayers, & Mason, 1994; Fahrbach, Morrell, & Pfaff, 1985). It is hypothesized that the OXT-receptor gene expression is controlled by estrogen and progesterone. Rises in the plasmatic level of estrogens are associated with increased OXT-receptor binding in the hypothalamus and MPA and with a different density of the OXT-receptor in the bed nucleus of the stria terminalis (BNST) and in the central nucleus of the amygdala it has been associated with quantitative and qualitative differences in maternal care (Francis, Champagne, & Meaney, 2000; Francis, Young, Meaney, & Insel, 2002). The role of OXT in maternal behavior was recently questioned when some studies demonstrated intact maternal behavior in knockout mice. Newer evidence, in a more naturalistic environment, shows that OXT-knockout mice have significant deficits in social behavior, which include spontaneous and parturient maternal nurturing. It is also described that OXT injections reduce infanticidal behavior in this species (Mccarthy, 1990; Nishimori et al., 1996).

Prairie voles (*Microtus ochrogaster*) are monogamous rodents that display biparental care of the young and pair bond formation between adult mates. Differently from rats, in this species maternal behavior is also present in juveniles and virgin females while half of the female adults are spontaneously maternal (Solomon, 1991; Wang & Novak, 1994). Also in this species, OXT is critical for the expression of spontaneous maternal care in both adult females and juveniles. In juveniles, the density of OXT-receptor binding in the nucleus accumbens correlates positively with the time they spend crouching the pups whereas in adults, microinjections of OXT receptor-antagonists in this area prevent its appearance. The nucleus accumbens is part of the reward and reinforcement system, which could explain the maintenance and arousal of maternal behavior and pair bonding in monogamous species as an increase in motivation through enhanced sensitivity to oxytocin in this nucleus. According to this hypothesis, it was confirmed that monogamous rodents have higher levels of OXT-receptor binding in the nucleus accumbens in comparison to non-monogamous species (Olazabal &

Young, 2006b; Olazabal & Young, 2006a). Similarly, several studies demonstrated the influence of another nanopeptide in parental behavior in males. Vasopressin (AVP) is associated with the appearance of parental behavior in prairie voles, and their antagonists, like oxytocin antagonists in females, block it. In addition, different distribution and density of V1a receptors seem to determine quantitative and qualitative differences in parental care (Wang et al., 1994; Hammock & Young, 2005; Parker, Kinney, Phillips, & Lee, 2001).

The mother-infant interaction has also been investigated in OXT-receptor-knockout models. Knockout pups have longer latency to crawl back to the mother when displaced from the nest. During this task they also emit less ultrasonic vocalizations, which can be interpreted as a lower distress when separated from the mother. Less ultrasonic vocalizations in the same circumstances and lack of preference for mother's scent were equally observed in μ-opioid receptor knockout mice. This suggested that oxytocin and the opioid system are involved in the motivational aspects that control infant

approach and contact to the mother (Young et al., 1997; Moles & D'Amato, 2004).

Social contact between male and female adults has also been investigated. Infusion of OXT and AVP directly in the brain increases the amount of social investigations and contact in rats, voles and Mongolian gerbils (Witt, Winslow, & Insel, 1992; Razzoli, Cushing, Carter, & Valsecchi, 2003; Young, Nilsen, Waymire, Macgregor, & Insel, 1999). Similarly, comparisons between two macaque species demonstrated that the social and gregarious bonnet monkey has higher levels of CFS oxytocin than the non-social pigtail macaque (Rosenblum et al., 2002). At least for vasopressin in voles it was shown that the social contact among adults and juveniles depends on the distribution of V1a receptors in different areas of the brain. Viral expression of V1a receptors increase the contact time among rats and it is also postulated that oxytocin receptor distribution may influence the motivation and affiliation in different species (Landgraf et al., 2003).

1.3.2.1.2.2. Social bond formation

Generally, two different interesting models have been used to study the social bond arousal in animals. One of them is used to investigate the selective bond between ewes and their lambs while the other model examines the behavior and neurobiological underpinnings of adult monogamous prairie voles during mating. Bond formation, unlike the maternal care of some species, is highly selective and depends on the integration of several cognitive processes, i.e. motivation and interest, social memory and a reward-punishing system that are important to the maintenance of a given relationship. In the monogamous prairie vole, it also encompasses rejection and aggressive behavior directed at other conspecifics. The neurobiology of bond formation is not completely understood but in recent years, considerable progress has been made (Lim & Young, 2006).

In sheep, it was also demonstrated that the physiological modifications that occur during pregnancy and labor are important to the development of maternal behavior after parturition. Administration of estradiol and progesterone

followed by vagino-cervical stimulation induce maternal behaviors in virgin ewes (Keverne, Levy, Poindron, & Lindsay, 1983). Higher levels of OXT in the CSF and in the brain are found after vagino-cervical stimulation, which is a common practice of farmers to induce maternal acceptance between ewes and lambs even after the parturition. Ewes that give birth and receive additional vagino-cervical stimulation have a higher probability of exhibiting maternal care and acceptance of other ewe's offspring (Kendrick, Levy, & Keverne, 1991; Kendrick, Keverne, Chapman, & Baldwin, 1988b; Kendrick, Keverne, Chapman, & Baldwin, 1988a; Kendrick, Keverne, Baldwin, & Sharman, 1986). Although these effects can be blocked by ephidural anesthesia, intracerebroventricular OXT injections can reverse it, suggesting that oxytocin release in the brain triggered by the natural vagino-cervical mechanic stimulation during labor is one of the important neurobiological modifications associated with the appearance and development of the infant-maternal bond (Levy, Keverne, Kendrick, Piketty, & Poindron, 1992). The evidence that the injection of oxytocin alone can

produce acceptance of lambs in virgin ewes confirms this fact (Kendrick et al., 1987).

At the onset of maternal behavior in sheep, oxytocin may work as a modulator of the release of other neurotransmitters, such as noradrenalin, GABA, glutamate and acetylcholine. In the bulb olfactory, it may promote reorganization of cells which culminates with selectivity of odors and selective maternal care. After that, the initial unselective acceptance of lambs is substituted by a high selective maternal behavior directed to their own offspring. It is of interest to observe that some mitral cells are selectively stimulated by their lambs' odors after parturition and show increased glutamate and GABA release just in response to them (Levy, Kendrick, Goode, Guevaraguzman, & Keverne, 1995; Kendrick, Levy, & Keverne, 1992; Keverne, Levy, Guevaraguzman, & Kendrick, 1993). Thus, it is hypothesized that the vagino-cervical stimulation during labor is transmitted through the spinal cord to the PVN which, after depolarization, releases OXT in the posterior pituitary and in different parts of the brain that were primed by estrogen and progesterone and have higher

density of OXT receptors. These areas may include the medial pre-optic area, ventral tegmental area, bed nucleus of the stria terminalis, medial amygdala and olfactory bulb and are, therefore, involved in the appearance and maintenance of this important social bond. The reorganization that takes place in the olfactory bulb seems to be important to the learning process that culminates with social selectivity and recognition resulting in a long-lasting relationship (Lim et al., 2006).

Oxytocin also facilitates mating between adult male and female prairie voles as well as sexual intercourse. It is hypothesized that oxytocin release occurs after vaginal stimulation during mating. Similarly, intracerebroventricular injection of oxytocin in males and females promotes pair bond-formation in the absence of mating (Cho, DeVries, Williams, & Carter, 1999; Williams, Carter, & Insel, 1992) and intracerebroventricular infusion of oxytocin antagonists inhibits pair bond formation in the presence of prolonged mating (Williams, Insel, Harbaugh, & Carter, 1994; Cho et al., 1999).

Comparisons between two monogamous voles' species (*M. orchrogaster* and *M. pinetorum*) with two non-monogamous species (*M. pennsylvanicus* and *M. montanus*) have been elucidative. While monogamous species present long-lasting pair bond formation and care for the pups for a long time, non-monogamous species are solitary and abandon the offspring after just two weeks of care. Although all four species have the same distribution of oxytocin neurons throughout the brain, the distribution of oxytocin receptors is significantly different between monogamous and promiscuous voles (Insel et al., 1992; Insel, Wang, & Ferris, 1994). Monogamous prairie voles have elevated levels of OXT receptors and V1aR (vasopressin 1a receptor) in the brain regions implicated in reward and reinforcement such as the NAcc and ventral pallidum (Lim & Young, 2004b). In contrast, promiscuous montane and meadow voles have, in these regions, lower levels. Microinjections of OXT antagonists in the NAcc impair pair bond formation in female prairie voles, while injections of V1aR antagonists in males produce the same results (Lim et al., 2004b; Young, Lim, Gingrich, & Insel, 2001).

Correspondingly, the artificial expression of V1aR in these brain regions of promiscuous meadow voles is capable of inducing long-lasting pair bond in this species (Lim et al., 2004a). For this reason, it is hypothesized that the distribution of OXT receptors and V1aR are responsible for the observable differences in social bond formation between these species and that these nanopeptides regulate mesolimbic dopamine pathways in a similar way as observed in place-conditioning in drug addiction experiments.

1.3.2.2. Behavioral studies in humans with emphasis in social behavior

Several studies report a correlation between peripheral levels of OXT and behavioral and physiological responses in humans. Among them, peripheral levels of OXT were correlated with positive physical contact with a partner, reduced hormonal response to a given psychosocial stressor, lower levels of anxiety in patients with depression (Grewen, Girdler, Amico, & Light, 2005;

Taylor et al., 2006; Scantamburlo et al., 2007; Taylor, 2006), and male and female sexual response (Baskerville & Douglas, 2008). In addition, higher levels were also found in subjects who received a monetary transfer that reflects a human intention of trust in comparison to unintentional computer-made monetary transfer. This fact leaded some authors to postulate that OXT levels are important for the development of trust and trustworthiness in human relationships (Zak, Kurzban, & Matzner, 2005). In the same study, OXT plasma levels were also correlated to the amount of money returned, which could be interpreted as a measure of altruistic behavior. Interesting to note is that the isolated receipt of money does not produce any significant enhancement in the OXT levels, suggesting the necessity of human interactions to elicit such an increase. Although it is still debatable if the peripheral OXT circulation is an accurate measure of neuropeptide function, other studies have also found lower OXT peripheral levels in autistic spectrum disorders, schizophrenia and depression (Cyranowski et al., 2008; Keri, Kiss, & Kelemen, 2009; Goldman,

Marlow-O'Connor, Torres, & Carter, 2008; Green et al., 2001).

The relationship between OXT levels and bonding has also been investigated in humans. There is a general consensus that prenatal and postpartum OXT release are both involved in the bonding formation between mothers and their infants and associated to reduced maternal stress reactivity (Nelson et al., 1998). During birth, there is a significant increase of OXT levels in the cerebrospinal fluid of the parturient mother, and postpartum levels are positively correlated with positive feelings and reduced anxiety (Takagi et al., 1985). Oxytocin is also released into the blood circulation by mechanic stimulation of the nipple during breastfeeding, which is in turn associated with lower negative feelings, lower stress and cortisol levels, and attenuated ACTH response in nursing mothers (Mezzacappa & Katkin, 2002; Altemus, Deuster, Galliven, Carter, & Gold, 1995). During sucking, there is a negative correlation between ACTH and plasma OXT levels (Chiodera et al., 1991), and a study that measured OXT levels during the pregnancy and postpartum found that a pattern of

increase is associated with higher maternal-fetal bonding (Levine, Zagoory-Sharon, Feldman, & Weller, 2007). Finally, OXT levels in early pregnancy and in postpartum have been positively correlated to several other measures of maternal bonding, which include attachment-related thoughts, gaze at the infant, affectionate touch, and frequent infant checking (Feldman, Weller, Zagoory-Sharon, & Levine, 2007).

The limitations of plasma levels as an indicator of CNS activity have been pointed out and other methods, like lumbar puncture and sampling of CSF, have been adopted. Unfortunately, these methods also have limitations and are not so easily executed in humans. The weak correlation between peripheral and central concentrations could partially explain the divergent findings regarding long-term relationships and OXT plasma levels, which were positively and negatively correlated with dissatisfaction with the current relationship. Plasma OXT levels were negatively correlated with marriage quality, physically affectionate partner contact, partner relations, quality of other relationships and the frequency of social contacts (Taylor

et al., 2006). Another study found positive associations with greater self-reported interpersonal distress (Turner, Altemus, Enos, Cooper, & McGuinness, 1999). In contrast, some studies pointed out that oxytocin could be an indicator of positive feelings associated with long-lasting relationships. Positive correlations with the capacity to express feelings and share emotions, with a more supportive relationship with the partner and reported frequency of partner massages and hugs were also found (Light, Grewen, & Amico, 2005; Tops, van Peer, & Korf, 2007; Grewen et al., 2005).

The studies mentioned above show that oxytocin is associated with the emergence and maintenance of social behavior in humans, i.e. trust and trustworthiness and mother-infant bonding. The conflicting results of plasma OXT levels regarding marital relationships sustain two contradictory hypotheses. OXT may be enhanced in long-lasting satisfactory relationships and is associated with bond strength, or OXT may be associated with couple dissatisfaction and work as an anxiolytic and motivational neurotransmitter, which enhances the possibility of finding new social contacts. Regarding the

limitations of plasma levels and their weak association with CNS activity, new approaches need to be employed.

A number of recent studies have investigated the influence of OXT on facial-expressions and other subtle social signal processing. Some of these studies employed intra-nasal injections of OXT to overcome the limitations of studies with plasma OXT measurement cited above. A quantity of this peptide is inhaled, achieving the subarachnoid space through the olfactory epithelium. Cerebrospinal concentrations rise within 10 minutes and remain elevated for 80 to 120 minutes (Campbell, 2010). In The Reading the Mind in the Eyes test, which was developed to access social cognition in patients with autistic spectrum disorders, subjects need to identify, what a person was thinking and feeling while looking at different pictures of their eyes region. Compared against placebo intranasal administration of OXT improves the performance of male subjects, mainly for difficult stimuli. Other studies that evaluated the effect of OXT on the recognition and processing of emotional signals have inconsistent results. There is evidence that OXT improves recognition of positive facial expressions and

decreases aversion to angry faces (Marsh, Yu, Pine, & Blair, 2010; Di Simplicio, Massey-Chase, Cowen, & Harmer, 2009; Evans, Shergill, & Averbeck, 2010). It was also reported that oxytocin improves only the recognition of fearful faces and has no effect on emotion recognition in a visual search task (Unkelbach, Guastella, & Forgas, 2008; Guastella, Carson, Dadds, Mitchell, & Cox, 2009).

The investigation through a semantic task oriented to interpersonal relationships also produced some interesting results. In one study, words from five different categories (relationship, sex, safety and threat, happiness and sadness, and others) were presented for 8 seconds and participants were instructed to rate them as positive or negative. Although any improvement in accuracy was observed, subjects that received OXT had a shorter latency for words of the sexual and relationship categories (Unkelbach et al., 2008). A recent study evaluated detection of briefly presented emotional faces after OXT intranasal administration and reports a significant improvement of accuracy in detection of happy faces. This study also suggests that oxytocin is

involved in the early stages of visual processing of emotional stimuli (Schulze et al., 2011). As mentioned earlier, OXT may have different effects in different populations i.e. in subjects with less emotional skills or who suffered parental separation. Additionally, a cross-over double blind study using accuracy in an empathy task as the dependent variable and OXT administration and score in an autistic traits inventory as independent variables found that accuracy was only improved in subjects with high levels of autistic traits (Bartz et al., 2010a). Although the authors of this study defend that oxytocin could have a restrict effect in the general population, many of the studies cited above contradict this statement.

Studies that evaluated the cognitive aspects of empathy are more numerous than studies that evaluated emotional aspects. One of these studies evaluated the learning performance conditioned by social and non-social reinforcers in men and women after administration of intranasal OXT. As social reinforcers, these authors used happy and angry human faces and as non-social reinforcements green and red circles. The evaluation of

emotional empathy was done through the Multifaceted Empathy Test which, besides the identification of emotions (cognitive empathy), has a subtest for emotional empathy (identification of arousal). OXT potentiated learning conditioned by social reinforcers and enhanced emotional empathy but not cognitive empathy. Furthermore, emotional empathy levels in treated men were similar to empathy levels in untreated women, which suggest that oxytocin attenuates the normal discrepancy in emotional empathy observed between men and women (Hurlemann et al., 2010). Another study also investigated emotional empathy after intranasal OXT and reports that participants with higher self-criticism and lower self-reassurance, social safeness, and attachment security have less positive experiences on the Compassioned Focused Imagery under OXT than placebo. However, analyzing the whole group, oxytocin increased relaxed positive affects while decreasing activated positive affects. In general, oxytocin enhanced the ease of imagining, receiving compassion from another person and receiving various compassionate

qualities for the self, which is also considered a measure of emotional empathy (Rockliff et al., 2011).

Attachment security is associated with reduced levels of psychological stress and insecure patterns are associated with some psychiatric disorders (Ditzen et al., 2008). The effects of OXT on the subjective experience of attachment security in humans were investigated in at least one study. The authors report that OXT can increase the subjective experience of attachment security in subjects with insecure attachment patterns and that a single dose of OXT is sufficient to show it (Buchheim et al., 2009). It is hypothesized that OXT may promote more adaptive attachment patterns and reduce the psychological stress associated with insecurity, which could be an important achievement in some psychiatric disorders as social phobia and borderline personality disorder.

Some studies cited above verified that oxytocin intra-nasal injection is associated with enhanced recognition of faces but the mechanism involved has not been completely investigated. Three studies found that

intranasal OXT enhances gazing time to the eye region when observing neutral and emotional facial expressions (Andari et al., 2010; Guastella, Mitchell, & Dadds, 2008; Gamer, Zurowski, & Buchel, 2010). However, the challenging hypothesis that OXT improve on facial recognition is mediated by increased attention in the highly informative eye region needs to be still investigated. In contrast to this hypothesis, one study found no increase in gazing time to the eye region in women (Domes et al., 2010) and does not support this evidence.

Pioneer studies on the influence of OXT on cognitive aspects pointed out that OXT, unlike vasopressin, may have amnesic effects. More recently, some studies suggest that OXT can alter the memory in a socially selective manner. In one study, intranasal OXT improved recognition of faces, but not of non-social stimuli. Previously presented faces were more correctly assessed as “know” as in the control condition (Rimmele, Hediger, Heinrichs, & Klaver, 2009). Another study demonstrated that OXT selectively reduced the implicit memory of socially relevant words, but not neutral words in males

(Heinrichs, Meinlschmidt, Wippich, Ehlert, & Hellhammer, 2004). The short and long-term effects of OXT as a single post-learning dose on memory were also investigated in a single-blind placebo-controlled study. After thirty minutes and 24h, participants that received OXT showed improved identity recognition for neutral and angry faces and had a lower bias while identifying not previously presented faces (Savaskan, Ehrhardt, Schulz, Walter, & Schachinger, 2008). In contrast, it has also been shown that intranasal OXT given before learning improves memory for happy faces, but not for neutral and angry faces (Guastella, Mitchell, & Mathews, 2008). Finally, there is also evidence that OXT modulation of recollection of mother-infant care styles or attachment representations is dependent on the predominant attachment representations people possess. Less anxiously attached individuals remember their mother as more caring and close after OXT administration, whereas more anxiously attached individuals remember their mother as less caring. Regarding some apparent contradictory findings and an effect modulation by personality traits, early experience

and gender, some authors suggest that OXT may therapeutically benefit only some individuals while it could harm others and, therefore, should not be considered an all-purpose attachment panacea (Bartz et al., 2010b).

The effects of OXT on social behavior also seem to be dependent on the available social information. Declerck, Boone, & Kiyonari (2010) investigated cooperation in two different social games after intranasal administration with strong and weak incentives to cooperate. Participants who received OXT cooperated more only when social information, i.e. social incentives, were present. When social information was lacking, OXT decreased cooperation. Like some studies that assessed OXT plasmatic levels, some studies investigated trust in social relationships during intranasal administration. One of the pioneer studies in this field was executed by Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr (2005), which demonstrated that OXT promotes trust in taking social risks in comparison to placebo and to non-social risks. This finding has been corroborated by more recent studies (Mikolajczak, Pinon, Lane, de Timary, &

Luminet, 2010; Mikolajczak et al., 2010). Complementarily, subjects that receive OXT show no change in their trusting behavior if their trust is breached several times while subjects receiving a placebo decrease their trust when exposed to betrayal (Baumgartner, Heinrichs, Vonlanthen, Fischbacher, & Fehr, 2008). This is in accordance with the hypothesis that OXT promotes social behavior through a positive social bias, which in some circumstances could be disadvantageous.

