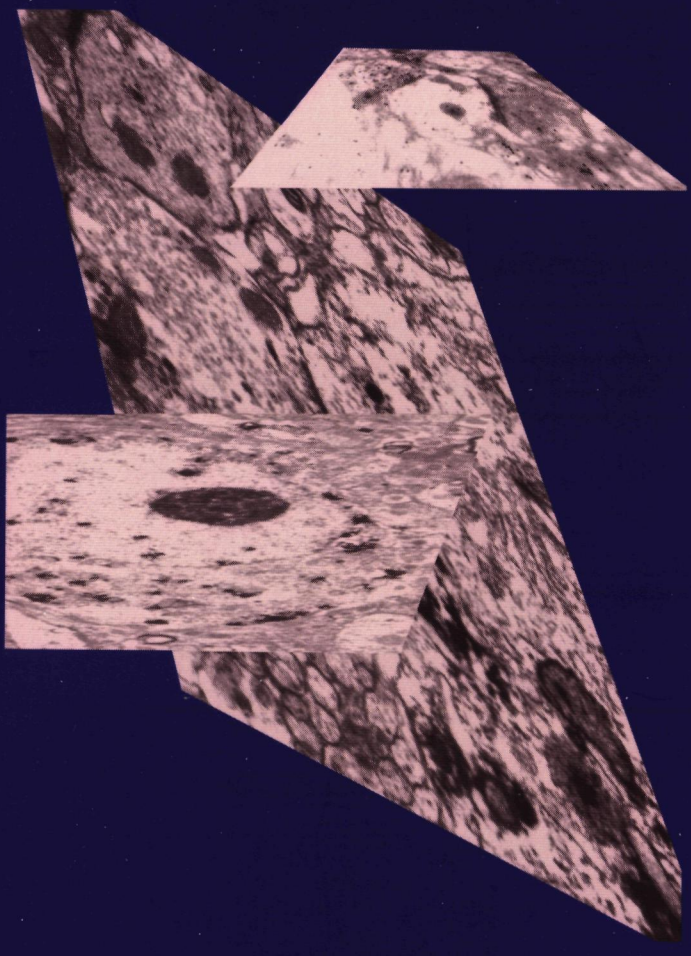
Corticotropin-Releasing Hormone Neurons in the Hypothalamic Paraventricular Nucleus: Morphological and Functional Aspects



W.H.A.M. Mulders

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Corticotropin-Releasing Hormone Neurons
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Morphological and Functional Aspects

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op het gebied van de Medische Wetenschappen

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Chapter 1

General Introduction

PREFACE

Since the isolation and characterization of corticotropin-releasing hormone (CRH) in 1981 by Vale and co-workers (1981) many studies have been performed to investigate the localization, function, and neuroanatomical characteristics of this neuropeptide. CRH occurs throughout the brain including areas such as the cortex, amygdala, bed nucleus of the stria terminalis, locus coeruleus, parabrachial nucleus and hypothalamic areas (Joseph and Knigge 1983, Swanson et al. 1983). The most prominent cluster of CRH neurons is located in the hypothalamic paraventricular nucleus (PVH). The majority of these neurons is involved in the activation of the hypothalamo-pituitary-adrenal axis (HPA-axis) regulating the organism's response to stressful stimuli.

This thesis describes neuroanatomical studies in the rat set up to gain more insight in the regulation and organization of the CRH neurons in the PVH. In this first chapter the following aspects of the CRH neurons will be discussed to provide a framework for the next chapters:

- morphological structure of the PVH, including the localization of CRH in the PVH,
- function of CRH in stress response,
- origin and composition of afferent input,
- interactions with the immune system,
- the different animal models used.

Finally, we will present the main goals and outline of this thesis.

