In vivo antisense Targeting von Vasopressin mRNA in Ratten

In vivo antisense targeting on vasopressin mRNA in rats

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To my Family

List of abreviations

ACN	:	Anterior commissural nucleus
AL	:	Ansa lenticularis
ATG	:	Adenine, Thymine, Guanine
AUG	:	Adenine, Uracil, Guanine
BST	:	Bed nucleus of the stria terminalis
BW	:	Body weight
cDNA	:	Complementary deoxyribonucleic acid
cDNAs	:	Complementary deoxyribonucleic acids
CNS	:	Central nervous system
DI	:	Diabetes insipidus
DNA	:	Deoxyribonucleic acid
H_2O_2	:	Hydrogen peroxide
HCL	:	Hydrochloric acid
HNS	:	Hypothalamo-neurohypophyseal system
HPLC	:	High Performance Liquid Chromatography
i.c.v	:	Intracerebroventricular
IgG	:	Immunoglobulin G
ispi	:	Intersupraoptic-paraventricular islands
LSN	:	Lateral subcommissural nucleus (LSN=ACN)
M oligos	:	Methylphosphonate oligos
mpoa	:	Medial preoptic area
mRNA	:	Messenger ribonucleic acid
NaCl	:	Sodium Chloride
NP	:	Neurophysin
ODN	:	Oligodeoxynucleotide
ODNs	:	Oligodeoxynucleotides
oligo	:	Oligodeoxynucleotide
OT	:	Oxytocin
OVLT	:	Organum vasculosum of the lamina terminalis
P oligos	:	Phosphodiester ODNs
PBS	:	Phosphate buffer saline
Pe	:	Periventricular nucleus

pf	:	Perifornical region
PVN	:	Paraventricular nucleus
RNA	:	Ribonucleic acid
Rnase-H	:	Ribonuclease H
RT PCR	:	Real time polymerase chain reaction
S oligos	:	Phosphorothioates oligos
SCN	:	Suprachiasmatic nucleus
SON	:	Supraoptic nucleus
V2	:	Vasopressin 2 (receptor)
V2R	:	Vasopressin 2 receptor
VP	:	Vasopressin
Zi	:	Zona incerta

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1 INTRODUCTION

The impact of certain therapeutic agents, whose mechanism of action is based on its influence over the genetic code could certainly revolutionize contemporary pharmacology. Antisense targeting, or antisense strategies are among the several proposed approaches. This technology theoretically offers the exciting possibility of selectively modifying the expression of a particular gene without affecting the function of others (Akhtar and Juliano, 1992). Such selective inhibition of a gene expression allows the study of physiological and pathophysiological cellular processes on a molecular basis (Schlingensiepen and Schlingensiepen, 1997). The first definitive demonstration of a block in translation due to the use of antisense RNA in cell-free extracts was carried out in 1963 by Singer, Jones, and Nirenberg (Pestka, 1992). However, it was not until 1978 that this novel concept became popular with the work of Zamecnik and Stepheson. At that time, these authors published their results on blocking the replication of Rous Sarcoma Virus in infected chicken fibroblasts by adding a synthetically assembled oligodeoxynucleotide (oligo) directed against a specific sequence of a viral genome (Zamecnik and Stepheson, 1978). Since then, these pioneering works and many subsequent studies have shown impressive results about the effects of this technology modulating biological processes. In the last few years, the antisense technology has been considered not only a useful tool to study a gene function but also it aroused interest about its possible use as therapeutic agents to treat many diseases (Sharma and Narayanan, 1995; Galderisi, Cascino and, Giordano, 1999). The possibility of blocking specific gene expression without multiple side effects projects towards drugs of a new generation, which would exert their action

at the molecular level (Monteith and Levin, 1999), hence some authors have considered antisense strategies, as the "magic bullet" for the treatment of certain illnesses (Stein and Cheng 1993; Ma and Doan, 1994).

Antisense therapeutics using synthetic oligodeoxynucleotides (ODNs) are currently being evaluated in clinical trials for cancer, inflammation, and viral diseases (Monteith and Levin, 1999; Yuen et al., 1999; Nemunaitis et al., 1999). In neuropharmacology, antisense technology has also become a very valuable tool to block the expression of specific genes in vitro as well as in the intact brain of animals (Heilig and Schlingensiepen, 1996). A potential future use of this technique on the central nervous system (CNS) of humans could include several neurological disorders (Telleria-Diaz, 2000) such as Brain tumors (Engelhard, http://www.moffitt.usf.edu /cancjrnl/v5n2/article7.html), the ischemic Stroke (Wahlestedt et al., 1993), and some neurodegenerative disorders like Alzheimer or Parkinson disease (Soreq and Seidman, 2000; Van Kampen, McGeer, and Stoessl, 2000). However, it should be pointed out that the actual cascade of cellular and molecular events in antisense targeted tissues is still highly hypothetical to date. On the other hand, there is little information regarding the side effects these compounds could produce during a long term administration. Therefore more investigations are required before the introduction of this therapeutic modality on the human CNS can be implemented.

1.1 The antisense concept.

Cell must synthesize proteins by means of the expression of thousands of genes to

maintain its function. The specific information of each gene is encoded in the DNA. To be expressed, a gene is transcribed from the DNA into messenger RNA (mRNA) and subsequently translated into the corresponding protein (Schlingensiepen and Schlingensiepen, 1997). The DNA molecule consists of two associated polynucleotide strands that wind together in a helical fashion. DNA has a sense strand (5'→ 3'), which encodes the synthesis of a protein; the complementary strand is antisense (3'→5'), and it does not code for the synthesis of any protein. The single stranded Pre-mRNA is copied from the antisense strand and has the same sequence of nucleotides as the sense strand of DNA except that the thymine is replaced by uracil. A short complementary synthetic antisense oligo will hybridize to the mRNA leading to a translational arrest (Phillips and Gyurko, 1997) (Fig.1). Non-homologous or mismatched sequences of other genes do not form stable enough hybrids for the antisense oligo to inhibit protein translation. Thus, it is possible to target a unique sequence present in a single gene. Since by definition, the mRNA is the "sense" strand, any complementary sequence is "antisense" to it. The antisense orientation is a prerequisite for sequence-specific inhibition of translation, therefore this term is attributed to the technique as a whole (Schlingensiepen and Schlingensiepen, 1997).

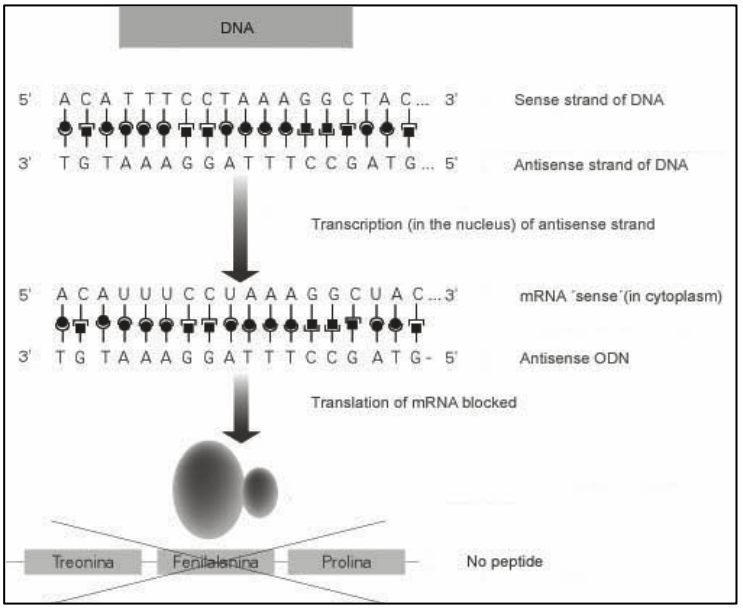


Fig1. An antisense ODN contains the same bases sequence as the DNA no coding strand.

1.2 Uptake and intracellular fate of antisense ODNs.

The pioneering work of Zamecnik and Stephenson, and many other further studies demonstrate that despite their large size and polar nature, oligonucleotides do indeed enter cells. However, ODNs in their natural form have a phosphodiester backbone that renders them subject to rapid degradation in the blood, intracellular fluid, or cerebrospinal fluid by exo- and endonucleases. The half-life of phosphodiester ODNs (P oligos) is in the range of minutes in blood and tissue culture media. In cerebrospinal fluid, the half-life of ODNs is a few hours (Phillips and Gyurko, 1997), therefore several chemical modifications have been proposed to prolong the half life of ODNs in biological fluids while retaining their activity and specificity (Wagner 1995) (Fig.2).

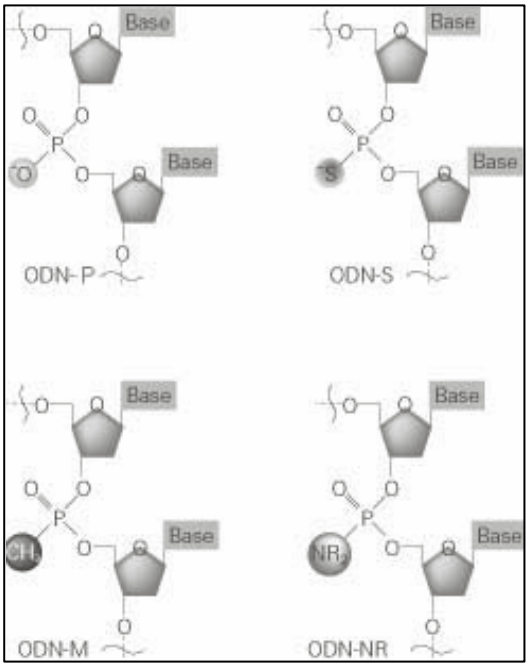


Fig.2. Since unmodified DNA oligonucleotides are very unstable in the biological milieu, a number of number of structural analogues have been developed with improved stability towards nucleases. Backbone-modified ODN analogues include the phosphorothioates (S-oligos), methylphosphonates (M-oligos), and phosphoramidates (NR-oligos) among others.