In addition to increased perceived trustworthiness and attractiveness of faces, generosity, trust and cooperation (Zak, Stanton, & Ahmadi, 2007; De Dreu et al., 2010; De Dreu, Greer, Van Kleef, Shalvi, & Handgraaf, 2011; Chen, Kumsta, & Heinrichs, 2011), OXT also enhanced envy and gloating in social games. There is also additional evidence that OXT facilitation of social approach may be context specific and sensitive to positive social cues. Using an ostracism paradigm Alvares, Hickie, & Guastella (2010) demonstrated that ostracized participants reported negative affective and attachment related reactions, as well a significant motivational change with increased desire to be involved

in the game, and any of these effects were influenced by OXT. However, in included participants, OXT enhanced the desire to play again with the same participants, which also suggests that OXT may function as a strengthening positive social reinforcer. Regarding paternal behavior, there is evidence that a single intranasal administration promotes positive behavior and diminishes hostility exhibited by fathers in a 15 min section of play with their children (Naber, van Ijzendoorn, Deschamps, van Engeland, & Bakermans-Kranenburg, 2010).

Regarding the research reviewed in this and prior sections, OXT should not be viewed as a nanopeptide that invariantly improves social cognition or prosocial behavior. As an alternative to this simple point of view, some authors suggest that OXT can alter the basic processing of social stimuli, for example the salience of interpersonal cues that in turn could produce a wide variety of behavioral effects depending on situational and/or dispositional factors (Bartz, Zaki, Bolger, & Ochsner, 2011; Brune, 2012).

1.4. CD38/cADP ribose system and the regulation of oxytocin secretion

The release of oxytocin from dendrites and axons terminals is considered to be independent and probably has different intracellular mechanisms. Axons release their vesicles in response to electrical stimulation, resulting in oxytocin release in their terminals in the posterior pituitary, whereas release from dendrites in the brain is less dependent on electrical activity (Sabatier, 2006). However, it is accepted that both types of oxytocin release require increases in intracellular calcium concentration but only dendritic release can be primed for further activity-dependent release by mobilizing calcium from intracellular stores (Tobin & Ludwig, 2007).

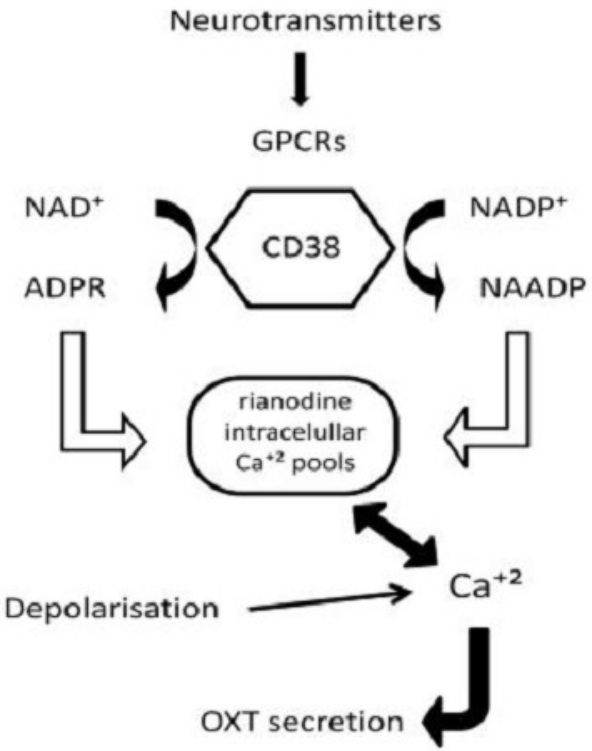
Depolarization-secretion coupling of oxytocin release has been reported in both somatodendritic and neurohypophysial terminals of hypothalamic neurons. Ca^{2+} released from inositol 1,4,5-trisphosphate (IP_3) and/or ryanodine-sensitive intracellular Ca^{2+} stores is involved in stimulated oxytocin release (De Crescenzo et

al., 2004). It is probable that exocytosis from axonal terminals in the posterior pituitary results from Ca^{2+} entry via voltage-gated channels after depolarization of the terminals whereas oxytocin release from dendrites results from Ca^{2+} release from intracellular stores into the cytosol, which triggers dendritic but not axonal oxytocin release (Landgraf & Neumann, 2004).

Recently, a novel mechanism of intracellular Ca^{2+} mobilization that plays a key role in oxytocin release from soma and axon terminals of hypothalamic neurons was identified. This mechanism is linked to the activity of NAD^{+}-glycohydrolase/CD38 which is a multifunctional molecule with enzymatic and receptor activity and plays a key role in various physiological processes in different tissues (proliferation, migration, differentiation, adhesion and secretion) (Salmina, Lopatina, Ekimova, Mikhutkina, & Higashida, 2010). In the brain, CD38 is found in neurons and glial cells, shows intracellular and plasma membrane location, and is abundant in neuronal perikarya and dendrites (Ceni et al., 2003; Ceni et al., 2003; Mizuguchi et al., 1995).

Activation of CD38 with its substrate ligand NAD^+ results in the hydrolysis of NAD^+ and cADPR to ADP-ribose or in cyclisation of NAD^+ to cADPR (Higashida et al., 2001). In addition to the production of cADPR, the enzyme can use $NADP^+$ to produce nicotinic acid adenine dinucleotide phosphate ($NAADP^+$). cADPR mobilizes Ca^{2+} from ryanodine-sensitive intracellular Ca^{2+} stores in the endoplasmic reticulum and NAADP liberates it from other pools located in lysosomes or secretory granules. Both molecules function as second messengers independent of IP_3 (Salmina et al., 2010; Higashida et al., 2007). Additionally, binding to non-substrate ligands or CD38 ligation results in the activation of intracellular signaling cascades or in an increase of intra-cellular Ca^{2+} (Higashida et al., 2007; Higashida et al., 2001). In excitable cells, cADPR potentiates Ca^{2+} liberation through ryanodine receptors and accelerates its subsequent sequestration by sarcoendoplasmic reticulum Ca^{2+}-ATPase (Yamasaki-Mann, Demuro, & Parker, 2009).

Figure 1. Scheme illustrating CD38 activity in oxytocin-producing neurons. Neurotransmitters control CD38-dependent Ca^{2+} release from ryanodine receptor-regulated intracellular stores, thus affecting the secretion of oxytocin (OXT) and social behavior. GPCR, G protein-coupled receptor.



Jin et al. (Jin et al., 2007) investigated CD38 gene knockout mice and discovered that CD38-dependent cADPR- and NAADP-sensitive intracellular Ca^{2+} mobilization plays an important role in oxytocin release from soma and axon terminals of hypothalamic neurons, exerting profound actions in social behavior. CD38 knockout mice demonstrated significantly greater locomotor activity, but no or little emotional abnormalities such anxiety and fear compared to their wild type littermates. However, their maternal behavior was significantly impaired and social amnesia in males was evident. Depolarization-induced increases in intracellular Ca^{2+} concentration were significantly lower in $CD38^{-/-}$ mice, whereas, in wild-type mice, this was effectively blocked by inhibitors of cADPR-signaling pathways. These experiments confirm the role of CD38 enzymatic activity in the regulation of Ca^{2+} homeostasis in this cell type. In addition, oxytocin secretion from hypothalamic neurons and from axon terminals in the posterior pituitary gland was significantly reduced. Several findings indicate that the metabolites of ADP-ribosyl cyclase are essential for oxytocin secretion and

suggest that the deficit in oxytocin secretion in CD38 knockout mice may be a result of reduced ADP- rybosil cyclase activity.

Regarding the lower secretion of oxytocin in CD38 knockout mice, other mechanisms involving acidic lysosomes or granules have been proposed and other possibilities, such as unknown interactions of CD38 with the neurosecretory machinery or a channel effect of CD38 to facilitate release of neuropeptides, cannot be excluded. In wild mice, the highest CD38 mRNA levels were found in the hypothalamus, which is also characterized by high levels of ADP-ribosyl cyclase activity (Jin et al., 2007). Complementarily, CD38 knockout mice show reduced CD38 immunoreactivity in the hypothalamic periventricular region and lower plasmatic concentrations of oxytocin. In contrast, CD38 knockout mice also showed higher concentrations of oxytocin in the hypothalamus and posterior pituitary, indicating overstorage. This overstorage is probably associated with impaired release, which leads to the observable deficits in social behavior.

1.5. Genetical studies

1.5.1.Autistic traits, behavioral phenotype and intermediate phenotypes.

The participation of the oxytocin system in several psychiatric conditions was investigated and positive associations were found with autistic spectrum disorders, obsessive-compulsive disorder, eating disorders, addiction, schizophrenia, post-traumatic stress disorder and Prader-Willy syndrome. Among them, the autistic spectrum disorders have been studied with special interest due to their significant impairment in social behavior (Marazziti & Dell'Osso, 2008).

The term autistic spectrum disorders describes a series of neurodevelopmental disorders characterized by deficits in social reciprocity, impaired communication, and repetitive restricted patterns of behaviors and interests. The symptoms can be identified before the age of 3 years, and result in functional impairment. The first description of autism was made by Kanner in 1943, who presumed a very low prevalence of the disorder in the general population. Nowadays, the prevalence of autistic

spectrum disorders in the general population is thought to be around 1:110 people and is evidently higher than the prevalence of 5:10.000 described in the 1990s.

Although autism spectrum disorders (ASD) probably manifst themselves when an array of possible genetic vulnerabilities are present, possible in concert with epigenetic factors and gene-environment interactions, the concordance of autism in monozygotic twins is around 88% (Blenner, Reddy, & Augustyn, 2011). In addition, the hereditability of autistic traits (i.e. problems or peculiarities in sociocommunicative behavior, perception of others and self, and adaption to the environment that do not meet formal criteria for an ASD) and of social behavior were demonstrated and are well accepted (Scourfield, Martin, Lewis, & McGuffin, 1999; Knafo & Plomin, 2006; Hoekstra, Bartels, Verweij, & Boomsma, 2007). The last cited study evaluated a sample of twins aged 18 years, their siblings and parents and found that autistic traits are continuously distributed in the general population, men have higher scores than women and individual differences in autistic traits scores show substantial heritability (57%). In addition, relatives of

individuals with ASD show elevated levels of autistic traits (Bishop, Maybery, Wong, Maley, & Hallmayer, 2006; Constantino et al., 2006), suggesting that subclinical autistic traits share genetic factors with diagnosed ASD. The distribution of autistic traits in the general population is demonstrated to be a normal curve with extremes representing a small quote of this population, and common genetic variants present in a significant percentage of the general population are thought to play a role in the etiology of autism (Chakrabarti et al., 2009; Wang et al., 2009; Ronald et al., 2010; Anney et al., 2010). Recently, Lundström et al. (2012) demonstrated that ASD and autistic traits have the same etiology and suggested that ASD is the lower extreme of a continuum of social and communication skills. Although it was recently suggested that environmental factors common to twins explain around 55% of the liability to autism (Hallmayer et al., 2011), the heritability or the proportional liability attributed to genetic factors in former twin studies was around 80%-90% and genetic factors have been more extensively

investigated (Bailey et al., 1995; Lichtenstein, Carlstrom, Rastam, Gillberg, & Anckarsater, 2010).

Some twin studies report that autistic traits, as assessed using quantitative scales such as the Childhood Autism Spectrum Test (CAST) (Williams et al., 2008), Autism-spectrum Quotient (AQ) (Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001), and Social Responsiveness Scale (SRS) (Constantino et al., 2003a), show a smooth distribution in community samples, and heritability estimates range from 36% to 87% in twin samples. Other studies in middle-to-late childhood report modest shared environmental influences ranging from 10% to 32% (Constantino & Todd, 2003b; Constantino & Todd, 2005; Constantino & Todd, 2000; Ronald, Happe, & Plomin, 2008). The studies mentioned above give support to the idea that autistic traits are continuously distributed in the general population and are genetically transmitted as championed by a number of autism researchers (Baron-Cohen et al., 2001; Constantino et al., 2003b; Skuse, Mandy, & Scourfield, 2005; Ronald et al., 2006; Allison et al., 2008; Hoekstra, Bartels, Cath, & Boomsma, 2008). These authors also defend that the

strong heritability is not limited to the clinical autism spectrum disorders, but also accounts for variance in autistic traits in the general population.

Among the several behaviors investigated as possible heritable autistic traits, special attention was given to language and communication, reciprocal social interaction and repetitive stereotyped behavior and interests, which are the core symptoms of autistic spectrum disorders. The current consensus indicates that language delay, social-pragmatic problems, and impairments in spontaneous narrative discourse and reciprocal social interaction are heritable autistic traits, since studies that evaluated families and relatives of children with autistic spectrum disorders found a higher prevalence of these traits in relatives of autistic probands than expected by chance. A small number of studies evaluated repetitive and stereotypic behavior and included only a broadly defined set of behavior like reports of real-life non social-skills and preferences and a rigid/perfectionist personality. Nevertheless, they also found that problems in these behavioral dimensions are

shared among relatives and probably have a high heritability (Sucksmith, Roth, & Hoekstra, 2011).

With regard to the cognitive functioning, relatives of people with autism perform poorer on tasks that assess social cognition, i.e. “Theory of Mind” or the ability to process information relating to other people’s mental states. Relatives of people with autism perform poorer on the “Reading the Mind in the Eyes” Test (Baron-Cohen, Wheelwright, Hill, Raste, & Plumb, 2001), in which the participants have to identify complex psychological states from looking at pictures of the eye region of people’s faces (Dorris, Espie, Knott, & Salt, 2004; Gokcen, Bora, Erermis, Kesikci, & Aydin, 2009). Palermo, Pasqualetti, Barbati, Intelligente, & Rossini (2006) asked parents of autistic probands to identify schematic facial patterns representing five basic emotions and fathers performed worse than mothers, while both performed worse than the control group. Likewise, two additional studies report that relatives of people with autism have lower performance in emotion-recognition (Wallace, Sebastian, Pellicano, Parr, & Bailey, 2010; Bolte & Poustka, 2003). There is also evidence that

relatives of people with autism have lower scores on tasks that evaluate face memory and recognition, and eye gaze processing (Duchaine & Nakayama, 2006; Wallace et al., 2010). Several studies with some methodological problems and contradictory findings suggest that in the executive functions domain, superior performance on the spatial span task, higher level planning deficits and ideational fluency difficulties may be additional heritable cognitive phenotypes associated with autistic spectrum disorders. Others provide support for visual attention difficulties in the first degree relatives of autistic probands, especially attentional engagement/ disengagement, divided attention and oculomotor abnormalities, with mixed findings for local visual attention biases (Sucksmith et al., 2011). Finally, research on personality traits, which are believed to reflect an underlying genetic liability for autism, lacks specificity and is currently inconclusive. The personality characteristics more commonly described in the relatives of autistic probands compared to relatives of typically developing children or children with another medical condition include rigid, impulsive, aloof, shy, tactless,

reserved /schizoid, irritable, hypersensitive to criticism, neurotic, undemonstrative, and anxious. A factor analysis detected three clusters of personality traits that were more common in relatives of autistic probands compared to relatives of Down syndrome probands; these were called withdraw, difficult and tense. However, only the withdraw factor was significantly associated with the broader behavioral phenotype of autism (Murphy et al., 2000). Additionally, one study suggests that some personality traits are associated with poor performance in social cognition tasks (Losh et al., 2009).

Beside the study of behavioral and cognitive phenotypes, several authors have investigated functional and anatomical changes in relatives of people with autism. These alterations may represent the physiological counterpart of genetic predisposition and maybe be correlated with performance in different domains, including social tasks and behavioral problems. Parents of autistic probands have atypical brain activity during the “Reading the mind in the eyes” task compared to matched controls (Baron-Cohen et al., 2006). There was reduced activity in the mid-temporal gyrus and the

inferior frontal gyrus during completion of the mentalising task. Likewise, siblings of autistic probands have significantly reduced fMRI activity when responding to happy vs. neutral faces during an emotion recognition task (Spencer et al., 2011) and reduced levels of gaze fixation, which were associated with decreased activity within the right hemisphere in the fusiform gyrus, an important structure for face processing and gaze direction (Dalton, Nacewicz, Alexander, & Davidson, 2007). Commonalities between children with autism and their siblings were also found during tasks that assess sensitivity to biological motion. The results implicate shared areas of atypical function in the left dorsolateral prefrontal cortex, the right inferior temporal gyrus and the bilateral fusiform gyrus (Kaiser et al., 2010). Abnormal activity during visual attention was also identified in relatives of autistic probands. Their parents showed reduced activation of the right middle occipital gyrus whereas their siblings performed significantly less well than controls on a visual-divided attention task, which was associated with atypical fronto-cerebellar activation (Baron-Cohen et al., 2006; Belmonte, Gomot,

& Baron-Cohen, 2010). Consequently, some authors suggest that at a neurobiological level, relatives of individuals with autism share subtle disruptions in brain function that are not necessarily identified at a behavioral level. Some of the alterations found in these studies could be compensations of a genetic vulnerability, and the others, the shared hereditary functional abnormalities.

Additional findings, which describe possible intermediate phenotypes for autism, are the absence of right-hemisphere lateralized augmentation of the N170 event related potential to faces as well of the shorter latency N170 to faces (versus object) (Dawson et al., 2005), a reduction in steady state gamma-band responses in magnetencephalographie (Rojas et al., 2011), larger left hippocampus volumes (Rojas et al., 2004) and increase gray matter in the inferior and medial frontal gyri and cerebellum (Peterson et al., 2006). All these alterations cited above were found in parents of autistic probands. A prolonged latency in the P400 component in response to direct eye gaze (Elsabbagh et al., 2009), a significant reduction in the volume of amygdala (Dalton et al., 2007) and significantly reduced white matter fractional

anisotropy values (Barnea-Goraly, Lotspeich, & Reiss, 2010) were found in autism and in their siblings.

These findings as a group suggest that autistic traits are genetically transmitted and that functional correlates of low social functioning may be found in people who are not classified as having an autistic spectrum disorder but as having a mild to moderate impairment in communication and social skills. It is also hypothesized that these people would have a greater chance of having children with autistic spectrum disorders or children with mild to moderate impairment in social behavior, which may also show functional and structural abnormalities found in autistic spectrum disorders. Like the autistic traits, functional and structural abnormalities are also normally distributed in the general population and would reflect the neurophysiological counterpart of poor social skills.

1.5.2. OXTR and CD38 gene and social behavior

As demonstrated above, the heritability of social behavior is well described and the preservation of neuropeptides along the evolution is observed (Scourfield et al., 1999; Knafo et al., 2006). Given that, genetical studies on social behavior could reveal the genetical variants associated with individual differences on social behavior and shed some light on the causes of some pathological conditions such as ASD. Linkage data and disease association with common variants of OXTR confirmed their role as risk factors for ASD (Yrigollen et al., 2008; McCauley et al., 2005; Jacob et al., 2007; Ylisaukko-oja et al., 2006; Wu et al., 2005; Wermter et al., 2010; Lerer et al., 2008). Among these studies, two genome wide scans highlight the 3p25 region, containing the OXTR, as a putative linkage site for ASD (Lauritsen et al., 2006; McCauley et al., 2005). Concomitantly, three single nucleotide polymorphisms are described as the most promising candidates in the pathophysiology of ASD: rs53576 (G to A), rs2254298 (G to A) and rs2270465 (C to G). These more recent alleles are overtransmitted to offspring diagnosed as having an ASD

and form a central component in ASD-related haplotypes (Wu et al., 2005; Wermter et al., 2010). Lerer et al. (2008) showed in an association study that certain variants of SNPs and haplotypes in the OXTR confer risk for ASD and were associated with lower IQ and social adaptive scores whereas a study with 195 Chinese Han trios found a significant association between autism and two OXTR SNPs (rs2254298 A and rs53576 A) (Wu et al., 2005). Jacob et al. (2007) found significant association with a single SNP variant (rs2224298) in a sample of Caucasian children and adolescents with autism and Yrigollen et al. (2008) examined three OXTR SNPs and observed an association between a rs2268493 variant and ASD. Positive results were also found in the Japanese population by Liu et al. (2010). Despite negative results in the family-based association test, these authors report significant differences in allelic frequencies of four SNPs, including the rs2254298, between patients and controls in a population based case-control study. In contrast, one study on 18 polymorphisms of the OXTR in 436 subjects showed negative results and does not support the role of common

genetic variation in the OXTR in the etiology of autism spectrum disorders in Caucasian samples (Tansey et al., 2010). Together with studies on OXTR knockout mice (Pobbe et al., 2012) and social behavior in human beings highlighted bellow, these data support the hypothesis that variations in this gene are associated with significative individual differences on social behavior and may be implicated in the pathogenesis of autism spectrum disorders.

Regarding social behavior and different OXTR polymorphic variations, there is also evidence that supports the association of risk allele carriers with childhood aggression (Malik, Zai, Abu, Nowrouzi, & Beitchman, 2012), aggressive behavior and trait anger modulated by alcohol consumption in women (Johansson et al., 2012), lower tendency of exculpating accidentally committed harms (Walter et al., 2012), anxiety and depression in adolescent girls (Thompson, Parker, Hallmayer, Waugh, & Gotlib, 2011), poor social ability in children diagnosed with attention deficit hyperactivity disorder (Park et al., 2010), and lower allocations in social games as Dictator Game (Israel et al., 2009). Inoue

et al. (2010) examined seven SNPs of OXTR and amygdala volume in 208 socially intact subjects without neuropsychiatric history and current diagnosis. Carriers of the rs2254298A allele showed larger bilateral amygdala volume, which was proportional to the allele's dose and gave additional support to the hypothesis that the oxytocin effect on behavior is also modulated by the amygdala function.

