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

Ph.D. Dissertation

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Abbreviations used in the text and/or Figures

5-HT	serotonin (5-hydroxytryptamine)
AC	absolute control
ACTH	adrenocorticotrophic hormone
AdH	adenohypophysis
AH	anterior hypothalamic nucleus
ALT	alanine transaminase
AR	arcuate nucleus
AST	aspartate aminotransferase
ATL	atenolol
AVP	arginine-vasopressin
AVPR	arginine-vasopressin receptor
BNST	bed nucleus of stria terminalis
cAMP	cyclic adenosine monophosphate
CAS	Chemical Abstracts Service
CAT	coynanthine
ClB	chlorobenzene mixture
CNS	central nervous system
CO	chiasma opticum
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
DA	dopamine
dae	days after exposure
diBro	diagonal brand of Broca
DM	dorsomedial hypothalamic nucleus
doe	days of exposure
E	epinephrine
ECF	extracellular fluid
Em	eminence median
EPM	elevated plus maze
GAL	galanin
GALR	galanin receptor
GGT	gamma-glutamyl transpeptidase
GP	globus pallidus
HA	histamine
HC	hippocampus
HCB	hexachlorobenzene
HPA	hypothalamo-pituitary-adrenal axis
i.c.v	intracerebroventricular
ISA	intrinsic sympathomimetic action
LC	locus coeruleus
LS	lateral septum
LT	lateral hypothalamic nucleus
M15	galantid
MB	mamillary nucleus
MeA	medial amygdala
Meynert	nucleus basalis of Meynert
mRNA	messenger ribonucleic acid
NE	norepinephrine
NH	neurohypophysis
OF	open-field
OXT	oxytocin
OXTR	oxytocin receptor
PCN	parvicellular nuclei
PDL	pindolol
PNL	propanolol
PO	medial preoptic nucleus
POP/EDCs	persistent organic pollutants with endocrine disruptor effects
PTA	phentolamine
PVN	paraventricular nucleus
RI	resident-intruder
RIA	radioimmunoassay
SC	stress control
SCN	suprachiasmatic nucleus
SEM	standard error of mean
SON	supraoptic nucleus
TCB	1,2,4-trichlorobenzene
VM	ventromedial hypothalamic nucleus
YOB	yohimbine

SUMMARY

The arginine-vasopressin (AVP)-ergic and oxytocin (OXT)-ergic neuroendocrine systems have multiple functions in homeostatic maintenance. Their (co)existence, the interactions between them and the regulatory elements involved, *e.g.* the neurogenic systems related to monoamines such as serotonin (5-HT), norepinephrine (NE) or epinephrine (E) or the neuropeptide galanin (GAL) are at the center of scientific interest and have been partially described to date. However, these studies are related mainly to the higher levels of release located in either intrahypothalamic or other central nervous superior areas, and only slightly to the neurohypophysis (NH), which could also be a site of AVP and OXT expression. We have estabished that primary NH cultures may be used to study the release of AVP and OXT, and our group has already partially identified the involvement of some neurogenic monoamine regulators.

We set out to acquire a more profound understanding of the roles of a wider range of neurogenic systems, under basal and stimulated conditions at the level of NH. We therefore attempted to elucidate the roles of NE and E and to identify the acting receptors. We set out to reveal the interactions between GAL and the monoamines of interest in AVP and OXT release. To attain these aims, we performed incubation procedures with related receptor agonists and/or antagonists on cultured pituicytes.

If the secreted amounts of AVP and OXT are modulated, physiological or behavioral disturbances may appear. Homeostasis can be implemented by behavior including the largely AVP- and OXT-mediated anxiety and aggression. Independently or in interaction with neurogens and/or others, and incidentally influenced by internal-external stimuli, AVP and OXT are involved in a broad range of behavioral traits. Various external impacts affect hormonal release at different regulatory levels in the brain. Nevertheless, besides chemization, we should increasingly calculate with the relevance of ambient endocrine disruptor substances as possible external-environmental stimuli which may be capable of disturbing behavior and the underlying AVP and OXT mechanisms. We have therefore examined the consequences of a chosen environmental stimulus on anxiety and aggression and on AVP and OXT release *in vivo* and *in vitro*. We wished to know, how this stimulus can disturb the release, and how the changes can be interpreted in terms of a NH model. To provoke the stimulated condition, adult male rats were exposed to chlorobenzenes for 30, 60 or 90 days, after which AVP- and OXT-mediated behavior was examined in open-field, elevated plus maze and resident-intruder tests. NH cultures were prepared from the rats, and further incubation procedures with some relevant neurogenic regulators (5-HT and NE) were performed to study the release *in vitro*. The AVP and OXT levels of NH supernatants and the AVP, OXT and adrenocorticotrophic hormone (ACTH) levels of the blood were measured with immunologic methods. Similarly to the monoamines examined earlier, NE and E increased the AVP and OXT release in NH cultures *in vitro*. We concluded that α_1-receptors are involved in E-stimulated AVP and OXT release, and β_2-receptors in NE-stimulated AVP and OXT release. GAL interacted with all the monoamines, decreasing or inhibiting their effects enhancing AVP and OXT release. The plasma AVP, OXT and ACTH levels were increased, to extents depending on the duration and dose of the exposure. The 5-HT- and NE-stimulated release of AVP and OXT in our NH model was disturbed following the external impact. Several anxiety-related and aggressive behavioral elements were also enhanced following exposure, while certain explorative and locomotive elements of the animals were decreased. As both physiological and behavioral elements were modulated by chronic, subtoxic doses of chlorobenzenes, it was concluded that low doses of such environmental endocrine disruptors may pose potential risks of anxiogenic and/or aggressive consequences in exposed subjects.

1. INTRODUCTION

Homeostasis is a property of organisms that regulates and tends to maintain their internal *milieu*. It involves a variety of neural, immune and endocrine elements and is implemented by both physiological and behavioral patterns [1, 2]. The arginine-vasopressin (AVP)-ergic and oxytocin (OXT)-ergic neuroendocrine systems play a dominant role in homeostatic maintenance [3], via the amounts of these mediators secreted into the bloodstream or the extracellular and cerebrospinal fluids (ECF/CSF). AVP and OXT are needed for the adaptation to stress [4], and impacts affecting the AVP- and OXT-ergic systems may therefore influence the stress responses, and even disturb the homeostasis.

Homeostasis can be maintained *inter alia* via AVP- and OXT-mediated behavioral forms, including anxiety and aggression [4-6]. Anxiety is a basal inborn trait, which is vital in life-threatening situations, as it enhances the capability and motivation to stress [7] and prepares the individual to attempt to cope with upcoming negative events. Major changes in it can be detrimental, as it is indispensable when affected, *e.g.* in escape or avoidance. Aggression, and especially intermale aggression, is also critical for survival, as it promotes better access to resources such as food, territories or mating partners [8]. Additionally, aggression and many AVP- and OXT-mediated behavioral forms are crucial for effective communication in societies [6]. Behavior results from the interactions of homeostatic regulators and their underlying physiological mechanisms. The AVP- and OXT-ergic systems interact with various central (neurogenic) and/or peripheral (non-neurogenic) regulators; incidentally influenced by internal or external impacts, they are involved in a broad range of behavior, such as cognition, emotionality and social attachments [subsections 1.1.4 and 1.2.4], and affect a number of important physiological functions [subsections 1.1.3 and 1.2.3]. Thus, if the amounts of AVP and OXT (or their involved regulators) expressed and secreted are altered by impacts in relevant central areas, this may lead to physiological consequences and/or behavioral disturbances, and even disorders.

The coexistence and interactions of the AVP- and OXT-ergic and many of the involved regulatory neurogenic systems, such as the monoaminergic systems related to dopamine (DA), serotonin (5-HT), histamine (HA), norepinephrine (NE) and epinephrine (E), or the peptidergic system related to galanin (GAL), have been discussed in numerous papers to date. Studies on such regulators in AVP and OXT secretion (or production or transmission) have mainly been performed with regard to levels in intra- or extrahypothalamic superior brain areas [subsection 1.3], 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 [9], the neurohypophyseal pituicytes may have roles in AVP and OXT expression. AVP and OXT were earlier dogmatic considered to be only stored and not produced in the NH [10], but our group [subsection 1.3] and others [11, 12] have demonstrated that the NH is also the site of AVP and OXT expression. We have reported that radioimmunoassay (RIA) and spectrometry reveal that the secretion of these hormones in a NH model *in vitro* was elevated during 2 weeks [13]. Additionally, pituicytes are sensitive to osmotic changes [14, 15]; indeed, it is presumed that the activation of AVP expression may be due to the osmosensitivity of NH glial cells [16, 17]. The receptors, mediators, innervations and related functions of monoaminergic and peptidergic neurogenic regulators have been proven to be involved in neuropeptide secretion at higher, regulating levels in the central nervous system (CNS), and this has also been partially verified within the hypothalamo-neurohypophyseal system under *in vivo* conditions, as reviewed by Sladek *et al*. [18] and detailed below [subsection 1.3]. We concluded that a NH culture could be used as model system to investigate basal AVP and OXT secretion and that stimulated by osmosis or neurogens (monoamines), because of the ability of nonapeptide release (and synthesis), and the presence of neurogenic regulators (their receptors on the pituicytes) in *in vivo* functional superior structures [subsection 1.3]. Many advantageous technical features, such as reproducibility, simplicity, low costs, *etc.* may increase the usefulness of the NH model. We hypothetized that the involvement of DA, 5-HT or HA found earlier could appear at the level of the NH, independently of hypothalamus, under *in vivo* conditions. Our recent findings and published data indicate that analogous roles of the neurogens to those observed in *in vitro* NH cultures exist at higher regulatory levels (”superior” neurons). These areas could mainly be affected in the evolution or background of AVP- and OXT-related behaviors.

Certain external impacts can cause central and peripheral changes in the neuroendocrine systems, and abnormal behavior may occur. There is evidence that *inter alia* some pollutants may alter AVP- and OXT-mediated behavioral forms [subsection 1.4]. The available data suggest that such stressor agents, similarly to others, may stimulate the secretion of underlying hormones. The synchronous (central and peripheral) effects of stressors may be explained by the presence of centrally or peripherally involved same or similar neurogenic regulators and the route and duration of exposure, the dose and the physicochemical properties of these agents.

We also investigate the secretion of AVP and OXT under stimulated conditions [section 2], with the use of environmental pollutants for stimulation.

1.1. The AVP-ergic system

1.1.1. The central and peripheral AVP-ergic system

AVP is synthetized in many areas of the CNS (Fig. 1), but mainly in the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei. Smaller, neurosecretory parvicellular nuclei (PCN) of the PVN terminating in the areas of the ventromedial nucleus (VM), the eminence median (Em) and suprachiasmatic nucleus (SCN) can also produce and secrete AVP and other neuropeptides. In extrahypothalamic areas, the bed nucleus of the stria terminalis (BNST), the diagonal band of Broca (diBro), the nucleus basalis of Meynert (Meynert), the medial amygdala (MeA), the locus coeruleus (LC), the hippocampus (HC), the globus pallidus (GP) and the choroid plexus are able to produce AVP. Many such areas can also express OXT, as mentioned in subsection 1.2.1 or *e.g.* in [19]. AVP-ergic neural pathways originating mainly in the SON and PVN have been found to project to several extrahypothalamic targets throughout almost the whole brain or spinal cord, and are known to be projiced to the NH across the pituitary stalk [5].

Functions of AVP are expressed via the receptors AVPR1A, AVPR1B and AVPR2. AVPR1A is widely distributed in the CNS, on both the neurons and the astrocytes, and also in the extra- and intraparenchymal blood vessels, even outside the CNS, *e.g.* in the vascular smooth muscles, liver, spleen and kidneys. AVPR1B is located mainly in the hypothalamus, amygdala, cerebellum and circumventricular organs, such as the NH. AVPR2 is rare in the CNS; it has been found only in the cerebellum. AVPR2 is distributed in the peripheral tissues, *e.g.* in the renal distal tubules and collecting ducts [20, 21], and elicits antidiuretic effects. AVP can likewise bind weakly to the OXT receptor.