The first significant investigations on the mechanism of cellular uptake of DNA oligonucleotides (with P oligos) showed that these probably bind to a specific cell surface binding protein before being internalized by endocytosis (Akhtar and Juliano, 1992). The existence of a specific cell surface receptor for uptake of P oligos by endocytosis was also confirmed by other authors (Yakubov et al, 1989). Phosphorothioate oligos (S oligos) are also thought to enter cells by receptor mediated endocytosis. Using phosphorothioate homopolymers conjugated to a fluorescent acridine at the 5'-end, it was demonstrated that uptake of S oligos was somewhat slower than that of corresponding P oligos, but was dependent on chain length, with shorter S oligos being taken up faster than longer ones (Stein et al., 1988). Since

methylphosphonate oligos (M oligos) are neutral and fairly hydrophobic, these molecules were initially thought to enter cells by passive diffusion, but certain studies suggest that this is unlikely to be their predominant mode of cellular uptake (Akhtar and Juliano, 1992). A study assessing efflux (transport) kinetics of M oligos across model membranes (liposomes) showed that they were transported across model membranes at similar rates to P oligos and S oligos -thought to enter cells via endocytosis- (Akhtar S et al., 1991).

Upon cellular entry and escape from endosomal compartments, ODNs migrate to the cytoplasm and nucleus. However, it is unlikely that they exert their action solely in the nucleus because antisense activity has been observed with oligomers targeted to vesicular stomatitis virus, whose replication cycle is entirely cytoplasmic (Lemaitre, Bayard and Lebleu, 1987; Kulka et al., 1989).

1.3 **Pharmacokinetics of antisense ODNs.**

Comparison of different antisense oligo compounds shows that pharmacokinetics depend on the chemistry and to a lesser extent on length or sequence. P oligos are degraded rapidly in the plasma with a half-life of about 5 min if injected intravenously into monkeys (Agrawal, Temsamani and Tang, 1991) or rats (Inagaki et al., 1992). After 14 min the entire dose is degraded so that organ uptake of the intact compound is virtually impossible (Schlingensiepen and Schlingensiepen, 1997). The half-life of M oligos in mice plasma is 6 min for body distribution and 17 min for elimination (Chen et al., 1990).

Intravenously administered S oligos are cleared from plasma biphasically with an alpha phase of about 50 min and a beta phase of 40 hours. Initial short half-life is due to distribution into most major organs, with the brain having the lowest concentration. S oligos are then slowly eliminated from the body, primarily with urine. Up to 30% is excreted within 24 hours and 70% within 10 days after single administration. Up to 10% is excreted in the faeces. Constant plasma concentrations are obtainable by one daily injection. S oligos are very stable in all tissues except liver and spleen where 50% of any particular S-ODN is degraded within 2 days. Breakdown products appear to be eliminated preferentially since almost no intact oligo is found in the urine whilst plasma contains predominantly undegraded oligo (Schlingensiepen and Schlingensiepen, 1997).

Since ODNs enters the brain only in minute quantities after intravenous, subcutaneous, or intraperitoneal administration (Engelhard, http://www.moffitt.usf.edu /cancjrnl/v5n2/article7.html), there are two main strategies for applying ODNs to brain tissue. One is to infuse ODN into cerebral ventricles, and the second is to inject them intracerebrally (Szklarczyk and Kaczmarek, 1995). While a P oligo (unmodified oligo) was rapidly degraded following intra-ventricular injection, a S oligo remained stable (Whitesell L et al., 1993). In another study (Yaida and Nowak, 1995), a P oligo exhibited a limited and strictly periventricular distribution after intraventricular administration. In contrast the S oligo showed significant penetration into and accumulation within brain, with extensive uptake in ipsilateral striatum and dorsal hippocampus, as well as in midline periventricular structures.

1.4 Mechanism of action of the antisense ODNs.

Although the exact mechanisms of antisense ODNs is still not known, there are at least 5 different mechanisms proposed which include: 1) Inhibition of translation by hybrid formation that inhibits peptide synthesis; 2) formation of a ribonuclease H (RNase-H) substrate; 3) inhibition of mRNA transport from nucleus to cytoplasm because of a partially double-stranded structure formation; 4) inhibition of RNA processing by interference of splicing events; 5) triplehelix formation (Akhtar and Juliano, 1992; Phillips and Gyurko, 1997; Schlingensiepen and Schlingensiepen, 1997) (Fig.3).

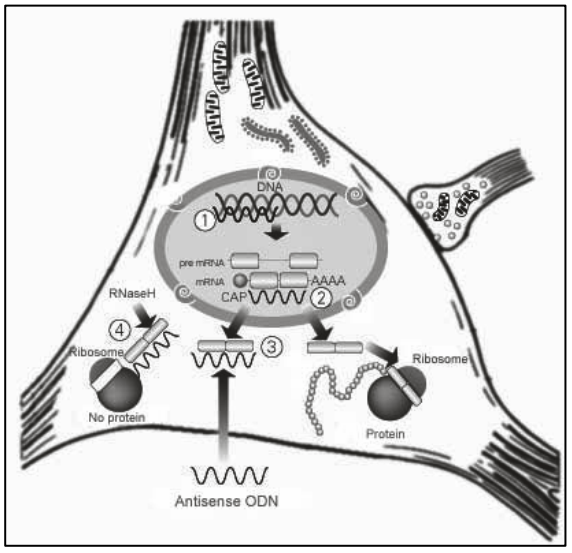


Fig.3. Principle of antisense inhibition. *Right*: normal gene activity in which mRNA is produced in nucleus and transported to cytoplasm where it enters ribosome and dictates sequence of amino acids to form a protein. *Left*: in principle ODN should bind to mRNA and prevents translation of message for proteins from ribosome assembly (3). Alternatively, antisense ODN can produce triple-helix formation (1), hybridization to formed mRNA preventing its transport from the nucleus to cytoplasm (2), or destruction in cytoplasm of the mRNA by the RNase stimulation (4).

Antisense inhibition could be considered as another example of the pharmacological receptor theory where, the ODN is the drug and the target sequence is the receptor, in this case, the concept relies on the strength and efficiency of specific base-pairing to mRNA through Watson-Crick hybridization. For binding to occur between the two, a minimum level of affinity is required based on the hydrogen bonding between base pairs. To achieve pharmacological activity, a minimum number of 15 bases provides a minimum level of affinity (Crooke, 1993). Increasing the length of the antisense should result in a higher level of specificity, but there is an upper limit of ~25-30 nucleotides above which ODNs are not incorporated effectively by cells. Therefore, instead of increasing length, it is best to test the specificity of the antisense sequence against the genetic database (GenBank) for possible cross-reaction with sequences other than the target sequences (Phillips and Gyurko, 1997).

Antisense ODNs are designed and synthesized in the hope they recognize specific nucleic acid sequences contained within a mRNA, so that they bind predominantly to cytosolic mRNA sense strands through hydrogen bonding to complementary nucleic acid bases, then, the resulting hybrid could not be translated into the encoded protein (translational arrest) because either translation initiation is inhibited or double strand is a substrate for RNase H. Both mechanisms are thought to lead to a selective inhibition of protein synthesis. However it should be pointed out that evidences for a specific translation arrest have been provided by several studies showing that the antisense oligos can block the synthesis of a single protein without reducing the level of the correspondent mRNA (Boiziau et al., 1991; Brysch et al, 1994). *In vivo*, the importance of RNase H for the antisense effect remains to be proven for most cell types (Schlingensiepen and Schlingensiepen, 1997). High RNase H activity naturally occurs

in tissues of very high proliferative activity like embryonic tissue or oocytes (Dagle, Weeks and Walder, 1991). However, in other cell-types the role of the enzyme appears to be minor. It has been demonstrated, in various non-embryonic cultured cells, that down regulation of protein translation is not enhanced by degradation of the respective mRNA even after exogenous addition of RNase H (Schlingensiepen and Schlingensiepen, 1997).

1.5 Design of antisense agents and target selection.

ODNs can be designed to target different parts of the DNA-RNA machinery. To make an ODN that will be effective, the conventional wisdom is that it has to be at least 11-15 nucleotides long but not longer than 20-25 nucleotides. Longer sequences decrease uptake into cells and shorter sequences lack specificity (Phillips and Gyurko, 1997). However, other authors (Schlingensiepen and Schlingensiepen, 1997) remark that the minimum length of an oligo has to be 12 bases to recognize a single specific sequence in the genome. This assumption is based on the estimated number of base pairs in the human genome and a random distribution of bases. For them, it would be more difficult to define a maximal length for an antisense compound since its uptake is time and temperature dependent and it is also influenced by cell type, cell culture conditions, media and sequence, etc. Furthermore, it has been shown that antisense molecules of more than 30 bases trigger a genetic cascade of cellular antiviral response (Manche et al, 1992).

A straightforward way to design an antisense ODN is to target the initiation codon (AUG) and the neighboring bases and compare them with a sequence upstream (5'

end) and downstream (3' end) from that site. The initiation codon of the mRNA is the site that triggers protein production as the message is translated into amino acids in the ribosomes. This strategy is also convenient because the majority of the cDNAs reported in the GenBank contain the AUG region. Therefore one may access the selected DNA on GenBank and begin by localizing the initiation codon DNA triplet (ATG). Then, a 15-18 sequence of bases overlapping the site (e.g., -1 to +14) is chosen, one upstream (e.g., -20 to -35) and one downstream (e.g., +63 to +77). The complementary bases of the selected sequence from 5' to 3' are read to give the antisense sequence in the 5' to 3' direction; then the antisense sequence is synthesized by an ODN manufacturer (Phillips and Gyurko, 1997).

Although typically the coding region is targeted, sometimes, it does not work; in addition, many other targets can be inhibited equally or more efficiently by ODNs targeted to sequences located further downstream in the coding region (West and Cooke, 1991; Jachimczak, 1993; Spampinato et al., 1994). Examples of non-coding targeted regions would be untranslated or intron sequences; or to the border region between these and the coding sequence (Heilig and Schlingensiepen, 1996).