Allele variability on the OXTR polymorphism rs53576 has also been implicated in observable differences in social behavior by several authors. Male individuals with the OXTR rs53576 A/A genotype showed low positive affect scores and adolescents with this same genotype presented lower levels of non-verbal IQ. Haplotypes including this SNP were associated with lower positive affect, higher negative affect and emotional loneliness (Lucht et al., 2009). A allele carriers also show lower behavioral and dispositional empathy, higher physiological and dispositional stress reactivity (Rodrigues, Saslow, Garcia, John, & Keltner, 2009) and reported more attachment insecurities (Gillath, Shaver, Baek, & Chun, 2008). Complementarily, GG genotype

was related to less self-reported difficulty in hearing and understanding people in the presence of background noise (Tops, van Ijzendoorn, Riem, Boksem, & Bakermans-Kranenburg, 2011). Controlling for differences in maternal education, depression and marital discord, parents with the possibly less efficient variants of the OXTR polymorphism rs53576 (AA/AG) also showed lower levels of sensitive response to their toddlers (Bakermans-Kranenburg & van Ijzendoorn, 2008). Kim et al. (2010) examined the behavior of Korean and American participants regarding their tendency to seek social support in times of distress and found a three-way interaction between the OXTR polymorphism rs53576, amount of stress and culture. Among the distressed Americans, those with the GG/AG genotypes reported seeking more emotional support, compared with those with the AA genotype, whereas in the whole group of Korean participants and Americans under conditions of low stress, significant differences between the groups were not shown. Additionally, carriers of the A allele have lower levels of optimism, mastery and self-esteem (Saphire-Bernstein, Way, Kim,

Sherman, & Taylor, 2011), exhibit less affiliative cues during social interactions and thence are judged to be less sociable by their observers (Kogan et al., 2011). Accordingly, male homozygotes for the allele G show higher levels of trust behavior (Krueger et al., 2012) and a reduced cortisol level in a standardized psychological stress procedure when accompanied by social support of a female partner or of a close female friend, which indicates the occurrence of a suppressive social modulation on the HPA axis (discussed in details bellow) that is not evident in A carriers (Chen et al., 2011). As a result, carrying the A allele is considered a risk for impairments in social behavior and Tost et al. (2010) reported a decrease in hypothalamic gray matter volume, increase right amygdala, increased coupling between hypothalamus and amygdala, lower scores for reward dependence (an indirect measure of social engagement) and lower amygdala activation during emotional face processing in A allele carriers. In conjunction, these studies point out that the brain circuitry associated with oxytocin release and action is conspicuously involved in the regulation of social behavior and that the common

variations of the OXTR explain part of the observed differences in social functioning in clinical and non-clinical populations. However, studies that evaluate concomitantly genetic variations, brain function and behavioral phenotypes are not numerous and a recent meta-analysis on the two most studied SNPs did not confirm their role as determinants of social behavior, personality and psychopathology in humans (Bakermans-Kranenburg & van Ijzendoorn, 2013).

The CD38 gene is located on the short arm of chromosome 4(4p15) and consists of eight exons. A recent genome-wide linkage scan found the 4p15 region to have suggestive evidence for linkage to autism spectrum disorders (Ebstein et al., 2009). A variety of polymorphisms have been described in the CD38 gene and some were associated with Type II diabetes, systemic lupus erythematosus, and premenopausal and postmenopausal bone mineral density (Ferrero, Saccucci, & Malavasi, 1999; Yagui et al., 1998; Gonzalez-Escribano, Aguilar, Torres, Sanchez-Roman, & Nunez-Roldan, 2004; Drummond et al., 2006). Unlike the relatively large number of genetical studies on OXTR

and social behavior, investigations on the CD38 gene are recent and comprise a smaller number of studies. Ebstein et al. (2009) genotyped 12 tagging SNPs spanning across the CD38 gene in 170 trios diagnosed with ASD and found an association of two categorical measures of ASD and CD38 SNPs. Measures of social skills were associated with four SNPs variants (rs4634217:A, rs4516711:G, rs4508877:T and rs3796867:T) while CD38 mRNA levels in lymphoblastic cells correlated with scores in the Vineland Adaptive Behavior Scales, which indicates the level of social support the probands need. A highly significant reduction in CD38 expression in human lymphoblastic cell lines derived from ASD subjects in comparison to their unaffected parents was also found (Lerer, Yirmiya, Salomon, & Ebstein, 2010). Riebold et al. (2011) replicated the finding that CD38 mRNA levels in lymphoblastic cells are correlated with scores in the Vineland Adaptive Behavior Scales and implied one polymorphism (rs6449182, C>G variation) as one of the possible causal factors.

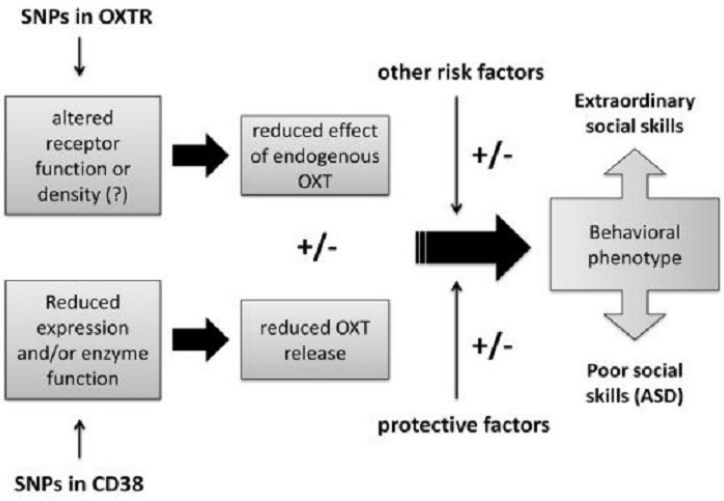
Munesue et al. (2010) examined the immunohistochemical expression of CD38 in the

hypothalamus of post-mortem brains of non-ASD subjects and found that CD38 was colocalized with OXT secretory neurons. Additionally, they analyzed 10 single nucleotide polymorphisms and mutations on the CD38 gene from Japanese and Caucasian cases. Two polymorphisms (rs6449197 and rs3796863) showed significant associations with the subset of high functioning autism (IQ>70) in the Caucasian population but not in the Japanese subjects. A mutation that caused tryptophan to replace arginine at amino acid residue 140 (rs1800561, 4693C>T) was found in 0.6-4.6% of the Japanese population and was associated with ASD and with lower levels of plasma OXT. The association of the polymorphism rs3796863 with ASD was replicated in 170 Israeli. But in contrast to previous findings, the association was found in low functional autistic probands (IQ<70) (Lerer et al., 2010). In a recent study, carriers of the risk allele of the polymorphism rs3796863 showed lower levels of plasma OXT and less parental touch during interaction with their infants while the interaction of high plasma OXT and low-risk allele predicted longer

duration of parent-infant gaze synchrony (Feldman et al., 2012).

Finally, Sauer, Montag, Worner, Kirsch, & Reuter (2012) demonstrated that homozygotes for the risk alleles of this same polymorphism (CC) have slower reaction times and greater activation of the left fusiform gyrus during visual processing of visual stimuli. This difference was enhanced with the administration of intra-nasal oxytocin, although the reaction times of risk allele carriers were still evidently reduced, which suggests that the availability of oxytocin may modulate fusiform gyrus activity and consequentially social behavior. In conclusion, the presence of some risk alleles of CD38 and OXTR genetic polymorphisms could lead to reduced OXT plasma levels or abnormal oxytocin action and consequentially lead to ASD, when associated with additional risk factors and/or lack of protective factors, or to mild to moderate social deficits and the presence of autistic traits (figure 2).

Figure 2 (below). Hypothesis of how single nucleotide polymorphisms lead to altered social behavior. SNPs of human CD38 may cause lower levels of oxytocin in the brain or plasma through lowering the expression or enzymatic activity of the CD38 gene or receptor. SNPs of OXTR could be hypothetically associated with impairments of OXT receptor function with consecutive reduced OXT function. This system is also under influence of other environmental and non-environmental factors. ASD may be triggered by other strong risk factors and/or other weaker protective factors, while ASD may not be induced by other weaker risk factors and/or other strong protective factors, including estrogens.



1.6. Oxytocinergic Modulation of Stress Reaction

1.6.1. The classical stress reaction and its indicators

Stress is defined as a threat to the organism's homeostasis which goes together with an activation of the sympathetic-adrenomedullary system and the hypothalamic-pituitary-adrenal axis (Selye, 1955). The entire stress response is dependent on a complex system, the stress system, which is located in both the CNS and the periphery of the body. In the CNS, the main components of the stress system are located in the hypothalamus and in the brain steam and include the parvocellular neurons of the hypothalamus, which produce the corticotrophin-releasing-hormone (CHR), the CRH containing neurons of the paragigantocellular and parabranchial nuclei of the medulla and the locus coeruleus (LC), the arginine vasopressin (AVP) neurons of the paraventricular nucleus (PVN) of the hypothalamus, and other predominantly noradrenergic (NE) cell groups in the medulla and the pons, including the LC. The peripheral components of the stress system include the peripheral parts of the hypothalamic-

pituitary-adrenal (HPA) axis (i.e. adrenal gland), the efferent sympathetic-adrenomedullar (SAM) system, and components of the parasympathetic system (Campeau, Day, Helmreich, Kollack-Walker, & Watson, 1998; Pacak & Palkovits, 2001; Stratakis & Chrousos, 1995).

A variety of stressors can stimulate the release and production of CRH in the PVN of the hypothalamus. CRH, via the portal circulation, in conjunction with arginine vasopressin, stimulates the pituitary to produce adrenocorticotropic hormone (ACTH), which is released into the blood stream and triggers the release of glucocorticoids by the adrenal glands (cortisol in humans and other primates and corticosterone in mice and rats). Theses hormones are essential to life and regulate or support a variety of important cardiovascular, metabolic, immunologic, and homeostatic functions, which help the organism adapt to stressful circumstances. Glucocorticoids exert their actions via intracellular receptors, which exist in their inactive state in the cytosol within a complex assembly of heat shock proteins that serve to stabilize the unbound receptor. Once glucocorticoids are bound to these receptors, the

translocation to the nucleus occurs. There, these complexes (receptor-glucocorticoid) either interact with other transcription factors or bind to specific DNA response elements with a resultant up- or down-regulation in the expression of various genes (Nater, 2004). Glucocorticoids have an inhibitory effect on CRH and ACTH secretion (negative feedback), which serves to limit the duration of exposure to glucocorticoids and minimizes their catabolic, lipogenic, anti-reproductive and immunosuppressive effects (Charmandari, Kino, Souvatzoglou, & Chrousos, 2003).

Several different stimuli can activate the HPA axis and psychological stress is among the most potent. Situations that are perceived as new, unpredictable or uncontrollable are closely related to an increased secretion of cortisol and the two most eminent psychological factors eliciting this response are emotional ego-involvement and tense anticipation of a potentially harmful event. Considering this, Kirschbaum and colleagues elaborated a potent laboratory stress paradigm, the Trier Social Stress Test (TSST), which has been evaluated extensively (Brody, 2002; BuskeKirschbaum et al., 1997; Clow, Patel, Najafi,

Evans, & Hucklebridge, 1997; Domes, Heinrichs, Reichwald, & Hautzinger, 2002; Chen et al., 2011; Pierrehumbert, Torrisi, Ansermet, Borghini, & Halfon, 2012; Maruyama et al., 2012; Morris, Rao, & Garber, 2012). Psychologically stressful situations activate the HPA axis through the limbic system, amygdaloid nuclei, and the bed nucleus of the stria terminalis while the hippocampal and prefrontal regions are responsible for inhibiting CRH, ACTH and cortisol release (Sapolsky, 2003; Herman & Cullinan, 1997; Ledoux, 2000; Davidson & Irwin, 1999).

Beside the HPA axis, the autonomic nervous system (ANS) is also activated during stress and responsible for the relatively rapid changes in cardiovascular, gastrointestinal, electrodermal, respiratory, endocrine, and exocrine functions observed in fight or flight reactions. The main substances acting within the ANS are the three endogenous catecholamines norepinephrine (NE), epinephrine (EP) and dopamine (DA). Noradrenergic neurons are of utmost importance in eliciting physiological and behavioral modification during stress. They synthesize the catecholamines from

the precursor thyrosin, which circulates in the cell plasma. These neurons are located in the locus coeruleus, the lateral tegmental cell system and in the pontodorsal medullary cell group. The locus coeruleus is responsible for the greatest production of catecholamines and targets the amygdala, olfactory bulb, medial septum nucleus, nuclei periventricularis and paraventricularis in the hypothalamus, and the whole cortex. Norepinephrine (NE) is a neurotransmitter released at the postganglionic synapses of the sympathetic nervous system (SNS), which is responsible for a series of peripheral physiologic responses in several tissue types. The adrenal medulla is a special part of the autonomic nervous system (ANS). Its glandular cells are modified cells of the SNS and release norepinephrine and epinephrine in the blood stream upon activation by preganglionic sympathetic nerve cells. The response of the ANS triggered by stress can be artificially divided into central and peripheral responses which in conjunction determine the behavioral and physiological modifications observed (Nater, 2004). The release of catecholamines influences the response of the endocrine system and the hypothalamic CRH and

ACTH secretion by the pituitary gland are controlled by different groups of afferent impulses in the CNS, including the locus coeruleus complex. Central noradrenalin seems to stimulate the secretion of ACTH via activation of alpha-1-receptors and influences a series of cognitive functions, mediating an increase of vigilance (Goldstein, 1995).

Salivary and plasmatic concentrations of cortisol and catecholamines have been used as indicators of stress reactivity and, respectively, as indicators of HPA and SNS activity when several methodological aspects are observed (Kirschbaum & Hellhammer, 1994; Kirschbaum, Wust, Faig, & Hellhammer, 1992; Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). More recently, the salivary alpha-amylase concentration has also been investigated as an indicator of stress and significant positive correlations between alpha-amylase and catecholamines concentrations were also established, which indicates that alpha-amylase could be seen as a measure of adrenergic activity (Morse, Schacterle, Esposito, Furst, & Bose, 1981; Morse, Schacterle, Furst, & Bose, 1981; Bosch et

al., 1996; Bosch et al., 1998; Chatterton, Vogelsong, Lu, Ellman, & Hudgens, 1996; Chatterton, Vogelsong, Lu, & Hudgens, 1997). Alpha-amylase is one of the most important salivary enzymes, and was first isolated in this secrete in 1831 and was found in serum and urine in 1846 and 1863 (Zakowski & Bruns, 1985). It is a calcium-containing digestive metalloenzyme that hydrolyzes the alpha 1,4 linkages of starch to glucose and maltose and has bacterial interactive function. The secretion of alpha-amylase occurs mainly in the salivary glands and pancreas although amylase activity has been found in different tissues and fluids including lung, sweat, leukocytes and thrombocytes, colostrum and milk, tears, tonsils, thyroid, endometrium, semen, the female genital tract etc. (Nater, 2004). During stress, sympathetic and parasympathetic stimulations lead to an increase of salivary alpha-amylase concentration. Studies investigating the isolated action of one of these systems are not considered physiologic, since these two branches of the autonomical nervous system do not act independently. While sympathetic stimulation (via norepinephrine), probably through β-1 receptors, leads to

increased alpha-amylase concentration, parasympathetic stimulation (via acetylcholine) leads to intense fluid secretion. Other evidences that this enzyme could be used as a maker of autonomic response were shown by several authors, who described increases of its concentration during physical stressors such as treadmill exercise (Gilman, Thornton, Miller, & Biersner, 1979), exposure to a high pressure chamber (Gilman, Fischer, Biersner, Thornton, & Miller, 1979), bicycle exercise (Walsh et al., 1999), running (Nexo, Hansen, & Konradsen, 1988), or cold exposure (Chatterton et al., 1996).

1.6.2. Oxytocin as a modulator of stress response

Animal studies demonstrated that a variety of stressful stimuli, including fear conditioning stimuli, immune challenge with interleukin-1β, restrain stress and electric shocks activate hypothalamic oxytocinergic neurons and promote oxytocin release in the blood circulation (Onaka, 2004). In the first report of an oxytocinergic response to stress in humans, Sanders et al. (Sanders, Freilicher, &

Lightman, 1990) described an increase in oxytocin plasmatic concentrations in women during stimulation with uncontrollable noise in contrast to controllable noise. Adults that had cancer during childhood in comparison to women who experienced child sexual abuse and controls exhibit higher mean plasmatic levels of OXT during the Trier Social Stress Test (Pierrehumbert et al., 2010). Increased levels of OXT were found in people with relational distress, romantic attachment anxiety and subclinical depression and were also associated with greater social dissatisfaction and severity of the disorder in social phobia (Taylor, Saphire-Bernstein, & Seeman, 2010; Marazziti et al., 2006; Holt-Lunstad, Birmingham, & Light, 2011; Hoge, Pollack, Kaufman, Zak, & Simon, 2008). Nonetheless, it has been shown that patients with major depression have reduced plasma oxytocin concentration (Frasch, Zetzsche, Steiger, & Jirikowski, 1995; Ozsoy, Esel, & Kula, 2009) and that oxytocin concentration in the cerebrospinal fluid is low in women with a history of childhood abuse (Heim et al., 2009). Recently, low CSF oxytocin concentration was

also related to high intent in suicide attempters (Jokinen et al., 2012).

Release of oxytocin into the peripheral circulation during stress seems to be dependent on the task and varies considerably in different species. Exercise of prolonged endurance induces oxytocin release into the blood circulation in humans as well exercise of short duration and high intensity in horses (Hew-Butler, Noakes, Soldin, & Verbalis, 2008; Hada, Onaka, Takahashi, Hiraga, & Yagi, 2003). Oxytocin is not only secreted into the blood by axons terminals in the posterior pituitary but also by dendrites in different brain regions. Such release was shown within limbic brain regions including the septum, the hippocampus and the central amygdala, and also within the brain stem and the suprachiasmatic nucleus. Importantly, oxytocin is also released within its nuclei of origin, i.e. the hypothalamic supraoptic and paraventricular nuclei (Neumann, 2007a). Oxytocin release caused by different stressful stimuli supports the hypotheses that this neuropeptide is involved in the regulation of stress response in different species. The intracerebral release is also stimulated by

pharmacological stressors like local or peripheral administration of hyperosmotic solutions (Landgraf & Ludwig, 1991; Ludwig, Callahan, Neumann, Landgraf, & Morris, 1994), treatment with cholecystokinin octapeptide (Neumann, Landgraf, Takahashi, Pittman, & Russell, 1994), interleukin-1β (Landgraf, Neumann, Holsboer, & Pittman, 1995) and naloxone-induced morphine withdrawal in morphine dependent rats (Russell, Neumann, & Landgraf, 1992). In male rats, social defeat by a large and aggressive conspecific selective stimulates oxytocin release within the supraoptic nucleus while blood concentration remains unchanged. Virgin females exposed to maternal defeat, a social defeat by a lactating female, have an increased concentration of oxytocin within the paraventricular nucleus. The same is observed in lactating females with high levels of aggressive behavior and anxiety traits during exposure to a virgin intruder and defense of their offspring (Bosch, Kromer, Brunton, & Neumann, 2004; Wigger & Neumann, 2002). Forced swimming provokes oxytocin release within both nuclei and into the blood circulation while 10 minutes of shaker stress promotes

release within the paraventricular nucleus (Wigger et al., 2002; Wotjak et al., 1998; Nishioka, nselmo-Franci, Li, Callahan, & Morris, 1998). The dendritic release does not always correspond to the release into the peripheral circulation and these two systems do not always work in perfect consonance, which could explain why some of the observed behavioral and physiological effects are not accompanied by increased concentration in blood.

The influence of oxytocin on the HPA axis has also been investigated. Single injections of oxytocin in rats (1mg/kg s.c.) are associated with acute transient increases in ACTH and corticosterone plasma concentrations followed by decreases under the baseline after 6 hours. The same dose injected once a day for five days decreases corticosterone for ten days after the last injection (Petersson, Hulting, & Uvnas-Moberg, 1999). Intracerebroventricular injections of oxytocin for 5 days in rats reduce cortisol release during stress in a dose dependent manner as well anxiety and rearing behaviors (Windle et al., 1997). Similarly, intracerebral infusion of oxytocin antagonists is associated with increased activity of the HPA axis as indicated by enhanced basal and

stress-induced secretion of corticosterone and ACTH into the blood (Neumann, Wigger, Torner, Holsboer, & Landgraf, 2000). The mechanism whereby oxytocin modulates the HPA axis function is not completely understood but one study on rats demonstrated that intracerebral infusion attenuates neuron activity in the paraventricular nucleus, ventrolateral septum and dorsal hippocampus during stress (restraint) (Windle et al., 2004). The intranasal administration for 8 days is also able to reduce the blood concentration of ACTH 90 minutes after social isolation in adult female squirrel monkeys (Parker, Buckmaster, Schatzberg, & Lyons, 2005).

