Study of neurogenic involvement in arginine-vasopressin and oxytocin release under basal and environmentally stimulated conditions

Ph.D. Thesis

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Abbreviations

5-HT	serotonin (5-hydroxytryptamine)
AC	absolute control
ACTH	adrenocorticotrophic hormone
AVP	arginine-vasopressin
CAT	corynanthine
cAMP	cyclic adenosine monophosphate
ClB	chlorobenzene mixture
DA	dopamine
E	epinephrine
EPM	elevated plus maze
GAL	galanin
HA	histamine
HCB	hexachlorobenzene
NE	norepinephrine
NH	neurohypophysis
OF	open-field
OXT	oxytocin
PDL	pindolol
POP/EDCs	persistent organic pollutants with endocrine disruptor effects
PVN	paraventricular nucleus
RI	resident-intruder
RIA	radioimmunoassay
SC	stress control
SON	supraoptic nucleus

1. INTRODUCTION

The arginine-vasopressin (AVP)-ergic and oxytocin (OXT)-ergic systems are crucial in homeostatic maintenance, via the amounts of these mediators secreted into the bloodstream or extracellular/cerebrospinal fluid. Homeostasis is implemented by both physiological and behavioral patterns, and can be maintained *inter alia* via AVP- and OXT-mediated behavior, including anxiety and aggression. Anxiety enhances the capability and motivation to stress, while aggression helps gain better access to resources. Behavior results from the interactions (*e.g.* neuroendocrine) of homeostatic regulators and the underlying mechanisms. The AVP-ergic and OXT-ergic systems interact with central (neurogenic) and/or peripheral regulators; incidentally influenced by internal-external impacts, they are involved in a broad range of behavior, such as emotionality or social attachments, and various systematic functions. Accordingly, if the secretion of AVP and/or OXT in the relevant central areas is altered, behavioral and/or physiological abnormalities may appear.

The coexistence and interactions between AVP-ergic and OXT-ergic and neurogenic systems related, for example the monoaminergic regulators dopamine (DA), serotonin (5-HT), histamine (HA), norepinephrine (NE), epinephrine (E) or the peptidergic galanin (GAL) systems, have been partially studied to date. However, the studies concerning these regulators with regard to AVP and OXT secretion have been performed practically only from the aspect of levels located within intra- or extrahypothalamic superior brain areas, and much less so at the level of the neurohypophysis (NH), which is a lower regulating area of hypothalamo-neurohypophyseal AVP and OXT release. Besides their glial functions, pituicytes in the NH may also have roles in AVP and OXT expression. In an *in vitro* NH model, AVP and OXT were revealed to be secreted by radioimmunoassay (RIA) and mass spectrometry. Pituicytes are sensitive to osmotic changes, and the activation of AVP expression may also be due to the osmosensitivity of NH glial cells. The existence (receptors, mediators, innervations or the related functions) of the monoaminergic and peptidergic neurogens mentioned above (which have been proven to be involved in neuropeptide secretion at higher regulating levels in the brain) has been partially verified within the hypothalamo-neurohypophyseal system under *in vivo* conditions. We concluded earlier that NH cultures could be used as a model to investigate basal (non-stimulated, intact) AVP and OXT secretion, and that stimulated by osmosis or certain neurogenic monoamines. Such

cultures can be used because of their ability to release (and synthesize) nonapeptides, and the presence of neurogenic regulators (*e.g.* monoaminergic receptors on the surface of pituicytes) found earlier in "*in vivo* functional" superior brain structures. Its simplicity and low costs may enhance the usefulness of the model. We hypothetized that the involvements of DA, 5-HT or HA found earlier could be occur at the level of the NH *in vivo* independently of the hypothalamus. Our recent findings and related published data suggest that roles of the neurogens analogous to those observed in *in vitro* NH cultures exist at higher regulatory levels ("superior" neurons). These brain areas could mainly be affected in the evolution or background of AVP- and OXT-related behavioral forms, including anxiety and aggression, and/or in some of their known physiological functions.