1.1.2. Regulation of the secretion and production of AVP

The encoded neuropeptides, including AVP and OXT and their mRNAs, were found „virtually” in all types of neurons [22] and glial cells [23] in most regions of the CNS. Neuropeptides are synthetised on the ribosomes in the cell somas and dendrites [24]. Post-translational processing (proteolytic cleavage, glycosylation, phosphorylation, acetylation and amidation) of the precursors occurs in the neurosecretory vesicles into which they are packaged by the Golgi apparatus [25]. The axonal compartment appears to lack synthetising capacity, despite the presence of mRNAs encoding the neuropeptides [5]. After the synthesis, central (preferentially from the dendrites in hypothalamic nuclei and axonal terminals of neurons in other brain areas) or peripheral (preferentially from the axonal terminals of

neurons in the PVN/SON) secretion occurs into the ECF and the portal circulation within the Em, targeting the adenohypophysis (AdH) or within the NH (into the systemic circulation to their target cells in the peripheral tissues). From the ECF, the secreted neuropeptides are transported to the CSF or target central neurons [5], mainly via diffusion. The neuropeptides are more persistent than other mediators [5]. Thus, AVP (and OXT) can act as either a neurotransmitter (synaptically) or a neuromodulator (for relatively distant CNS targets via the CSF) or a neurohormone [5] (via the bloodstream). The central and peripheral secretion are generally regulated in an independent manner because of the blood-brain barrier, but they may sometimes be associated [7]. Several intrinsic-extinsic parameters and regulators may themselves affect the mechanisms of central and/or peripheral release or synthesis of AVP (and OXT), as reviewed by Landgraf *et al*. [5]. Besides the various physiological, behavioral or stressor stimuli, other related factors, *e.g.* neurogenic systems (DA, HA, 5-HT, *etc.*) are involved in different brain areas [5]. Various of these features, focusing mainly on AVP and OXT release within the hypothalamo-hypophyseal area or in the NH model system, are described in detail below [subsections 1.3.1 and 1.3.2].

1.1.3. Physiological roles of AVP

AVP is involved as a neurohormone in the regulation of blood pressure, influencing vasoconstriction, water and ion homeostasis [26] and body temperature [27], and in many OXT-related functions, mainly during parturition and the adaptation of the fetus to the stress of labor [19]. AVP, together with other hypothalamic neuropeptides transported to the AdH, acts as a conductor of the hormonal orchestra, thereby influencing almost all the body functions related to homeostasis, metabolism and growth. The parvicellular AVP, synergistically with the corticotropin releasing hormone (CRH) released into the hypophyseal portal system, is involved in regulation of the hypothalamo-hypophyseal-adrenal (HPA) axis, and AVP is therefore essential for the control and production of suitable responses to different stresses [4, 28]. The parvicellularly released AVP (and to lesser extents OXT and other neuropeptides) and AVP released within the NH into the short portal vessels [4] involving the adrenocorticotrophic hormone (ACTH) finally induce the adrenal corticosterone/cortisol-mediated cellular processes within the target cells, *e.g.* the expression (or repression) of key enzymes, mediators, receptors or membrane elements.

1.1.4. Behavioral consequences of AVP

Behavior expressed via AVPR1A and/or AVPR1B and mainly related to central AVP

secretion is discussed in many papers, *e.g.* by de Wied *et al*. [29] and Landgraf *et al*. [5, 7]. AVP is often discussed in concert with OXT [5] because AVP and OXT (and other neuropeptides) are mutually (both functionally and/or locationally) involved in the various forms of social behavior, learning, memory and anxiety. AVP plays roles in social and non-social memory and in both non-spatial and spatial learning and memory [8]. Most papers reporting on social behavior have demonstrated its roles in aggression. AVP affects mainly intermale aggression, and AVP-facilitated aggression appears to be dependent upon prior experience, suggestive of synaptic learning and/or the epigenetic regulation of gene expression. The AVP-ergic projections from the BNST and MeA to the lateral septum (LS) are also important for aggression and are strongly androgen-dependent, as surveyed by Caldwell *et al.* [8]. Numerous studies suggest mediating functions of AVPR1B receptors in aggression [30] and indicate that the interconnecting 5-HT-ergic elements decrease AVP-facilitated aggression [31]. Furthermore, AVP is involved in affiliation such as pair bonding, or paternal or maternal care [8]. Anxiety and depression are specific behavioral states associated with stress. Pharmacological and transgenic studies in rodents allow the modeling of anxiety- and depression-related behavior. The role of AVP via AVPR1A and/or AVPR1B is discussed in relation to the modulation of anxiety and depression, and to action on the HPA stress axis. Several anxiety disorders are associated with an elevated AVP level [32, 33]. Depressed patients exhibit elevations in the plasma level of AVP [34], the mRNA of AVP in the SON [35], the number of AVP-expressing cells and the level of AVP in the PVN [36].

1.2. The OXT-ergic system

1.2.1. The central and peripheral OXT-ergic system

OXT, a widely investigated peptide mediator, has been reviewed, among others, by Neumann *et al*. [37]. OXT is confined mainly to the PVN and the accessory neurosecretory groups (PCN) that bridge between the SON and the PVN (Fig. 1). Smaller amounts of OXT are also detectable in the SON [19, 38]. The OXT-ergic trajectories innervate various extrahypothalmic targets [5]. The main physiological functions involved in lactation and parturition are ascribed to the peripheral secretion originating from the PVN and SON and terminating in the systemic circulation in the NH. OXT-ergic neurons are found in correlation with or located close to AVP-ergic neurons in the CNS. Although these neurons usually constitute separate populations, there are some plasticity-inducing conditions, such as parturition, in which the co-storage of AVP and OXT is observed [39]. The PCN are also

capable of producing OXT, but these cells project mainly to the brainstem [4] instead of the Em. OXT is also produced and released outside the CNS in several peripheral locations, *e.g.* in the uterus, ovary, corpus luteum, testes, epididymis, prostate, heart, thymus and adrenal glands, due to the presence of mRNA transcript and a high local concentration of OXT supporting the local effects. The functions of OXT are mainly transmitted via the OXT receptor (OXTR), but AVP receptors (and *vice versa*) are also capable of binding OXT slightly. The OXTR is distributed in most CNS areas or peripherally, *e.g.* in the uterus, heart and testis [20, 40].

1.2.2. Regulation of the secretion and production of OXT

OXT is synthetised both dendritically and somatically [41], and post-translational processing of the precursors occurs in the neurosecretory vesicles throughout the intracellular, axonal transport [25]. While undergoing the complex maturation process, the neurosecretory vesicles are targeted to their sites of release [5, 22]. Its production is followed by central (from dendrites in the hypothalamic nuclei and axonal terminals in other brain areas) and/or peripheral (from axonal terminals of magnocellular neurons) secretion into the ECF/CSF or the bloodstream within the Em or NH. Various elements are involved in the release or production of OXT [5, 41]. The AVP-related neurogenic regulators also have dominant roles. Features of neurogens concerning OXT release within the hypothalamo-hypophyseal system or the NH model are described in detail [subsections 1.3.1 and 1.3.2.].

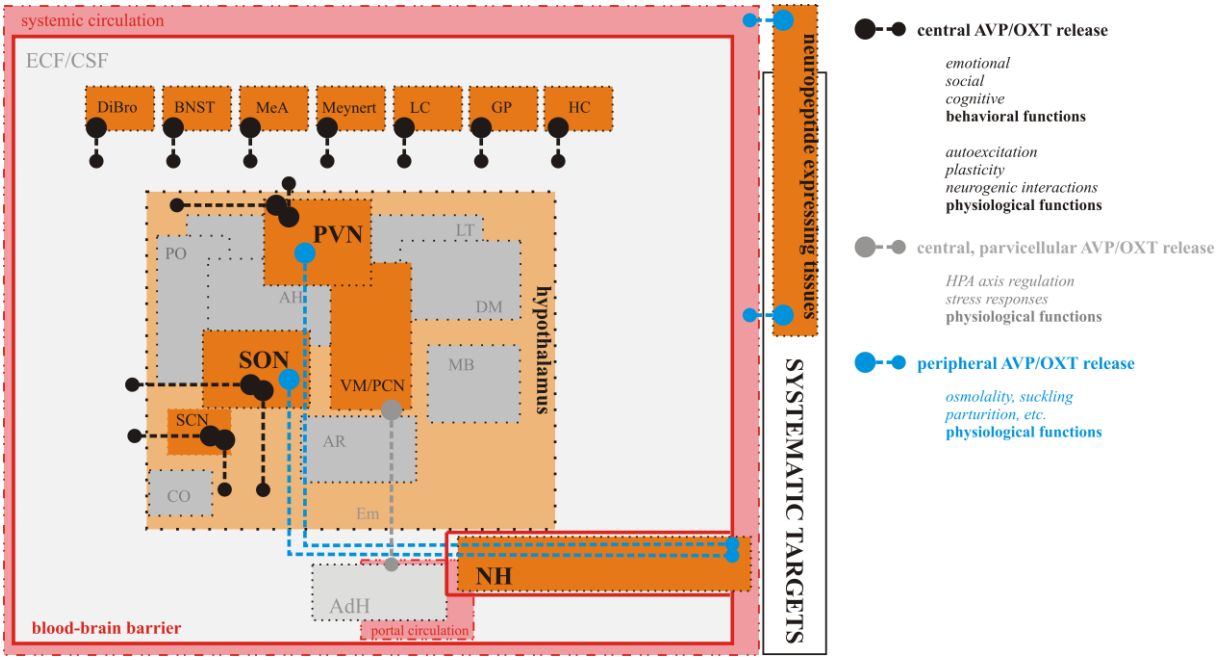
1.2.3. Physiological roles of OXT

OXT triggers both lactation and parturition [42]. AVP and OXT from the PVN participate in the autonomous nervous regulation of the endocrine glands and fat tissue under the influence of the SCN [43]. Its participation in the somatic and autonomic regulation of the cardiovascular system, thermoregulation, pain, gastric motility and osmoregulation has also been reported [40]. OXT is involved in the regulation of responses to stress with blunting of the effects of the HPA axis, mainly during lactation [4, 44]. Several peripheral endocrine, paracrine and autocrine actions of OXT are also known. For example, OXT contributes to sperm transfer through the reproductive tract by inducing smooth muscle contraction [45]. In the mammary gland, OXT is secreted in response to suckling. OXT acts on myoepithelial cells to induce contraction of the ducts and the ejection of milk [40].

1.2.4. Behavioral consequences of OXT

OXT has been proposed as the ”hormone of love” [37, 40]. OXT and AVP are mutually involved in behavior [5, 42, 46, 47]. OXT has various central functions (via both dendritic and axonal release, mainly within limbic areas) which are affected, for instance, in the facilitation of maternal behavior, lordosis behavior, and inhibition of learning and memory. OXT is also implicated in eating behavior. It is widely accepted that both AVP and OXT are important for various emotions, *e.g.* anxiety [48, 49] or social attachments such as aggression or affiliation [5, 50, 51].

Fig. 1. Schematic presentation of the central and peripheral AVP-ergic and OXT-ergic systems



Orange rectangles: main nuclei/areas involved in AVP and OXT production and release. Black dashed lines with circle ends indicate central, gray dashed lines with circle ends indicate central, but parvicellular, and blue dashed lines with circle ends indicate the peripheral production and/or release and (bidirectional) transmission.

Abbreviations: PO: medial preopticus nucleus, AH: anterior hypothalamic nucleus, PVN: paraventricular nucleus, LT: lateral nucleus, DM: dorsomedial hypothalamic nucleus, VM/PCN: ventromedial hypothalamic nucleus/parvicellular neurons, SON: supraoptic nucleus, SCN: suprachiasmatic nucleus, AR: arcuata nucleus, MB: mamillary nuclei, CO: chiasma opticus. Em: median eminence, NH: neurohypophysis, AdH: adenohypophysis, ECF/CSF: extracellular/cerebrospinal fluids, DiBro: diagonal band of Broca, BNST: bed nucleus of stria terminalis, MeA: medial amygdala, Meynert: nucleus basalis of Meynert, LC: locus coeruleus, GP: globus pallidus, HC: hippocampus.

1.3. Neurogenic impacts on the AVP- and OXT-ergic systems

Acting as neurotransmitters or modulators, systems of homeostatic neurogenic regulators innervate most of the CNS areas and are involved in vegetative functions (*e.g.* cardiovascular regulation, nutrition, intake of food, thermoregulation, circadian rhythm, sleep,

inflammation, immune responses, *etc.*) and behavioral traits (anxiety, depression, social attachments, *etc.*). They are also located outside the CNS, where they usually regulate local processes.

1.3.1. Monoaminergic regulation of the AVP- and OXT-ergic systems

The PVN and SON receive moderate 5-HT-ergic innervation from the B7, B8 and B9 raphe nuclei and are also innervated by the NE-ergic system arising from the A1/A2 and A6 cell groups of the brainstem. 5-HT receptors are located in both the PVN and the SON [52]. These inputs play a role in the release of AVP and OXT when an increased hormone level is necessary. Enhanced release is additionally linked to increased peptide synthesis. Tg8/KO mice (a knockout strain for the monoamine oxidase-A gene, which presents high levels of NE and 5-HT in the brain) have been used for immunohistochemical evaluations. The results suggested that, in comparison with control C3H/HeJ mice, NE increased the levels of AVP and OXT in the PVN and SON, and 5-HT is proposed to stimulate the expression of AVP and OXT expression in the PVN, and only that of OXT in the SON [53, 54]. NE and 5-HT stimulate AVP expression and participate in differentiation of the neurochemical phenotype in the SCN [55] of Tg8/KO mice too. Human and animal studies suggest a mutual role of AVP and 5-HT [31] and/or NE [56] in various aspects of emotional behavior or disorders. Other papers have reported mainly on 5-HT receptors in different brain areas related to AVP or OXT expression or release [57, 58]. The involvement of 5-HT receptors in the mediation of stress-induced AVP and OXT secretion [59] or of NE receptors to in AVP secretion from the PVN in pain modulation [60] has also been identified.