MORPHOLOGY OF THE PVH

The PVH is situated in the dorsal part of the hypothalamus and lies just adjacent to the third ventricle (Swanson 1992) (fig. 1). In transverse sections the PVH is visible as a triangularly shaped nucleus and can be easily distinguished from the surrounding hypothalamus by a relatively cell poor zone. The average total PVH volume on both sides of the brain is estimated to be 0.36 mm^3 (Kiss et al. 1991). Estimations on the number of neurons in the PVH result in about 10,000 neurons on one side of the brain (Kiss et al. 1983, Swanson and Sawchenko 1983, Kiss et al. 1991). Most authors grossly divide the PVH in two divisions on the basis of neuronal size, the magnocellular and the parvocellular division. Kiss et al. (1991) distinguish also a third group, the mediocellular division, which has also been suggested previously by Swanson et al. (1986). These divisions have been further subdivided on the basis of chemoarchitectonic and morphometric criteria. In the next paragraphs a description will be given and a comparison will be made of the subdivisions as distinguished by Armstrong et al. (1980), Swanson and Kuypers (1980), Swanson et al. (1986) and Kiss et al. (1991).

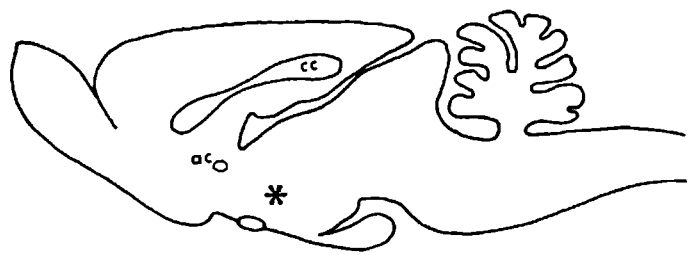

FIGURE 1 Sagittal view of the rat brain illustrating the position of the PVH (asteriks) within the brain. cc = corpus callosum, ac = anterior commisure

THE MAGNOCELLULAR SUBDIVISION The magnocellular subdivision has been subdivided into three parts by Armstrong et al. (1980) on the basis of neuronal shape and staining intensity: the medial paraventricular nucleus (PVM), the lateral paraventricular nucleus (PVL) and the posterior subnucleus of the paraventricular nucleus (PVPO).

Swanson and Kuypers (1980) and Swanson et al. (1986) distinguish also three parts in the magnocellular division, that are only slightly different from the subdivisions made by Armstrong et al. (1980). These are the medial magnocellular part (MM), a small unit that is situated in the rostral periventricular PVH and the posterior magnocellular part (PM), which is further subdivided on the basis of neurochemical criteria in a medial and lateral region. The first is a mainly oxytocinergic region, the latter a predominantly vasopressinergic region. The PM corresponds to the PVM and PVL of Armstrong et al. (1980).

In contrast to the subdivisions in the magnocellular division described above Kiss et al. (1991) consider the magnocellular division as a single division on the basis of quantitative histological measurements, which show that all neurons in this part are of equal size. Furthermore, the cell-packing density was found to be homogenous throughout this subdivision. The magnocellular division as defined by Kiss et al. (1991) corresponds with the PM of Swanson and Kuypers (1980) and Swanson et al. (1986) and the PVM and PVL of Armstrong et al. (1980).

THE PARVOCELLULAR SUBDIVISION Armstrong et al. (1980) distinguish only two parvocellular subdivisions. The first is the parvocellular subdivision of the paraventricular nucleus (PVPA), which starts immediately rostral to the PVM and extends to the rostral pole of the PVPO. The parvocellular neurons in the PVPA have a smaller diameter than the magnocellular neurons. The second parvocellular subdivision is the dorsomedial cap (PVDC) which borders the dorsal surface of the PVH and extends to its caudal limit. The neurons in the PVDC are intermediate in size compared to the parvocellular and magnocellular neurons.

Swanson et al. (1986) divide the parvocellular division in six subdivisions based on cell size, cell density and projection. These subdivisions are the anterior parvocellular part (AP), the medial parvocellular part (MP) which has been subdivided in a dorsal and a medial region, the dorsal parvocellular part (DP), the lateral parvocellular part (LP) and the periventricular parvocellular part (PV).