Ubiquitous external-environmental impacts can alter the neuroendocrine systems, both centrally and/or peripherally, and indeed abnormal behavior may occur. Some pollutants may also alter AVP- and OXT-mediated behavior. The available data indicate that such stressors may stimulate the secretion of various underlying hormones. The synchronous (central and peripheral) effects of these stressors may be explained by the presence of central and peripheral (target) neurogenic regulators (such as DA, 5-HT or HA) and the (environmental) impact features (such as the physico-chemical properties, the route of action, the duration and the dose*)*. Many semi-volatile, bioaccumulative pollutants have the ability to interfere with central and/or peripheral endocrine elements (POP/EDCs). Because of the numerous possible endocrine targets in the central nervous system, POP/EDCs are capable of disturbing behavior directly, via action on hormones either alone or additively or synergistically in ambient mixtures. POP/EDCs are usually found in tissues and, especially in complex mixtures, such agents may be biologically active even at extremely low doses *in vivo*. Chlorobenzenes, and especially the hexachlorobenzene (HCB), are often applied as a model to study POP/EDCs though little information is available on the neurobehavioral effects of HCB or other chlorobenzenes on emotional or other AVP- and/or OXT-mediated behavior. HCB contamination causes not only neurotoxicological symptoms, but also neuropsychiatric signs such as an increased frequency of schizophrenia and hypochondria. The effects of long-term dietary exposure to HCB on aggressive behavior and regional brain biogenic mediators, including the concentrations of the neuropeptide-related (and the interacting) monoamines (*e.g.* NE and 5-HT), have already been examined. Following exposure, the subjects displayed

abnormal aggressiveness, and changes were also observed in the monoaminergic mediator concentrations in various superior brain areas.

A topical question is an investigation of neurogenic involvement in AVP and OXT secretion under ”environmentally stimulated” conditions. A POP/EDC (*e.g.* HCB), or such agents in a mixture, may be used for stimulation.

2. AIMS

The involvement of monoaminergic regulators and interactions between monoamines and GAL in AVP and OXT release have been investigated, in part through use of an *in vitro* NH model. We set out to acquie a deeper understanding of neurogenic involvement, under both basal (aims 1 and 2) and environmentally stimulated conditions (aims 3 and 4).

- **Aim 1.** To clarify the roles of NE and E in AVP and OXT secretion, using the NH model, and to identify the specific adrenoceptors involved on the pituicytes.

- **Aim 2.** To reveal the interactions between GAL and the monoamine neurogens in the secretion of AVP and OXT, using the NH model.

Certain environmental impacts (ubiquitous stressors), even at low doses, may alter behavior, including AVP- and OXT-dependent forms, affecting related neuroendocrine elements at the different regulating levels. This led to:

- **Aim 3.** To examine the consequences of exposure to a POP/EDC mixture (orally applied chlorobenzene agents) on anxiety-related behavior and aggression, and on peripheral AVP and OXT secretion *in vivo*.

- **Aim 4.** To examine whether the changes in AVP and OXT secretion can be interpreted with the NH model, and whether neurogenic involvement in AVP and OXT secretion is modified under environmentally stimulated conditions.

3. MATERIALS AND METHODS

3.1 Animals, experimental groups and environmental impacts

Male Wistar rats were used, maintained under controlled conditions, with food and water provided *ad libitum*. Subjects for aims 1 and 2 were not exposed, but those for aims 3 and 4 were divided into experimental groups and exposed environmentally. Smaller, unrelated male rats, maintained separately, were used as intruders [section 3.3.3]. For the examinations in aims 1 and 2, no groups were created. For the examinations in aims 3 and 4, subjects divided into groups were subjected to environmental exposure. Orally administratered 0.1 (D1) or 1 (D2) μg/kg/day 1:1 1,2,4-trichlorobenzene + HCB (ClB) was used. Rats were exposed to ClB for 30, 60 or 90 days (groups D1/D2-ClB-30/60/90) or to stomach tube insertion only (as stress controls, groups SC) for 30, 60 or 90 days (as groups SC-30/60/90) or were not exposed (absolute controls, group AC).

3.2. Protocols

3.2.1. Neurogenic involvement in AVP and OXT secretion in non-stimulated NH cultures

Rats (for aims 1 and 2) were killed and NH cultures were prepared [section 3.4.1]. Confluent cultures were incubated [sections 3.4.2.1-2]. The supernatant was harvested and AVP and OXT levels were assayed [section 3.5.1].

3.2.2. Behavioral and endocrine effects of external-environmental impact, neurogenic involvement in AVP and OXT secretion in stimulated NH cultures

Subjects (for aims 3 and 4) were exposed [section 3.1], the behavior related to anxiety, locomotion and exploration was observed in open-field (OF) and elevated plus maze (EPM) tests and the aggressive behavioral elements were evaluated with the neutral cage paradigm of the resident-intruder (RI) tests [section 3.3]. Following the tests, the rats were sacrified, and the plasma levels of AVP, OXT, adrenocorticotrophic hormone (ACTH) [sections 3.5.1-2] and toxicity markers [section 3.5.4] were determined. Hypophyses were dislodged and NH cultures were prepared [section 3.4.1]. Confluent cultures were incubated [section 3.4.2.3]. The AVP and OXT levels of the harvested supernatant were measured [section 3.5.1].