1.6 Toxicological properties of the ODNs.

A significant number of ODNs have now been evaluated in animals and in humans for potential toxicological effects. In rodents, the most prominent toxicities appear to be related to cytokine release induced by these drugs. In monkeys, complement activation, abnormalities in clotting, and hypotensive events have been reported, while in humans, doses of up to 2mg/kg intravenously administered over 2 h have been

observed to be without significant toxicity (Crooke, 1996; Galbraith et al., 1994; Iversen, Copple and Tewary, 1995). Some of these effects were found to be dependent on ODN base sequence, backbone modification, and/or dosage schedule and could be avoided (Akhtar and Agrawal, 1997). However other authors sustain that insufficient purity is one of the main reasons for toxicity even of the otherwise properly designed oligos (Schlingensiepen and Schlingensiepen, 1997).

1.7 Prospects for the future.

Many diseases ultimately result from the production of a mutant protein or because the overproduction of a normal protein. The potential use of ODNs as therapeutical agents is attractive due to their theoretical specificity, relative ease of production and, to date, paucity of reported adverse effects. Embracing the idea that tomorrow's drugs will not merely treat symptoms, but also attack disease-causing genes, scores of biotechnology companies are developing compounds to intervene in the cell's genetic machinery in order to prevent the expression of specific genes involved in AIDS, cancer, and inflammatory disease, among other disorders (Sharma and Narayanan, 1995; Mercola and Cohen, 1995; Metzger and Nyce, 1999; Monteith and Levin, 1999; Brysch and Schlingensiepen, 1994).

The era of the oligos in the clinical environment seems to have already begun with the approval by the FDA of the first antisense oligonucleotide (Vitravene®) for cytomegalovirus retinitis in AIDS patients as well as, with the proceeding of several clinical trials for certain inflammatory diseases and some cancers (Marcusson et al., 1999; Yuen, Halsey and Fisher, 1999; Nemunaitis et al.,1999).

In the case of the CNS, the antisense technology has been also established as an important neuropharmacological tool for targeting neurotransmitter receptors (Karle and Nielsen, 2000; Heilig, 1995; Zhang and Creese, 1993; Weiss et al., 1993), neuropeptides (Skutella et al., 1994a; Skutella et al., 1995; Jirikowski et al.,1995) and inducible transcription factors (Hooper, Chiasson and Robertson, 1994; Schlingensiepen and Heilig, 1997). For a possible use in the clinical environment on the CNS, neurooncology is the most intensively researched area at the moment (Tellería-Díaz, 2000), however, many obstacles still exist in the development and the immediate use of this technology as possible therapeutic option for certain neurological diseases. Delivery of ODNs to the CNS, toxicity problems with some oligos, and lack of information about the side effects that ODNs could induce within the brain upon repeated administration on the CNS are among the major problems. Therefore more investigations are required before a therapeutic applications of these molecules as treatment for certain neurological disorders seem feasible. For this goal, targeting certain neuropeptidergic system represents a very useful tool.

1.8 The magnocellular neurons of the hypothalamo-neurohypophyseal system as standard model for the study of neuropeptidergic systems.

For decades the magnocellular neurons of the hypothalamo-neurohypophyseal system (HNS), in which either vasopressin (VP) or oxytocin (OT) are produced, have been playing a pivotal role in fundamental discoveries in the nervous system. The magnocellular neurosecretory cells in the hypothalamus have been the most intensively studied peptidergic neurons in the nervous system since the primary

structure of VP and OT was the first of all neuropeptides to be published in the 1950s by the Nobel prize laureate Du Vigneaud (van Leeuwen et al., 1998). The HNS expresses abundantly and specifically these two neuropeptides, which clearly display their function and is relative ease of manipulation. In addition, isolation of perikarya, axons, and nerve terminals of these cells for anatomical, biochemical, and physiological analysis is also of relative easy (Gainer et al., 1995) as well, the assessment of the blood levels of OT and VP.

1.9 The HNS. Anatomical and physiological remarks on the vasopressinergic system.

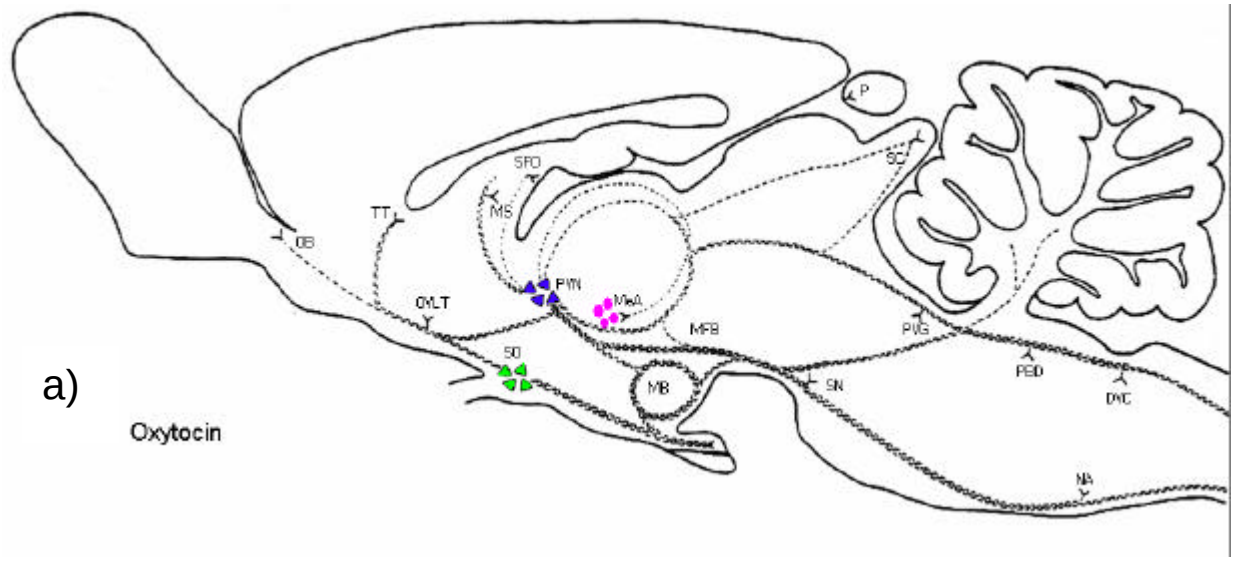
The HNS consists of magnocellular neurons in which either the VP or OT precursor is synthesized, packaged and transported towards the terminals in the median eminence and the neural lobe of the pituitary, where the processed products (i.e., VP-neurophysin II and glycopeptide; OT-neurophysin I) are released into the bloodstream in response to certain stimuli. While the function of vasopressin is important for osmoregulation, OT regulates smooth muscle contractility in female and male reproductive tract, as well as milk ejection during lactation.

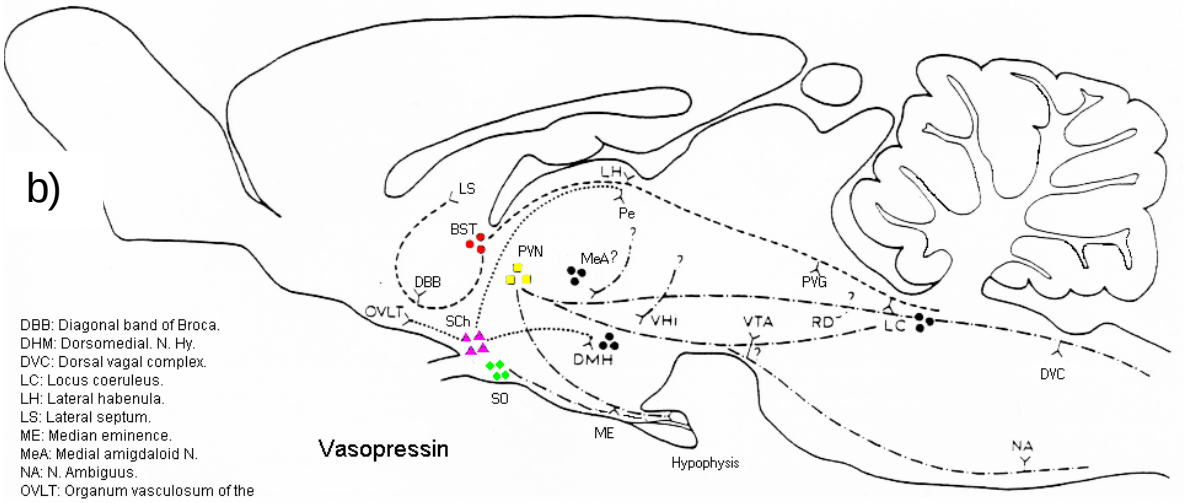
VP and OT are nonapeptides that not only exert their actions as neurohypophysial hormones but also as important neurotransmitters or neuromodulators in different parts of the brain (Buijs, 1990). VP is synthesized as a precursor prepropeptide, principally in magnocellular neurons whose cell bodies are located in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the mammalian hypothalamus (Swanson and Sawchenko, 1983). The VP precursor is packaged into secretory

granules and is subject to cleavage and other modifications as it is transported from the cell bodies to storage in posterior pituitary nerve terminals (Brownstein, Russell and Gainer, 1980). The mature peptide products are the nonapeptide VP, a putative carrier molecule termed neurophysin (NP) and a 39 aminoacid glycopeptide (copeptin;CPP). These are stored in equimolar amounts in posterior pituitary terminals until neural inputs governed by physiological stimuli elicit their release (Renaud and Bourque, 1991). VP is a central component of the systems that regulate osmotic balance (Reeves and Andreoli, 1992). The physiological challenge of dehydration results in a rise in plasma osmolality that is detected by to date undefined osmoreceptor mechanisms. Subsequent excitation of the magnocellular hypothalamic neurons leads to a release of VP into the general circulation from posterior pituitary stores. VP travels through the blood stream to its targets that exhibit specific receptors. Particularly, through an interaction with V2-type receptors located in the kidney, VP increases the permeability of the collecting ducts to water (Murphy et al., 1998). Thus, the hypothalamus (trough VP) promotes water conservation by decreasing the amount of water lost in the urine. The related nonapeptide hormone OT is also expressed in magnocellular neurons of the SON and PVN, but VP and OT are rarely found in the same cell under normal conditions (Vandesande and Dierickx, 1975).