DA-ergic input has been observed in the PVN and SON. The DA innervation was uniform, and more important in the PVN in than the SON [61] Both DA and NE terminals synaptically connected to somata or dendrites of magnocellular neurons have been detected morphologically [61]. DA is important in the regulation of AVP and OXT secretion [62, 63]. DA-ergic innervations have been found in the SCN on AVP and vasoactive intestinal peptide-containing neurons [64]. The intermediate and the neural lobe of the pituitary gland are also innervated by two, virtually independent groups of DA-ergic neurons [65].

HA, which acts as a neurotransmitter in the hypothalamus, increases the plasma levels of AVP and OXT and activates mainly AVP-ergic neurons in the SON [66]. Furthermore, HA augments c-fos expression in AVP and OXT neurons, and also messenger RNA for AVP or OXT in both the PVN and the SON [67]. HA-ergic blockade moderates the AVP secretion

enhancement induced by dehydration [68] or the OXT response to suckling in lactating rats [69]. Dehydration has been shown to increase the neuronal turnover of HA in the hypothalamus [68]. Various data have revealed that not only HA, but also NE and E are involved in the regulation of AVP secretion. Both HA-ergic neurons and NE-ergic neurons activate AVP-ergic and OXT-ergic neurons and increase the release of AVP and OXT into the peripheral circulation, and they seem to be involved in the mediation of the same physiological events that lead to the release of AVP or OXT [67]. The data also demonstrate that HA stimulates the expression of c-fos hypothalamic CRH and the formation of mRNA of CRH in the hypothalamic CRH neurons, via their receptors and hence indirectly the release of AVP [70].

The NE-ergic system is able to activate mRNA expression of AVP and OXT [53] and increase the central and/or systemic release of AVP and OXT, mainly with the participation of HA-ergic neurons [67]. However, NE has been reported to inhibit AVP release too [71, 72]. This contradiction can be explained in that adrenergic receptors may be distributed differentially on the surface of AVP-ergic cells, allowing the different adrenergic inputs to be excitatory or inhibitory [73]. Several studies suggest that NE may regulate the systematic, peripheral release of OXT. It has been proved that there are large amounts of noradrenergic varicosities in contact with both AVP and OXT in hypothalamic magnocellular neurons under basal conditions, but there is a significant increase in the density of noradrenergic varicosities apposed to the somata of OXT neurons in the PVN and SON during lactation [74]. Moreover, the depletion of hypothalamic NE decreases the systemic release of OXT in response to footshock, the peripheral administration of cholecystokinin and hypertonic saline, and suckling during lactation [75].

Our group has demonstrated, that elements related to DA, 5-HT, or HA may be (co)located in tissues cultured from the rat NH, and the AVP and/or OXT secretion of cultures may be modulated directly and/or via interactions of monoaminergic and GAL-ergic elements [subsection 1.3.2], as proved by the data relating to treatment with different receptor specific agonists or antagonists. DA [76], HA [77] and 5-HT [78] are involved in the release of AVP and OXT observed in *in vitro* NH cell cultures, which may be influenced by monoaminergic systems (or related interactions, *e.g.* with GAL), this control occuring at the level of the posterior pituitary too under *in vivo* conditions.

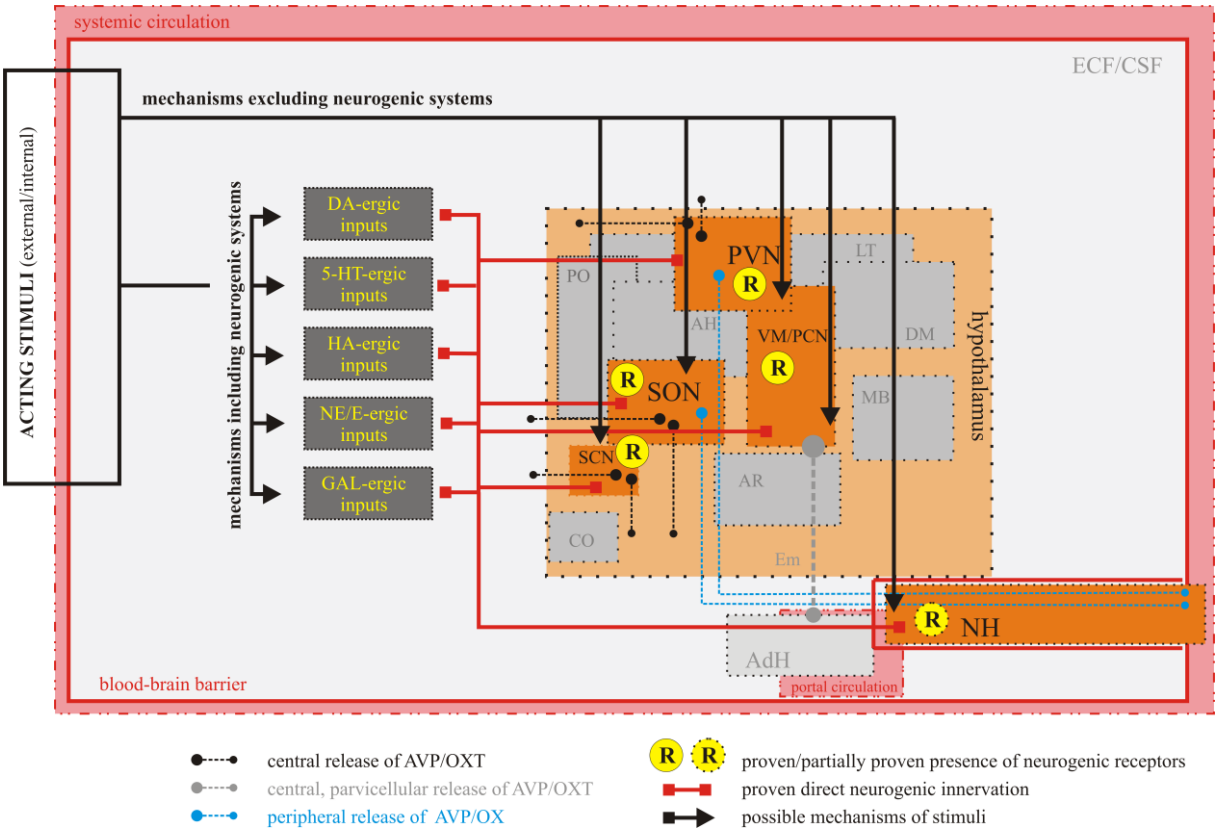
1.3.2. GAL-ergic regulation of the AVP- and OXT-ergic systems

The peptide GAL [79] can act as a neurotransmitter or a neuromodulator. It is widely

distributed within the CNS, where it modulates ascending neurotransmitter systems, including the cholinergic, NE-ergic, 5-HT-ergic and different neuroendocrine pathways [80]. The highest densities of GAL-like immunoreactivity are found in the amygdaloid complex, the hypothalamus, the brainstem and the NH [81]. GAL may coexist and be coexpressed in AVP- and OXT-ergic cells of the hypothalamic magnocellular nuclei [82, 83] and the PCN [81]. Neurons containing both GAL and AVP are very common in the SON and also occur in the PVN and the SCN. The SON and PVN contain neurons with immunoreactivity for both GAL and OXT [84]. GAL receptors (GALR1, GALR2 and GALR3) are widespread throughout the body, often colocalized with AVPR1-2 and OXTR [80].

We have partially mapped possible monoaminergic-peptidergic interactions in neuropeptide secretion, and reported an interaction between GAL and DA relating to the AVP [85] and OXT [86] secretion of NH cultures. Moreover, the effects of intact rat, porcine, and human GAL and human GAL fragments (the N-terminal 1-16 and C-terminal 16-30 sequences) on peripheral AVP secretion induced by a non-osmotic (HA pretreatment) or an osmotic stimulus (hypertonic NaCl solution) were examined in Wistar rats. Intracerebroventricularly (i.c.v.) injected intact GAL or the N-terminal 1–16 fragment prevented the plasma AVP enhancement. Following the intravenous administration of rat GAL or i.c.v. injection of the C-terminal 16–30 fragment, the AVP concentration did not return to the basal level. I.c.v. administration of the GAL antagonist galantid (M15) before the i.c.v. injection rat of GAL prevented the inhibitory effect on the increased plasma AVP level following a stimulus with NaCl solution or HA. We have suggested that GAL, as a peptide modulator, may be physiologically involved in the regulation of AVP release following stimulation [87] under *in vivo* conditions.

Fig. 2. Schematic presentation of the regulation of the hypothalamic AVP-ergic and OXT-ergic systems



Stimuli (physiological-behavioral, external-internal), *e.g.* induced by a stressor impact, can influence the related neuropeptide elements at the level of transcription/expression or release, either involving the neurogenic elements or excluding them (in these cases via other mechanisms). The proven, direct neurogenic innervations (red line with rectangle ends) and the presence of neurogenic receptors (yellow circles) related to the AVP and OXT-producing/secreting areas are also shown.

Abbreviations: PO: medial preopticus nucleus, AH: anterior hypothalamic nucleus, PVN: paraventricular nucleus, LT: lateral nucleus, DM: dorsomedial hypothalamic nucleus, VM/PCN: ventromedial hypothalamic nucleus/parvicellular neurons, SON: supraoptic nucleus, SCN: suprachiasmatic nucleus, AR: arcuata nucleus, MB: mamillary nuclei, CO: chiasma opticus. Em: median eminence, NH: neurohypophysis, AdH: adenohypophysis, ECF/CSF: extracellular/cerebrospinal fluids.

1.4. Environmental impacts on neuroendocrine systems

Some pollutants (external stressor impacts) affect various regulators of the homeostasis and/or alter behavior. Major amounts of such chemicals have been manufactured and used in industry and agriculture. Their physicochemical properties or the by-product reactions have resulted global enrichments. Many of these ubiquitous pollutants are semi-volatile, bioaccumulative, stable and toxic [88], most of them (hereafter POP/EDCs) have the ability to interfere with the central and/or peripheral endocrine systems [89]. Because of the possible endocrine targets in the CNS, POP/EDCs are capable of disturbing behavior directly via action on hormones, or indirectly on other traits [90], either alone or additively or synergistically in ambient mixtures [90, 91]. POP/EDCs may also affect neural and immune elements, and they are actually neuro(immuno)endocrine disrupters [91]. POP/EDCs have

been found in most tissues of exposed subjects [92], and evidence is increasing that such POP/EDCs, especially in complex mixtures, may be biologically active even at extremely low doses [93] *in vivo*. Their actions can be mediated mainly via nuclear, *e.g.* aryl-hydrocarbon receptors that affect mainly gene expression [94].

Chlorobenzenes, and especially hexachlorobenzene (HCB), have often been applied as models to study POP/EDCs [89, 95]. Most of the literature data on chlorobenzenes relate primarily to HCB. Although the production and use of HCB and many other chlorobenzenes have been banned or strictly controlled, they are still present in detectable levels in the environment [96-98]. Following accidental or occupational exposures of humans who subsequently exhibited overt symptoms or disorders, *e.g.* porphyria turcica [99], use of HCB was virtually eliminated, but it is still found in the biosphere [96]. Subjects may be exposed via the inhalation or ingestion of contaminated foodstuffs [100], but occupational exposure may often occur via inhalation or dermal contact [101]. Placental or lactation-mediated transfer to fetuses or suckling offspring [102, 103] can also arise. HCB (and other chlorobenzenes), as a dioxin-like compound bound to aryl-hydrocarbon receptors [104], can accumulate in lipid-rich tissues, *e.g.* in the endocrine glands [105].

To date, many consequences of HCB or other chlorobenzenes that affect various homeostatic regulators have been investigated [98, 104, 106]. As an endocrine disruptor, HCB is a widely profiled contaminant which can affect parathyroid, thyroid [104] or various steroid [89, 107] endocrine elements. Many active, industrial, intermediate- or by-product chlorobenzenes, *e.g.* the 1,2,4-trichlorobenzene (TCB) have also been reported to be able to disturb certain thyroid functions [108].

The POP/EDCs, chlorobenzenes, HCB or other chemically active agents such as TCB, applied alone or in complex mixtures, have mainly been investigated with regard to their physiological effects, though possible behavioral consequences could also influence the homeostasis [97, 98, 107].