3.3. Measurements of behavioral elements

3.3.1. OF tests

To determine anxiety-related behavior, locomotion and exploration, OF tests were performed. The following behavioral elements were observed: the percentages of time spent in the centre and the periphery, the total distance moved, the mean velocity, and the total duration and numbers of rearing, grooming, sniffing, freezing and defecation episodes.

3.3.2. EPM tests

EPM tests were performed to determine anxiety-related behavior. The following behavioral elements were measured: the total duration in all open or closed arms, the preferred site of the apparatus, the numbers of entries into the various zones, the number of head-dipping episodes, and the total duration and numbers of rearing, grooming, sniffing and freezing episodes.

3.3.3. RI tests

RI tests were applied to determine intermale aggressive behavior. The resident was placed in the arena for area habituation, and was left to explore it. The parameters relating to the locomotion, exploration and self-care as measured in the OF tests were recorded throughout the habituation and later, until the end of the test. After habituation, an intruder was introduced to the resident. The offensive (the total duration and numbers of aggressive grooming episodes, lateral threats, menacing postures, chasing and biting attacks), defensive (the total duration and numbers of defensive upright posture and immobility episodes). and social (naso-nasal and naso-genital contacts) elements of the resident were recorded.

3.4. Cell culture techniques

3.4.1. Preparation of NH cultures

Primary NH culturing was performed. Cultured cells were kept in a humidified atmosphere of 5% CO_2 in air at 37 °C.

3.4.2. Incubation procedures with neurogen mediators

Conditions, methods, dose/time effects, kinetic curves and relations between DA, 5-HT, HA, GAL and AVP and OXT secretion were in part as described earlier. For incubation procedures, only confluent monolayers were used, because the basal AVP and OXT contents of the supernatant had become constant. Functionality was checked, and the medium was changed and then left untouched before manipulations.

3.4.2.1. Adrenergic involvement in NH cultures (aim 1)

For the study of NE/E involvement in the AVP and OXT secretion of non-stimulated, intact NH cultures, receptor-specific mediators (such as adrenoreceptor agonists; α- and β-antagonists) were used. The dose-time kinetic characterization was designed and performed as applied earlier in the cases of DA, 5-HT or HA.

3.4.2.2. GAL-monoamine interactions in NH cultures (aim 2)

For examination of the interactions between the monoaminergic agonist mediators and GAL, incubation procedures were performed (in all possible variations) on NH cultures. Doses and times were chosen on the basis of (partially published) kinetic examinations.

3.4.2.3. Neurogenic involvement in NH cultures prepared from ClB-exposed (stimulated) rats (aims 3 and 4)

NH cultures prepared from rats (AC or exposed for 30, 60 or 90 days) were incubated with either medium vehicle only or 10^{-6} M 5-HT or 10^{-6} M NE. The choice of 5-HT and NE was based on their importance in the evolution of AVP/OXT-related behavior. After manipulation, the supernatant was collected and stored at -70 °C until RIA assay.

3.5. Measurements and assays

3.5.1. Determination of AVP and OXT secretion of NH cultures and plasma AVP and OXT levels

Sample collection and the conditions for measurement of the supernatant AVP and OXT levels from NH cultures were as described earlier. Measurements were based on modified RIA methods for AVP and OXT detection. The sensitivity of the assays for

AVP and OXT was 1 pg/tube. Following extraction with a recovery of ≥95%, the plasma AVP and OXT levels were assayed by the same RIA method.

3.5.2. Determination of plasma ACTH

Plasma ACTH levels were measured by an immunochemiluminescence assay with an Immulite 2000 apparatus, using DPC kits.

3.5.3. Determination of total protein concentration

Total protein concentration was measured by spectrophotometry with a modified Lowry method and/or using a commercial kit.

3.5.4. Determination of toxic characteristics of exposure to the POP/EDC impact

Toxicity marker liver transferases were measured by standard kinetic methods. The subjects were weighed throughout, and the brain and main organs were removed and weighed. Signs indicative of HCB or 1,2,4-trichlorobenzene toxicity were monitored, and general morphological examinations were performed with conventional stains on the specimens prepared from excised organs.