Immunohistochemical work from a number of laboratories has shown that both VP and OT neurons are present in each of the two principal hypothalamic magnocellular nuclei, the SON and PVN, and are not segregated into separate nuclei as some early studies had suggested. Nevertheless, it is now clear that there are large numbers of VP and OT neurons in various locations outside of this classical localization, and that these neurons are heterogeneous in morphology and probably in function as well (Sofroniew,

1983) (Fig.4a and 4b). Such clusters of cells are usually referred to as accessory nuclei (Fischer et al., 1979) and in their composition there are magnocellular and parvocellular neurons as well.





DBB: Diagonal band of Broca.
DHM: Dorsomedial. N. Hy.
DVC: Dorsal vagal complex.
LC: Locus coeruleus.
LH: Lateral habenula.
LS: Lateral septum.
ME: Median eminence.
MeA: Medial amigdaloid N.
NA: N. Ambiguus.
OVLT: Organum vasculosum of the laminae terminalis.
Pe: Periventricular N.
PVN: Paraventricular N.
PVG: Paraventricular grey.
RD: Dorsal raphe N.
SCh: Suprachiasmatic N.
SO: Supraoptic N.
VHi: Ventral hippocampus.
VTA: Ventral tegmental area.

Fig.4a and 4b. Distribution of main vasopressinergic and oxytocinergic cell groups and their connections into the rat brain (Buijs, 1990).

In the rat brain was Peterson the first who described the majority of such nuclei more than 30 years ago, and termed as anterior commissural nucleus (ACN), fornical nuclei and the nucleus of the medial forebrain bundle (Peterson, 1966). Perhaps the most conspicuous of the "newly" identified locations is the suprachiasmatic nucleus (SCN), located bilaterally in the midline at the base of the third ventricle, just dorsal to the optic chiasm. In this nucleus, a portion of the neurons (parvocellular cells of 10-15µm in diameter) contain VP (but not OT) and its associated neurophysin (Sofroniew, 1983); this structure is currently being considered as the site of the master pacemaker controlling circadian rhythms (the neural or internal clock) and it has no connections with the posterior lobe (van Esseveldt, Lehman and Boer 2000; Ingram, 1998). Of particular interest are the studies showing that while osmotic perturbations increase the VP mRNA levels in the SON and PVN nuclei, the vasopressin neurons in the SCN do not increase their mRNA content under these conditions (Burbach et al., 1984).

A large number of so called accessory magnocellular VP and OT neurons have been found also throughout the hypothalamus in different mammals including humans. As yet there is no accepted nomenclature for them, and there is considerable species variation regarding their precise location (Sofroniew, 1983; Grinevich and Polenov, 1994) . The best represented groups in different mammals are: (1) a group in the dorsal anterior hypothalamus extending towards the stria terminalis; (2) diffuse cells and small groups in the anterior hypothalamus between the SON and PVN; (3) diffuse cells and small groups in the posterior hypothalamus; and (4) a group near the fornix in the posterior hypothalamus. Many species have unique groups of such neurons. For example, one prominent group in the rat is the ACN, which is composed of OT neurons but is not present in most other mammals, including other rodents such as the guinea

pig. The function of these dispersed groups is poorly understood. Neurons in different groups appear in some cases to have different projections. It also appears that different groups may be sensitive to different afferent stimuli (Sofroniew and Schrell , 1982).

In addition to the previous, other neurons containing neurophysin and VP are located outside the hypothalamus. These include perikarya in the medial amygdala, in the bed nucleus of the stria terminalis (BST), and in the ventral lateral septum. These neurons are parvocellular and their immunostaining is far weaker than the one obtained for hypothalamic neurons but increases under colchicine treatment (Sofroniew, 1983).

1.9.1 Morphological aspects of the SON and the PVN in the rat hypothalamus.

The SON contains only magnocellular neurons and is divided into two parts, principal or anterior and retrochiasmatic or tuberal. The principal anterior division has been the subject of most experimental investigations. In the rat it begins with loosely packed neurons just dorsolateral to the optic chiasm as far rostral as the organum vasculosum of the lamina terminalis (OVLT) and extends caudally into the posterior hypothalamus as a dense cluster of deeply staining, large cells adjacent to the optic chiasm, and its most posterior aspect, ventrolateral to the ascending optic tract. The retrochiasmatic portion is a densely packed, thin sheet of cells close to the pial surface between the median eminence and the optic tract (Armstrong, 1995).

The PVN consists of two parts with different functions. One contains parvocellular neurons which project to the median eminence, brain stem and spinal cord and may influence the regulation of anterior pituitary secretion and the autonomic nervous

system. The other part contains the magnocellular neurons, which project to the neurohypophysis (Bisset and Chowdrey, 1988). According to Swanson and Kuypers, the magnocellular division of the PVN in the rat can be viewed most simply as three dense clusters of magnocellular neurons embedded in a larger shell of parvocellular neurons that consists of five components. The anterior magnocellular part lies just ventromedial to the fornix at the level of the medial preoptic area; it corresponds for the most part to the ACN of Peterson. The medial magnocellular part consists of a small, compact group of large neurons in the periventricular and anterior parvocellular parts of the nucleus, between the anterior magnocellular part and the posterior magnocellular cell group, this medial magnocellular part may correspond to the posteromedial part of the ACN of Peterson. The large posterior magnocellular part corresponds to the magnocellular part of the PVN of most authors. It should be pointed out that the anterior part of the posterior magnocellular part contains a zone in which magnocellular and parvocellular neurons are intermixed (Swanson and Kuypers, 1980).

A clear immunocytochemical localization of the VP and OT-containing neurons has been observed in different parts of the SON and PVN in the rat. The anterior and the medial subnuclei of the magnocellular part of the PVN consist almost exclusively of OT neurons. In the posterior subnucleus OT neurons are concentrated anteroventromedially and the VP neurons posterodorsolaterally (Swanson and Sawchenko, 1983). In the principal part of the SON OT neurons are concentrated anterodorsally and VP neurons posteroventrally; the majority of cells in the retrochiasmatic part contain VP (Rhodes, Morrell and Pfaff, 1981).

1.10 Aims of the present study.

Since the magnocellular neurons of the rat hypothalamus represent an optimal model for the study of neuropeptidergic systems, the employment of an antisense oligonucleotide against the VP mRNA could provide us with profitable information about the effects of these compounds upon its application to the CNS. No studies on the repeated administration of a 19-base antisense molecule against the VP mRNA has been performed before. In the present study we evaluate the effect of an antisense oligonucleotide against such transcripts on the expression of VP, but also on the VP and OT immunoreactivity of the magnocellular neurons.

2. MATERIAL AND METHODS

2.1 Animals and housing conditions.

Intact adult Wistar male rats (n=16) weighing 250 to 300 g served as experimental subjects. Animals were initially in plastic cages on a 12 hour dark, 12 hour light cycle with free access to standard lab chow and water. Twelve hours before oligo administration, rodents were placed individually in metabolic cages to measure water consumption, and urine output.

2.2 Surgery.

For intracerebroventricular (i.c.v.) injections, a chronic polyethylene cannula (PP 20; LHD Heidelberg) was implanted under chloralhydrate anesthesia (400 mg/kg BW, intraperitoneally) into the left lateral ventricle 7 to 9 days before the experiment. The stereotaxic coordinates were took from a stereotaxic atlas (Paxinos and Watson, 1982) and they were: 1.3 mm lateral to the midline, 0.6 mm posterior to the bregma and 5 mm vertical from the skull surface. Five or seven days after surgery, angiotensin II (25 pmol) was injected i.c.v. to each rat. Only those animal which responded with an immediate drinking were included in further experiments. The animals were anesthetized again, and a polyethylene catheter (PP 50, HDL, Heidelberg, Germany) filled with heparinized saline was inserted in the femoral vein through a subcutaneous tunnel, sealed and secured at the back of the neck.

The correct position of the i.c.v. cannulae was verified histologically by postmortem dissection at the end of the experiment.

2.3 Antisense oligo.

On the basis of the sequence of VP cDNA, we designed a 19-base 5'-3' end capped phosphorothioate antisense ODN that corresponded to the beginning of the initiation codon of the VP mRNA (5'-CAT GGC GAG CAT AGG TGG A-3'). Synthesis was performed at the Institut für Molekulare Biotechnologie, Jena, by Dr. Birch Hirschfeld. ODNs were purified by gelfiltration and HPLC. Mismatch ODN bases and saline solution were used as controls. Both the antisense and the mismatch constructs were compared to the GenBank database (*Entrez*) and found to have little or no homology to mRNAs registered in GenBank.

2.4 Intracerebroventricular infusions.

Animals were handled and sham injected frequently so that actual i.c.v injections and blood withdrawal could be performed without anesthesia. ODNs or vehicle were slowly infused i.c.v. using a Hamilton syringe connected to a 25-gauge injection needle via polyethylene tubing. ODNs were administered slowly over a 120 seconds period, with the injection needle left in the i.c.v. cannula for 3 to 4 min to ensure complete injection of the ODNs.

Animals received two i.c.v. injections at 4-hour intervals of either antisense oligo [n=5], mismatch oligo [n=5] (15 µg of each) or saline[n=6]. The first i.c.v injection was given between 2:00 and 3.00 pm, and the second one between 6:00 and 7.00 pm.
ODNs were dissolved in sterile saline solution (0.9% NaCl) and injected at a dose 15 µg/1.5 µL (1.5 µL flushed with 5 µL of saline).

2.5 Water consumption and urine osmolality assessment.

Water intake was determined by weighing of water which the rat drank during a 12-hour time period (from 6.00 pm to 6.00 am). Urine osmolality was measured at the beginning and at the end of the experiment with an osmometer.