However, despite the prevalence of AVP- and OXT-ergic elements, insufficient data are available on the possible effects of most POP/EDCs, including the chlorobenzenes found in the environment near human dwellings, on the AVP- and OXT-related endocrine functions or behavioral traits. Most data on the neurobehavioral effects of chlorobenzenes in general also relate to HCB. Relevant publications emphasize only its neurotoxicity (*e.g.* Peters *et al*. [109]), the discrete disturbances caused in locomotion (*e.g.* deDuffard *et al*. [110]) or its involvement in some forms of cognitive behavior (*e.g.* Lilienthal *et al.* [111]). Such effects have usually been observed in cases of long-term exposure and/or larger (at least mg or

higher) exposure doses, in both humans and animals. More data exist on humans and the neurodevelopmental effects of pre- or postnatal exposure to HCB on infants or children have often been examined. For example, discrete correlations have been detected between exposure to organochlorines, including HCB, and impaired neurodevelopment in infants or children [112]. Decreases in social competence scores and enhanced attention-deficit hyperactivity disorder symptomatology have been observed in children aged under 4 years exposed prenatally to chronic HCB [113]. Less information is available on the neurobehavioral effects of HCB or other chlorobenzenes on emotional or other AVP- and/or OXT-mediated (and noncognitive) behavior, such as aggression. HCB contamination is known to cause not only neurotoxicological symptoms [114], but also neuropsychiatric signs, such as an increased frequency of schizophrenia and hypochondria in patients with porphyria turcica [109]. Effects of chronic, dietary exposure to HCB on aggressive behavior and regional brain biogenic mediators, including the concentrations of neuropeptide-related (and interacting) monoamines, were examined in minks and European ferrets by Bleavins *et al.* [115]. Following exposure, the animals displayed abnormal aggressiveness and changes in monoaminergic mediator concentrations in different brain areas, *e.g.* in the cerebellum or hypothalamic nuclei.

2. AIMS

The involvement of the monoaminergic regulators and the interactions between the monoamines and GAL in AVP and OXT secretion had previously been investigated in part by our group, using the *in vitro* NH model. We now set out to understand the involvement of the monoaminergic systems in AVP and OXT secretion. We therefore examined the roles of several neurogens under basal [aims 1 and 2] and subsequently stimulated conditions [aims 3 and 4].

Aim 1. We set out to clarify the roles of NE and E in AVP and OXT secretion by using the NH model. In addition, we wished to identify the adrenoceptors involved on the pituicytes.

Aim 2. Similarly as for the earlier investigated DA, we aimed to identify a wide range of interactions between GAL and the monoamines (5-HT, HA, NE and E) in the secretion of AVP and OXT, using the NH model.

Numerous studies have revealed that certain environmental compounds, even in low doses, may alter behavior, including some AVP- and OXT-dependent behavioral forms, affecting the neuroendocrine elements at different regulatory levels and resulting in behavioral and physiological consequences or disorders. The stressor effects of such POP/EDCs on AVP- and OXT-ergic elements may also influence neuropeptide secretion, and therefore alter AVP- and OXT-related physiological functions and behavioral traits.

Aim 3. We further set out to examine the consequences of a realistic, external POP/EDC mixture of frequent chlorobenzene agents on anxiety-related behavior and aggression, and on peripheral AVP and OXT secretion *in vivo*.

Aim 4. We wished to discover

i. whether the changes in neuropeptide secretion can be interpreted via the NH model, and
ii. whether the known neurogenic involvement in AVP and OXT secretion is modified under environmentally stimulated conditions.

3. MATERIALS AND METHODS

3.1. Materials

Chemicals and cell culture materials were obtained from Sigma-Aldrich (St. Louis, MO, USA), Invitrogen Corporation (Carlsbad, CA, USA) or BD Biosciences (San Jose, CA, USA), unless otherwise indicated. All chemicals were of analytical grade. The cell culture medium was Dulbecco's Modified Essential Medium supplemented with 20% fetal calf serum and conventional components and antibiotics as described earlier [13].

3.2. Animals

Medically certified, adult male Wistar rats (Charles River, Isaszeg, Hungary) weighing 300-350 g, aged 6-8 weeks at the beginning of the experiments, from different litters were used. The animals were maintained in different rooms of the Animal House of the Department of Physiology, Anatomy and Neuroscience under controlled (and constant) conditions (22±2 ºC, relative air humidity 55-65%, automated 12-h dark – 12-h light cycle, lights on at 6:00 a.m). After arrival, the animals were randomly housed into standard cages (5 rats/cage maximum). Rat chow (CRLT/N, Charles River, Gödöllő, Hungary) and tap water were provided *ad libitum*.

The rats used for aims 1 and 2 were kept regularly (no different treatments/experimental groups) until their sacrifice. Further rats using were divided into experimental groups for aims 3 and 4 [subsection 3.3]. Smaller, unrelated Wistar male rats weighing 200-250 g, initially aged 4-6 weeks, maintained in an isolated room under the same conditions as mentioned above, were used as intruders for the resident-intruder (RI) tests [subsection 3.6.3].

The acclimation to the staff and the experimental environment lasted for 2 weeks. The level of animal suffering during the experiments was minimized as much as possible. The care and procedures were carried out in accordance with European Communities Council Directive 86/609/EEC. Formal approval to conduct the experiments was granted in advance by the Animal Experimentation Committee at the University of Szeged.

3.3. Experimental groups and environmental impacts

For the examination of neurogenic involvement (NE/E and interactions between GAL and monoamines 5-HT, HA, NE and E) in AVP and OXT release from NH cultures (aims 1 and 2), healthy unexposed rats were used. The related experimental protocol [subsection 3.4.1] is outlined in Fig. 3A.

For the examinations in aims 3 and 4, the following exposures and experimental groups were used. An orally administratered, 0.1 (D1) or 1 (D2) µg/kg/day dose of a mixture of TCB (Chemical Abstracts Service /CAS/ registry number: 120-82-1) and HCB (CAS registry number: 118-74-1) in a ratio of 1:1, diluted in 0.015% ethanol in tap water (referred to below as /D1 or D2/-ClB) was used. These agents were arbitrarily chosen to model ambient mixtures of POP/EDCs, as relevant, active compounds with known endocrine disruptor potential. The dosage and duration of ClB exposure were selected on the basis of our earlier nonbehavioral studies and the lowest reported observed effects [97, 98, 107]. The rats were exposed to ClB for 30 (n=10, group D1-ClB-30; n=5, group D2-ClB-30), 60 (n=10, group D1-ClB-60; n=5, group D2-ClB-60) or 90 (n=10, group D1-ClB-90; n=5, group D2-ClB-90) days or were exposed to only the insertion of a stomach tube (stress controls, SC) for 30 (n=5, group SC-30), 60 (n=5, group SC-60) or 90 (n=5, group SC-90) days or were not treated (n=10, absolute controls, AC). The related experimental protocol [subsection 3.4.2] is outlined in Fig. 3B.

3.4. Protocols

3.4.1. Neurogenic involvement of AVP and OXT secretion in intact NH cultures

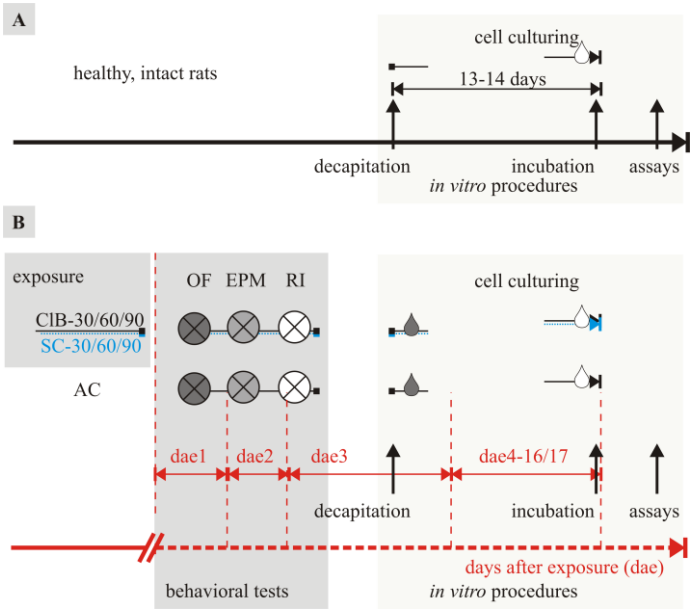
The protocol is outlined in Fig. 3A. The subjects were killed by rapid decapitation, NH cultures were prepared [subsection 3.6.1] from their hypophyses, and the confluent primary cell cultures were incubated (with neurogens) [subsections 3.6.2.1 and 3.6.2.2]. Subsequently, medium supernatant samples were harvested and AVP and OXT concentrations were measured [subsection 3.7].

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

The protocol is illustrated in Fig. 3B. After *in vivo* exposure [subsection 3.3], behavior relating to anxiety, locomotion and exploration were observed in open-field (OF) and elevated plus maze (EPM) tests and the (intermale) aggressive behavioral elements were evaluated with the neutral cage paradigm of RI tests [subsection 3.5]. Following the behavioral tests, the rats were immediately killed by rapid decapitation, always at approximately the same time of day. Trunk blood was collected to determine the plasma levels of AVP, OXT, ACTH [subsections 3.7.2 and 3.7.3] and aspartate aminotransferase (AST), alanine transaminase (ALT) and gamma-glutamyl transpeptidase (GGT) as conventional markers of liver damage [subsection 3.9]. The hypophyses were dislodged quickly and carefully under sterile conditions, NH cultures were prepared [subsection 3.6.1], and the confluent primary cultures

were subjected to incubation procedures [subsection 3.6.2.3], after which the AVP and OXT concentrations of the harvested supernatant samples were measured [subsection 3.7.1].

Fig. 3. Experimental protocols



A. Protocol for study of neurogenic involvement in AVP and OXT secretion in NH cultures (aims 1 and 2). **B.** Protocol for study of the behavioral and endocrine effects of external, environmental POP/EDC impact (aims 3 and 4). The red axis (time axis) denotes the duration of the study in days: the solid part relates to the days of exposure (doe) and the dashed part to the days after exposure (dae). The experimental groups: groups D1/D2-ClB (exposed to ClB, black line), SC groups (exposed to stress, blue line) and AC group (doe=0, no exposure). Exposure duration: 30 (ClB/stress until doe=30, ClB-30/SC-30 groups), 60 (ClB/stress until doe=60, ClB-60/SC-60 groups) or 90 (ClB/stress until doe=90, ClB-90/SC-90 groups) days. Following exposure (until dae=0, maximum doe=90), and OF tests (on dae=1), EPM (on dae=2) tests, and RI tests (on dae=3), the rats were killed on dae=3 immediately after the RI tests and various *in vitro* procedures were performed (preparation of NH pituicyte cultures from dae=3 to dae=16-17 and monoamine incubation of confluent NH cultures on dae=16 or dae=17). Following incubation, the AVP and OXT and ACTH levels of plasma collected and prepared on dae=3 (dark drop symbols) and/or NH culture supernatant samples prepared on dae=16-17 (white drop symbols) were measured by RIA or immunochemilumiscence assay.

3.5. Measurements of behavioral elements

Animals occurred only once in each test. Tests were performed under constant conditions in test room in the early hours of the dark (active) phase. The test room was illuminated by dim light. The animals were transferred to the test room and allowed to become habituated for 1 h. They were removed from their cages in a random sequence for the testing. The apparatus was cleaned with 70% ethanol before each trial, to eliminate odorous cues. Behavior was videotaped by a camera mounted on the ceiling directly above the apparatus and recorded by behavioral software (EthoVision v2.3, Noldus, Wageningen, The Netherlands). Each test was (re-)analyzed by an observer who was unaware of the nature of the experiments.

3.5.1. OF tests

OF tests [116] were performed to determine the effects of ClB exposure on the anxiety-related behaviors, spontaneous locomotor activity and explorative behavior of individual animals. Each rat was placed at the center of a circular (diameter 80 cm, height 45 cm), plastic, black-painted arena open at the top, and the following behavioral elements were detected by the EthoVision and/or staff members manually: the percentages of time spent in the centre and periphery, total distance moved and the mean velocity, and the total durations and numbers of rearing, grooming, sniffing, freezing and defecation episodes. The zones applied were determined in advance with the software, using its standard options. Each lasted for 5 minutes.

3.5.2. EPM tests

EPM [117] tests were performed to determine the effects of ClB exposure on anxiety-related behavior. The maze apparatus (wood, painted brown) consisted of two opposing open arms (50x8 cm) and two opposing closed (50x8x15 cm) arms, and a central area (8x8 cm). The floor under the apparatus was covered with dark cloth to reduce height cues. Each animals was placed at the center, always facing the same open arm, and the following behavioral elements were measured by the EthoVision and/or re-analyzing observer: the total durations in open or closed arms, the preferred site of apparatus (calculated with a ratio of total duration % in all open arms and total duration % in whole apparatus), the numbers of entries into different zones, head-dipping, and the total durations and numbers of rearing, grooming, sniffing and freezing episodes. The zones applied were determined in advance with the software, using its standard options. Each test was performed for 3 minutes.

3.5.3. RI tests

RI tests with the neutral cage paradigm were applied to determine the possible changes in intermale aggressive behavior following exposure to ClB. The resident animal was placed at the center of an arena for habituation, and was left to explore the area for 5 minutes. The arena was an empty, neutral cage, strewn with unused, clean wooden chips and covered with a plastic, transparent lid. The parameters relating to locomotion, exploration and self-care, as measured in the OF tests [subsection 3.5.1], were recorded throughout the habituation and also throughout the RI test. After habituation, an intruder animal was introduced to the resident and various offensive (the total durations and numbers of aggressive grooming episodes, lateral threats, menacing postures, chasing and biting attacks), defensive (the total

durations and numbers of defensive upright posture and immobility episodes) and social (naso-nasal and naso-genital contacts) behavioral elements of the residents were recorded, as described earlier [118]. The behavior of the intruder animals was not analyzed. Each test lasted for 5 minutes.