The physiologic effects of oxytocin in the HPA axis are accompanied by behavioral modifications, which are caused by the direct effect of oxytocin in the brain and by the attenuation of the physiological stress response. For example, oxytocin is released in the paraventricular nucleus of male rats during mating with a receptive female and is followed by reduced anxiety during psychological stress, which is observable for at least 4 hours after mating (Waldherr & Neumann, 2007). The

loss of social preference and the social avoidance observed in rats after a social defeat can be reverted by intracerebroventricular infusion of oxytocin (Lukas et al., 2011). Beyond oxytocinergic inhibition of the physiological responses to stress, the intracerebroventricular infusion of oxytocin antagonists also stimulates the secretion of the excitatory amino acids glutamate and aspartate in the central amygdala during forced swimming, increasing swimming and decreasing floating time (Ebner, Bosch, Kromer, Singewald, & Neumann, 2005). Social support following intense psychological stress (i.e., immobilization stress) promotes the release of oxytocin in the paraventricular nucleus and attenuates the physiological and behavioral stress response toward a subsequent stressor (i.e., elevated plus maze) in pair bounded female prairie voles (Smith & Wang, 2012). Furthermore, some of the behavioral effects attributed to oxytocin might be enhanced or diminished by the indirect action of the HPA. To illustrate this assumption, partner preference is stimulated in adrenalectomized female prairie voles and inhibited by the administration of corticosterone and

exposition to psychological stress (forced swimming test) (DeVries, Devries, Taymans, & Carter, 1995; DeVries, Devries, Taymans, & Carter, 1996).

The influence of oxytocinergic activity on stress response has also been investigated in humans. Legros, Chiodera, Geenen, & Vonfrenckell (1987) investigated the influence of low-dose oxytocin i.v. infusion in 8 men and reported decreased plasma ACTH and cortisol in comparison to control saline injection. More recently, Ditzen et al. (2009) demonstrated that intranasal administration of oxytocin promotes positive behavior in a couple-conflict task and is associated with a reduced cortisol salivary concentration in comparison with placebo. Evidence that oxytocin modulates the stress-induced cortisol response is similarly presented by other authors. Heinrichs, Baumgartner, Kirschbaum, & Ehlert (2003) compared salivary cortisol levels during the Trier Social Stress Test in a randomized placebo-controlled design in which subjects received a placebo or oxytocin and social support from a close friend or not. Their main findings were that social support buffers the cortisol response to stress, that oxytocin has anxiolytic effects and that the

group, which received oxytocin and social support, presented the lowest levels of cortisol and anxiety. Likewise, subjects with impaired emotional regulation abilities benefit from intranasal administration of oxytocin during social stress and also present lower plasmatic concentrations of cortisol when compared with group treated with placebo (Quirin, Kuhl, & Dusing, 2011). Complementarily, breast feeding women who have increased levels of OXT, also have an attenuated cortisol response to psychological stressors (Altemus et al., 1995; Heinrichs et al., 2001). Environmental factors also seem to play a role in the regulation of stress reaction modulated by oxytocin since early parental separation has been shown to reduce the buffering effects of oxytocin on cortisol response. This study suggests that oxytocin sensitivity in the HPA regulation may be set early in life (Meinlschmidt & Heim, 2007) and varies in accordance with life events and personality traits.

The effect of social support on the HPA axis response to stress could be modulated by the release of oxytocin and its actions on its respective receptors. Supporting this idea, Rodrigues et al., 2009) tested how one

polymorphism of the oxytocin receptor gene (rs53576) relates to empathy and stress reactivity. In comparison to homozygotes GG, individuals with one or two copies of the A allele (AG/AA) exhibit lower behavioral and dispositional empathy and displayed higher physiological and dispositional stress reactivity, as determined by heart rate response during a startle anticipation task and an affective scale. This same polymorphism also modulates the buffering effects of social support on the cortisol response to stress since only individuals with at least one copy of the G allele show lower cortisol levels during the Trier Social Stress test when compared with individuals with the same genotype receiving no social support (Chen et al., 2011). This suggests that social contact does not attenuate the stress reaction in AA homozygotes. Regarding the sympathetic activity during stress, one study showed that GG subjects generally display heightened stroke volume and cardiac output. On the other hand, A allele carriers displayed higher awakening cortisol levels and more variation in salivary cortisol across the day (Norman et al., 2012).

The release of oxytocin in different stressful circumstances, its higher and lower plasmatic and CSF concentrations associated with stressful life events, the inhibition of ACTH and cortisol release promoted by its intracerebral and venous administration in different species, the behavioral studies with intranasal administration and on the rs53576 SNP of the OXTR gene, support the idea that oxytocin is involved in the regulation of stress reaction and shed some light on the mechanisms through which social support diminishes anxiety and HPA responses to stress. However, the number of studies that describe the sympathetic response to stress modulated by the oxytocin system are less numerous and two studies on the SNP rs53576 of the OXTR present contradictory findings, showing that critical allele carriers have a higher and lower sympathetic response to stress.

2. Statement of purpose

It is a well accepted fact that the oxytocin system regulates the social behavior of different species, including human beings which is demonstrated by several studies cited above. However, it is also assumed that social behavior is a complex series of actions that involves different cognitive domains, perception modalities and emotional response, which are determined by the interaction of several individual and environmental biological factors. It was also demonstrated that the OXT receptor and CD38 common genetic variants are associated with the hereditability of autism (Yrigollen et al., 2008; McCauley et al., 2005; Jacob et al., 2007; Ylisaukko-oja et al., 2006; Wu et al., 2005; Wermter et al., 2010; Lerer et al., 2008) and several authors support the idea that autistic traits are normally distributed in the general population (Baron-Cohen et al., 2001; Constantino et al., 2003b; Skuse et al., 2005; Ronald et al., 2006; Allison et al., 2008; Hoekstra et al., 2008). Regarding this, classical autism would represent the lower extreme of a normal curve while people with extraordinary social ability would

represent the higher. Factor analytic approaches had also failed to show discontinuity between autistic spectrum disorders and autistic-like traits (Constantino et al., 2004) and in two twin studies no genetic or environmental thresholds could be discerned, which also indicates a continuum of symptoms (Ronald et al., 2006; Lundstrom et al., 2012). Additionally, a series of studies demonstrated that the same common genetic variants associated with autism are associated with differences in social ability in the normal population, which includes processing of social information (Tops et al., 2011), personality traits (Rodrigues et al., 2009) and different brain anatomy and activity during social tasks (Tost et al., 2010). Among these genetic variants, three of the most studied are the SNPs rs53576 and rs2254298 of the OXTR and rs3796863 of the CD38 gene. For these SNPs, several studies indicate an association between risk alleles and impairments on social behavior in the general population (Lucht et al., 2009; Bakermans-Kranenburg et al., 2008; Kim et al., 2010; Feldman et al., 2012; Sauer et al., 2012) and at least three studies pointed out that the critical alleles of these SNPs are overtransmitted in ASD

(Wu et al., 2005; Munesue et al., 2010; Lerer et al., 2010). However, the investigation of these genetical variants as determinants of autistic traits as measured by scales such as the Autism-spectrum Quotient (Baron-Cohen et al., 2001) and Social Responsiveness Scale (Constantino et al., 2003a) in the general population has not been a focus of attention of many studies. If the autistic traits are normally distributed in the general population and these genetical variants associated with autism are also associated with social deficits in the same population, it is presumed that these genetical variants may also be associated with the presence of autistic traits in individuals without a diagnosis of a mental disorder.

Among the different traits and deficits that an individual with autism can exhibit, three of them were systematically investigated in the general population by Baron-Cohen et al.: Empathizing, Systemizing and specific traits of autism (Wheelwright et al., 2006). Empathizing is one such specific component of social cognition and is defined as the drive to identify another person's emotion and thoughts, and to respond to these with an appropriate emotion. Empathy consists of an

affective and cognitive component, which respectively includes an observer's emotional response to the affective state of another and a "theory of mind" (attribution of a mental state and prediction of behaviors and feelings of someone else) (Baron-Cohen & Wheelwright, 2004). Systematizing is defined as the drive to analyze, understand, predict, control and construct rule-based systems, which is higher prevalent in autistic probands and in men. In contrary, females score higher on empathy while autistic probands have the lowest score (Wheelwright et al., 2006). Finally, specific autistic traits of autism can be understood as those traits that are important to the definition and diagnosis of the disorder, i.e. the criteria in the diagnose manuals. They include traits like attention to details and deficits in social skills, communication, attention switching and imagination (Baron-Cohen et al., 2001). Of interest is the fact that men tend to have a higher prevalence of specific autistic traits than women, which in association with the higher prevalence of autism in males, lead some authors to consider autism as an extreme expression of a "male brain" phenotype (Baron-Cohen, 2002). This extreme

male brain phenotype is normally characterized as a higher prevalence of specific autistic traits, higher tendency to systematizing and lower levels of empathy, which in fact, described the phenotype observed in ASD. Whether these heritable phenotypes, which are characterized by being extremes of a continuum in a given population, are determined by the SNPs rs3796863, rs2254298 and rs53576, has not been a subject of any study. The only genetic-wide association study of autistic traits in the general population, using a dimensional measure of autistic-like behaviors, (Ronald et al., 2010) found an association of three SNPs with autistic behaviors but none of them are directly related to oxytocin and linkage disequilibrium was not described in one autistic sample. The interaction of both SNPs in the determination of social behavior has not also been studied until this moment. Regarding the cited studies, it is plausible to assume that critical alleles of both SNPs are associated with the presence of autistic traits in a "dose-dependent" manner and that the interaction between them has a significant effect.

Furthermore, animal studies show that a variety of stressful circumstances are associated with the activation of oxytocinergic neurons in the hypothalamus and with oxytocin release in the blood circulation (Onaka, 2004). The experience of traumatic events is also associated with alterations in oxytocin secretion in humans (Pierrehumbert et al., 2010; Heim et al., 2009). In conjunction these findings led to the confirmed hypothesis that oxytocin is also involved in the regulation of the physiological stress response.

Some animal studies show that oxytocin, when administered for some days, can inhibit the activity of the HPA and the release of ACTH and corticosterone (Windle et al., 1997; Windle et al., 2004). In humans, oxytocin regulates the stress response and its intranasal administration reduces the release of cortisol and anxiety (Ditzen et al., 2009; Heinrichs et al., 2003; Quirin et al., 2011). It is well accepted that oxytocin release is one of the ways by which social support modulates the magnitude of the stress reaction and exerts a protective role. Carriers of the critical allele A of the SNPs rs53576 have higher physiological and dispositional stress

reactivity and do not benefit from social contact as a protective factor during stress tests. Unlike GG homozygotes, a suppressive effect of social support on the stress reaction (i.e. lower cortisol levels) has not been indentified (Rodrigues et al., 2009; Chen et al., 2011). Considering the suppressive effect of oxytocin on the HPA, it is also plausible that an effect of the critical alleles on the SNPs rs3796863, rs2254298 and rs53576 on the modulation of the HPA and sympathetic activity can be demonstrated during participation in a social stress test. Presumably, if the polymorphisms have additive effects, an effect of their interaction will be also observed. In this respect, carriers of the critical alleles would present a relatively intense stress reaction, which would be demonstrated by higher levels of cortisol and alpha-amylase, and report of negative emotions.

The purpose of this thesis is to investigate the effect that the SNPs rs3796863, rs2254298 and rs53576 have in the prevalence of autistic traits in a sample of healthy adults, and the individual differences on the stress response during a standardized stress test in male participants. As a secondary objective, the impression that the participants

cause on blind raters will also be investigated. Regarding this, the main hypotheses of this thesis can be so formulated:

H1: Critical alleles (rs53576A, rs2254298A and rs3796863C) have a dose-dependent effect on the prevalence of autistic traits and on the empathy and systematizing quotients.

H2: Homozygotes for the OXTR rs53576 (GG) and rs2254298 (GG) have an attenuated HPA and sympathetic response to social stress when compared with critical allele carriers (AA/AG).

H3: Homozygotes for the CD38 rs3796863 (CC) critical alleles have an enhanced HPA and sympathetic response to social stress in comparison with carriers of the A allele (AC/AA).

H4: There are differences in the intensity of the emotional states evoked by the Trier Social Stress Test associated with different genotype groups mentioned above, in which critical alleles carriers may report more

negative emotions like anxiety or anger and less positive emotions like joy.

H5: Carriers of critical alleles cause a worse impression on blind raters during public speaking.

H6: The selected SNPs interact in an additive manner to determine the mentioned behavioral phenotypes, in which a larger number of critical alleles is associated with worse social abilities.

H7: The selected SNPs interact to determine the magnitude of the HPA and sympathetic response to social stress in the same manner reported on H6.

3. Methods

The performed investigation of genetical correlates of social behavior was divided into two independent but associated studies. The first study investigates if there is any difference on self-reported autistic traits between groups of participants, which carry different alleles of the SNPs on the genes coding the OXT receptor and CD38 (rs53576, rs2254298 and rs3796863). Similarly, the second study was also designed to investigate individual differences under the influence of the same SNPs but instead of personality traits, the physiological stress response triggered by the Trier Social Stress Test and the impression on blind observers were investigated.

3.1. Study one

3.1.1. Participants

Participants were recruited by advertisement within the Justus-Liebig University's facilities through printed public advertisement or by contact via e-mail found in

the data bank of the Department of Personal Psychology and Individual Differences, which contains the contact details from approximately 2000 subjects. The majority of male subjects were selected from the screening phase of an fMRI study, in which they were genotyped. In this study, 600 healthy males needed to be screened and each received 25,-€ as compensation of time expense. The female and the smaller part of the male sample were selected from the genetic databank from our department or recruited within university facilities specifically for this study, in both cases they received a correspondent amount of credits required in their curriculum as volunteers in research projects. Therefore, this convenience sample was basically composed of current or former students of this university.

The following criteria were adopted for exclusion of participants from study 1:

- Presence of mental disorder and physical illness
- Use of substances, which alter the function of the central nervous system

- Chronic diseases

Participants suffering from mental disorders, physical illness and substance or psychotropic medication use were excluded because these conditions are associated with impaired or altered social behavior and empathy and would have added different biological and environmental confounding variables into the analysis (Thoma, Friedmann, & Suchan, 2013).

3.1.2. Behavioral Assessment

After agreement and signature of the terms of consent and observance of exclusion criteria, the participants received a link per e-mail, through which they had access to the behavioral questionnaires including the German translation of the following scales: Autism Spectrum Quotient, The Cambridge Behavior Scale and the Revised Cambridge Personality Questionnaire.

3.1.2.1. The Autism Spectrum Quotient (AQ)

The AQ is a short, easy to use, easy to score and self-administered scale, which was developed for identifying the degree to which any individual adult of normal IQ may have autistic traits. It comprises 50 questions, made up of 10 questions assessing 5 different domains:

- *social skills* (items 1, 11, 13, 15, 22, 36, 44, 45, 47, 48)
- *attention switching* (items 2, 4, 10, 16, 25, 32, 34, 37, 43, 46)
- *attention to detail* (items 5, 6, 9, 12, 19, 23, 28, 29, 30, 49)
- *communication* (items 7, 17, 18, 26, 27, 31, 33, 35, 38, 39)
- *imagination* (items 3, 8, 14, 20, 21, 24, 40, 41, 42, 50)

Each of the items usually scores 1 point if the respondent designates the abnormal or autistic-like behavior either mildly or strongly. The abnormality or autistic-like phenotype is defined as poor social skill, poor communication skill, poor imagination, exceptional attention to detail and poor attention-switching/strong

focus of attention. Around half of the items were designed to produce an “agree” response, and the other half a “disagree” response, in a high scoring person with autism spectrum condition (high functioning autism or Asperger syndrome). In the final version of the scale questions of different domains were randomized.

Test-retest reliability within two weeks demonstrated that their means do not differ statistically and that the tests are highly correlated ($r = 0.7$, $p = 0.002$). Self versus parent report of people with autism spectrum conditions indicates that self-report is more conservative and has the tendency to result in lower scores. The internal consistency (Cronbach’s alpha) of items in each of the five domains in the validation study was considered high to moderate (Communication = 0.65; Social behavior = 0.77; Imagination = 0.65; Attention to Details = 0.63; Attention Switching = 0.77) and regarding the decision to score “slightly agree” and “definitely agree” responses using 1 point only, a reanalysis scoring these as respectively 1 and 2 points led to similar results (Baron-Cohen et al., 2001). The most used scoring method is to designate 1 point to “slightly agree” and “strongly agree”

answers for the items 1, 2, 4, 5, 6, 7, 9, 12, 13, 16, 18, 19, 20, 21, 22, 23, 26, 33, 35, 39, 41, 42, 43, 45, 46, and 1 point to “definitely disagree” and “slightly disagree” answers for the items 3, 8, 10, 11, 14, 15, 17, 24, 25, 27, 28, 29, 30, 31, 32, 34, 36, 37, 38, 40, 44, 47, 48, 49, 50. Therefore, the minimal and maximal possible score are respectively 0 and 50 for this method and 0 and 100 if the alternative method, which codes 2 and 1 for the different degrees of agreement and disagreement, is used.

In the above cited validation study, different AQ scores were observed between a group of participants with high functioning autism and the general population, including students. Excepted for the group of people with high functioning autism, mean differences were also observed between men and women, with the mean of female participants being lower. In a complementary analysis the authors demonstrated that students of science have a higher prevalence of autistic traits than students of humanities and social sciences while students of mathematics were pointed out as the group with the highest prevalence of autistic traits. This fact indicates that this instrument is able to detect differences in non-

clinical samples, which was later confirmed by a report of an association between scientific/mathematical skills, and autistic conditions (Baron-Cohen, Wheelwright, Burtenshaw, & Hobson, 2007).

Considering the fact that 79.3% of male participants with autistic spectrum conditions, 2% of controls, 92.3% of female participants with autistic spectrum conditions and 1% of female controls score 32 or more on the AQ, the authors suggest a cut-off of 32 when the instrument is used for screening in the general population. In the general population sample, the percentage of males and females scoring above or equal 20 also differs (40% vs. 21% respectively), which indicates that a greater number of males also have moderate levels of autistic traits and not only a higher prevalence of severe forms (2 vs. 1%) (Baron-Cohen et al., 2001).

As can be seen, the Autism Spectrum Quotient is a validated instrument to access autistic traits in clinical and non-clinical samples and is sufficiently sensitive to discriminate differences in the general population. An alternative but similar instrument as the Adult Social

Responsiveness Scale (Constantino et al., 2005), which has the same function as the AQ, would not have brought any advantage and, differently from the AQ, is only available for use under the payment of a license.

3.1.2.2. The Cambridge Behavioral Behavior Scale (Empathy Quotient (EQ))

Empathy is an important social ability which allows us to tune into how someone else is feeling or thinking. Empathy describes the capacity to understand and predict the intention and behavior of others and to experience the emotion triggered by their emotion. Without this ability, an effective social interaction is hindered. It is also the "glue" of the social world, leading us to help others instead of hurting them (Baron-Cohen et al., 2004). In recent terminology, most authors recognize an affective and a cognitive component of empathy. The affective component can be described as the appropriate emotional response to someone's emotion. In contrast, the cognitive component is the ability of setting aside one's own

current perspective, attributing a mental state to the other person while inferring the likely content of their mental state, given the experience of that person. This process is currently known as the capacity to formulate a “theory of mind” or “mindreading” and has been studied in several psychiatric and neurological conditions including autism (Boucher, 1989; Biedermann, Frajo-Apor, & Hofer, 2012; Poletti, Enrici, Bonuccelli, & Adenzato, 2011).

The EQ is also a short, self-administered, easy to use and easy to score scale, which comprises 60 questions broken down into two types: 40 questions tapping empathy and 20 filler items not relevant to empathy itself and, therefore, not taken into account for scoring. These 20 items were included to distract the participant from a relentless focus on empathy. Each of the 40 items cited above scores 1 point if the respondent records the empathy behavior mildly and 2 points if he records the empathy behavior strongly. Like the AQ, half of the items are designed to induce an agreement and the other half a disagreement, in case a respondent has the highest or the lowest level of empathy. This avoids response bias in either way. In addition, the items are randomized and the

EQ has a forced choice format, which is straightforward to score because it does not rely on any interpretation (Baron-Cohen et al., 2004).