2.6 Blood collection for vasopressin determination in plasma.

Blood sampling was carried out in conscious and unrestrained rats 12 h after the last i.c.v vehicle or ODN injection. The grid cage tops were removed and the femoral vein catheter was connected to an extension catheter (PP 50) with a syringe, both filled with heparinized saline. The rats were allowed to adapt to the new environment (open cage) for at least 30 min. Blood (1 ml) was slowly withdrawn from the femoral vein catheter in ice-cold heparinized Eppendorf tubes. Blood samples were centrifuged at 3,000 x g in a refrigerated centrifuge, plasma was removed and stored at –75°C until assayed for AVP content. VP radioimmunoassays were performed in the routine laboratory of the Dept. of Pediatrics, Dr. Rascher, University of Erlangen

2.7 Immunocytochemistry.

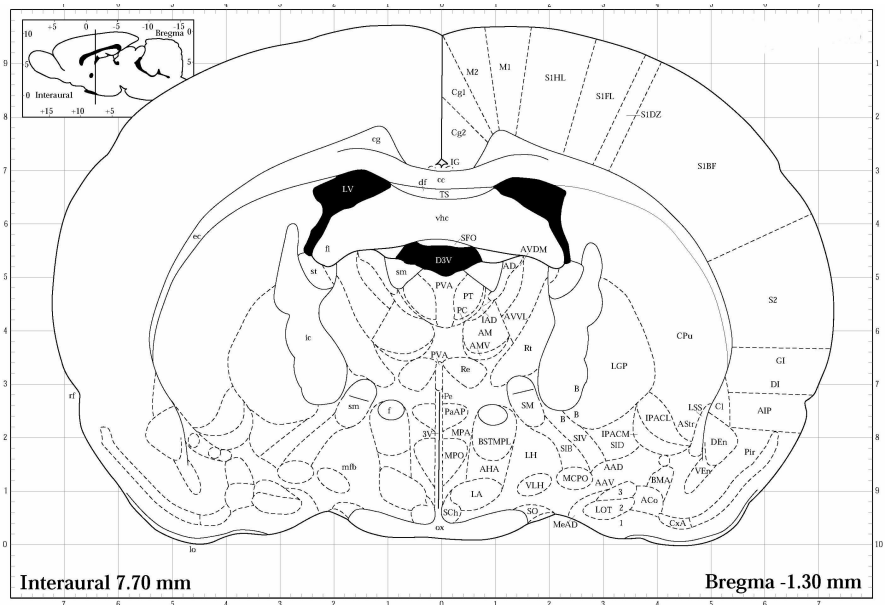
After the blood sampling, animals were killed by prolonged ether anesthesia, followed by cardiac perfusion with 4% paraformaldehyde in PBS. Brains were removed and postfixed in the same fixative over night. Serial frontal sections (100µm) were cut on a vibratome and collected in PBS. Pituitaries were dehydrated in ascending series of ethanol and embedded in EPON. Serial semithin sections (0.5µm) were cut on a Reichert Ultracut microtome and affixed onto glass slides. Epoxy resin was removed with sodium methoxide, for details see (Jirikowski et al., 1990). Floating vibratome sections were incubated overnight at 4°C with rabbit anti VP (Chemicon) diluted 1:1000 in PBS containing 0.05% Triton X-100.

After incubation with anti-rabbit IgG (Sigma) diluted 1:100 in PBS for 30 min at room temperature, sections were incubated with rabbit peroxidase-antiperoxidase (Sigma) diluted 1:100 in PBS for 30 min at room temperature. Immunoprecipitates were stained with 3´, 3´-diaminobenzidine and H_2O_2. Some of the sections were mounted in PBS for brief microscopical evaluation. Thereafter immunocomplexes were removed by incubating the sections in HCL (1mN) for 15 minutes, followed by washing in PBS 1X 15min. Sections were incubated overnight at 4°C with Anti-OT (Chemicon) diluted 1:500 in PBS containing 0.05% Triton X-100. Subsequently sections were rinsed in PBS 1X10min and incubated for 2 hours at room temperature with CY3-labeled anti rabbit IgG (Jackson Immuno Research Laboratories, Inc.) diluted 1:200. After washing in PBS (3X10 min) sections were affixed onto slides, coverslipped with Moviol (Sigma) and evaluated with epifluorescence or phase contrast ilumination with an Olympus photomicroscope.

2.7 Statistical analysis.

Counting of cells was performed in supraoptic (SON) and paraventricular (PVN) nuclei. For standardization, we selected sections at identical planes [~ interaural 7.70 mm, Bregma-1.30 mm for SON, and interaural 7.20 mm, Bregma-1.80 mm for PVN] for quantification (Fig.5 and Fig.6). VP and OT cell populations were determined by counting the total number of immunoreactive neurons (soma).

The data from the water intake and the blood levels of VP were analyzed (mean, SD, SEM) using the a statistical software package (SPSS 10.0 for windows). The results are presented as mean and SEM. In order to compare two means, the Mann-Whitney Test was employed. The level for statistical significance was set at $p < 0.05$.



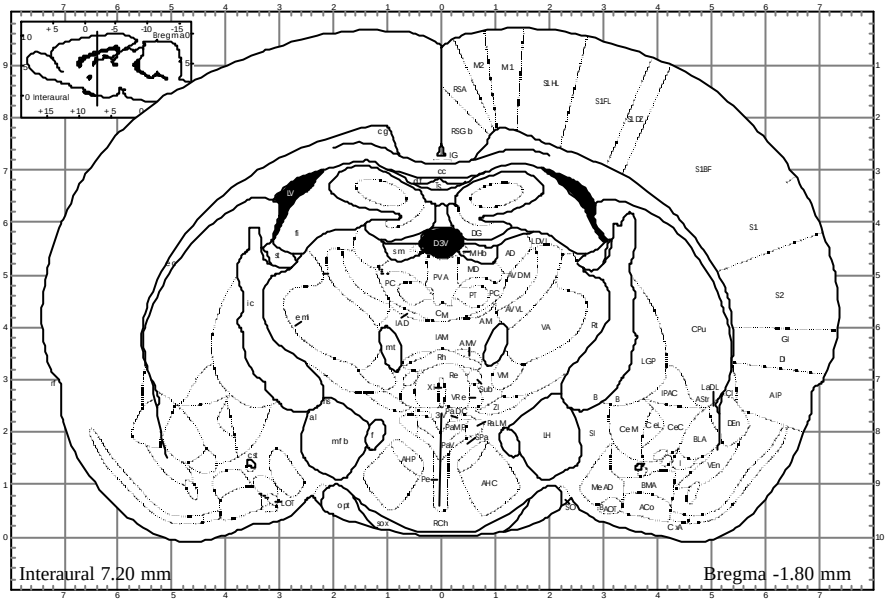


Fig.5 and 6. SO: Supraoptic nucleus, PaV: Paraventricular nucleus with the PaVP (parvocellular) and PaLM (magnocellular) components (Paxinos and Watson, 1997).

3. RESULTS

3.1 Water consumption and urine osmolality.

Rats injected with the antisense and mismatch oligo showed within 2h of the first injection reduced drinking behavior in comparison to the saline injected control animals. Total water consumption was determined at the end of the experiment. Animals injected with the antisense oligo showed significantly reduced water intake as compared with saline controls ($p<0,05$). Water intake was also reduced in comparison to animals injected with the mismatch oligo but this difference was not significant (Fig.7). Symptoms of diabetes insipidus were not observed in the experimental group.

3.2 Vasopressin blood levels.

Immunoassays of serum, from the blood collected trough the femoral catheter at the end of the experiment showed increased vasopressin levels in the antisense group compared to saline ($p=0,05$) and mismatch ($p=0,15$) treated animals, but this however was not significant (Fig. 8).

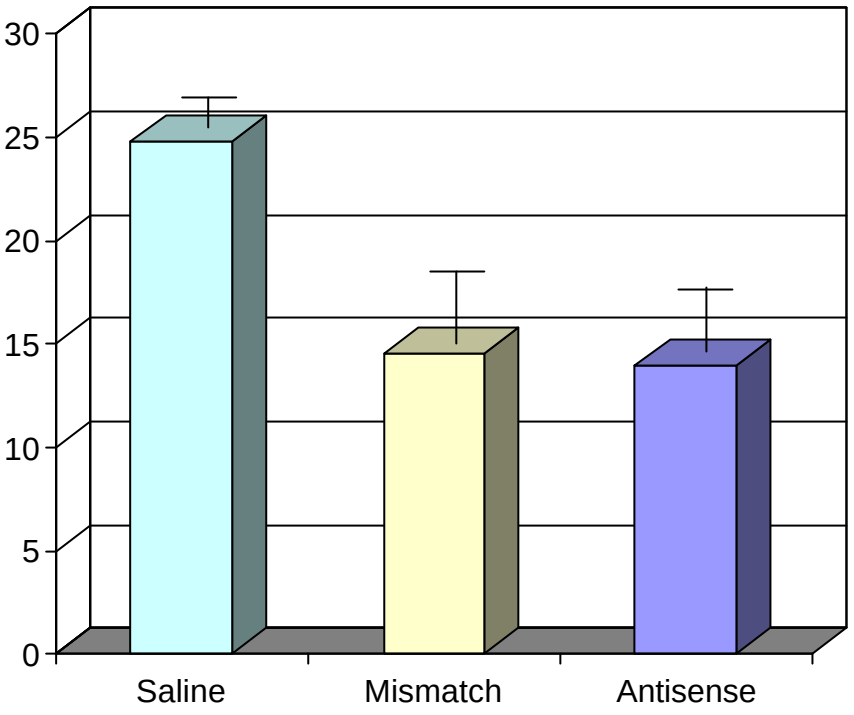


Fig.7: Water consumption(ml/12h)