3.6. Statistical analysis

Data for aims 1 and 2 were analyzed with the Kruskal–Wallis test. Data for aims 3 and 4 were processed by factorial analysis of the variance. The factors were: treatment (/AC/, ClB or SC) and duration of treatment (30, 60 or 90 days). Groups were compared by using Fisher's LSD *post hoc* test and the Dunnett *post hoc* test. Changes were considered statistically significant at $p<0.05$.

4. RESULTS AND DISCUSSION

Aim 1. We found that pretreatment with corynanthine (CAT, as an α_1-receptor antagonist) or phentolamine (as an $\alpha_1+\alpha_2$-receptor antagonist) effectively inhibited the E-stimulated AVP and OXT release of NH cultures. After pretreatment with E, these antagonists did not block the increasing effects of E. The α_2- (and slightly α_1)-receptor antagonist yohimbine was ineffective before treatment with E for both AVP and OXT. Pretreatment with CAT before treatment with NE reduced the NE-stimulated AVP release (for OXT, data are not yet available) because NE has an α_1-receptor agonist character

besides its β-receptor agonist profile. However while the elevation of AVP secretion was totally blocked by pretreatment with CAT before treatment with E, CAT administratered before treatment with NE exerted only a partial blocking effect. Pretreatment with propanolol (a β_1+ β_2 -receptor antagonist) was sufficiently effective for both AVP and OXT, but pretreatment with atenolol (a β_1-receptor antagonist) did not prevent either NE-stimulated AVP or OXT secretion. It was surprising that pretreatment with pindolol (PDL, a β_1+ β_2-receptor antagonist) alone (or both pretreatment with PDL and treatment with NE) significantly increased NE-stimulated AVP secretion. This apparetly contradictory effect can be explained in that PDL not only acts as a blocker, but also exerts intrinsic sympathomimetic action and a strong adrenergic agonist effect. The molecular mechanisms of the intrinsic sympathomimetic action of PDL have not been clarified. It was hypothesized that the receptor loss induced by β-antagonists with intrinsic sympathomimetic action is mediated through cyclic adenosine monophosphate (cAMP). PDL stimulated cAMP accumulation 100-fold over the basal rate, and the increase in cAMP formation is the rate-limiting step for the biological response of partial agonists. We conclude that mainly α_1-receptors may be involved in E-stimulated AVP and OXT secretion, and β_2-receptors in NE-stimulated AVP and OXT secretion *in vitro*.

Aim 2. Our present and eariler findings indicate that AVP and OXT secretion can be influenced directly by the GAL-ergic system, and that GAL-ergic control of the AVP and OXT secretion in rats occurs independently of the hypothalamus, at the level of the NH. Our results permit the supposition that GAL receptors do exist on pituicytes cultured *in vitro*. We presume that the effects of GAL–monoamine interactions on AVP and OXT secretion can develop through the monoaminergic receptors in the NH. This hypothesis is supported by the present findings, *i.e.* GAL had no effect on the increases induced in the levels of AVP and OXT by K^+ administration, which causes nonspecific, receptor-independent hormone secretion. We have demonstrated that the administration of GAL before E or NE prevents the enhancement of AVP and OXT secretion. Before 5-HT or HA administration, GAL has a moderate decreasing effect. The changes induced in AVP and OXT secretion by the monoaminergic system can be directly influenced by the GAL-ergic system.

Aims 3 and 4. POP/EDCs, including chlorobenzenes, can cross the blood-brain barrier and accumulate. They can reach a critical dosage and induce cellular mechanisms which may disturb the endocrine elements/functions, including behavior-related hormonal

mechanisms. The measured parameters in the AC and SC animals did not differed significantly. It may therefore be conclude of that the observed consequences did not originate from the (neuro)toxicity of the applied exposure and/or the stressing effects of handling or the oral gavage.

Aggression and anxiety-related behavior are appreciably affected by AVP and OXT. AVP has anxiogenic effects and OXT has anxiolytic effects, while AVP increases intermale aggression and OXT is involved particularly in maternal care and aggression. Regulation of the neuropeptides is implemented by many factors, including the neurogenic 5-HT- or NE-related elements. Their effects are involved in AVP and OXT regulation in higher brain levels *e.g.* in the hypothalamic paraventricular nucleus (PVN) and the supraoptic nucleus (SON). It has been reported that NE increases both AVP and OXT expression in the PVN and SON, and 5-HT is postulated to stimulate both AVP and OXT expression in the PVN, but only OXT in the SON. Other papers have identified the involvement of 5-HT receptors in different areas related to AVP or OXT production or secretion. NE can increase the central and peripheral release of neuropeptides, mainly via the involvement of HA-ergic neurons. Nevertheless, NE has also been reported to inhibit AVP release. Correlative roles of AVP and 5-HT and/or NE in various aspects of emotional or social behavior or the related disorders too have been described.