For scoring the EQ “definitely agree”, responses score 2 points and “slightly agree” responses 1 point on the following items: 1, 6, 19, 22, 25, 26, 35, 36, 37, 38, 41, 42, 43, 44, 52, 54, 55, 57, 58, 59 and 60. Complementarily “definitely disagree” responses score 2 points and “slightly disagree” responses 1 point on the following items: 4, 8, 10, 11, 12, 14, 15, 18, 21, 27, 28, 29, 32, 34, 39, 46, 48, 49 and 50. The remaining 20 filler items are discarded. The maximal and minimal scores correspond to 80 and 0, which indicate the most and least empathic behavior pattern.

In its validation study (Baron-Cohen et al., 2004) the authors confirmed that people with autistic spectrum disorders and normal intelligence exhibit lower levels of empathy than controls matched for age and gender (mean EQ scores of 20.4 and 42.1, respectively). Additionally, they also demonstrated that healthy female participants have a higher score than healthy males (47.2 vs. 41.8).

Finally, the test-retest reliability ($r = 0.97$ ($p < 0.001$)) and internal consistency (Cronbach's alpha = 0.92) were high. Finally, EQ scores are negatively correlated with AQ scores ($r = -0.56$, $p < 0.0001$) and positively correlate with a self-report scale assessing reciprocity and intimacy in relationships, the FQ (Friendship Questionnaire) ($r = 0.59$, $p < 0.001$). Although future studies to evaluate the validity of EQ in different populations and psychiatric disorders are needed, and the individual's belief about his own empathy being the real psychological domain accessed by the instrument, the EQ is sensitive enough to differences on empathy in non-clinical samples and can be used as a tool in these settings.

3.1.2.3. The Revised Cambridge Personality Questionnaire (Revised Systemizing Quotient (SQ-R))

In contrast with empathizing as the most powerful way of understanding and predicting the social world, systemizing is held as the most powerful way of understanding and predicting the law-governed inanimate

universe. Systemizing is also known as the drive to analyze the variables in a system, to derive the underlying rules that govern the behavior of a system. It also refers to the drive to construct systems and allows you to predict the behavior of a system, and control it. A system is defined as something with accepted inputs, which can be operated in a rule-governed way to deliver different outputs. While systemizing is required to understand and predict a series of inanimate systems such as mathematical, mechanic, social and technical systems, empathizing is also required to predict human behavior routinely (Baron-Cohen, Richler, Bisarya, Gurunathan, & Wheelwright, 2003).

Men have superior ability in systemizing than females, who have superior ability in empathizing. To test the empathy-systemizing theory and the extreme male brain theory of autism, which postulates that autism is the extreme accentuation of male systemizing drive, the SQ was developed. The male brain is characterized by a better systemizing than empathizing (S > E – also called type S) whereas the female brain by a better empathizing than systemizing (E > S – also called type E). Individuals

diagnosed with high functioning autism on the other hand have a much better systemizing ability and a much worse empathy (S >> E – also called type extreme S) (Baron-Cohen et al., 2003; Wheelwright et al., 2006).

SQ was meant to be an instrument that assesses the individual's interest in systems across the range of different classes of systems. It is short, easy to complete and easy to score and the first version comprised 60 items, being 40 scoring items and 20 filler items. To the current version, the Revised Systemizing Quotient (SQ-R), 37 new items, which cover social and domestic systems, were added and 2 old items erased due to redundancy. Additionally, the filler items were also removed in order to make the scale shorter (Wheelwright et al., 2006). Like the AQ and EQ, the SQ-R has a forced-choice format, can be self-administrated, is straightforward to score and sensitive to differences observed in the general population, confirmed by the fact that physical scientists score higher than biological, social and humanities scientists, with the last being the group with lowest scores. These findings are in accordance with the fact that physical sciences require a

greater capacity for systemizing. This study also demonstrated that the correlation of EQ with SQ-R is weak but significant (r = -0.09) in the general population, and suggested that they must be considered independent domains (Wheelwright et al., 2006). Regarding this, it is a sounded approach to use these scales together to evaluate autistic traits in non-clinical samples. The final versions of these three scales are available on the website of the Autism Research Center (http://www.autismresearchcentre.com/).

3.1.3. Genetical analysis

The cells for DNA isolation were obtained from check cell swabs and mouthwash with an antibacterial solution (Listerine® – Johnson & Johnson) and stored at less than -18˚C until transportation to our laboratory. In the laboratory, the samples were centrifuged at 4000 rpm for 5 minutes. The upper two-thirds were discarded and the lower pellet was mixed in the remaining solution by shaking. From this suspension 200 µl were pipetted for

DNA isolation and the remaining sample was stored again at -20˚C.

For the isolation of DNA, an automatized process in the MagnaPure System (Roche, Germany) with magnetic particles and specialized reagent kits was used (MagNA Pure LC DNA Isolation Kit I; Roche Diagnostics, Mannheim, Germany). This method relies on the fact that DNA binds to Magnetic Glass Particles (MGPs) in the presence of a chaotropic salt at a pH > 7.0. MGPs have a glass (silica) surface and a magnetic core. Nucleic acids are absorbed into the silica-surface of the MGPs in the presence of isopropanol and a high concentration of chaotropic salts. Polysaccharides and proteins do not attache to the beads and are removed by sequential washing steps. After washing, nucleic acids are then eluted from the beads by applying low salt conditions and heat. At the end of the isolation process we obtained 100µl of purified DNA solution.

The purified DNA was finally genotyped through PCR amplification in a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) with an end point plate reader using

TaqMan® Genotyping Assays for the OXTR rs53576 and rs2254298 and CD38 rs3796863 SNPs (C_3290335_10, C_1337916_10 and C_1216944_10, Applied Biosystems, Carlsbad, United States). During the first step, the target DNA is amplified by the AmpliTaq Gold® DNA polymerase (Applied Biosystems, Carlsbad, United States) using sequence-specific primers. Detection is achieved by means of an exonuclease cleavage of the allele-specific predesigned annealed probes containing an allele-specific 5' dye label, which generates the permanent assay fluorescence signal.

3.2. Study two

3.2.1. Participants

For this study, 80 male participants were originally selected from the first study's sample or from the genetic data bank of the department of Personal Psychology and Individual Differences according to their genotype (Table 2.2.1) on the SNPs OXR rs53576 and CD38 rs3796863. In order to test the hypotheses that the three studied SNPs

are associated with altered cortisol and alpha-amylase release, and altered impressions on blind raters during social stress, a partially balanced distribution on the two above mentioned SNPs would be the best approach. However, considering the very small minor allele frequency (MAF) of 0.068 of the OXTR rs2254298 (NCBI, 2013) and the necessity of a larger sample for reaching a similar frequency on the eight possible genotypes, it was not taken into account in sample selection. Participants were contacted and invited to participate in the study, without being informed about the test they would take part in. Each participant received a compensation of 25€ for the time spent on the test. In addition to the exclusion criteria mentioned above for study 1, participants who mentioned the practice of sport activities during more than 7 hours per week, abuse or dependence of tobacco or psychotherapeutic treatment in the last 12 months were excluded from the study due to a presumably altered cortisol level.

Table 1: Participants distribution based on combined genotypes.

Genotype			OXTR rs53576	
			AG/AA	GG
CD 38	rs3796863	AC/AA	20	19
		CC	20	21

After agreement, the participants met the appointment, in which the Trier Social Stress Test was performed accompanied by sequential saliva sampling.

3.2.2. The Trier Social Stress Test - TSST

The TSST is a task protocol that combines high levels of social-evaluative threat and uncontrollability and consists of a brief preparation period followed by a test period in which the participant is required to deliver a free speech

about his/her suitability for employment in a mock job interview. Following this, the participant is instructed to perform mental arithmetic in front of a trained audience, which withholds verbal and non-verbal feedback (Kirschbaum, Pirke, & Hellhammer, 1993; Dickerson & Kemeny, 2004).

The TSST is a proven potent stimulator of the acute stress response and can produce a two to three-fold rise in salivary cortisol levels in 70-80% of tested subjects and additional rise in other markers of HPA axis activity such as ACTH (one- to three-fold), salivary alpha-amylase, adrenaline and noradrenaline, and heart rate response. Furthermore, the TSST has also been associated with elevations in the concentration of cytokines, which suggest an activation of the immune system under these circumstances (Foley & Kirschbaum, 2010). Among these markers, cortisol has been considered the best indicator of HPA axis activity. Salivary and blood concentration of cortisol increase gradually after stimulation onset and reach peak concentrations 10-30 minutes after stress cessation. Firstly detected in healthy subjects, the cortisol response

to the TSST has been analyzed in several groups and shows variations according to several factors, which include gender, age, corticosteroid binding globulin levels, genetic polymorphisms, personality, chronic stress, stress inoculation and physical training. Additionally, cortisol response triggered by the TSST is attenuated in breast-feeding women (Heinrichs et al., 2001), by social support and intranasal administration of oxytocin in healthy subjects (Heinrichs et al., 2003), in women after having physical contact with their partners (Ditzen et al., 2007), and after administration of intranasal oxytocin in subjects with borderline personality disorders (Simeon et al., 2011). Higher concentrations of oxytocin and a moderate negative correlation with salivary cortisol levels were also observed during the TSST in subjects who had a life-threatening illness during adolescence or childhood in comparison with abused and control subjects (Pierrehumbert et al., 2010). In a recent study, Unternaehrer et al. (2012) found that the exposition to the TSST is associated with dynamic changes in DNA methylation of the OXTR, which in conjunct with the

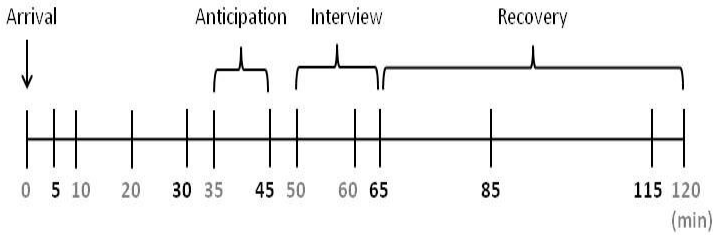
studies cited above evidences that this test is also an important tool to elucidate the effect of oxytocin in the reactivity of the HPA axis and the modulation of oxytocinergic activity by stress itself.

The TSST was performed during the afternoon (between 3 and 7 pm) to avoid sampling in different periods of the day, which is associated with a larger variability of cortisol and alpha-amylase concentrations, mainly due to the physiological circadian rhythm. At arrival, (time 0) the participant was taken to room A and instructed on the experiment and saliva sampling without being informed about the content of the TSST. In this same room, all the saliva samples were collected and the behavioral scales were filled out along the whole experiment. In the beginning of the anticipation phase, 35 minutes after arrival (time 35 minutes), the participant was informed that he should give a talk to an audience in approximately 10 minutes, the content of which he would be informed about shortly before the talk. At this moment, the participants were also accompanied to room B, where the talk was to take place, and were briefly introduced to the three evaluators. Participants spent the next 15 minutes of

the anticipation phase in room A. Just before being left in room B by the investigator (time 45 minutes), the participant was informed that he should take over the role of job applicant who had been invited for a personal 5 minute-interview with the company staff's managers and that he should introduce himself and convince the audience that he would be the most suitable candidate for a fictitious position. The evaluators were the same along all the sections, and trained to interact with the participant a minimal and in a standardized form. They were also introduced as being particularly able to monitor non-verbal behavior. Additionally, participants were also informed that the talk would be recorded by a video-camera for posterior analysis. Following the interview the participant was asked to perform mental arithmetic for another 5 minutes, in which he should subtract the value 13 from 2054 and from the result of each subtraction, the same amount successively. Each time the participant made a mistake, he was asked to start from the beginning, i.e. 2054 minus 13. At time 65 minutes the task was interrupted and the participant was taken to room A for resting, which marked the beginning of the

recovery phase. The course of the TSST is schematized below.

Figure 3. Time course of the TSST and its phases. Numbers in black correspond to time points in which saliva was sampled.



Saliva was sampled in the following time points (figure 3): 5, 30, 45, 65, 85 and 115 minutes, together with mood assessment through the Differential Affect Scale (Zerssen & Koeller, 1976; Zerssen & Petermann, 2011), which is employed to evaluate one's current mental state and maps the full range of normal and pathological changes of well-being. The Differential Affect Scale is a self-report questionnaire of a series of emotional states including interest, anxiety, anger, stress, joy,

astonishment, sorrow and shame, and is suitable for detecting differences in the experienced psychological burden between and within subjects at different time points.

3.2.3. Behavioral assessment during the TSST

In addition to the data obtained from the participants with the Differential Affect Scale, the raters were asked to observe each participant during the interview, rate their behavior and state their opinion about them on a behavioral scale. For this purpose the raters were kept blind to the participants' genotypes and three not-validated scales were used. The first scale assessed the frequency of the following behaviors: eye contact with the interviewers, looks at the ceiling, self grooming, not correctly finished spoken sentences, number of "ahmmmmss" and "mmhhhss" etc., looks to the ground, speech pauses, stuttering and stammering, looks at the watch and request to continue talking. The second and the third scales were designed to gather information

about the impression given by the participants and were scored with a Likert scale from 0 (not at all) to 4 (very much). The first of these two scales assessed the global impressions of the raters about the participants and the following items were included: arm movement (gesticulation), whole body movement (move around), head movement, convincing, self-confident, structured, motivated, strained, familiarity with speeches, nervous, angry, even-tempered, witty, charming, insecure, anxious, friendly, calm, experienced and annoyed. The third and last scale investigated the speculative opinion of the raters about the participants' social competence and academic performance through the following characteristics: intelligent, successful, integrative, helpful, cooperative, honest, trustworthy, loyal, sympathetic, ambitious, success-oriented, career-oriented, and if the rater considered the participant as a potential candidate for the final phase of a selective process of a company.

3.2.4. Additional behavioral measures

All the participants also completed the Social Phobia Scale and 75 of them the Autism, Systematizing and Empathy Questionnaires described above. The Social Phobia Scale was developed and validated by Mattick and Clarke (Mattick & Clarke, 1998) and assesses fears of being scrutinized during routine activities (eating, drinking, writing, etc) and possesses high levels of internal consistency (Cronbach's alpha = .92) and test-retest reliability (r = .92). It can differentiate social phobia from agoraphobia and simple phobias and between social phobia and normal samples. It comprises 20 items, which describe social anxiety in different circumstances and are rated with a five-point (0-4) Likert rating scale (not at all, slightly, moderately, very, extremelly), being the maximal and minimal score 80 and 0, respectively.

3.2.5. Saliva sampling

Saliva was obtained by instructing the subjects to put a roll-shaped synthetic saliva collector into the mouth for 3 minutes without swallowing the excess of saliva (Salivette®, Sarstedt, Nümbrecht, Germany). All participants were instructed not to brush or floss their teeth and not to drink or eat 30 minutes prior to the beginning of the experiment. All samples were frozen at -20 ˚C prior to laboratory testing. After thawing, they were mixed and centrifuged for 10 minutes at 3000 r.p.m. to remove particular material.

3.2.6. Determination of free cortisol concentration

The salivary free cortisol concentration was determinated by solid phase enzyme-linked immunosorbent assay (Cortisol ELISA, IBL, Hamburg, Germany) based on the completion principle. An unknown amount of antigen present in the sample and a fixed amount of enzyme labeled antigen compete for the binding sites of the antibodies coated onto the wells. After incubation the

wells were washed to stop the competition reaction. After substrate reaction, the intensity of the developed color, read by the photometer at 450nm, was inversely proportional to the amount of antigen in the sample (concentration). Results were determinated using a standard curve with a 4 Parameters Logistics fit.

The process was completely automated in the Nexgen Four™ analyzer (Adaltis, Rome, Italy) reducing human intervention to a minimum. Following the instructions of the fabricant, 50µl of each standard, control and centrifuged sample was pipetted into the respective wells of the microtiter plate and 100µl of enzyme conjugate was added. After incubation of 2 h (18-25˚C) the plate was washed 4 times and 100µl of the substrate solution was pipetted into each well. To stop the enzymatic reaction, a stop solution was used after 30 minutes. Finally, the optical density at 450nm was read 15 minutes after the addition of the stop solution. With the help of the intern software available, the calibration curves were plotted and the concentrations read.

3.2.7. Determination of alpha-amylase concentration

The alpha-amylase concentration was determined using a standard curve of a liquid phase enzymatic assay (Salivary alpha Amylase Enzymatic Assay, IBL, Hamburg, Germany). In this kinetic process, a substrate (CNPG3, 2-chloro-4-nitrophenyl maltotriose) is added to the samples and its degradation by the investigated enzyme results in a product (CNP, 2-chloro-4-nitrophenol), whose absorbance can be read in a photometer at 405nm. The measured variation in absorbance per minute is proportional to the alpha-amylase activity, i.e. its concentration.

10µl of centrifuged saliva were previously diluted with 3ml buffer and standards and controls were additionally diluted following the instructions of the fabricant. 10µl of each prediluted standard, controls and samples were pipetted into the respective wells of the microtiter plate and 200µl of CNPG3 solution was added into each well. After 3 minutes incubation, the plate was placed into the photometer (Multiskan* FC Microplate Photometer, Thermo Scientific, Vantaa, Finland) and the optical

density was measured at 0 and 2.5 minutes. Alpha-amylase concentrations of unknown samples were read from the calibration curve created with the variation in the measured optic density observed in these two measures plotted from the 5 provided standards with a 4 Parameters Logistics fit.

4. Results

4.1. Study one

This study was designed to investigate the hypothesis that critical allele carriers of the three genotyped SNPs show higher prevalence of autistic traits, lower tendency to demonstrate empathy during social interactions and a higher rate of systematizing. For this purpose, 333 subjects were enrolled, 205 men and 128 women. All subjects completed the Autism Questionnaire and were genotyped. From the entire sample, 14 men and 5 women did not complete the Empathy Questionnaire and 9 men and 18 women did not complete the Systematizing Questionnaire. The statistical analyses depicted in the sections below were all conducted with the software package SPSS 15.0 for Windows (SPSS Inc., Chicago, IL) and the level of significance for all tests was set at 0.05.

Before starting with the group comparisons, the internal consistency of the used scales was examined in the whole sample. As can be seen in table 2, the internal

consistency of the tested questionnaires ranged from .735 on the AQ to .861 on the SQ, which is considered good. However, the subscales of the AQ have on average only an acceptable reliability since the internal consistency of the subscales on Attention Switching and Imagination were poor (.547 and .586) and on Communication, unacceptable (.395).

Table 2: Reliability tests for all the scales used in study 1 and AQ-subscales

Reliability's Tests			
	Cronbach's alpha	number of items	N
Autism Questionnaire	.735	50	333
- Social Skills	.760	10	333
- Attention Switching	.547	10	333
- Attention to Details	.631	10	333
- Communication	.395	10	333
- Imagination	.586	10	333
Empathy Questionnaire	.838	40	314
Systematizing Questionnaire	.861	75	306

4.1.1. Comparisons between male and female samples

The mean age of the overall sample was 22.49 (SD=3.62) and the difference between the mean age of men (M = 22.52, SE = .24) and women (M = 22.45, SE = .33) was not significant (t(331) = .186, p = .852). To test the hypotheses that males have a higher prevalence of autistic traits, lower levels of empathy and higher levels of systematizing, independent t-tests with the scores on the three scales (AQ, EQ, SQ) and gender respectively as independent and dependent variables were performed. As observed in table 3, significant differences in the mean scores on the Empathy and Systemizing Quotient were found, whereby women have a higher mean score on empathy (M = 43.62, SE = .763 vs. M = 39.24, SE = . 732) and a lower mean score on systemizing (M = 48.25, SE = 1.19 vs. M = 58.65, SE = 1.13). Regarding the scores on the Autism Questionnaire, the assumption of equality of means in these two groups could not be discarded.

Table 3: Results of independent t-tests on the scores on AQ, EQ and SQ. For the t-test on SQ, equality of variances was not assumed. (***) : $p < .001$

	Levene's Test for Equality of Variance		T-Test for Equalitiy of Means				
	F	Significance	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Autism Quotient (AQ)	0.104	.747	1.449	331	.148	.816	.563
Empathy Quotient (EQ)	2.054	.153	-3.983***	312	.000	-4.377	1.099
Systemizing Quotient (SQ)	8.253	.004	6.338***	270.5	.000	10.403	1.641

Taking into consideration previous reports, which have indentified gender differences on the AQ, independent t-tests were also conducted on their subscales in an attempt to identify balanced gender-specific autistic traits that could not be evidenced by the comparison of total scores. From this analysis, the only significant difference was observed on the trait imagination, in which higher scores reflect lower capacity to form mental images and representations. In this sample, men reported a poorer imagination than women ($M = 2.45$, $SE = .122$ vs. $M = 1.70$, $SE = .118$; $t(318.5) = 4.39$, $p < .001$). Therefore, the reported findings that men in general, have more autistic traits than women were not confirmed in this sample, with poorer imagination being the only difference observed on the Autism Questionnaire. However we could replicate the findings that men score higher in the SQ and lower in the EQ.