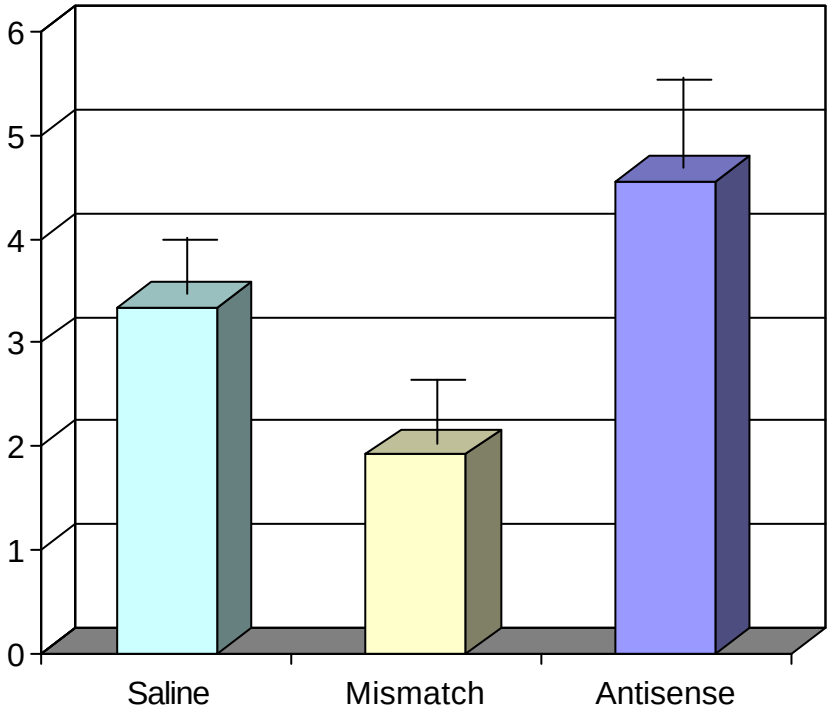


Fig.8: Plasma Vasopressin levels (pg/ml)

3.3 Immunocytochemistry.

3.3.1 Vasopressin immunostaining.

Neurons immunostained with Anti-VP could be visualized in the hypothalamus of all animals in the SON including in its retrochiasmatic portion, the magnocellular and the parvocellular PVN, suprachiasmatic nucleus (SCN) and also in various regions outside of the classical magnocellular nuclei, e.g, in the intersupraoptic-paraventricular islands (ispi). Scattered VP neurons were also seen in the BST, medial preoptic area (mpoa), perifornical region (pf), in the ansa lenticularis (AL) and the zona incerta (Zi) and occasionally in the periventricular nucleus (Pe) of some animals in all groups. Antisense treated animals appeared to have a similar distribution of vasopressin immunoreactive perikarya in the SON and the PVN as the controls. VP immunostaining was also present in bundles of nerve fibers in the median eminence and in the posterior lobe. No VP neurons were seen in the medial amygdala of any group. In the antisense group, the overall intensity of VP staining in magnocellular neurons and Herring bodies appeared to be similar to the intensity observed in control groups (Figs 9A, 10A and 10E).

3.3.2 Oxytocin immunofluorescence and colocalization of VP and OT.

OT immunofluorescence was mostly seen in perikarya and processes of magnocellular neurons from the SON, PVN, the lateral subcommissural nucleus (LSN) (LSN=ACN) and in varicosities of the median eminence. Few cells from the Pe nucleus

and perivascular groups also showed OT immunofluorescence. The number of neurons that showed OT immunoreactivity were usually much higher in the SON and PVN of the antisense treated group (Fig. 10B, 10D) as compared with control animals (Figs 9B, 10F). OT immunofluorescence was also observed in many of the magnocellular neurons that stained also for VP (Figs 10A, 10B, 10C, 10D). In the SON of antisense injected animals coexistence of both peptides was found in about 30% of the neurons, while in the magnocellular portion of the PVN colocalization was evident in more than 40% of these cells. Coexistence of both immunoreactivities was seldom observed in few and scattered neurons in saline- or mismatch probe injected controls. Double staining for VP and OT was observed occasionally in scattered cells in the antisense injected rats in the ispi, BST, mpoa, pf, AL, Pe and Zi. The parvocellular portion of the PVN and the SCN contained only VP positive neurons in all experimental groups, LSN was only oxytocinergic in all animals examined.

3.3.3 VP and OT in the posterior lobe.

Some of the neurosecretory terminals in the posterior lobe contained both immunoreactive peptides in the animals treated with the antisense oligo. Comparison of consecutive semithin sections of the posterior pituitary lobe also revealed coexistence of VP and OT in a fraction of the Herring bodies in these animals (Figs 11A, 11B). In both controls we observed OT and VP immunostaining in distinct populations of nerve terminals and Herring bodies (Figs 11C, 11D).

Fig 9: Control animals showed distinct patterns for VP immunostaining in the SON (Fig. 9A), with no coexistence for OT, as visible by immunofluorescence (Fig. 9B), neurons containing OT did not stain for VP (Fig. 9B. arrows). Similar observations could be made for the PVN (Fig. 9C for VP and Fig 9D for OT). There was not immunostaining for OT in the SCN, emphasizing specificity of the methods employed (Fig. 9E-F).

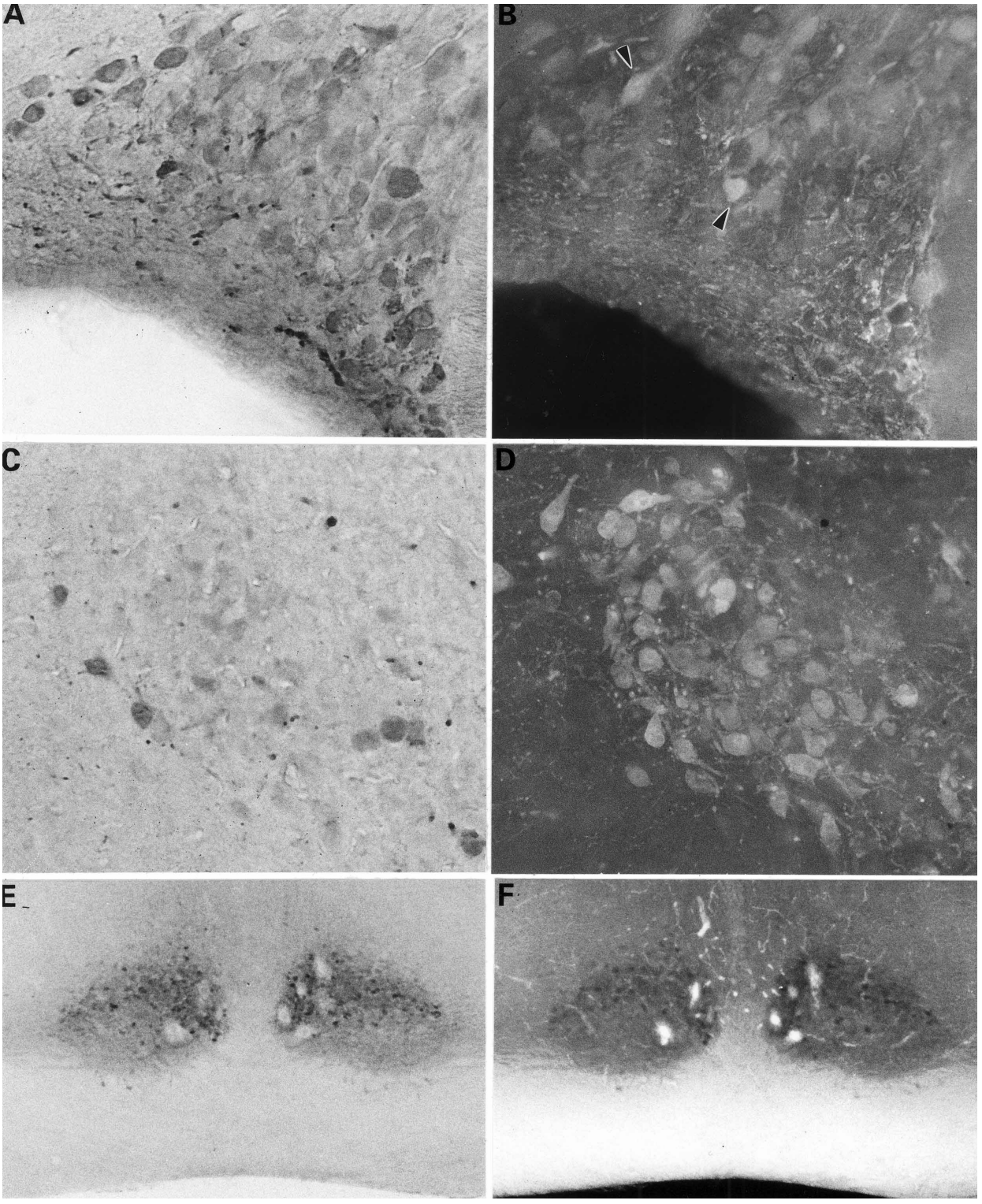


Fig. 9

Fig 10: SON of a rat treated with the antisense oligo. VP immunostaining (Fig. 10A) corresponds to a large extent with OT immunofluorescence (Fig. 10B. arrows). Similar phenomena was also observed in the PVN (Figs. 10C and 10D). SON of a control animal (Mismatch probe) showed distinct pattern for VP immunostaining (Fig. 10E) and OT immunofluorescence (Fig. 10F). The number of perikarya with OT immunofluorescence is much smaller than in the antisense treated group (Figs. 10D and 10B).