Incubation procedures were performed to examine monoamine-mediated AVP and OXT secretion and the changes under stimulated conditions caused by exposure to ClB. Even the basal levels of secreted AVP and OXT were altered. In the NH cultures prepared from ClB-exposed rats, elevated neuropeptide secretion was found, depending on the duration of exposure. Moreover, ClB for 90 days resulted in significant elevations of the secreted AVP and OXT levels. Our published data indicated that 5-HT or NE as specific monoaminergic receptor agonists, significantly increased the secretion of both AVP and OXT from NH cultures prepared from unexposed animals. Such stimulation can be inhibited with specific antagonists, and the receptors acting in the NH cultures were therefore clarified in detail by our group. In NH cultures prepared from exposed rats, neuropeptide secretion was also induced following 5-HT or NE incubation, but the increase was higher. We suspect that the basal and monoamine-mediated secretion of AVP and OXT was affected environmentally. ClB administrated chronically by stomach tube may pass into the behavior-related brain areas and concurrently the hypothalamo-neurohypophyseal system, including the pituicytes of the posterior pituitary. In cells,

HCB may modulate gene expression and induce various process. We suspect that changes observed *in vitro* in the basal and monoamine-mediated secretion (with the environmental influences) could appear at various levels of neuropeptide secretion simultaneously.

After exposure, plasma AVP and OXT levels were measured as results of peripheral (hypothalamo-neurohypophyseal) neuropeptide secretion. Exposure for 30 or 60 days did not change the AVP levels considerably, whereas ClB for 60 days enhanced the OXT level, and ClB for 90 days elevated the levels of both AVP and OXT in the plasma. Our findings may result from modulated peripheral secretion caused by alterations of similar regulations due to HCB mechanisms acting on the SON and/or the PVN or in the NH. Our results may correlate with the findings of increased plasma and/or cerebrospinal AVP levels in patients with anxiety disorders. Various anxiety disorders are associated with elevated AVP concentrations *e.g.* an increased plasma AVP level was measured in patients with post-traumatic stress disorder or obsessive–compulsive disorder. Our study revealed that the SC groups did not exhibit increases in OXT (or AVP) levels, though the stress caused by ClB may have induced peripheral OXT release. Few data are available concerning a correlation between the plasma AVP (or OXT) and aggressive behavior. Instead of neuropeptides, mainly the plasma levels of 5-HT, testosterone or glucocorticoids are mentioned as correlating with regulatory amounts of aggressive behavior. However, in the cerebrospinal fluid the AVP (and OXT or other regulators) concentration seemed to correlate, usually dose-dependently with the relevant aggression. The plasma ACTH levels were elevated directly proportionally to features of the exposure. Increasing ACTH levels may be related to parvicellular AVP-related elements affected by exposures. AVP and corticotropin releasing hormone released into the portal circulation at the median eminence, or even the AVP released into the short portal vessels within the NH, are known to stimulate ACTH secretion in the adenohypophysis, which triggers the adrenal release of glucocorticoids and facilitates physiological and behavioral adaptation to stressors.

5. CONCLUSIONS

NE and E increased AVP and OXT secretion in NH cultures *in vitro*. The α_1-receptors are probably involved in E-stimulated AVP and OXT secretion, the β_2-receptors are probably involved in NE-stimulated AVP and OXT secretion.

GAL interacts with monoamine neurogens, decreasing or inhibiting their effects increasing AVP and OXT secretion.

Our findings may relate to disturbances in AVP and OXT secretion at different regulatory levels. Chlorobenzenes act as discrete anxiogenous factors and have the potential to influence behavioral elements of aggression. These pollutants may pose potential risks in the etiology of psychiatric disorders with symptoms of abnormal anxiety and/or aggressiveness.

Besides the suggested changes in superior AVP- and OXT-related brain areas, the basal secretion of AVP and OXT in NH cultures prepared from exposed animals was also influenced to extents depending on the duration of exposure. The 5-HT-, and NE-stimulated neuropeptide secretion in our NH model seemed to be disturbed following the external impact. Various features of the exposure, and mainly the presence of similar neurogenic regulators in behavior-related brain areas, suggest that the changes observed in the NH model may be interpreted as possible, underlying mechanisms of the behavioral phenomena exhibited.

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