4.1.1.2. Effects of the investigated SNPs on the AQ, EQ and SQ scores

To investigate the hypothesis that the critical alleles (rs53576A, rs2254298A and rs3796863C) have a dose-dependent effect on the prevalence of autistic traits, empathy and systematizing quotients and that their interaction explain part of the variance of these traits observed in the normal population, several factorial ANOVAs where performed. Regarding the different prevalence of autistic traits observed in previous studies and the assumed gender specific differences on pathophysiology and brain function (Beacher et al., 2012; Rhodes, Jeffery, Taylor, & Ewing, 2013) the analyses were performed separately in the male and female samples as an attempt to identify gender specific genetic determinants.

From the 333 enrolled subjects, 292 were genotyped (177 men and 115 women) and all genotype frequencies were consistent with the Hardy-Weinberg equilibrium (rs53576: $X^2 = .650$, $p = .420$; rs2254298: $X^2 = .350$, $p = .553$; and rs3796863: $X^2 = .101$, $p = .750$). In order to

enhance the power of the analyses and considering the sample size and lack of previous studies analyzing the effect of these three SNPs on AQ, EQ and SQ scores, explorative analyses on the allele-level, which were associated with a less unbalanced distribution, were conducted. Table 4 shows the frequencies of the selected grouping for men and women within the different SNPs.

Table 4: Selected grouping on the three SNPs and their frequencies in the male and female samples. The initial explorative analyses were conducted in these groups.

	CD38 rs3796863		OXTR rs53576		OXTR s2254298	
	A- (CC)	A+ (AC/AA)	A- (GG)	A+ (AA/AG)	A- (GG)	A+ (AG/AA)
Men	92	85	74	103	139	38
Women	63	52	56	59	90	25

4.1.2.1. Male sample

In the male sample, separated factorial ANOVAs with the scores on AQ, EQ and SQ as dependent variables and the SNPs in the allele levels as grouped in table 4 as independent variables produced the results summarized in table 5.

Table 5: Results from independent factorial analyses of variance on the scores of the Autism, Empathy and Systematizing Questionnaires with polymorphisms of the OXT Receptor (rs53576 (AA/AG vs. GG) and rs2254298 (AA/AG vs. GG)) and CD38 (rs3796863 (CC vs. AC/AA)) as factors in males. (*): $p < .05$

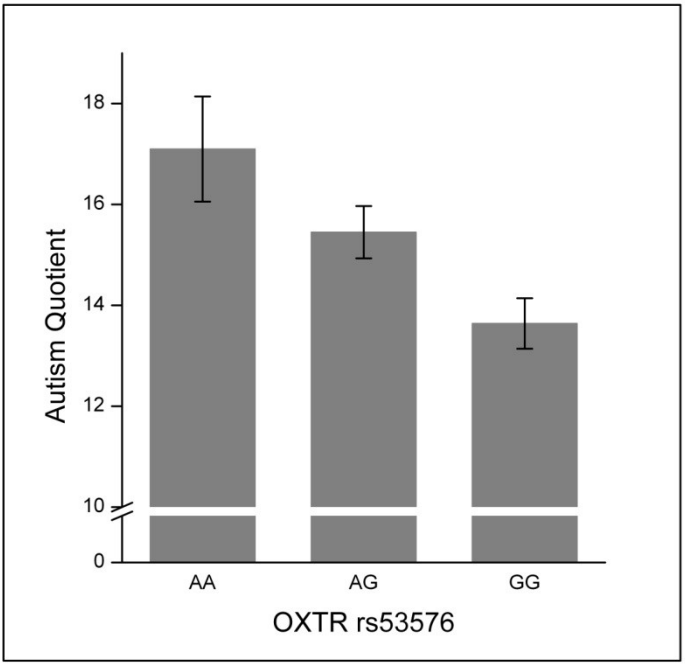
	AQ			EQ			SQ		
	Df	F	Sig.	Df	F	Sig.	df	F	Sig.
rs3796863	1	.424	.516	1	.026	.872	1	.367	.546
rs53576	1	5.367*	.022	1	.086	.770	1	1.941	.165
rs2254298	1	.395	.530	1	.043	.836	1	1.165	.282
rs3796863 * rs53576	1	1.036	.310	1	.911	.341	1	.258	.612
rs3796863 * rs2254298	1	.019	.892	1	2.336	.128	1	3.056	.082
rs53576 * rs2254298	1	.308	.580	1	1.001	.319	1	.482	.489
rs3796863 * rs53576 * rs2254298	1	0.828	0.364	1	0.085	0.771	1	1.118	0.292
Error	169	-	-	164	-	-	164	-	-
Total	177	-	-	172	-	-	172	-	-

As can be observed in the table above, the null hypothesis that the mean scores in the Autism Questionnaire are equal in the different genotype groups of the OXTR rs53576 can be rejected ($F(1,169) = 5.367$, $p = .022$) and the calculation of the partial eta squared (η_p^2) indicates that the presence or absence of at least one critical allele (A) can explain a small part of the partialed out variance observed in the scores ($\eta_p^2 = SS_{(rs53576)}/(SS_{(rs53576)}+SS_{(error)}) = .0308$). In contrast, the F-tests on the remaining factors and their interactions did not reach statistical significance.

The comparison of the group means supported one of our preliminary hypotheses that the presence of a critical allele (AA or AG) on the OXTR rs53576 is associated with higher scores on the Autism Spectrum Questionnaire ($M = 15.93$, $SE = .479$ vs. $M = 13.64$, $SE = .501$) and suggests that carriers of the A allele have an increased risk of having autistic traits. However, by means of this analysis it is still not clear if there is a dose-dependent effect of the A allele or if the group of heterozygotes AA is the one with the most accentuated autistic traits. In order to answer this question and to test the hypothesis of

a linear trend associated with the absence, presence of one, and presence of two critical alleles, a one-way ANOVA with the factor genotype of OXTR rs53576 with its three levels (GG, AG and AA) was run. In this manner this analysis confirmed that the assumption of equality of means is not sustainable ($F(2,174) = 6.679$, $p = .001$) and is associated with a medium effect size ($\eta_p^2 = .072$). Additionally, the hypothesis of a linear trend, which states that the mean of AQ increases proportionally to the number of critical alleles, can also be considered valid ($D_{lin} = 2.45$, $SE = .696$, $p = .001$) as illustrated by the differences observed between the means ($M_{AA} = 17.10$, $SE_{AA} = 1.042$; $M_{AG} = 15.45$, $SE_{AG} = .518$; $M_{GG} = 13.64$, $SE_{GG} = .501$) and by the graph with their error bars in figure 4.

Figure 4: Mean scores in the Autism Spectrum Questionnaire (AQ) for the genotypes on OXTR rs53576 and their respective error bars. The linear trend is clear.



Considering the results observed above and the different dimensions of autistic traits, an additional multivariate ANOVA on the subscales of AQ having the OXTR rs53576 with its three levels (AA, AG and GG) as a factor was performed. Using Pillai's trace, there was a

significant effect of genotype on the traits included on the model ($V = .146$, $F(10,342) = 2.688$, $p = .004$) and separate univariate ANOVAs on the outcome variables revealed significant effects on the traits attention to details ($F(2,174) = 6.549$, $p = .002$) and attention switching ($F(2,174) = 4.024$, $p = .02$), suggesting that the higher prevalence of autistic traits associated with the critical allele A of the OXTR rs53576 is mainly due to altered attentional function. Analysis of polynomial contrasts revealed a linear trend, in the same direction observed on AQ scores, on the trait attention to detail ($D_{lin} = 1.12$, $SE = .310$, $p < .001$; $M_{AA} = 5.43$, $SE_{AA} = .364$; $M_{AG} = 4.40$, $SE_{AG} = .250$; $M_{GG} = 3.85$, $SE_{GG} = .223$). For all ANOVAs, Levene's Tests of Equality of Error Variance were performed. Since none of them reached statistical significance, the error variances were all assumed to be equal.

4.1.2.2. Female sample

Likewise, separated factorial ANOVAs on the AQ, EQ and SQ using the SNPs with allele levels as factors (see table 4) were executed on the female sample. Table 6 summarizes their results.

In contrast with the male sample, in the female sample the investigated SNPs had any effect on the prevalence of autistic traits assessed by the Autism Questionnaire. However, the effects of the CD38 rs3796863 ($F(1,105) = 4.010$, $p = .048$, $\eta_p^2 = .037$) and the interaction between this SNP and the OXTR rs2254298 ($F(1,105) = 4.071$, $p = .046$, $\eta_p^2 = .037$) were significant on the Empathy Quotient and produced small to medium effect sizes. The examination of the group means suggests that the findings on the CD38 rs3796863 are in accordance with our preliminary hypotheses, since the presence of at least one protective allele (A) is associated with a higher score on empathy ($M_{CC} = 43.08$, $SE_{CC} = 1.449$; $M_{AC/AA} = 44.69$, $SE_{AC/AA} = 1.145$). On the other hand, the effects of the interaction are not completely in accordance with our hypotheses, since a clear dose-dependent effect was not

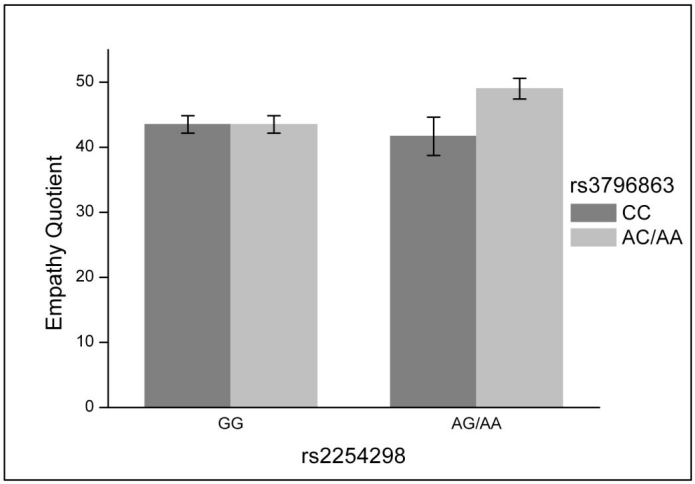
observed. The difference on the mean scores on empathy was only evident among carriers of at least one critical allele of the OXTR rs2254298 (A). In this group, women with at least one protective allele of the CD38 rs3796863 (A) had a higher empathy score than homozygotes CC ($M_{A+/A+}$ = 49.00, $SE_{A+/A+}$ = 1.589; $M_{A+/CC}$ = 41.69, $SE_{A+/CC}$ = 2.946). In homozygotes GG on the OXTR rs2254298 the presence of the protective allele on the CD38 rs3796863 is not associated with an increase on the frequency of empathic behavior ($M_{GG/A+}$ = 43.50, $SE_{GG/A+}$ = 1.340; $M_{GG/CC}$ = 43.45, $SE_{GG/CC}$ = 1.239) (figure 5).

Table 6: Results from independent factor analyses of variance on the scores of the Autism, Empathy and Systematizing Questionnaires with polymorphisms of the OXT Receptor (rs53576 (AA/AG vs. GG) and rs2254298 (AA/AG vs. GG)) and CD38 (rs3796863 (CC vs. AC/AA)) as factors in females. (*): $p < .05$

	AQ			EQ			SQ		
	df	F	Sig.	Df	F	Sig.	Df	F	Sig.
rs3796863	1	0.087	0.768	1	4.010*	0.048	1	1.292	0.258
rs53576	1	0.012	0.913	1	0.197	0.658	1	0.404	0.526
rs2254298	1	0.065	0.799	1	1.331	0.251	1	0.051	0.822
rs3796863 * rs53576	1	0.702	0.404	1	0.600	0.440	1	0.509	0.477
rs3796863 * rs2254298	1	0.714	0.400	1	4.071*	0.046	1	1.327	0.252
rs53576 * rs2254298	1	0.576	0.450	1	1.101	0.297	1	0.047	0.830
rs3796863 * rs53576 * rs2254298	1	0.064	0.801	1	0.463	0.498	1	0.028	0.868
Error	107			105			105		
Total	115			113			113		

Regarding the finding that the presence of at least one protective allele (A) on CD38 rs3796863 was associated with higher empathy scores, a one-way ANOVA on genotype level (H_0: $\mu_{AA} = \mu_{AC} = \mu_{CC}$) was also performed and produced a non-significant result ($F(2,113) = .846$, $p = .432$).

Figure 5: Bar-plot interaction chart from Empathy Quotient scores by the SNPs OXR rs2254298 (x axis) and CD38 3796863 (different colors) in women.



4.2. Study two

4.2.1. Sample

The main objective of study two was the investigation of the effects of the three mentioned SNPs on the salivary concentration of alpha-amylase and cortisol, on the current mental states of participants and on the impression caused by them during the Trier Social Stress Test. For this purpose, 80 men aged from 19 to 33 years (M = 24.03, SD = 3.146) were included in the study. The stress situation consisted of a mock job interview and an arithmetic task in front of a panel of three raters, which took place 35 to 45 minutes after arrival.

4.2.2. Effects of the studied SNPs on stress reaction

As illustrated in figure 5, saliva was sampled at different times within the course of the experiment (5, 30, 45, 65, 85 and 115 minutes) in order to monitor the cortisol and alpha-amylase secretion, which are well-established markers of the HPA axis and adrenergic function and

correlate with the intensity of the stress reaction (Kirschbaum et al., 1994; Kirschbaum et al., 1993).

To test the hypothesis that the dependent variables are distributed normally, Kolmogorov-Smirnov tests on the 6 different times- of measurement of cortisol and alpha-amylase and on their area under the response curve were employed. The area under the response curve was calculated by the trapezoidal method using the concentration at different times- after subtraction of the baseline (30 minutes). As the distribution of more than half of the cortisol measurements could not be considered normal and the assumption of homogeneity of residual variance was violated, logarithmic transformation ($y = \log(x)$) of the data was necessary. After transformation, no deviation of normality was observed and the heteroscedasticity was effectively corrected. Since the distribution of the majority of alpha-amylase measurements and of the areas under the response curve did not violate the model assumptions, raw data was used for their analysis. However, it was necessary to exclude an outlier from the statistical analysis of alpha-amylase response due to concentrations 4 to 6 times higher than

the overall mean. The participant was the only subject with the combined genotype OXTR rs53576 GG, OXTR rs2254298 A+ and CD38 rs3796863 A+, and produced extreme effects in all group means associated with these levels and their combination. Additional to the effects on cortisol and alpha-amylase release, effects of these SNPs were also investigated on the intensity of emotional states evoked by the TSST and on the impressions of the participants on blind raters.

4.2.2.1. Effects on cortisol release

In order to identify possible effects of the studied SNPs on the HPA axis function during the Trier Social Stress Test (TSST) a repeated-measures ANOVA having the logarithmic transformed cortisol concentration at the 6 different times- as dependent variables and the polymorphisms' genotypes grouped in the allelic levels mentioned above as fixed factors was employed. The levels of the three included factors were the same used in the initial explorative analyses in sections 3.1.2.1 and

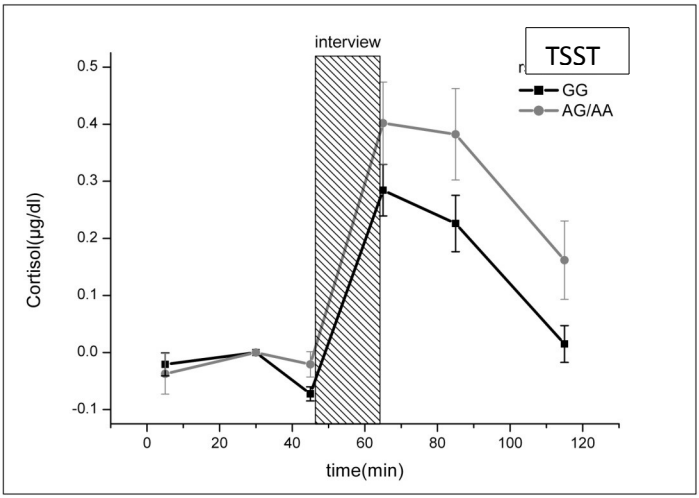
3.1.2.2, since the selection of participants was approximately balanced for OXTR rs53576 and CD38 rs3796863 and the division of OXTR rs2254298 in GG vs. AG/AA rendered the less unbalanced design. All the reported means are derived from the raw data and are expressed in µg/dl.

Mauchly's test indicated that the assumption of sphericity had been violated for the different times in which cortisol concentration was measured ($X^2(14) = 241.43$, $p < .001$). Therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .405$). As expected, there was a significant effect of time on the cortisol concentration ($F(2.02,145.84) = 39.361$, $p < .001$, $\eta_p^2 = .353$), which indicates that the TSST was capable of eliciting the previously reported stress reaction with a stress dependent release of cortisol in the whole sample. The interaction between time and the OXTR rs2254298 was equally significant ($F(2.02,145.84) = 3.32$, $p = .038$, $\eta_p^2 = .044$) and contrasts indicated that the concentration of cortisol at time 45 and 115 minutes in relation to base-line (30 minutes) was significantly lower in homozygotes GG, which can be confirmed by the observation of the F-

tests and their means after subtraction of the base-line at 45 ($F(1,72) = 4.187$, $p = .044$, $\eta_p^2 = .055$; $M_{GG} = -.072$, $SE_{GG} = .012$; $M_{A+} = -.020$, $SE_{A+} = .022$) and at 115 minutes ($F(1,72) = 4.299$, $p = .042$, $\eta_p^2 = .056$; $M_{GG} = .015$, $SE_{GG} = .015$; $M_{A+} = .162$, $SE_{A+} = .068$). The course of the cortisol response after subtraction of the base-line in both groups (GG and AA/AG) is illustrated with error bars on figure 6.

Additional difference contrasts also showed that the increase in cortisol concentration observed between the measures before (45 minutes) and after (65 minutes) the interview was also significantly larger on carriers of at least one critical allele (A+) ($F(1,72) = 4.019$, $p = .049$, $\eta_p^2 = .053$; $D_{GG} = .357$, $SE_{GG} = .042$, $D_{A+} = .423$, $SE_{A+} = .068$).

Figure 6: Time course of cortisol response after subtraction of the base-line (time 30 minutes) for both levels of the factor OXTR rs2254298 during the Trier Social Stress Test. Error bars represent the standard error of the means.



In summary, carriers of the critical alleles are exposed to higher concentrations of cortisol during the social stress test, which can be described as a smaller decrease from base-line levels before the interview, a larger increase during the interview and higher end-point concentration. These effects do not seem to be dependent on the

investigated personality traits since the inclusion of the scores on the Social Phobia Scale ($F(2.02,133.23) = 3.54$, $p = 0.31$, $\eta_p^2 = .051$) and on the Autism ($F(2.04,157.37) = 3.852$, $p = .022$, $\eta_p^2 = .048$), Empathy ($F(2.08,160.12) = 4.173$, $p = .016$, $\eta_p^2 = .051$) and Systematizing ($F(2.14,164.7) = 3.736$, $p = .023$, $\eta_p^2 = .046$) Questionnaires on new separated repeated-measures ANOVA as covariates does not reduce the effect.

Finally, an ANOVA of the area under the response curve by the three studied polymorphisms did not reveal significant results and supported the assumption that the total amount of cortisol released during the test in relation to the base-line at 30 minutes does not differ on the different genotype groups.

4.2.2.2. Effects on alpha-amylase secretion

In contrast to the effects observed in the cortisol response, a significant effect of the polymorphisms on the secretion of alpha-amylase was not observed. A similar repeated-measures ANOVA on the 6 measures of-

alpha amylase by the three different polymorphisms with the same allelic level produced negative results for the interaction of time and the different fixed factors (table 7).

Nevertheless, as reported in previous studies, the effect of the TSST in eliciting an increase in alpha-amylase concentration was observed ($F(3.94,284.02) = 4.544$, $p = .002$, $\eta_p^2 = .059$)), which can be explained by a significant difference between base-line level (30 minutes) ($M = 124.03$, $SE = 10.88$) and that observed after the TSST (65 minutes) ($M = 168.07$, $SE = 12.06$) ($F(1,72) = 23.089$, $p < .001$, $\eta_p^2 = .243$)) (figure 7). Since the assumption of sphericity was violated, the degrees of freedom presented in this analysis were corrected according to Greenhouse-Geisser estimates of sphericity ($\varepsilon = .789$). The mean concentrations are reported in Units/ml.