3.6. Cell culture techniques

3.6.1. Preparation of NH cultures

Primary NH cultures were prepared as described earlier [13]. All details of the procedures with the glial cells/pituicytes were also reported previously [76, 119, 120]. Cultured cells were kept in a humidified atmosphere of 5% CO_2 in air at 37 °C. The medium was changed daily on cells.

3.6.2. Incubation procedures with neurogenic mediators

The conditions, methods, dose-time effects, kinetic curves and relations between DA, 5-HT, HA, GAL and AVP and OXT secretion were in part described earlier [76-78, 85, 86] For incubation procedures, confluent (about 13-14-day-old) NH cultures were used: the basal hormone content of the medium had become constant by that age [13]. The functionality of cells was always checked before manipulations, through an aspecific stimulus with 30 mM K^+, which triggered the release of both AVP and OXT from the NH cultures [121]. The medium was always changed and culture then left untouched for 2 hours before any manipulations.

3.6.2.1. Adrenergic involvement in NH cultures

For the study of adrenergic (NE/E) involvement in AVP and OXT secretion from NH cultures (aim 1), the following receptor-specific mediators were used for the incubation procedures: E (an $\alpha + \beta_2$ -receptor agonist), NE (an $\alpha_1 + \beta$-receptor agonist); phentolamine [122] (PTA; an $\alpha_1 + \alpha_2$-receptor antagonist), corynanthine [123] (CAT; an α_1-receptor antagonist), yohimbine [124] (YOB; mainly α_2-receptor antagonist); propanolol [125] (PNL; an $\beta_1 + \beta_2$-receptor antagonist), atenolol [126] (ATL; an β_1 -receptor antagonist) and pindolol [127] (PDL; an $\beta_1 + \beta_2$ -receptor antagonist). The dose-time kinetic characterization of the agonists and antagonists was performed similarly for DA [76], 5-HT [78] or HA [77], and the applied dose and duration were chosen for the incubation procedures according to the protocol outlined in Fig. 4A. For investigation of the adrenergic monoaminergic agonist-antagonist effects, the NH cultures were always pretreated with 10^{-6} M mediator for 20 min at 37 °C, and

then treated with 10^{-6} M mediator for 1 hour at 37 °C.

3.6.2.2. GAL-monoamine interactions in NH cultures

For the examinations of the interactions between the monoaminergic agonist mediators (10^{-6}M) and GAL (aim 2), the incubation procedures depicted in Fig. 4B were performed on the NH cultures (duration of pretreatment and treatment 60 min). GAL (rat, 1-29) and the GAL antagonist galantid (M15) [128] were synthetized by Lajos Balazspiri, M.D., Ph.D..

Fig. 4. Incubation protocols

A

PRETREATMENT	→	TREATMENT
-		agonist (*e.g.* NE)
-		antagonist (*e.g.* PNL)
agonist (*e.g.* NE)		antagonist (*e.g.* PNL)
antagonist (*e.g.* PNL)		agonist (*e.g.* NE)

B

PRETREATMENT		→	TREATMENT
-	GAL		monoaminergic agonist (*e.g.* NE)
-	monoaminergic agonist (*e.g.* NE)		GAL
M15	GAL		monoaminergic agonist (*e.g.* NE)

A. Incubation procedures for examining adrenergic involvement in NH cultures. **B.** Incubation procedures for examining GAL-monoamine interactions.

3.6.2.3. Neurogenic involvement in NH cultures prepared from ClB-exposed (stimulated) rats

NH cultures prepared from experimental rats (unexposed or exposed for 30, 60 or 90 days) were incubated with vehicle (nonstimulated), or 10^{-6} M 5-HT (5-HT-stimulated) or 10^{-6} M NE (NE-stimulated) for 1 hour. The choice of 5-HT and NE was based on their importance in the evolution of AVP- and OXT-related behavior. After any manipulation, supernatant aliquots were collected and refrigerated at -70 °C until RIA was performed.

3.7. Hormone assays

3.7.1. Determination of AVP and OXT secretion of NH cultures

The details of sample collection and conditions for measurements of the AVP and OXT levels in the NH culture supernatants were reported earlier [13]. Measurements were based on modified [129] RIA methods for AVP [130] and OXT [131] detection. The sensitivity of the assays for AVP and OXT was 1 pg/tube. Results were calculated with respect to the measured total protein concentrations [subsection 3.8] of the samples.

3.7.2. Determination of plasma AVP and OXT

Blood was collected in chilled polystyrene tubes coated with ethylenediaminetetraacetic acid disodium salt solution and centrifuged immediately. Plasma aliquots were separated and stored at -70 °C until measurements. Following extraction on Amprep C8 minicolumns (product code: Y2-VW-RPN1902; GE Healthcare, Buckinghamshire, UK) with a recovery of ≥95%, the plasma AVP and OXT levels were assayed by RIA [subsection 3.7.1].

3.7.3. Determination of plasma ACTH

Plasma ACTH levels were measured by an immunochemiluminescence assay with an Immulite 2000 apparatus (Siemens Healthcare Diagnostic, Deerfield, IL, USA), using DPC kits (product code: L2KAC-02; Euro/DPC Limited, Glyn Rhonwy, UK).

3.8. Determination of total protein concentration

Total protein concentration was measured spectrophotometrically by a modified Lowry method [132] and/or using a commercial kit (product code: 23225; BCA Protein Assay Kit, Fisher Scientific, Chicago, IL, USA).

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

AST, ALT and GGT were measured by standard kinetic methods based on the recommendation of European Committee for Clinical Laboratory Standards. These enzymes are often used as markers of liver damage induced by harmful impacts such as environmental agents. Moreover, GGT can be used to characterize HCB exposure [133]. The animals were weighed regularly throughout the experiments. After sacrifice, the brain, liver, spleen and kidneys were removed and weighed. Signs on the animals indicative of HCB or TCB toxicity, *e.g.* alopecia, skin thickening, scarring or erythema [107], were monitored, and general morphologic examinations with conventional histological stains such as hematoxylin-eosin

staining were performed on the specimens prepared from the excised organs.

3.10. Statistical analysis

Statistical software (SPSS for Windows 13.0, SPSS Incorporation, Chicago, IL, USA and Statistica 9.0, Statsoft, Tulsa, OK, USA) was used for analyses. Neuropeptide concentrations in experiments involving neurogenic involvement in AVP and OXT secretion in NH cultures (aims 1 and 2) were analyzed with the Kruskal–Wallis, test using SPSS. Data from other experiments (aims 3 and 4) were processed by factorial (two-way) analysis of variance. The independent factors were the treatment (/AC/, ClB or SC) and the duration of the treatment (30, 60 and 90 days). Groups were compared by using Fisher's LSD *post hoc* test, and the ClB and SC groups were compared with the AC group by using the Dunnett *post hoc* test. All results are presented as means ± standard error of mean (SEM). Changes were considered statistically significant at $p<0.05$.

4. RESULTS

4.1. Study of neurogenic involvement of AVP and OXT secretion in intact NH cultures

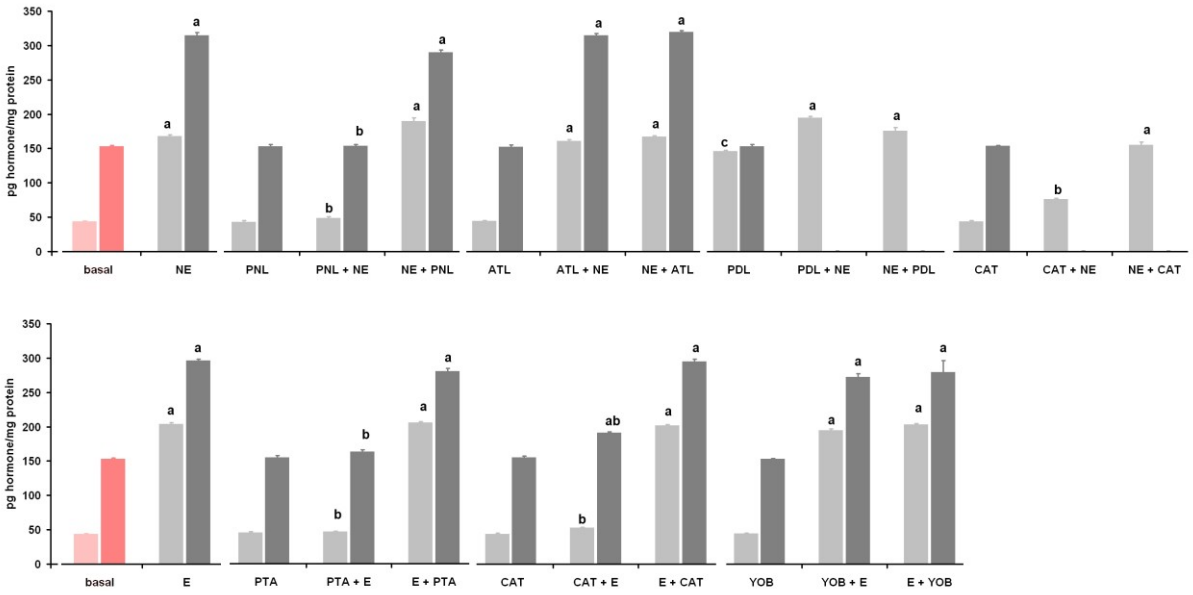
Effects of adrenergic involvement on AVP and OXT secretion in NH cultures

Our results are shown in Fig. 5. The results of the dose-time characterization of the mediators used are not shown presently (but were published earlier [134, 135]). Treatment with E or NE significantly increased both the AVP and OXT levels in the supernatant medium of the *in vitro* NH cultures. Treatment with α- (PTA or CAT or YOB) or β- (PNL or ATL) receptor antagonists merely had no effect on either AVP or the OXT secretion, but treatment with the β-receptor antagonist PDL enhanced the AVP levels relative to the basal (control) AVP level. Pretreatment with PNL or CAT prevented the NE-stimulated increase of AVP and OXT secretion successfully. Pretreatment with ATL or PDL applied before the treatment of NE did not block the increasing effects of NE on either AVP or OXT secretion; in fact the pretreatment of PDL applied before treatment of NE more significantly increased the AVP level. Pretreatment with NE and then PDL resulted in higher AVP levels than those seen with NE alone.

Pretreatment with PTA or CAT prevented the E-induced increase in both AVP and OXT release. Pretreatment with YOB did not significantly inhibit the E-induced AVP and OXT

secretion enhancement. After pretreatment with E, none of the α-receptor antagonists (PTA, CAT or YOB) significantly reduced the AVP and OXT levels.

Fig. 5. Adrenergic involvement of AVP and OXT secretion in NH cultures



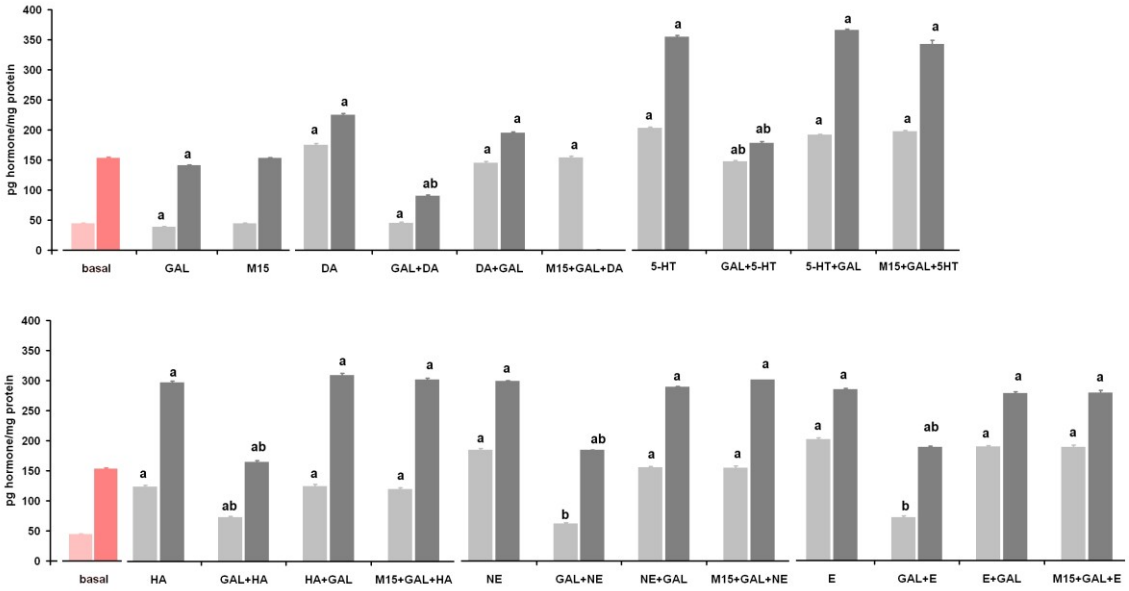
Means ± SEM, n=10 cultures/NH treatment scheme. Results are given in pg hormone/mg protein. AVP: lighter (gray) bars. OXT: darker (gray) bars. Significant differences: **a**: $p<0.05$ (vs. the controls); **b**: $p<0.01$ (vs. NE or E administration); **c**: $p<0.05$ (vs. PDL administration).