On the basis of quantitative measurements Kiss et al. (1991) distinguish a periventricular and a medial subdivision in the parvocellular division, which correspond to the PV of Swanson and Kuypers (1980) and Swanson et al. (1986) and the PVPA of Armstrong et al. (1980), respectively. The medial subdivision can be further subdivided in a anterior part (MAP), a lateral part (MLP) , a medial part (MMP) and a caudal part (MCP) on the basis of differences in cell density. The MAP is similar to the AP of Swanson and Kuypers (1980) and Swanson et al. (1986). The combined MLP, MMP and MCP are in agreement with the MP of Swanson and Kuypers (1980) and Swanson et al. (1986).

THE MEDIOCELLULAR DIVISION In contrast to the other authors Kiss et al. (1991) distinguish apart from the parvocellular and magnocellular division also a mediocellular division, located mediodorsally and caudally in the PVH. This mediocellular division contains neurons that are intermediate in size as compared to the magnocellular and parvocellular

division and consists of two parts, a dorsal subdivision (d) and a posterior subdivision (p). The d corresponds to the PVDC described by Armstrong et al. (1980). The p is comparable with the PVPO of Armstrong et al. (1980) and the LP of Swanson et al. (1986).

CYTOARCHITECTURE OF THE PVH As is explained above, the PVH contains two major groups of neurons, i.e. magnocellular and parvocellular ones. The morphological characteristics of these neurons, such as neuronal size and shape as well as dendritic and axonal organization, have been described by several authors.

The diameter of magnocellular neurons varies from 13 to 20 μm, while the diameter of parvocellular neurons varies from 6 to 12 μm (Armstrong et al. 1980, Kiss et al. 1991).

Magnocellular neurons typically have one or two primary dendrites regardless of their somatic orientation and location within a subdivision (Armstrong et al. 1980). Using Golgi-like and intracellular labeling techniques for visualization, these dendrites are observed to branch occasionally (Armstrong et al. 1980, Rho and Swanson 1989). They are mostly restricted to the subdivision in which they are situated (Armstrong et al. 1980, Van den Pol 1982) and tend to run parallel to the lateral border of the magnocellular division (Van den Pol 1982, Rho and Swanson 1989). The axons of the magnocellular neurons leave the PVH laterally or ventrolaterally, course above or below the fornix and then bend in the direction of the median eminence (Van den Pol 1982). Axon collaterals within the PVH have not been observed (Van den Pol 1982). Armstrong et al. (1980) also describe a separate group of magnocellular neurons in the caudal PVH, the PVPO. These neurons are more fusiform than the other magnocellular neurons and extend up to five dendrites which branch more often.

The parvocellular neurons can be divided in different groups with respect to their morphological characteristics. Rho and Swanson (1989), who used intracellular labeling to visualize the PVH neurons, make a distinction between the parvocellular neurons that project to the spinal cord (descending cells), located more dorsal in the PVH and parvocellular neurons that project to the median eminence (neuroendocrine cells), located more medial in the PVH. The descending cells give rise to three major dendrites in a tripolar arrangement. These dendrites are thin and branch more often than dendrites of neuroendocrine cells. The latter typically have two thick dendrites that extend from opposite sites of the cell body. These dendrites were observed to branch only once. The dendrites of both groups of parvocellular neurons run along the border of the subdivision in which they lie. The dendritic length is similar in both groups. The description of parvocellular neurons as given by Rho and Swanson (1989) above is in agreement with the work of Armstrong et al. (1980) and Van den Pol (1982). However, the two latter studies do not make a distinction between descending and neuroendocrine parvocellular neurons. The axons of the parvocellular neurons are thin and varicose and extend laterally towards the fornix (neuroendocrine cells) or continue towards the lateral hypothalamic area (descending cells) (Van den Pol 1982, Rho and Swanson 1989). Axon collaterals can be observed frequently towards the parvocellular as well as the magnocellular subdivisions (Van den Pol 1982, Rho and Swanson 1989).