Table 7: F-Test results of the repeated measures ANOVA on the 6 alpha-amylase measures by the three studied SNPs after Greenhouse-Geisser correction. The same factor levels of the section above were used. (**): $p < .01$

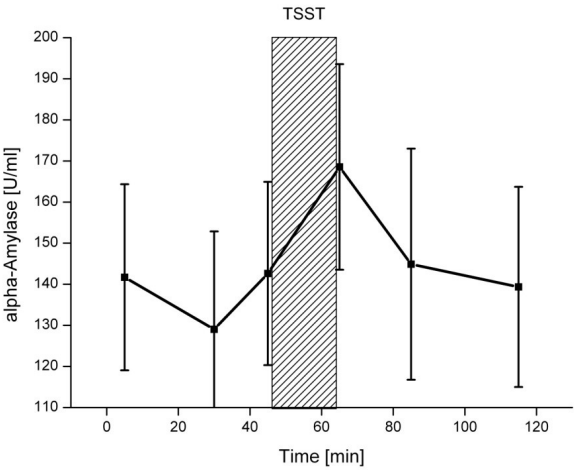
Source	Df	F	Sig.
Time	3.94	4.54**	0.002
rs53576*time	3.94	0.68	0.603
rs3796863*time	3.94	0.30	0.878
rs2254298*time	3.94	1.03	0.392
rs3796863 * rs53576*time	3.94	1.07	0.371
rs53576 * rs2254298*time	3.94	0.99	0.413
rs3796863 * rs2254298*time	3.94	0.65	0.628
Error	284.03		

Nevertheless, as reported in previous studies, the effect of the TSST in eliciting an increase in alpha-amylase concentration was observed ($F(3.94,284.02) = 4.544$, $p = .002$, $\eta_p^2 = .059$)), which can be explained by a

significant difference between base-line level (30 minutes) (M = 124.03, SE = 10.88) and that observed after the TSST (65 minutes) (M = 168.07, SE = 12.06) ($F(1,72) = 23.089$, $p < .001$, $\eta_p^2 = .243$)) (figure 7). Since the assumption of sphericity was violated, the degrees of freedom presented in this analysis were corrected according to Greenhouse-Geisser estimates of sphericity ($\varepsilon = .789$). The mean concentrations are reported in Units/ml.

The analysis of the area under the response curve of alpha-amylase did not produce any significant results, which supports the assumption that the released amount of alpha-amylase during the whole experiment did not differ between the analyzed allelic levels of the different investigated SNPs.

Figure 7: Time course of alpha-amylase salivary concentration. Mean of all participants on the TSST in different time points. A significant increase of alpha-amylase concentration is provoked by the social stress test.



4.2.2.3. Effects on emotional states evoked by the TSST

For each assessed domain on the Differential Affect Scale, the area under the curve during the 6 time-points was calculated. This measure can be interpreted as the intensity of a given emotion during the whole experiment and can be used in group comparisons. At first, a correlation matrix with these results, the scores on the four included social behavior scales (AQ, EQ, SQ and SPS), and the areas under response curve and maximal concentrations of cortisol and alpha-amylase was calculated. We could observe that the negative emotional states correlated together positively, with the correlation coefficient varying from .59 between sorrow and stress to .87 between anxiety and anger. Joy and interest correlate together positively ($r = .42$) and negatively with negative emotions.

The scores on the Social Phobia Scale correlated with the intensity of all emotional states assessed by the Differential Affect Scale, the correlation with joy and interest being negative and positive with negative

emotional states like stress and shame. In turn, the Systemizing Quotient correlated negatively with the intensity of reported sorrow, anxiety and shame and the Autism Quotient positively with shame, anxiety and with the scores on Social Phobia Scale. Noteworthy is the fact that the only correlation between behavioral measures and biological markers of stress was observed between the Empathy Quotient and maximal concentration of α-amylase ($r = -.30$, $p < .01$).

To test the hypothesis that the studied SNPs are associated with differences in the magnitude of reported emotional states during the TSST, multivariate analysis of variances on their areas under the curve and on their maximal intensity by the three SNPs' factors were performed. Using Pillai's trace, neither effects on the areas nor effects on the maximal intensity of the emotional states assessed by the DAS were significant. Additionally separated repeated measures analysis of variance with the 6 measures of each emotional state accessed during the TSST as dependent variables and the same SNPs' factors as independent variables were conducted and confirmed an effect of time on the

emotional states' intensity during the test for almost every state included in the model (table 8). Factors and interaction effects, in concordance with the multivariate ANOVAs, were non-significant.

Table 8: F-tests from repeated measures ANOVAs on the 6 measures of the subscales of the Differential Affect Scale by the factor time after respective Greenhouse-Geisser correction of the degrees of freedom according to Epsilon value. A significant effect of the TTTS on reported emotional states was for every analyzed emotion except Sorrow. (*) $p < .05$, (**) $p < .01$, (***) $p < .001$.

	Epsilon	Df	F	Sig.	η_p^2
interest	.781	3.904	22.529***	.000	.238
joy	.735	3.675	5.360**	.001	.069
astonishment	.586	2.931	10.406***	.000	.126
sorrow	.605	3.025	1.193	.314	.016
anger	.379	1.894	3.348*	.041	.044
anxiety	.406	2.029	4.711*	.010	.061
shame	.438	2.189	3.453*	.030	.046
stress	.624	3.122	16.926***	.000	.190

4.2.2.4. Effects on the impression given by participants on raters

To test the hypothesis that the participants with different genotypes give a different impression to raters during an interview, three behavioral scales were employed. One of them was a scale in which the raters counted the occurrence of eye contact with the interviewers, looks at the ceiling, self grooming, not correctly finished spoken sentences, number of “ahmmmmss” and “mmhhhss” etc., looks to the ground, speech pauses, stuttering and stammering, looks at the watch and requests to continue talking. The second scale was a global impression scale, in which the raters manifested their opinion using a 5-point Likert scale (not at all, a little, average, much and very much) regarding the following characteristics of the interviewee during the interview itself: arm movement (gesticulation), whole body movement (moving around), head movement, convincing, self-confident, structured, motivated, strained, familiarity with speeches, nervous, angry, even-tempered, witty, charming, insecure, anxious, friendly, calm, experienced and annoyed. The third and last scale investigated the speculative opinion of the

raters about the participants' social competence and academic performance through the following characteristics scored using also a 5-point Likert scale: intelligent, successful, integrative, helpful, cooperative, honest, trustworthy, loyal, sympathetic, ambitious, success-oriented, career-oriented, and if the rater considered the participant as a potential candidate for the final phase of a selective process of a company. All these three scales were specifically designed for this study and were not previously validated.

First, the inter-rater reliability was investigated by the calculation of Pearson product- moment-correlation coefficients between the items' scores from the three different raters. From the first scale, the item "not correctly finished spoken sentence" did not show any significant correlation among the raters while on the item "eye contact with interviews" a significant correlation was found only between two of them. A correlation between only two raters was also observed on the item "whole body movement" from the second scale and on the items "cooperative", "loyal" and "ambitious" from the third scale. The rest of the items showed significant

correlations ($p < .05$) between more than two or between all raters.

Before multivariate analysis of variance on the three scales were conducted, the data were subjected to factor analysis in order to reduce the dimensions of each scale. For this purpose, the mean scores from the different raters, between which a significant correlation was observed, were calculated. The item “not correctly finished spoken sentences” was not included in the analysis because no correlation was observed. For the items with only one significant correlation mentioned in the paragraph above, the mean between both correlated raters was calculated while the third non-correlated score was excluded. In cases with significant correlations between one and the other two raters but not between all three raters, means were calculated using the scores of all three raters in the same manner as for cases with correlation between all. These means were finally used as variables on the three factor analyses reported below.

4.2.2.4. Effects on the frequency of observed behaviors

The first analysis of the effects of the investigated SNPs on the impressions of blind raters was performed on the first scale mentioned in the section above, which was designed to measure the frequency of specific behaviors during the interview. A principal component analysis was conducted on the 9 items of this scale, which evidenced a significant correlation between a least two raters, with oblique rotation (direct oblimin). This method is considered more appropriate for factors with presumed significant correlations. The Kaiser-Meyer-Olkin measure, which verifies the sample adequacy for the analysis was “miserable” (KMO = .532) according to Hutcheson and Sofroniou (Hutcheson & Sofroniou, 1999). Two methods of extraction were compared. By Kaiser’s criterion, four factors had eigenvalues larger than 1 and explained in combination 65.07% of the variance. The communalities of the items after extraction, which represent the amount of variance in each variable that can be explained by retained factors, ranged from .42 to .90 with overall communality equal to .650. However,

the scree plot was ambiguous and the most evident point of inflexion justified the retainment of 2 factors, which in conjunct explained 40.71% of the variance. In this case, the communalities were considerably lower and varied between .032 and .806 with overall communality equal to .407. A table of factor loadings for these two solutions is presented below.

Table 09. Pattern matrix with extraction according to Kaiser's criterion and according to the scree plot point of inflexion. Loadings lower than .5 were omitted.

	Kaiser's criterion's factor loading				Inflexion's factor loading	
	1	2	3	4	1	2
requests to continue talking	.860				.898	
speech pauses	.849				.859	
“ahmmmmss” and “mmhhhss”	-.642				-.527	
stuttering and stammering		.743				.690
looks at the watch		-.665				
looks at the ceiling			. 528			-.507
self-grooming			-.875			
eye contact with the interviewers				.969		
looks to the ground						

Extraction Method: Principal Component Analysis

Rotation Method: Oblimin with Kaiser Normalization

As can be seen on the table above, both solutions contain factors that are difficult to interpret. Factor 1 is an exception, loaded similar in the same methods and seems to represent speech interruptions. Apart from the inadequacy demonstrated by the KMO and the difficulties in interpreting the factors, the first solution was kept for further analysis because it explains a significantly larger part of the variance and has a more appropriated overall communality.

According to this solution, means between the z-scores of the variables that loaded in the same factor were calculated respecting their direction (positive or negative loading) and were included as dependent variables in a multivariate analysis of variance by the SNPs with the same leveling adopted for initial analysis in the sections above (allelic level). Since the principal component analysis on these variables did not produce a satisfactory dimension reduction, a multivariate analysis on the 10 raw scores was also conducted. In both cases, significant effects of the SNPs and their interaction could not be observed.

4.2.2.4.2. Effects on global impressions

In order to investigate the effects of the studied SNPs on the global impressions, a similar procedure to that described above was adopted here. The means between the scores that showed at least one significant correlation between raters were included as variables in a principal component analysis with oblique rotation (oblimin). As at least one significant correlation was found in all items of this scale, all 20 items were included. The Kaiser-Meyer-Olkin measure of sample adequacy was "meritorious" according to Hutcheson and Sofroniou (1999) (KMO = . 862) and the communalities ranged from .54 to .88 with overall communality of .77. Four factors were extracted by Kaiser's criterion and the scree plot was not ambiguous and a clear point of inflexion, which justified retaining 4 factors, could be identified. With this solution, the four factors explained in conjunct 77.5% of the variance of the data. A table of the factor loadings is presented below (table 10).

Table 10: Pattern Matrix with factor loadings on each item of the global impression scale. Loadings lower than .5 were omitted.

	Components			
	1	2	3	4
calm	.793			
even-tempered	.778			
self-confident	.575			
insecure	-.749			
nervous	-.808			
anxious	-.845			
strained	-.906			
arm movement		.802		
whole body movement		.749		
head movement		.746		
witty		.513		
angry			.931	

annoyed	.869	
friendly	-.768	
charming	-.505	
convincing		.826
structured		.844
motivated		.930
familiarity with speeches		.846
experienced		.674

Extraction Method: Principal Component Analysis

Rotation Method: Oblimin with Kaiser Normalization

The interpretation of the four extracted factors is clear and they represent the dimensions security, body movement, irritability and persuasiveness, respectively. For the multivariate analysis of variance 4 means of each group of variables that loaded together in each factor were calculated. For this purpose, the scores of negative loaded variables were inverted. Despite of the good suitability and clearness of the principal component

analysis, the F-tests of the multivariate analysis of variance on above mentioned variables by the SNPs of interest (allelic level) were all non-significant. Similarly, an additional multivariate analysis of variance on the raw variables by the same independent variables produced no significant results.

4.2.2.4.3. Effects on the impression of social competence and academic performance

In this last statistical analysis of this thesis, the effect of the studied SNPs on the impressions of raters regarding the social competence and academic performance of the participants was investigated. As in the section above, all items were included in the principal component analysis because at least one correlation was significant between raters in all items. KMO measure of adequacy was "meritorious" (.865) and the communality ranged from . 64 to .87. The overall communality was .79 and can be considered appropriate. Kaiser's criterion and scree plot point of inflexion were not conflicting and suggested the

extraction of two factors, which in conjunct explained 79.76% of the variance on the data. The table below presents the items with their respective loadings in both factors.

The interpretation of both factors is straightforward and reduced the dimensions of this scale in a sympathy and performance factor. As in the analysis in the two sections above, means of the two different groups of variables that cluster together in one factor were used as dependent variable for a multivariate analysis. The item "potential candidate for the next phase" was excluded because of loading in both factors. Multivariate tests on the two variables derived from the principal component analysis by the SNPs were all negative. Using Pillai's trace, multivariate tests on the 13 separated variables created from the means between items' scores that correlated together by the same factors, there was a significant result for the interaction between CD38 rs3796863 and OXTR rs2254298 ($V = .34$, $F(13,58) = 2.368$, $p = .013$, $\eta_p^2 = .347$). However, separate univariate ANOVAS revealed non-significant effects for any of the dependent variables.

Table 11: Pattern matrix with factor loadings on the items of the speculative impressions scale. Loadings lower than .5 were omitted.

	Components	
	1	2
helpful	.933	
trustworthy	.912	
cooperative	.907	
sympathetic	.894	
integrative	.868	
honest	.861	
loyal	.806	
success-oriented		.953
career-oriented		.944
ambitious		.887
successful		.816

intelligent		.799
potential candidate for the next phase	.637	.532

Extraction Method: Principal Component Analysis

Rotation Method: Oblimin with Kaiser Normalization

5. Discussion

Although this study was not designed to test the validity of the employed questionnaires, we could observe that the internal consistence of the subscales of the Autism Questionnaire was significantly lower than reported in their validation study (Baron-Cohen et al., 2001). Extreme differences were observed on the subscales "Communication" (the reported Cronbach's alpha in the validation study was .65 and, and in our study .39), "Attention Switching" (.67 vs. .55) and "Imagination" (.65 vs. .58). The remaining subscales and scales alphas were classified as acceptable or good in our sample. One plausible explanation for these findings is the investigation of a smaller and more homogenous sample in our study. The inclusion of probands with autism, a much larger group of students from different disciplines and a control group not composed of students in the validation study was associated with wider score range, which can be observed in the isolated comparison of the standard-deviation of our sample (N = 333) with the sample students of different disciplines investigated by Baron-Cohen et. al (2001b). The standard-deviation on

the Autism Spectrum Questionnaire in our sample was smaller (s = 5.0 vs. s = 6.4) and comparisons between the subscales' dispersion measures lead to the same conclusions. Similarly, several studies pointed out that the internal consistency of the subscales of the Autism Spectrum Quotient in the sample of Baron-Cohen (2001b) can be an overestimation of the internal consistency in studies with a more homogeneous population. These authors also consider the factor structure with 5 factors (social competence, communication, imagination, attention switching and attention to details) fragile and suggest solutions with 2 to 4 factors, none of them being completely satisfactory (reviewed by Kloosterman, Keefer, Kelley, Summerfeldt, & Parker (2011).

In accordance with the criticism of some authors that the Autism Spectrum Quotient is not suitable for comparisons within a homogenous sample, we could not replicate the finding that, in general, men have more autistic traits than women. However, we confirm that men have a lower levels of empathy (E) and a higher tendency to systematize (S). Several studies confirmed

the hypothesis that women have a more pronounced empathic response and suggested that this difference is most evident in tasks that assess emotional empathy, and can be attenuated by intranasal administration of oxytocin to male subjects (Mestre, Samper, Frias, & Tur, 2009; Berthoz, Wessa, Kedia, Wicker, & Grezes, 2008; Hurlemann et al., 2010; Theodoridou, Rowe, & Mohr, 2013). The higher tendency to systemize observed in men is also in accordance with the few studies on this topic (Wheelwright et al., 2006; Baron-Cohen et al., 2003). The investigation of neurobiological factors associated with the development of extreme male-brain type (S>>E) was not a subject of many studies. The few available studies pointed out the role of testosterone and oxytocin on systemizing and empathy skills differences observed between men and women (Theodoridou et al., 2013; Valla et al., 2010). In our sample, the SNP CD38 rs3796863 and its interaction with the SNP OXTR rs2254298 could explain part of the variance observed on the EQ in the female sample. Here, the presence of at least one protective allele (A) on CD38 rs3796863 was associated with a higher score on the EQ and the analysis

of the interaction suggested that this effect is partially modulated by the presence of the critical allele (A) on the OXTR rs2254298. Only female carriers of at least one critical allele on the OXTR rs2254298 are susceptible to the effect of CD38 rs3796863 and are more or less empathic in dependency of the presence or absence of an adenine (C>A).

To date, three studies examined the direct effect of OXTR rs2254298 on empathy while one of them reported a positive result in a cumulative genetic risk approach which included the total number of critical alleles of this and 4 other SNPs of the oxytocin receptor gene (Schneiderman, Kanat-Maymon, Ebstein, & Feldman, 2013). Another study reported differences on the empathy scores related to genetical variability on this SNP on schizophrenic patients but, contrary to our hypothesis, patients carrying at least one critical allele scored higher on empathy tests than homozygotes GG (Montag et al., 2012). The only study that supports an association between lower level of empathy and the presence of the risk allele was conducted by Wu, Li, & Su (2012). Although association with autism and social

behavior have been described (Feldman et al., 2012; Higashida et al., 2012), direct examination of the effects of CD38 rs3796863 on the variability on empathy scale scores was not performed until the present date. Our study is the first to indicate a role of this SNP in the regulation of social behavior in women, which can be specifically characterized by altered empathy. The effect of the observed interaction was also not a subject of behavioral studies. However, genetic imaging studies demonstrated significant interactions of CD38 rs3796863 with the administration of oxytocin and with polymorphisms of the dopaminergic system (Sauer et al., 2012; Sauer, Montag, Reuter, & Kirsch, 2013). Sauer et al. (2012) demonstrated that oxytocin administration is only associated with shorter reaction times and activation in the fusiform gyrus during visual social processing among subjects with CC genotype. Since lower oxytocin plasmatic levels and lower transcription rates were identified on CC homozygotes on CD38 rs3796863 (Lerer et al., 2010; Jin et al., 2007), we can speculate that the genotype OXTR rs2254298 A+ is associated with an altered OXTR receptor function and/or transcription

which can be compensated or completely reverted by the increased oxytocin secretion associated with the genotype CD38 rs3796863 A+. Investigation of brain activation during social tasks and administration of oxytocin in different genotype groups as mentioned above for CD38 rs3796863 and their results give support to this preliminary hypothesis. Molecular and gene expression analysis, which can identify structural and functional changes on OXTR, are required and could explain how base substitution alter the neurophisyology of the social circuitry and determine behavior.

Similarly to OXTR rs2254298, several studies reported an association of the OXTR rs53576 with autism and social behavior but a recent meta-analysis was not able to demonstrate a significant effect for any of these two SPNs on these behavioral domains (Bakermans-Kranenburg et al., 2013). Our data contradicts this meta-analysis and shows an effect of OXTR rs53576 on the prevalence of autistic traits in male subjects and indicates the existence of a dose dependent effect of the critical allele (A). This effect can mainly be described as altered attentional functions, since the presence of the critical

allele (A) was associated to a stronger tendency to pay attention to details and with difficulties on attention switching and did not affect the scores of the remaining subscales. Our data also support the hypothesis that risk alleles from these three SNPs are associated with altered social behavior in a gender specific manner. While genetical variation on CD38 rs3796863 and on OXTR rs2254298 explains part of the variance observed on empathy scores in the female sample, the OXTR rs53576 was associated with the presence of autistic traits in males. The moderation of gender on the prevalence of depression associated with genetical variation of a polymorphism in the promoter region of the serotonin transporter gene (5-HTTLPR), the monoamine oxidase A-upstream variable number tandem repeat (MAOA-uVNTR), and negative life events (NLE) has already been described (Priess-Groben & Hyde, 2013; Hammen, Brennan, Keenan-Miller, Hazel, & Najman, 2010) and gender differences on the prevalence of autism, on social skills and on OXTR distribution in the ZNS indicate the presence of gender-specific risk factors and different susceptibility to them also in the development of

sociability. This hypothesis was not taken into account in the majority of studies and could explain part of the inconsistence of the findings and the negative results of the above cited meta-analysis.

The first study that investigated the genetic aspects of the larger sex bias in the prevalence of autism spectrum disorders found a significant excess in the total number of linkage peaks and identified a major male-specific linkage peak at chromosome 17q11, where the serotonin transporter gene (SLC6A4) is located (Stone et al., 2004). Based on this result, the authors suggest that gender-related phenotype heterogeneity should be taken into account in genetical studies. Another genome wide screen suggests that linkage to chromosome 7q and 16p is associated with autism in male-male affected sibling pairs while linkage to chromosome 15q appears to be attributable to male-female/female-male affected sibling pairs (Lamb et al., 2005). Genotype-gender interactions were also demonstrated on SPNs of the oxytocinergic system. Using the data from 2 large U.S. prospective cohorts with over 11,000 individuals, Chang et al. (2014) identified a gender-heterogeneous effect of the OXTR

SNP rs4686320 and a gender-specific effect of the OXTR rs53576. Male OXTR rs4686320T carriers scored higher on social connectedness while female T carriers scored lower. However, the observed gender-specific effect of the OXTR rs53576 is not in accordance with our data, since in this study the presence of the critical allele A was associated with social connectedness only in women.