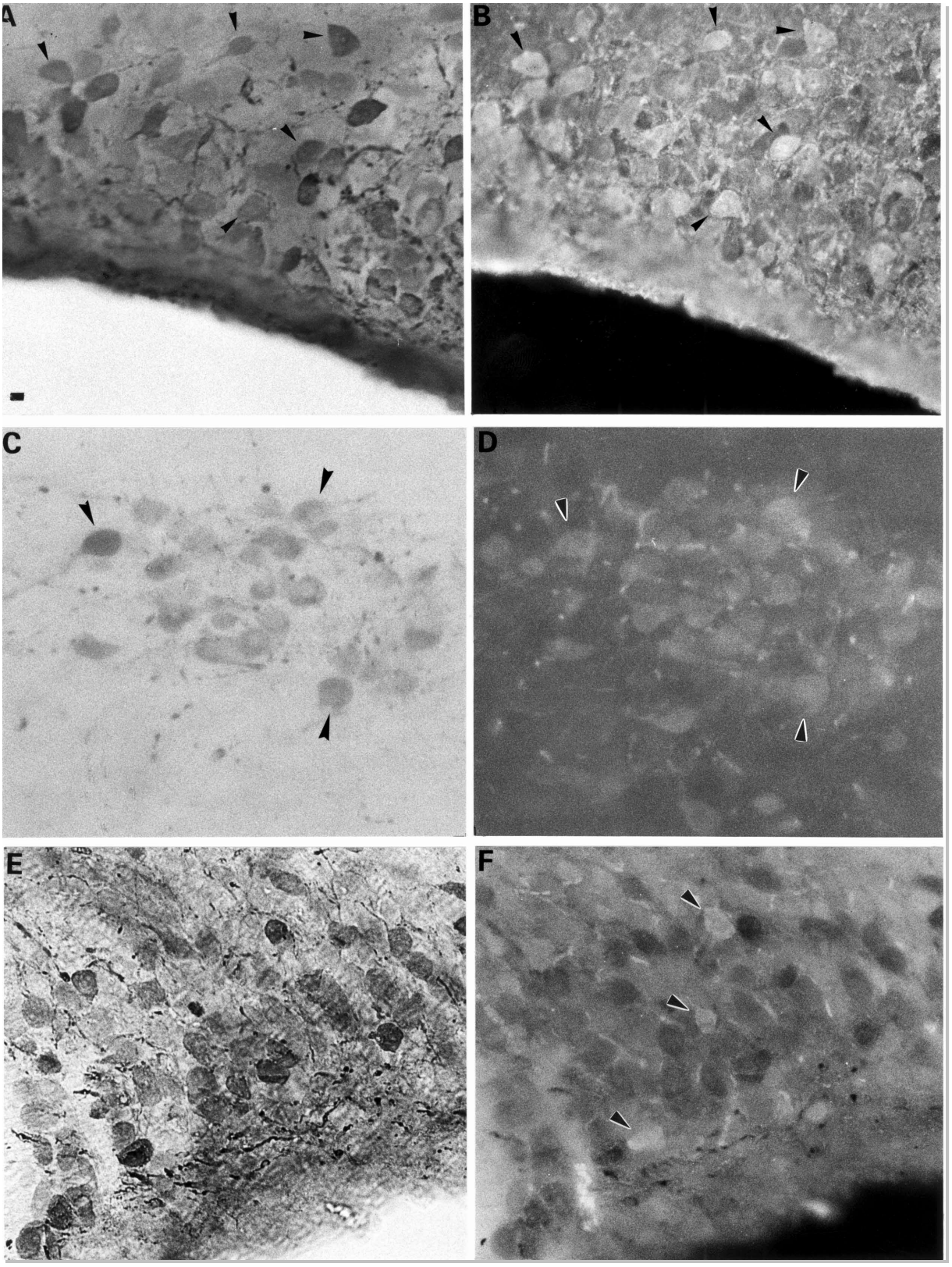


Fig. 10

Fig 11: Consecutive semithin sections of the posterior lobe, immunostained for VP (Figs.11A and 11C) and OT (Fig.11B and 11D). Coexistence of both immunoreactivities is observable in a fraction of the Herring bodies in the antisense treated group (Figs. 11A and 11B. Arrows), however, such phenomena of colocalization was not detected in all terminals (Figs. 11A and 11B. Asterisk). Control animals showed no coexistence in the Herring bodies (Figs. 11C and 11D). P: Pituicyte.

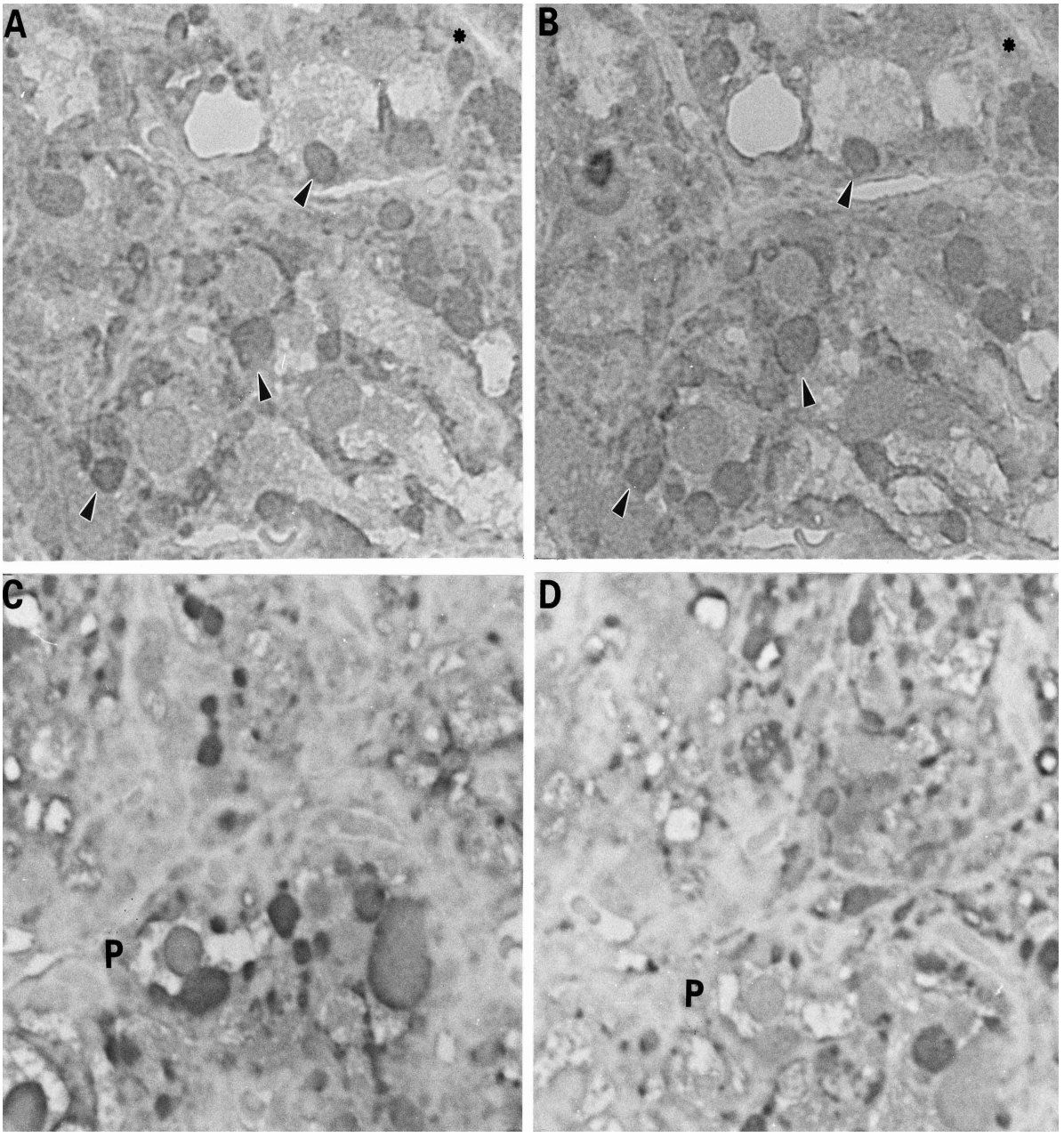


Fig. 11

4. DISCUSSION

4.1. Behavioral effects of antisense targeting on VP mRNA expression.

VP is a central component of the systems that regulate osmotic balance. Particularly, through an interaction with V2-type receptors located in the kidney, VP increases the permeability of the collecting ducts to water. However, little is known about the mechanisms that regulate VP gene expression, in terms of either the synaptic regulation, physiological status, or the downstream second messenger and transcription factor systems involved (Murphy et al., 1998).

In vivo antisense targeting with synthetic oligonucleotide probes has evolved as a useful tool to block neuropeptide expression on the translational level. In the present study we injected intracerebroventricularly a 19-mer phosphorothioate modified antisense oligonucleotide, which corresponded to the beginning of the initiation codon of the arginine vasopressin precursor. Single pulse injections of 50µg or more of this oligonucleotide have been shown to induce a transitory diabetes insipidus (DI) in rats (Skutella et al., 1994b). Meeker et al. (1995) found a decrease in AVP mRNA and a compensatory drinking response within the first 24 h upon the administration of an antisense AVP oligonucleotide. Here we injected only 15µg of this probe twice, and DI was not observed in any of our experimental animals, probably due to the lower dose. Unexpectedly we also observed a reduction of water intake in the animals who received antisense, but also in those who received mismatch oligonucleotides, therefore this finding can not be considered as an specific effect of such oligo, not even when the decrease on water consumption was higher in the antisense treated group.

This idea is also reinforced by the lack of a significant increase in the blood levels of VP in the antisense treated animals.

4.2. The phenomena of VP and OT colocalization in the HNS.

Most of the magnocellular neurons of the rat hypothalamo neurohypophysial system contain in intact rodents both vasopressin and oxytocin encoding transcripts as determined by single cell RT-PCR (Xi et al., 1999). Nevertheless immunocytochemistry and *in situ* hybridization fails to detect coexistence of both nonapeptides in most cells in intact animals (Mohr et al. 1993a), indicating that the coexpression of OT and VP neuropeptides does not occur under normal conditions and thus suggesting that the translation of OT mRNA may be repressed in VP-expressing neurons and vice versa in unchallenged animals.

Physiological challenge of oxytocinergic neurons occurs during parturition, resulting in a coexistence of both peptides in 20% of the magnocellular neurons (Jirikowski et al. 1991) and in 17% in lactating animals (Mezey and Kiss, 1991). Although the molecular mechanisms involved in the coexistence of OT and VP observed in such studies are not yet clear, it could be assumed that an increase in the VP translation during these situations could represent an essential mechanism to maintain the homeostasis, since VP could be required for maintaining blood pressure during the hemorrhage associated with parturition, or to retain water as compensatory mechanism due to a loss of fluid during lactation (Jirikowski et al. 1991; Mezey and Kiss, 1991). Another possible explanation could be also that, current VP neurons were recruited in to OT production as additional source for OT during lactation and parturition, perhaps as

consequence of receiving the same stimulus that elicit OT production from oxytocinergic neurons during these situations.