Effects of GAL-monoamine interactions on AVP and OXT secretion in NH cultures

All the previous and recent data are presented in Fig. 6. Treatments with monoamines (DA, 5-HT, HA, NE or E) merely significantly increased both the AVP and OXT levels. 5-HT exhibited the highest potency in the stimulation of AVP and OXT release. The decreasing effects of GAL treatment on AVP and OXT release were inhibited by pretreatments of M15 according to the published data.

Pretreatments with GAL inhibited the effects of all the monoamine on both the AVP and OXT levels. Pretreatment with M15 blocked the effects of the GAL, and the monoamine-stimulated neuropeptide levels were again observed. However, when the monoamines were applied (as pretreatments) before GAL treatments, GAL did not diminish AVP and OXT release.

Fig. 6. Effects of GAL-monoamine interactions on AVP and OXT secretion in NH cultures



Means ± SEM, n=10 cultures/NH treatment scheme. Results are given in pg hormone/mg protein. AVP: lighter (gray) bars. OXT: darker (gray) bars. Significant differences: **a**: $p<0.05$ (vs. the controls); **b**: $p<0.01$ (vs. monoamine /DA, 5-HT, HA, NE or E/ administration).

4.2. Study of behavioral and endocrine effects of external-environmental impact, neurogenic involvement in AVP and OXT secretion in stimulated NH cultures

Stress controls versus untreated animals

No statistical differences were found between the unexposed AC and the SC groups in any of the measured parameters. Significant differences between the ClB-exposed and AC groups [subsection 3.3] are indicated in Figs 7-11.

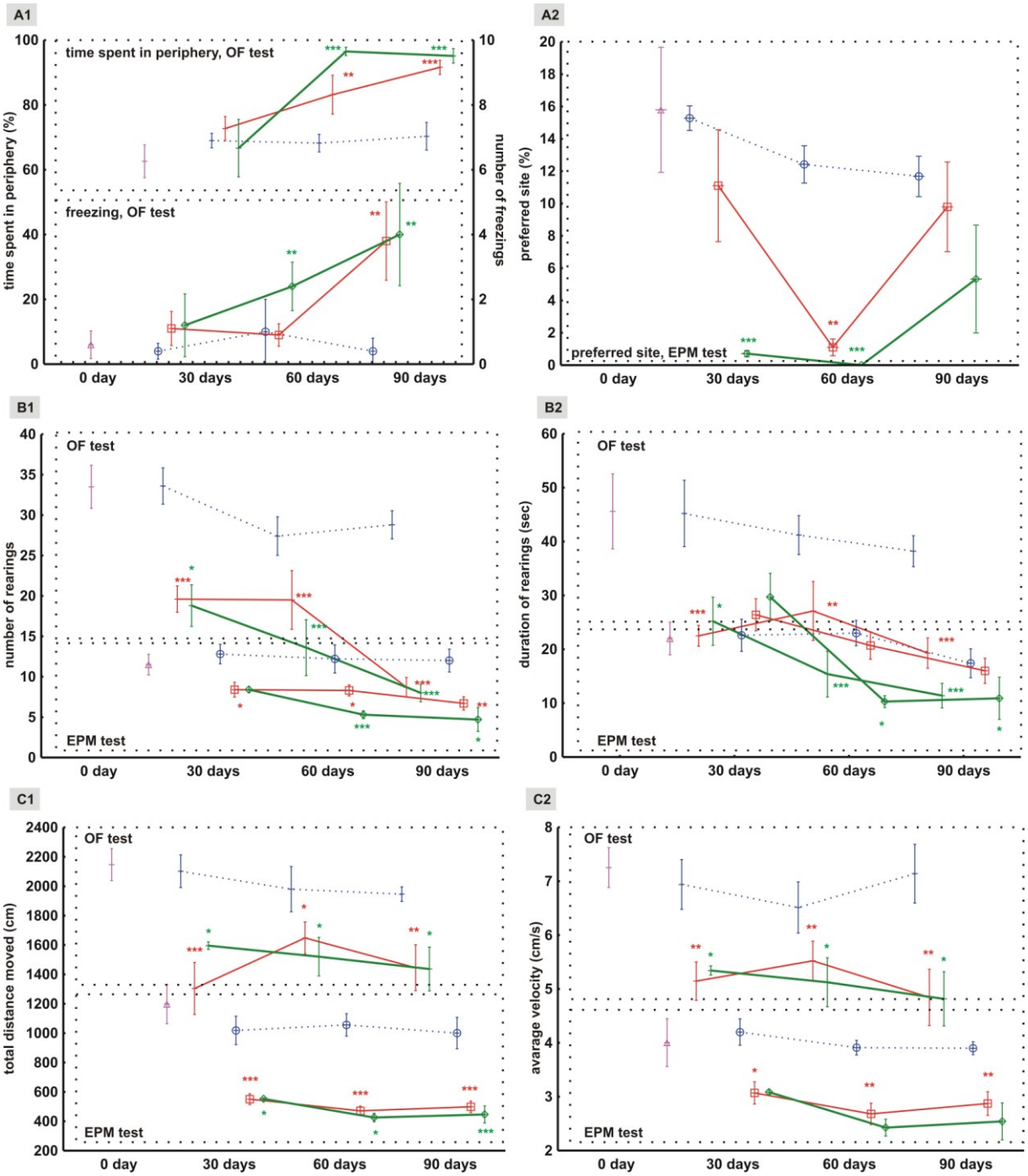
Behavioral changes following ClB exposure

OF, EPM and RI tests were applied to determine the ClB-induced changes in behavioral elements relating to anxiety, locomotor activity, exploration and intermale aggression of individuals. Results are summarized in Figs 7-8. In the OF tests, ClB exposure for 30 days did not cause a significant change, whereas in D1/D2-ClB-60 and D1/D2-ClB-90 the total duration of time spent in the periphery was statistically longer that for the controls. ClB exposure for 30 days did not have a significant effect on number of freezings, but in groups D2-ClB-60 and D1/D2-ClB-90 significant elevations were observed relative to the controls. In groups D2-ClB-30, D1/D2-ClB-60 and D1/D2-ClB-90, both the total duration and number of rearings were significantly reduced as compared with the controls. The total distance moved and the mean velocity (markers of spontaneous locomotor activity) were significantly

lower in groups D2-ClB-30, D1/D2-ClB-60 and D1/D2-ClB-90.

In the EPM tests, the anxiety-related “preferred site” was decreased significantly in the D2-ClB-30 and D1/D2-ClB-60 groups but not statistically in the D1/D2-ClB-90 compared to the control group. The total duration and number of rearings in the closed arms were modulated in the exposed groups relative to the controls.The mean velocity of the exposed groups was not significantly different from that of the controls, with the exception of the D1-ClB-60 group. The total distance moved was significantly less in the D1/D2-ClB-30, D1/D2-ClB-60 and D1/D2-ClB-90 groups.

Fig. 7. Behavior in OF and EPM tests

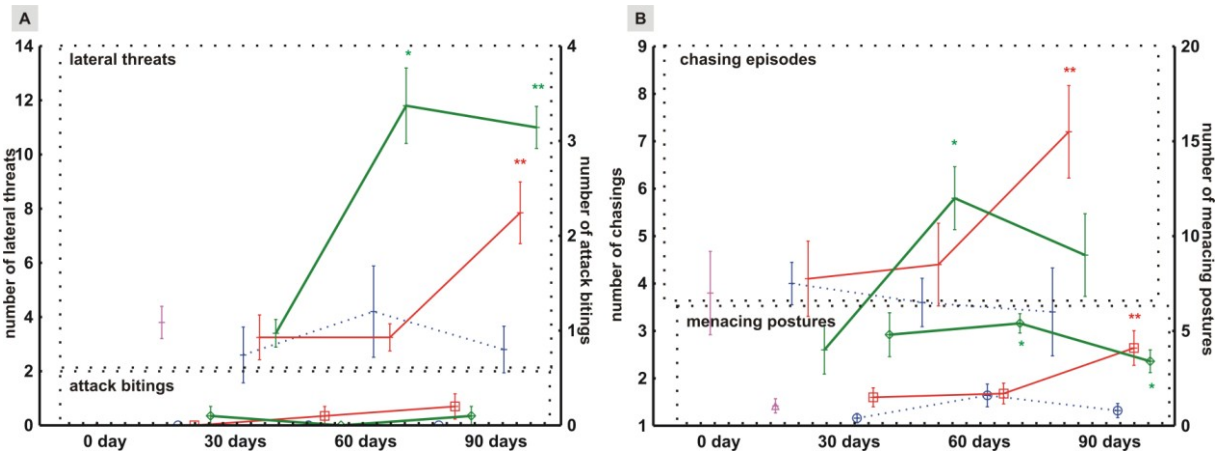


Means ± SEM, n=10/AC or D1-ClB or n=5/SC or D2-ClB groups. EPM: elevated plus maze, OF: open-field. **A1**: Anxiety-related elements: time spent in the periphery (upper part; ordered to left y axis) and freezing (lower part; ordered to right y axis), both measured in OF tests. **A2**: Anxiety-related element: preferred site, measured in EPM tests. **B1**: Explorative element: number of rearing episodes measured in OF (upper part) and EPM (lower part) tests. **B2**: Explorative element: duration of rearing episodes measured in OF (upper part) and EPM (lower part) tests. The explorative elements in the EPM test were measured in the closed arms. **C1**: Locomotive element: total distance moved, measured in OF (upper part) and EPM (lower part) tests. **C2**: Locomotive element: average velocity measured in OF (upper part) and EPM (lower part) tests. Absolute controls: day 0, purple marker. Stress controls: dashed blue lines and markers. D1-ClB-treated animals: solid red lines and markers. D2-ClB-treated animals: solid green lines and markers. Significance: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

The RI tests of behavior revealed significant increases in the number of lateral threats in the D2-ClB-60 and D1/D2-ClB-90 groups. Similarly, the number of chasing episodes was elevated in groups D2-ClB-60 and D1-ClB-90, as was the number of menacing postures in groups ClB-60 and ClB-90. However, no statistical differences were found in attack biting.

No significant differences were found in any of the other behavioral elements mentioned in subsection 3.5 and measured in the OF, EPM or RI tests (data not shown).

Fig. 8. Behavior in RI tests



Means ± SEM, n=10/AC or D1-ClB or n=5/SC or D2-ClB groups. **A**: Offensive elements: numbers of lateral threats (upper part; ordered to left y axis) and attack bitings (lower part; ordered to right y axis). **B**: Offensive elements: numbers of chasings (upper part; ordered to left y axis) and menacing postures (lower part; ordered to right y axis). Absolute controls: day 0, purple marker. Stress controls: dotted blue lines and markers. D1-ClB-treated animals: solid red lines and markers. D2-ClB-treated animals: solid green lines and markers. Significance: $^{*}p<0.05$, $^{**}p<0.01$.