Sex-dependent mechanisms impacting the structure and function of hypothalamic-limbic circuits that are of potential clinical and translational significance were complementarily identified in healthy subjects. At the level of brain structure, there is evidence that the presence of the OXTR rs53576 A is associated with social dysfunction and is reflected in morphometric alterations of the hypothalamus and amygdala. An allele-load-dependent decrease in gray matter volume in the hypothalamus, the oxytocinergic core of the brain, was associated with reduced reward dependence in males, which reveals a lower degree of human empathy, social communication and need for interpersonal contact (Tost et al., 2010). Correspondingly, Wang et al. (2013) indentified a lower functional connectivity in the

hypothalamus and a weaker resting state functional connectivity between the hypothalamus and the dorsolateral prefrontal cortex in male AA homozygotes on the same SNP.

In females, the presence of a critical allele A on the OXTR rs2254298 was associated with greater amygdala volume, decreased volume in a region of dorsomedial anterior cingulate cortex (ACC) and greater volume of the posterior brainstem (Furman, Chen, & Gotlib, 2011). Considering this and the results of a meta-analysis on empathy for pain which revealed consistent activation in the anterior and midcingulate cortex (Lamm, Decety, & Singer, 2011), it can be hypothesized that changes in the anterior cingulated cortex associated with genetic variability on the OXTR rs2254298 are responsible for altered empathy observed in females. However, the isolated effect of OXTR rs2254298 on empathy scores was not observed in our study and the observed effect of the interaction with CD38 rs3796863 revealed that the critical allele A on the first SNP was associated with higher empathy scores among CC homozygotes on the second SNP, which is in accordance with studies showing

a higher risk of autism associated with the critical allele A in Asian populations and with the allele G in Caucasians on the OXTR rs2254298 (Liu et al., 2010; Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2008). Additionally, some studies suggest that the presence of this critical allele is associated with greater amygdala volume in manual tracing whereas voxel based morphology techniques were not able to replicate these findings reviewed by Zink & Meyer-Lindenberg, (2012). Although the hypothesis of modulation of sex on the effects of different SNPs of the OXTR can still be refuted by the current state of knowledge, two additional studies indicate that correlations between OXTR rs2254298 genotype and hypothalamus gray matter are influenced by gender under the influence of ethnicity. In male Caucasian subjects, the presence of a critical allele A was associated with smaller hypothalamus gray matter volume and an effect of genotype in females was not observed (Tost et al., 2011). In contrast, an effect of this SNP was only observed on hypothalamus gray matter volume of Japanese females (Yamasue et al., 2011).

Ethnicity modulation is also supported by a preliminary behavioral study which investigates the interaction of distress and support-seeking in Korean and American participants. As hypothesized by the authors, a three-way interaction was confirmed. OTXR rs53576 AA American participants tend to seek less support under higher distress levels while between Korean participants and between participants under lower distress levels no genotype effect on support-seeking was observed (Kim et al., 2010). Likewise, an interaction between genetical variants on the OXTR and environmental factors has been reported. To illustrate this, Heim et al. (2009) found a decreased oxytocin concentration in the cerebrospinal fluid of women that suffered severe childhood abuse or neglect in comparison with women with no history of severe maltreatment. Complementarily, evidence of an interaction between child maltreatment, attachment style and emotional regulation, and OXTR rs53576 was also provided. African American male and female G allele carriers showed a more pronounced emotional dysregulation and a more frequent disorganized attachment style when exposed to child maltreatment

(Bradley et al., 2011). The additional lack of influence of negative experiences observed in homozygotes AA and the better sociability usually associated with the allele G is in accordance with the recently proposed differential susceptibility theory, which defends that the genotypes associated with a better outcome under positive experiences are also associated with functional impairments when exposed to negative life-events (Belsky et al., 2009). Although this theory describes only gene-environmental interactions, our study suggests that OXR rs225429A+ carriers can be susceptible to CD38 rs3796863 effects on the determination of empathy skills in females which can be characterized as a three-way interaction (gene-gene-gender interaction). Besides the description of the effect of the mentioned interaction, this study is also the first which evidenced a main effect of CD38 rs3796863 on empathy in females.

The research on the determinants of personality traits has been a challenge for modern neuroscience and has been facing the same methodological problems of research on complex diseases like diabetes, autism, asthma, bipolar disorder, Alzheimer's disease etc. These diseases are

likewise characterized by sexual-dysmorphic features and specific prevalence, relative low concordance between monozygotic twins, higher prevalence of sporadic cases in comparison with familiar occurrence, gender-dependent heritability and susceptibility to environmental factors. The investigation of their genetical predisposition must include the concomitant analysis of factors associated with differences on their prevalence. In addition to gender and environmental factors, which regulate gene expression through epigenetic modifications of DNA structure, gene-gene interactions should also be taken into account. These and other methodological problems, like the small sample size found in some studies including ours, are plausible explanations for the heterogeneity of findings and their inconsistence reported in the scientific literature.

Our study also provides evidence that the Trier Social Stress is suitable to identify changes in participants' emotional states and in the event-dependent secretion of cortisol and alpha-amylase. However, significant correlations between subjective emotional stress experience and concentration of both HPA- and SNS-

markers were not identified. The effect of the TSST on cortisol secretion was confirmed by several authors and this test is considered one of the most effective in eliciting physiological stress responses (Dickerson et al., 2004). Although the number of studies including the analysis of alpha-amylase concentration is smaller, the TSST test can also be considered an effective trigger of sympathetic activity and consequent salivary alpha-amylase concentration increase (Thoma, Joksimovic, Kirschbaum, Wolf, & Rohleder, 2012; Maruyama et al., 2012; Trueba, Mizrachi, Auchus, Vogel, & Ritz, 2012). Regarding the correspondence of emotional stress response with physiological responses, a review of 49 articles published up to August 2011 could not confirm a clear and robust association between them. In their conclusion, the authors suggest that dissociation between emotional and physiological responses should not be considered pathological, but could rather reflect a normal reactivity pattern and/or the result of several methodological issues. The authors also suggest that studies on this topic should include relevant factors which influence emotional-physiological correspondence

such as psychological traits and states and physiological disposition (Campbell & Ehlert, 2012). In our study, only correlations between the areas under the response curve of the 8 assessed emotional states and alpha-amylase and cortisol salivary concentration, and correlations between these two biological stress-markers and four personality traits were calculated. A detailed analysis of the correspondence of psychological and physiological responses should have included additional statistical analyses, which would have taken into account the delay of physiological over emotional response and the interactions between personality traits and emotional states. Since this was not one of the purposes of our study, our negative results on the psycho-physiological correspondence during the TSST should be considered weak.

Several articles mentioned in the introduction of this thesis have confirmed the role of oxytocin in the modulation of physiological and emotional stress-responses in animals and humans. Although a single *in vitro* study reported that oxytocin has an excitatory effect on the HPA (Gibbs, Vale, Rivier, & Yen, 1984), several *in*

vivo studies in different species have confirmed that the administration or release of oxytocin is associated with anxiolysis and decrease in cortisol concentration (Neumann et al., 2000; Chiodera & Legros, 1981). Moreover, Donaldson et al. (2008) collected robust evidence that the regulation of OTXR within certain brain areas may underlie variations of observed anxiety and fear behavior. The mechanisms by which oxytocin modulates anxiety and fear are still not understood completely but there is enough evidence that suggests that this effect occurs by modulation of the HPA axis (Neumann, 2002) and a facilitation of prosocial/affiliative behaviors concomitant with attenuation of other neural systems involved in fear and anxiety (Carter, 2007) by, for example, binding to medial and central subdivisions of amygdala (Huber, Veinante, & Stoop, 2005). However, as demonstrated by studies on the effects of different polymorphisms of OXTR, the effects of oxytocin on the HPA and stress modulation also seem to be sexually dimorphic and dependent on gonadal steroids. OXT has been shown to inhibit both basal and stressor-induced secretion of ACTH and corticosterone in

male and female rats, but not in pregnant and lactating female rats. Task-dependence has also been observed, with social fear-anxiety tests being associated with elevated OXT levels and inanimate-tests with lower levels (Litvin & Pfaff, 2013).

Our study confirms the role of oxytocin on the modulation of HPA stress reactivity since higher salivary cortisol levels just before the stress situation and at the endpoint were observed in OXTR rs225429A+. Additionally, this group also showed a higher increase in cortisol concentration during the stress-phase. One study evaluated the influence of the SNP OXTR rs53576 and reports that critical-allele carriers are exposed to higher levels of cortisol during stress under social support (Chen et al., 2011), while another demonstrated that this group exhibits higher physiological and dispositional stress reactivity, as determined by heart rate response during a startle anticipation task (Norman et al., 2012). Direct evaluation of the influence of genetic variants of CD38 and of the OXTR rs225429 on cortisol levels during the TSST in humans were not conducted until the present date and this is the first study which evaluated the role of

these three SNPs and their interaction together. Contrary to the results mentioned in this paragraph, our study did not confirm the effect of OXTR rs53576 on cortisol levels and also could not identify any effect of CD38 rs3796863 and of any interaction. Notwithstanding, this is the first study which evidences that variability on OXTR rs225429 is associated with different HPA activity during stress. Possible explanations for the negative results of OXTR rs53576 on HPA activity are differences on study design like the absence of social support and the investigation of younger participants in our study. Effects of any SNP on the SNS, indirectly measured by alpha-amylase concentration, as well as on behavioral measures were equally not observed, which contradicts the only study on this topic that indirectly revealed heightened sympathetic activity in GG homozygotes no OXTR rs53576 during stress (Norman et al., 2012).

Differently from cortisol levels, in our study, alpha-amylase levels were associated with a larger variability and with relatively wider ranges, larger variances and standard errors, which could reflect methodological problems and has made the identification of smaller

effects difficult. Although the more robust effect of TSST on the activation of the SNS and alpha-amylase secretion was identified, the effects of the SPNs, which are usually much smaller and more sensitive to the overall variability of parameters, did not reach statistical significance. Methodological differences between our study and that of Norman et al. (2012) could also partially explain the observed divergence of results. As reported above, our study evaluated much younger subjects and included alpha-amylase concentration instead of physiological cardiac parameters, like heart rate and stroke volume, as dependent variables.

In brief, the TSST test is appropriate for the investigation of the HPA- and SNS-social stress responses and our study was able to demonstrate the influence of a genetic variation of the OXTR rs225429 on cortisol but not on alpha-amylase secretion. This effect was independent of the personality traits assessed in this study (social phobia, empathy, autistic traits and systemizing), since the inclusion of these variables as covariates did not alter the results. Modifications on the classical TSST and adoption of other parameters employed by other authors make

comparisons with the available data difficult and further studies need to verify if our observed positive and negative findings are replicable. Nonetheless, our study is partially in consonance with physiological and behavioral studies published so far, which indicate that alterations in OXTR, including receptor density, affinity and structural changes in intracellular signal transmission, are associated with differences in HPA activity during stress. Since in our study the TSST was only conducted in male participants and gender-specificity characterizes most of the findings on the oxytocinergic system, female participants should be included in further studies. Gonadal steroids can influence the oxytocinergic system in different ways including their most direct classical action through the binding to their respective intracellular receptors such as ERα and ERβ for estrogens, and AR for androgens, via interaction with DNA through response elements in the promoter region of OXT or OXTR genes. In rats, the administration of estradiol or testosterone increases OXT binding and OXTR mRNA in different areas of the brain. The OXTR in rats and mice contains a classic estrogen response element (ERE) and humans

half-palindromic ERE motifs, and indirect action-sites of steroids (non-genomic) including a PKC-dependent pathway were also demonstrated (Dhakar, Stevenson, & Caldwell, 2013). Considering the findings of behavioral studies including our own and reports of sexual dimorphisms on the distribution of OXTR, results from physiological studies on male samples, should also not be generalized to females. Some authors also suggest that menstrual cycle should be taken into account by data collection and analysis of female samples.

Finally, the last aspect investigated in our study was the effect of the studied genetic variants on the impression provoked by participants on blind raters. For this purpose, three scales were developed which assessed the number of specific non-verbal behaviors, and the impression of raters about participants' social behavior, performance and achievement. To date, only one study examined this question and reported that OXTR rs53576 GG homozygotes are judged to have more pronounced social skills than A-allele carriers. The authors also described a moderation of sex, the observed effect being larger in males. OXTR rs53576 GG homozygotes also

displayed a larger number of non-verbal affiliative cues, which accounted for the difference on raters' judgments (Kogan et al., 2011). In contrast, our study was not able to identify differences attributed to the investigated SNPs in any of the employed scales. Critical allele-carriers on the included SNPs did not cause a worse impression on blind raters and did not exhibit different patterns of non-verbal behavior. In comparison with Kogan et al. (2001), our study included a larger number of participants (80 vs. 23) and a much smaller number of raters (3 vs. 116) and can be considered less powerful to identify such effects. Therefore, these negative results should also be interpreted with caution and additional research on this topic is required.

In summary, the research described in this dissertation confirms the role of genetical variants of the oxytocin receptor gene and CD38 gene in the modulation of social behavior and physiological response to stress in humans and suggests that the observed effects are modulated by gender. More specifically, our analysis shows that male critical-allele carriers of the OXTR rs53576 have, on average, more autistic traits, mainly due to altered

attention function, while empathic skills in females are under the influence of CD38 rs3796863 and its interaction with OXTR rs2254298. Additionally, we could also identify that male critical-allele carriers of OXTR rs2254298 present a higher cortisol concentration shortly before the stress-phase and at the end of the observation period, and a more pronounced increase during the stressful situation when submitted to one of the most studied social-stress-paradigms.

These results contribute to explain the difference prevalence of autistic traits and empathy among men and women, since the prevalence of the alleles are different and their effects, gender-specific. Future studies should also include these and other variables, like ethiniciy, which is also associated with different allelic distribution. An interesting confirmatory hypothesis that the prevalence of autistic traits or even disorders is proportional to the distribution of these alleles in different populations need to be tested. **6.**

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7. Appendix

7.1. Correlation-tables between emotional states, behavioral traits and biomarkers of HPA and SNS activity

Correlations		AUC cortisol	cortisol (Max.)	AUC α-amylase	α-amylase (Max.)	SPS
SQ	Pearson's r	0.21	0.187	-0.04	0.04	-0.22
	Significance (2-sided)	0.06	0.09	0.71	0.70	0.06
	N	80	80	80	80	80
AQ	Pearson's r	-0.01	-0.07	-0.06	0.13	0.50
	Significance (2-sided)	0.95	0.51	0.56	0.24	0.00
	N	80	80	80	80	80

EQ	Pearson's r	0.09	0.13	-0.15	-0.30	-0.18
	Significance (2-sided)	0.40	0.25	0.17	0.00	0.11
	N	80	80	80	80	80
SPS	Pearson's r	-0.11	-0.19	-0.14	-0.03	1.00
	Significance (2-sided)	0.34	0.08	0.21	0.80	
	N	80	80	80	80	80

Table 6.1: Correlations between social traits scales' scores and area under the response curve and maximal concentration of cortisol and α-amylase. (AQ) Autism Quotient, (SQ) Systemizing Quotient, (EQ) Empathy Quotient, (SPS) Social Phobia Scale.

Correlations		Interest	Joy	Astoni-shment	Sorrow	Anger	Anxiety	Shame	Stress
Interest	Pearson's r	1.00	0.42	0.12	-0.02	-0.05	-0.02	-0.02	-0.10
	Significance (2-sided)		0.00	0.29	0.86	0.65	0.85	0.86	0.37
	N	80	80	80	80	80	80	80	80
Joy	Pearson's r	0.42	1.00	0.22	-0.23	-0.18	-0.15	-0.17	-0.30
	Significance (2-sided)	0.00		0.05	0.04	0.11	0.17	0.14	0.01
	N	80	80	80	80	80	80	80	80
Astonishment	Pearson's r	0.12	0.22	1.00	0.32	0.35	0.50	0.40	0.45

	Significance (2-sided)	0.29	0.05		0.00	0.00	0.00	0.00	0.00
	N	80	80	80	80	80	80	80	80
Sorrow	Pearson's r	-0.02	-0.23	0.32	1.00	0.60	0.67	0.67	0.59
	Significance (2-sided)	0.86	0.04	0.00		0.00	0.00	0.00	0.00
	N	80	80	80	80	80	80	80	80
Anger	Pearson's r	-0.05	-0.18	0.35	0.60	1.00	0.87	0.77	0.64
	Significance (2-sided)	0.65	0.11	0.00	0.00		0.00	0.00	0.00
	N	80	80	80	80	80	80	80	80
Anxiety	Pearson's r	-0.02	-0.15	0.50	0.67	0.87	1.00	0.84	0.76

	Significance (2-sided)	0.85	0.17	0.00	0.00	0.00		0.00	0.00
	N	80	80	80	80	80	80	80	80
Shame	Pearson's r	-0.02	-0.17	0.40	0.67	0.77	0.84	1.00	0.76
	Significance (2-sided)	0.86	0.14	0.00	0.00	0.00	0.00		0.00
	N	80	80	80	80	80	80	80	80
Stress	Pearson's r	-0.10	-0.30	0.45	0.59	0.64	0.76	0.76	1.00
	Significance (2-sided)	0.37	0.01	0.00	0.00	0.00	0.00	0.00	
	N	80	80	80	80	80	80	80	80

Table 6.2: Correlation-matrix of the 8 area under the curve of the 8 emotional states assessed by the Differential Affect Scale during the Trier Social Test.

Table 6.3: Correlations between the 8 mental states investigated by the Differential Affect Scale during the TSST, social behavior traits including autistic traits, empathic behavior, systemizing and social phobia and area under the response curves and maximal salivary concentration of cortisol and α-amylase.

Correlations		SQ	AQ	EQ	SPS	AUC cortisol	cortisol (Max.)	AUC α-amylase	α-amylase (Max.)
Interest	Pearson's r	0.22	-0.15	0.22	-0.06	0.02	0.08	0.01	-0.20
	Significance (2-sided)	0.05	0.19	0.05	0.60	0.86	0.48	0.96	0.07
	N	80	80	80	80	80	80	80	80
Joy	Pearson's r	0.12	-0.19	-0.03	-0.29	0.05	0.06	-0.09	-0.09

	Significance (2-sided)	0.30	0.10	0.77	0.01	0.64	0.59	0.44	0.42
	N	80	80	80	80	80	80	80	80
Astonishment	Pearson's r	0.00	0.18	-0.17	0.32	-0.05	-0.16	-0.11	-0.11
	Significance (2-sided)	0.97	0.10	0.12	0.00	0.63	0.17	0.33	0.32
	N	80	80	80	80	80	80	80	80
Sorrow	Pearson's r	-0.22	0.18	-0.13	0.57	-0.15	-0.21	-0.13	-0.03
	Significance (2-sided)	0.05	0.10	0.25	0.00	0.19	0.06	0.25	0.80
	N	80	80	80	80	80	80	80	80

Anger	Pearson's r	-0.20	0.19	-0.03	0.58	-0.07	-0.09	-0.10	-0.01
	Significance (2-sided)	0.08	0.09	0.76	0.00	0.53	0.41	0.38	0.93
	N	80	80	80	80	80	80	80	80
Anxiety	Pearson's r	-0.22	0.24	-0.15	0.63	-0.14	-0.16	-0.11	0.00
	Significance (2-sided)	0.05	0.03	0.19	0.00	0.23	0.16	0.35	0.97
	N	80	80	80	80	80	80	80	80
Shame	Pearson's r	-0.23	0.29	-0.19	0.74	-0.12	-0.18	-0.08	0.04
	Significance (2-sided)	0.04	0.01	0.09	0.00	0.28	0.11	0.46	0.76

	N	80	80	80	80	80	80	80	80
Stress	Pearson's r	-0.13	0.28	-0.07	0.69	0.00	-0.08	-0.14	-0.12
	Significance (2-sided)	0.24	0.01	0.56	0.00	1.00	0.48	0.20	0.30
	N	80	80	80	80	80	80	80	80

8. Acknowledgements

First of all I would like to thank Prof. Dr. Jürgen Hennig for giving me the opportunity to work in his team and for the sucssesfull supervision during the last four years. Without his thrust, patience and knowledge these studies would not have been possible. Second, I would like to thank Dr. Yvonne Küpper for her key position in the organization, data collection and planning of the runned experiements. For the mutual assistance, friendly orientation in our Laboratory and respect, I would also like to thank Mrs. Meineke. For the collegial atmosphere and support in our shared Office, Mrs. Müller. And finally, for the lovely companion and close and distant care, respectively, Chirstl Szesny and my parents. You helped me to deal with the ubiquitous non-technical questions and problems during this period.