Stimulation of vasopressinergic neurons by osmotic stress through water deprivation also induces coexistence of both peptides in a certain numbrer of cells in the magnocellular population of the SON and the PVN (Telleria Diaz et al., 2001). The question whether OT neurons are recruited into VP expression upon prolonged osmotic challenge, to compensate the deficit of VP, VP neurons start to express OT or whether dormant populations of magnocellular neurons are activated to synthesize both neuropeptides is difficult to establish with this experiment. The role of OT in the regulation of water balance has been postulated since several years (Han et al., 1992). Previous and recent reports suggest that OT is able to bind VP V2 receptor (V2R) in kidney, thus stimulating levels of aquaporin 2, to cause antidiuresis. The activation of V2R by OT could partially compensate water conservation, however the affinity of OT for V2R is lower than VP (Terashima et al., 1999), so the role of this nonapeptide for osmoregulation seems to be less prominent than the are exerted by VP. Nevertheless, the observed coexistence of VP and OT immunoreactive peptides in the HNS, which in part seems to be reversible, is another example for the highly specific functional plasticity of the magnocellular hypothalamic system in response to special demands or during conditions of strong stimulation (Telleria Diaz et al., 2001).

Osmotic stimuli such as dehydration or salt-loading resulted in an increase in VP gene transcription (Murphy and Carter, 1990; Herman et al., 1991), a concomitant increase in VP mRNA abundance (Burbach et al., 1984; Zingg et al., 1986; Lightman and Young, 1987; Sherman et al., 1988) and an increase in the lenght of VP RNA poly (A) tail (Carrazana et al., 1988;. Carter and Murphy, 1989). OT gene has also shown similar

regulation by osmotic stimuli (Lightman and Young, 1987; Van Tol et al., 1987). Apparently the neuroendocrine cells in the hypothalamo neurohypophysial system are to a great extent malleable to functional changes, even to the extent that unrelated peptides are synthesized. These assumptions seem only to be true for peptidergic neurons that contribute to the posterior lobe and infundibular system. Centrally projecting neurons like the OT neurons in the LSN or the parvocellular VP cells in the PVN and the SCN did not show VP and OT coexpression under the experimental conditions, despite the fact that the neurons from the LCN are known to be osmosensitive (Grinevich and Akmayev, 1997).

The phenomena of VP and OT colocalization in a certain fraction of magnocellular neurons in the antisense treated group could respond to an increase in the VP mRNA transcription following and initial depletion in the levels of VP mRNA due perhaps to a RNase-H mediated mechanism. Maybe this lower dose of the antisense oligo initially decreased the amount of VP transcripts, which could be quickly followed by a compensatory increase of the VP mRNA and VP by means of de novo-transcription and translation, and thus avoiding the development of a DI in these animals. The lower or the remnant amount of the antisense oligo could be then insufficient to inactivate all of the new VP transcripts. However in this case, it should then be keep in mind that colocalization of both peptides should be also found in the ACN, which did not happen in our study group. Although the OT magnocellular neurons from the ACN have been considered by some authors the most anterior portion of the PVN (Swanson and Kuypers, 1980), these cells do not project to the posterior lobe, but to the median eminence. According to the evidence of some investigations (Arai et al, 1996; Fenelon et al, 1993; Laurent et al, 1989; Chernigovskaia et al 1985), it is very likely this group of

cell is a completely different structure from the PVN and therefore, the lacking of coexpression of VP and OT in the antisense treated animals could be explained by this functional differentiation.

Another possibility to rule out is the triple helix formation, since these structures could upregulate the expression of a gene (Imagawa et al., 1994). Triple helices can be both intra- and intermolecular in character (Gfrörer et al. 1993). In classical triple helices or intermolecular triplexes, the duplex involved in triplex formation must have a homopurine sequence in one strand (adenine or guanine), attending these are the nitrogen bases which offer additional junction sites for a third strand by means of Hydrogen bonds (TS´O POP et al. 1992). However in our case, neither the selected target sequence nor the designed oligo presented this feature. Therefore the possibility for triple helix formation in our antisense treated group is rather unlikely.

There is also a theoretical possibility of the antisense ODN binding to the locally opened loop created by RNA polymerase (Phillips and Gyurko, 1997). An opened loop could result in increased accessibility of binding sites for transcription factors and thus stimulate transcription of another genes. This possibility should be considered, since the coding genes for VP and OT they are located on the same chromosomal locus very close each other, only separated by 11KB but oriented in opposite transcriptional directions (Mohr et al., 1988b; Richter, 1988; Gainer and Wray, 1992). Therefore OT gene could be activated, resulting in the expression of the OT in a certain number of vasopressinergic cells, thus resulting in the colocalization of VP and OT observed in the antisense treated group. The lower dose of the employed oligo may not be enough to produce a translational arrest of the VP gene, sufficient to decrease VP production, but enough to increase the transcription of the OT gene through the mechanism

mentioned above. As a consequence a translational activation of OT is very likely. OT peptide would be stored in the soma of the magnocellular neurons since at the moment of the experiment there was not a real peripheral demand for the release of OT.

Another possibility to be considered is the production of a paradoxical effect. Such effects of ODN treatment have been encountered even in the most simple in vitro systems (Engelhard, http://www.moffitt.usf.edu /cancjrnl/v5n2/article7.html, Williard et al., 1994), and the mechanisms of these nonspecific effects of ODNs could be related to (1) the structure of the ODN itself, (2) hybridization to DNA or mRNA other than the target sequence, with subsequent RNase deavage, (3) binding to proteins or other molecules, and/or (4) ODN degradation products, which in themselves can affect cellular functions.(Hélène, 1994; Altmann et al., 1996, Milligan et al., 1993; Ma and Calvo, 1996; Wagner, 1994; Warzocha, 1997; Carter and Lemoine, 1993; Mahon et al, 1995; Weidner and Busch 1994). However this possibility seems to be very unlikely here, since a previous study has shown that this oligo with higher dose indeed induced DI in rats (Skutella et al., 1994b).

4.3. Conclusions.

In a previous study, the i.c.v administration of higher dose of this oligo probe against the initiation codon of VP was shown to produce DI in rats (Skutella et al., 1994b). In the present work, the double i.c.v injection of a lower dose of such probe did not affect the blood levels of VP, neither produced DI in the experimental group, but on the other hand, it seems that, the OT production was either upregulated in vasopressinergic magnocellular neurons or that, oxytocinergic cells started to produce VP, leading to the

coexpression of both peptides in a fraction of the magnocellular neurons in the HNS of these animals. With the available data is difficult to establish the origin and the actual underlying mechanism of this phenomenon of colocalization, however, this finding could be another evidence that the administration of an antisense oligo against certain proteins can modify the expression of a different gene than the one that was targeted. The overproduction of another protein could perhaps also explain some of the side effects observed in some experiments upon the administration of antisense probes. Therefore, before considering this technology as the ”Magic Bullet” and introducing any of these substances for clinical use, it should be ruled out in each experimental animal setting whether or not the oligo under investigation affect the expression of other genes.

5. Zusammenfassung

In vivo antisense Targeting mit synthetischen Oligonukleotid- Sonden, die komplementär zu spezifischen neuropeptidergen Transcripten sind, wird als Alternative zu transgenen Tiermodellen bei bestimmten Fragestellungen diskutiert. Die Technik des Antisense Targetings hat auch große Erwartungen hinsichtlich einer möglichen neuropharmakologischen Anwendung geweckt. Die tatsächlichen molekularen und zellulären Abläufe, die dieser Technik zugrunde liegen sind aber bis heute noch weitgehend unbekannt. In der vorliegenden Arbeit injizierten wir in Ratten intracerebroventrikulär eine synthetische Oligonukleotid-Sonde, die komplementär zur Startcodon Region des Arginin-Vasopressin Precursors war. Hochdosierte Einzelinjektionen dieser Sonde induzierten in Ratten vorübergehend Diabetes insipidus. Bei zwei aufeinanderfolgenden Injektionen von geringen Dosen der Oligonukleotid-Sonde wurden die Plasma-Vasopressin-Spiegel aber nicht verändert, die Wasseraufnahme dieser Tiere war sogar leicht reduziert. Mit Immunzytochemie beobachteten wir in den magnozellulären hypothalamischen Kerngebieten und im Hypophysen-Hinterlappen eine Koexistenz von Vasopressin und Oxytocin. Obwohl die tatsächlichen Ursachen der Koexpression dieser beiden hypothalamsichen Peptidhormone schwer zu bestimmen sind liegt der Schluß nahe, daß Oligonukleotid-Sonden direkt oder indirekt in der Lage sind, auch die Expression anderer als der eigentlich vorgesehenen Gene zu modulieren. Dieser Gesichtspunkt sollte bei einer möglichen späteren therapeutischen Anwendung von in vivo Antisense-Strategien im Auge behalten werden.

6. SUMMARY

In vivo antisense targeting with synthetic oligonucleotides complementary to specific neuropeptidergic transcripts has been proposed as a tool to replace transgenic animal models for certain experimental settings, thus having potentially great neuropharmacological implications. The actual molecular and cellular events associated with this approach however are mostly unknown to date. In the present study we injected intracerebroventricularly a synthetic oligonucleotide, complementary to the startcoding region of the Arg-vasopressin precursor. Single pulse injections of high doses of this oligo have been shown to induce a transitory diabetes insipidus in rats. Here we injected twice lower doses of this oligonucleotide which did not affect the plasma vasopressin levels and resulted in a non significant reduction of water intake. With immunocytochemistry we observed a coexistence of vasopressin and oxytocin in the magnocellular hypothalamic nuclei and in the posterior lobe. The underlying mechanism of such coexpression is difficult to establish here, however we can conclude that the employment of these compounds can modify the expression of a different gene than the targeted, which should be keep in mind for the use of this technology as modulator for biological processes or as therapeutic agents.

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Ehrenwörtliche Erklärung

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