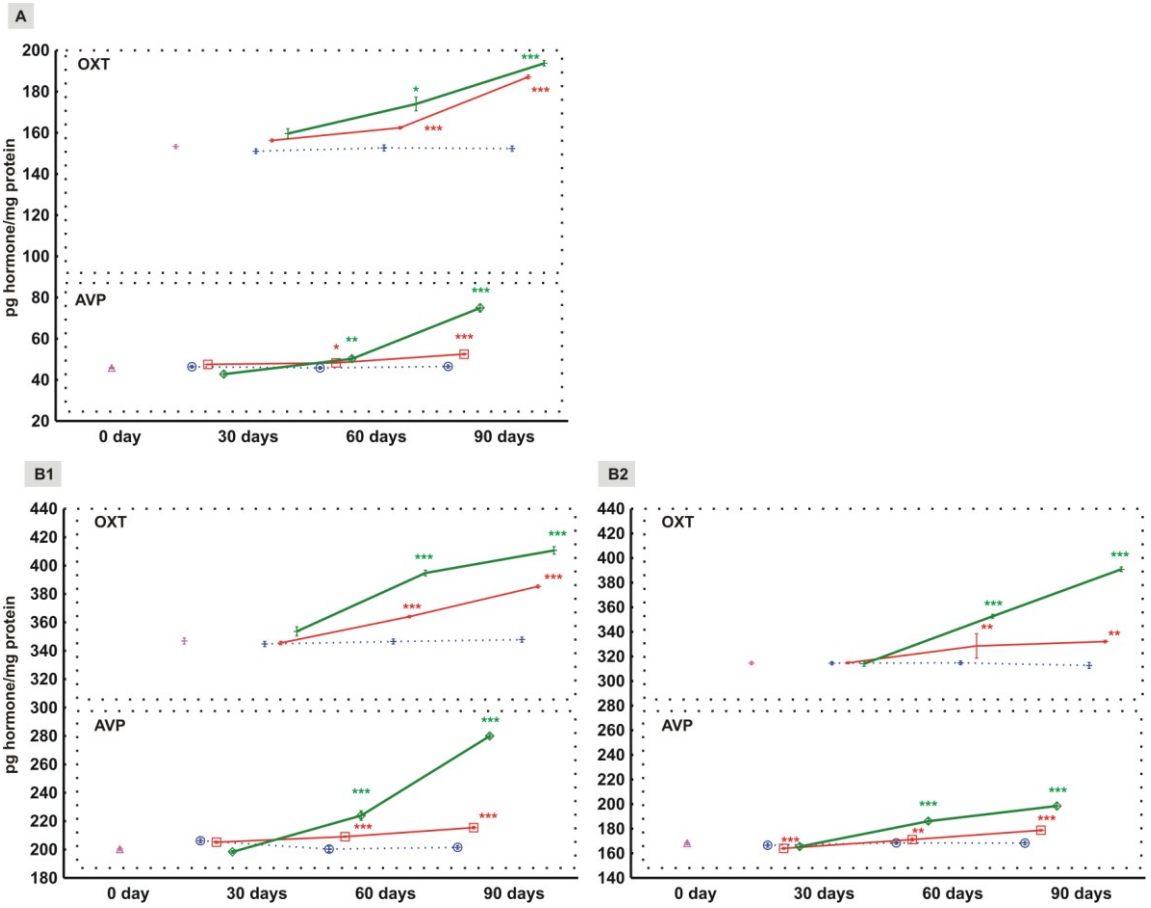
AVP and OXT secretion of NH cultures

The secreted AVP and OXT concentrations measured in the supernatants after incubation with monoamines [subsection 3.6.2.3] are depicted in Fig. 9.

Nonstimulated, basal AVP and OXT levels:

The basal AVP secretion was modulated by exposures to ClB in groups D1/D2-ClB-60, and even more so in groups D1/D2-ClB-90. Statistical differences in basal OXT secretion were also found between the controls and the ClB-exposed rats: the OXT level was elevated in groups ClB-30, and became significant in groups D1/D2-ClB-60 and D1/D2ClB-90.

Fig. 9. AVP and OXT secretion of NH cultures prepared from exposed rats



Means ± SEM, n=10 cultures/NH treatment/AC, SC or D1-ClB or n=5 cultures/NH treatment/D2-ClB groups. AVP (lower part) and OXT (upper part) concentrations. Results are given in pg hormone/mg protein. Absolute controls: day 0, purple marker. Stress controls: dotted blue lines and markers. D1-ClB-treated animals: solid red lines and markers. D2-ClB-treated animals: solid green lines and markers. Incubations: **A:** nonstimulated, **B1**: 5-HT-stimulated, **B2**: NE-stimulated. Significance: $^{**}p<0.01$, $^{***}p<0.001$.

5-HT-stimulated AVP and OXT levels:

The basal AVP and OXT levels were both significantly increased following administration of 10^{-6} M 5-HT, but differences were observed between the ClB-exposed groups and the non-exposed controls. In contrast with group D2-ClB-30, of non-exposed controls the 5-HT-induced levels of AVP and OXT secretion were significantly elevated relative to the non-exposed controls in the D1-ClB-30 (AVP only), D1/D2-ClB-60 and D1/D2-ClB-90 groups.

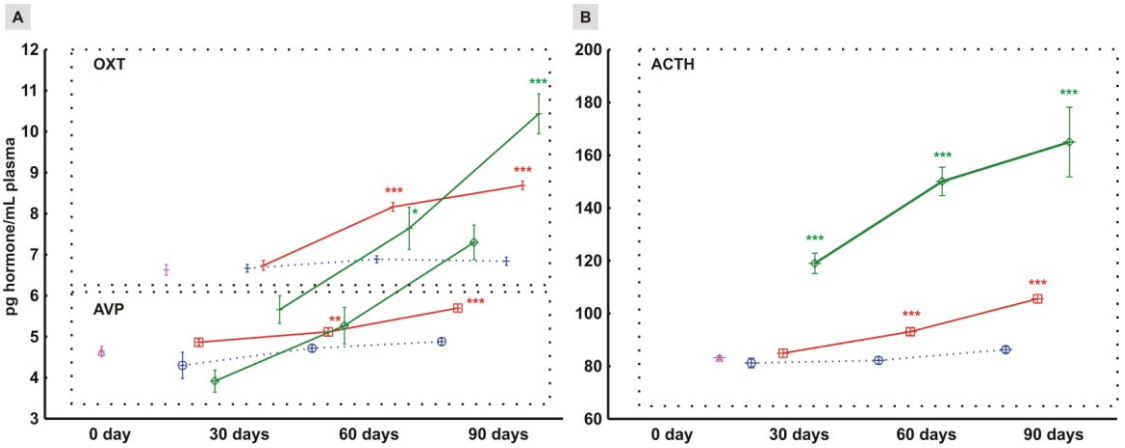
NE-stimulated AVP and OXT levels*:*

The basal AVP and OXT secretion was significantly increased by administration of 10^{-6} M NE, with differences between the ClB-exposed groups and the non-exposed controls. The difference was significant in the D1/D2-ClB-60 and D1/D2-ClB-90 groups.

Plasma AVP and OXT and ACTH levels

After exposure and behavioral tests, blood was collected and the plasma levels of AVP, OXT and ACTH were determined. Results are listed in Fig. 10. The plasma levels of AVP in groups D1/D2-ClB-30 and D2-ClB-60 and of OXT in groups D1/D2-ClB-30 did not differ significantly from the controls, whereas the levels of OXT in groups D1/D2-ClB-60 and of both AVP and OXT in groups D1/D2-ClB-90 were statistically elevated. The ACTH concentration was significantly increased in group D2-ClB-30, and highly elevated in groups D1/D2-ClB-60 and D1/D2-ClB-90.

Fig. 10. Plasma levels of AVP and OXT and ACTH following exposure to ClB

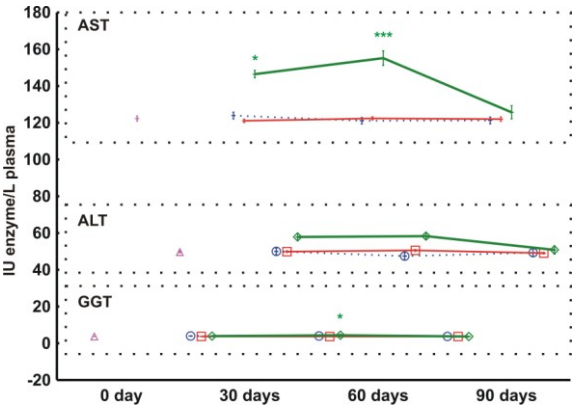


Means ± SEM, n=10/AC or D1-ClB or n=5/SC or D2-ClB groups. Hormones measured in trunk blood collected at endpoints of experiments. Results are given in pg hormone/mL plasma. **A**: AVP (lower part) and OXT (upper part) concentrations. **B**: ACTH concentrations. Absolute controls: day 0, purple marker. Stress controls: dotted blue lines and markers. D1-ClB-treated animals: solid red lines and markers. D2-ClB-treated animals: solid green lines and markers. Significance: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Toxicity of ClB exposure

The body and organ weight results did not reveal any significant differences between the experimental groups and the controls (data not shown). Nor were statistical differences detected in AST, ALT or GGT following exposure to D1-ClB. Despite some discrete alterations in the levels of liver enzymes, the measured concentrations remained in the normal, subtoxic ranges following exposure to D2-ClB [Fig. 11]. No malformations or other overt signs (*e.g.* alopecia) of HCB or TCB toxicity were observed. No structural differences were observed in the slides prepared from excised brain, liver, spleen or kidneys for standard microscopic examinations.

Fig. 11. Changes in plasma levels of liver damage markers following exposure to ClB



Means ± SEM, n=10/AC or D1-ClB or n=5/SC or D2-ClB groups. Markers were measured in trunk blood collected at endpoints of experiments. The results are given in IU enzyme/L plasma. Absolute controls: day 0, purple marker. Stress controls: dotted blue lines and markers. D1-ClB-treated animals: solid red lines and markers. D2-ClB-treated animals: solid green lines and markers. Reference (subtoxic) enzyme ranges: 50-200 IU/L for ALT, 30-250 IU/L for AST and 2-20 IU/L for GGT. Significance: $^{*}p<0.05$, $^{***}p<0.001$.

5. DISCUSSION

The AVP- and OXT-ergic neuroendocrine systems are essential for their miscellaneous functions maintaining of homeostasis in organisms [5, 29, 136].

The secretion of these neuropeptides is controlled by various regulators and may be influenced by internal and external factors. The neurogenic systems of monoamines and GAL are involved in the expression of both AVP and OXT in different brain areas. It is generally accepted that AVP and OXT are synthetised in various superior brain nuclei [Fig. 1] and released either centrally into the ECF/CSF or peripherally into the systemic circulation from the axon terminals of the magnocellular nuclei in the NH. As a result of the peripheral secretion, the AVP and OXT elicit their physiological roles as neurohormones, but the related behavioral consequences are caused by the centrally secreted AVP and OXT as neurotransmitters and neuromediators [5].

The pituicytes were earlier presumed to play a role only in the storage of AVP and OXT [10]. However, we have shown, that the pituicytes are able to produce and secrete both AVP and OXT [13] and can be used as a model to investigate the secretion of AVP and OXT. Many monoamines, such as DA [76], 5-HT [78] and HA [77] are known to be involved in the expression of AVP and OXT (in NH cultures). We have now performed various incubation procedures with E- and NE-related mediators [subsection 3.6.2, Fig. 3] in order to explain the involvement of adrenergic monoamine regulators (aim 1). Our results are summarized in

Fig. 5. Many data have been published on the effects of NE or E on AVP and OXT expression [subsection 1.3.1] at different regulatory levels, but usually without a consideration of the NH. There are three groups of adrenoceptors. The α_1-receptors mediate mainly the general responses in the effector organs or nuclei, the α_2-receptors are essential for the feedback regulations of synaptic neurotransmitter release, and the β-receptors have roles in the cardiovascular, uterine and peripheral metabolic functions [137]. We found that pretreatment with CAT (as an α_1-receptor antagonist [123]) or PTA (as an α_1+α_2-receptor antagonist [122]) effectively inhibited the E-stimulated release of AVP and OXT from NH cultures. After pretreatment with E, these antagonists did not block the enhancing effects of E. Pretreatment with the α_2- (and slightly α_1)-receptor antagonist YOB [124] before E was ineffective as concerns both AVP and OXT. Pretreatment with CAT before NE reduced the NE-stimulated AVP release (for OXT we have no data as yet) because NE has an α_1-receptor agonist character besides its β-receptor agonist profile. However, while the AVP secretion elevation was totally blocked by pretreatment with CAT before E, CAT administratered before NE exerted only a partial blocking effect. This is in accordance with the findings of others [73, 138]. Pretreatment with PNL (as a β_1 + β_2-receptor antagonist [125]) was sufficiently effective in the cases of both AVP and OXT, but pretreatment with ATL (as a β_1 receptor antagonist [126]) did not prevent NE-stimulated AVP and OXT secretion. It was somewhat surprising that pretreatment with PDL (as a β_1 + β_2-receptor antagonist [127]) alone significantly increased NE-stimulated AVP secretion. This contradictory effect can be explained in that PDL not only acts as a blocker, but also exerts intrinsic sympathomimetic action (ISA) and a strong adrenergic agonist effect [139]. The molecular mechanism of the ISA of PDL has not been clarified. It has been hypothesized that the receptor loss induced by β-antagonists with ISA is mediated through 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 [140, 141].

We conclude that mainly the α_1-receptors may be involved in the E-stimulated AVP and OXT secretion, and the β_2-receptors in NE-stimulated AVP and OXT secretion *in vitro*. Our observations are in accordance with the findings of De Souza and Kuyatt [142] on α_1-adrenergic receptors in rat pituicytes.

To examine the GAL-monoamine interactions (aim 2), additional incubation procedures were performed [subsection 3.6.2, Fig. 3]; the results are summarized in Fig. 6. GAL receptors are distributed throughout the body, often colocalized with AVPR1-2 and/or OXTR [80]. GAL acts via at least three receptor subtypes [80, 143]. On neuropeptide-producing cells

of MCNs, mainly GALR1 is expressed [144]. Low levels of GALR3 are expressed in the pituicytes [145]. Our present findings and earlier observations [85, 86] indicate that AVP and OXT secretion can be directly influenced by the GAL-ergic system, and that the GAL-ergic control of 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*. However, more subtype-specific GAL receptor antagonists were not available, and therefore we could not identify the (all) acting GAL receptors specifically. M15 has high affinity for GALR3, but can also bind more weakly to GALR1 and GALR2 [128]. In aggrement with the relevant literature [145], we presume that the blocking effect of GAL involves *inter alia* the mediation of the GALR3 receptors. The mechanisms of the effects of GAL–monoamine interactions on neuropeptide secretion can be explained in that the GAL receptors may be somehow coupled to the catecholaminergic receptors, as observed, for example, in the SON [82]. We presume that the effects of the GAL–monoamine interactions on AVP and OXT secretion can develop through the monoaminergic receptors in the NH. This is supported by the present finding that GAL did not influence the increases induced in the levels of AVP and OXT by K^+ administration, which causes nonspecific, receptor-independent hormone secretion [121]. The present study has demonstrated that pretreatment with GAL prevents the E- or NE- induced 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. The interactions between the monoaminergic and GAL–ergic systems from the aspect of AVP and OXT production occur independently of the hypothalamus, at the level of the posterior pituitary.

Certain impacts may be capable of altering AVP and OXT secretion in superior brain areas by inducing disturbances in the related (AVP- and OXT-mediated) behavioral traits and/or the peripheral release in the hypothalamo-neurohypophyseal system inducing the physiological consequences. These effects may be explained by the involved, same or similar (and impressionable) *inter alia* neurogenic regulators and the attributes of the (exposures to) impacts.

The POP/EDCs are potential external impacts that may affect neuroendocrine systems. POP/EDCs can cross the blood-brain barrier and accumulate in neural cells. If they attain a critical level, they may induce various cellular mechanisms which may disturb endocrine elements/functions, including the behavioral-related hormonal mechanisms [89, 94].

Chlorobenzenes such as HCB or the industrial by-product TCB are frequent POP/EDCs [89, 108], that are still detectable in the environment and food chain [89, 95]. Although they have many targets in the CNS, only limited data exist on the neurobehavioral (or related endocrine) effects of any doses of chlorobenzenes and usually lower doses of most POP/EDC chemicals. Low amounts of POP/EDCs often result in no overt symptoms or effects and are therefore neglected, whereas long-term exposure to discrete doses may involve potential health risks [146]. Modeling of dietary exposure (oral gavage) was one of our aims because (human) subjects may be primarily exposed via the food chain [100].

Our study included basic toxicological routines to decide whether ClB was subtoxic. General toxicological parameters such as the plasma levels of AST, ALT and GGT [Fig. 11] were measured following the exposure of experimental animals. The effects of exposure to 0.1 or 1 μg/kg per day doses of ClB on the examined toxicicological parameters were not significant or remained within the reference ranges after either 30, 60 or 90 days of exposure. Following exposure, the body and organ weights did not reveal significant differences between the ClB-exposed groups and the SC or AC groups (data not shown). Moreover, no other signs of overt toxicity were detected. We were unsuccessful in demonstrating published signs [98, 107] of toxic HCB or TCB contamination on the body or excised organs. The results indicate that the dose (0.1 or 1 μg/kg/day) and duration (30, 60 or 90 days) of exposure to ClB applied in this study must be considered subtoxic.

The measured behavioral-physiological parameters of the AC and SC animals did not differ significantly. We may postulate, therefore, that the observed consequences did not originate from the (neuro) toxicity of the exposure and/or the stressing effects of handling or oral gavage.

Besides their somatic effects, AVP and OXT influence a broad variety of behavioral forms [5, 7, 41] and aggression and anxiety-related behavior are considerably affected by these neuropeptides [6, 8, 47]. It is accepted that AVP has anxiogenic [21, 147], while OXT has anxiolytic [148] effects, and that AVP increases intermale aggression, whereas OXT is involved particularly in maternal care and aggression [6, 46]. Regulation of the neuropeptides is implemented by many factors [5], including the neurogenic systems of biogenic amines, *e.g.* the monoamine 5-HT- or NE-related elements. Their structures, mediators are connected or their effects are involved in AVP and OXT regulation in higher brain levels, *e.g.* the PVN and SON [18, 53, 58]. It has been reported that NE increases the expression of both AVP and OXT in the PVN and SON, and 5-HT has been proposed to stimulate the expression of both AVP and OXT in the PVN but only that of OXT in the SON [53, 54]. Other papers have

clarified the involvement of 5-HT receptors in different areas related to AVP or OXT production or secretion [58]. NE can increase the central and peripheral release of neuropeptides mainly via the involvement of HA-ergic neurons [67]. Nevertheless, NE has also been reported to inhibit AVP release [71-73]. Correlative roles of AVP and 5-HT [31] and/or NE [56] in various aspects of emotional or social behavior or the related disorders have likewise been described.

Incubation procedures were performed to examine monoamine-mediated AVP and OXT secretion and the changes caused under stimulated conditions by exposure to ClB [subsection 3.6.2.3, Fig. 9]. The basal levels of secreted AVP and OXT were altered following exposure. The NH cultures prepared from ClB-exposed rats demonstrated neuropeptide secretion elevations dependent on the dose and duration of the exposure. Exposure to ClB for 90 days resulted in significant elevations in the secreted AVP and OXT levels. We suspect that the basal neuropeptide secretion was disturbed environmentally. According to our formerly published data, as specific monoaminergic receptor agonists, 5-HT and NE significantly increase both the secretion of AVP [78, 134] and OXT [78, 135] from NH cultures prepared from unexposed animals. This stimulation can be inhibited with specific antagonists, and the receptors acting in the NH cultures were therefore investigated in details by our group [78, 134, 135]. In NH cultures prepared from exposed rats, the neuropeptide secretion was also induced following 5-HT or NE incubations, but the nature of the increase was altered. We suspect that the monoamine-mediated neuropeptide secretion was affected environmentally. HCB and chlorobenzenes can reach and accumulate in the brain and could be neurotoxic [109]. ClB chronically given by stomach tube may pass into the behavior-related brain areas and the hypothalamo-neurohypophyseal system, including the pituicytes of the posterior pituitary. In cells, HCB may act generally as a dioxin-like agent [104] modulating gene expression and inducing various processes [94]. Moreover, HCB has been reported to affect phospolipid patterns in the brain, altering the functional efficacy of the neuronal membranes, and to result in changes in the membrane properties and consequently in the activity of the proteins embedded in the membranes, such as receptors and ion channels [149]. Obviously, HCB (and certainly other chlorobenzenes) has the possibility to produce the neurological symptoms mentioned earlier [111, 149, 150]. We suspect that the changes in basal and monoamine-mediated secretion (with the environmental influences) that are observed could appear at various levels of neuropeptide secretion simultaneously. The changes may be caused by modifications of the membrane receptors or their sensitivities or the transmitter amounts or the other HCB-related mechanism mentioned above. Our assumptions may be supported by

the findings of Bleavins *et al*. Minks and ferrets were treated chronically with dietary HCB and the levels of different regional biogenic amines such as 5-HT and NE were measured [115]. Besides modulated behavior reflecting abnormal aggressiveness and hyperexcitability, elevated 5-HT and NE levels were detected in the hypothalamus following HCB exposure.

After ClB exposure, modulated plasma AVP and OXT concentrations were measured as a result of peripheral (hypothalamo-neurohypophyseal) neuropeptide secretion [Fig. 10]. Exposure for 30 or 60 days did not change the AVP level considerably, whereas exposure to ClB for 60 days increased the level of OXT, and ClB for 90 days elevated the levels of both AVP and OXT in the plasma. These findings may be due to the modulation of peripheral secretion caused by alterations of similar regulations due to HCB mechanisms (mentioned above) 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 [21]. Several anxiety disorders are associated with elevated AVP, *e.g.* in patients with post-traumatic stress disorder [32]. The AVP concentrations are elevated in both the CSF and the plasma of patients with obsessive–compulsive disorder [33]. However, our findings concerning the plasma OXT levels seem to disagree with earlier reported, usually negative correlations between the OXT levels and anxiety scores [151]. It is widely accepted that the plasma OXT level may be increased by a variety of stress-inducing conditions, and that the OXT released mainly from the PVN may play a role in the physiological responses to stress. For instance, in male rats stressed by shaking, the stress produced an increase in OXT secretion in the PVN, which resulted in a plasma OXT enhancement [152]. The effects of chlorobenzene-induced environmental stress on the OXT-related mechanisms have not been investigated to date. Our study revealed only that the SC groups showed no increase in OXT (or AVP) levels, though the stress caused by ClB may have induced peripheral OXT release. Few data are available that indicate a correlation between the plasma AVP (or OXT) level and aggressive behavior. Instead of neuropeptides, mainly the plasma levels of 5-HT, testosterone or glucocorticoids are mentioned as correlating with aggressive behavior. In the CSF however, the concentration of AVP (and OXT and some other regulators) usually seemed to correlate dose-dependently with the relevant aggression [6].

The plasma ACTH level elevations were directly proportional to the characteristics of the exposure [Fig. 10]. The increasing ACTH levels may be related to parvicellular AVP-related elements affected by the exposure. AVP and CRH released into the portal circulation at the Em, or even the AVP released within the NH into the short portal vessels, are known to stimulate ACTH secretion in the AdH, which triggers the adrenal release of glucocorticoids

and facilitates physiological and behavioral adaptation to stressors at both peripheral and brain levels [4]

Few papers report effects of POP/EDC on emotional or other AVP- and/or OXT-related behavior such as aggression, and there are insufficient data relating to chlorobenzenes. Zala *et al.* stated that many POP/EDCs are capable of influencing behavior [90]. In an OF test, rats exposed prenatally to 3,3',4,4',5-pentachlorobiphenyl (PCB 126) exhibited anxiogenic behavioral responses, including decreases in the time spent at the center of an OF, the number of rearings and the duration of grooming [153]. Organochlorine insecticide residues, mentioned as biomagnificable depos, may still be a cause of anxiety in women [154]. The published papers suggest that the long-term presence of POP/EDC agents (with features similar to those of chlorobenzenes) in considerable doses often leads to appreciable anxiogenic effects, as in the case of bisphenol A [155]. After ClB exposure, anxiety was measured in (OF and EPM) behavior tests [Fig. 7]. Both tests were mainly designed to determine anxiety-related behavioral elements in rodents [156]. The anxiety-related elements were altered following exposure to ClB. Alterations in behavioral elements relating to locomotion and/or exploration in the treated rats were also detected. These results suggest that, depending on their dose-time features, chlorobenzenes may affect the anxiety-related behavior of experimental animals discretely. To summarize our results from the RI tests [Fig. 8], it may be stated that the applied subtoxic doses of ClB had discrete effects on the (intermale) aggression. Probably the applied dose was too low because in our previous experiments when other (non-behavioral) consequences of higher doses of ClB exposures had been examining subsidiarily high(er) aggressive of animals were observed by our colleagues but these findings were not proven by suitable tests on those occasions. Various directions (positive, negative or uneffective) and features of the observed effects related to aggression were described in the papers depending on the properties of applied exposures, used POP/EDC chemical(s), subjects or other noteworthy conditions as discussed widely [90]. Our findings are in accord with those of Bleavins *et al*., who observed that a larger dose of HCB increased aggressiveness in minks and European ferrets [115].

6. CONCLUSIONS

We set out to achieve a better understanding of the involvement of monoaminergic systems in the secretion of AVP and OXT under basal and stimulated conditions.

Similarly to monoamines examined earlier (DA, 5-HT and HA), NE and E increased the secretion of both AVP and OXT in NH cultures *in vitro*. No significant differences were found between the effects of NE and E. The results of incubation with-adrenoceptor specific agonists or antagonists indicate that the α_1-receptors are probably involved in E-stimulated AVP and OXT secretion, while the β_2-receptors are probably involved in stimulation by NE.

GAL interacts with monoamine neurogens, and decreases or inhibits their enhancing effects on the secretion of AVP and OXT. Our results demonstrated that GAL receptors (probably GALR3) exist on the surface of the pituicytes, but the exact nature of the GAL receptor subtypes actually involved has not been satisfactorily elucidated. The GAL receptors are probably somehow coupled with the monoaminergic receptors, and the monoaminergic systems can therefore be directly influenced by the GAL-ergic system. We may postulate that the involvement of neurogens found in the NH model *in vitro* may also exist at the level of the NH, independently of the hypothalamus, *in vivo*.

In conclusion, it may be presumed that chronic exposure to POP/EDC chlorobenzenes may influence certain AVP- and OXT-related behavioral traits and the underlying neuroendocrine systems. Our findings may relate to disturbances in AVP and OXT secretion at different regulatory levels. Chlorobenzenes may act as discrete anxiogenic factors and may also have the potential to influence behavioral elements of aggression. Consequently, as frequent environmental pollutants, these chemicals 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 (accumulated dose) of ClB exposure. In our NH model, the monoamine 5-HT- and NE-stimulated neuropeptide secretion seemed to be disturbed following exposure to POP/EDC impact. Based on the attributes of applied ClB exposure and mainly the attendance of similar monoaminergic neurogenic regulators in the behavior related brain areas, the changes found in the NH cultures may possibly be interpreted in terms of the underlying mechanisms of the detected behavioral phenomena.

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APPENDIX