LOCALIZATION OF CRH NEURONS IN THE PVH

The most dense cluster of CRH neurons within the brain is found in the PVH. However, under normal conditions the peptide content of these neurons is too low to stain for CRH

with current immunohistochemical techniques. Therefore, most authors have used an intracerebroventricular (icv) injection with colchicine, which interrupts axonal transport, to increase the peptide content in order to visualize all CRH neurons in the PVH. After an icv injection of colchicine about 2000 CRH neurons can be counted on each side of the brain (Swanson et al. 1983, Sawchenko and Swanson 1985). The majority of these neurons is located in the parvocellular division, but a smaller number can be found in the magnocellular division as well (Swanson et al. 1983). A small percentage of the CRH neurons projects to the spinal cord (descending CRH neurons). However, the largest portion of the CRH neurons in the PVH projects to the external zone of the median eminence (Swanson and Kuypers 1980, Kawano et al. 1988), where CRH is released into the portal pituitary vessels (neuroendocrine CRH neurons) and transported to the anterior pituitary where it stimulates the production of adrenocorticotropic hormone (ACTH) (Antoni 1989) according to the circadian rhythm and following stressful stimulation as will be explained below.

FUNCTION OF CRH IN STRESS

After a physiological or psychological stress the organism prepares itself for the so-called fight or flight response by activation of the sympathetic system, stimulating the release of adrenaline and noradrenaline from the adrenal medulla. In addition, there is an increased production of corticosteroids, i.e. glucocorticoids (corticosterone) as well as mineralocorticoids (aldosterone), from the adrenal cortex. Corticosteroids are responsible for increasing the availability of glucose from the blood, inhibition of immune functions and changes in cardiovascular tone (Dallman et al. 1987). The production of corticosteroids is regulated by ACTH. The production and secretion of ACTH is in turn predominantly under the control of CRH located in the PVH (Antoni 1989) and to a lesser extend of other peptides in the PVH, such as vasopressin (Rivier and Vale 1983a,b, Jones and Gillham 1988). This cascade following stressful stimulation is called the hypothalamo-pituitary-adrenal axis (HPA-axis). Corticosteroids exert a negative feedback effect on the activity of the HPA-axis (fig. 2). This feedback effect of corticosteroids is exerted on different levels of the HPA-axis such as via a direct effect on CRH neurons in the PVH, via a direct effect on the ACTH-releasing corticotropes in the anterior pituitary (Keller-Wood and Dallman 1984, Jingami et al. 1985, Kovacs et al. 1986, Almeida et al. 1992), or indirectly via other brain areas, such as the hippocampus (Herman et al. 1989). Since corticosteroids decrease CRH mRNA (Jingami et al. 1985) as well as peptide content (Sawchenko 1987), removal of corticosteroids by adrenalectomy leads to an increase of CRH content in the parvocellular neurons in the PVH (Sawchenko 1987, Almeida et al. 1992). This inhibitory effect of corticosteroids is mediated through interaction with two different corticosteroid receptors, the mineralocorticoid and the glucocorticoid receptor (De Kloet and Reul 1987). The mineralocorticoid receptors have a higher affinity for corticosteroids than the glucocorticoid receptors and are thought to be involved in the basal regulation of the activity of the HPA-axis whereas the glucocorticoid receptors are thought to be involved in the stress-induced activity of the HPA-axis (Reul and De Kloet 1985, De Kloet and Reul 1987).

CIRCADIAN RHYTHM

During basal conditions the plasma corticosteroid levels change from low levels in the morning to high levels in the evening, which is regulated by CRH (Watts and Swanson 1989). The timing of this circadian rhythm is regulated by the suprachiasmatic nucleus, which projects to the PVH (Berk and Finkelstein 1981).

AFFERENT INPUT TO THE PVH

In addition to the feedback control of corticosteroids the activity of CRH neurons is also under the control of different brain structures, as is shown by physiological, functional and anatomical studies. The innervation of the PVH and the CRH neurons can be grossly subdivided in four groups: limbic structures, nuclei in the brain stem, hypothalamic afferents and circumventricular organs (fig. 3). In the following paragraphs these groups will be discussed in some detail.

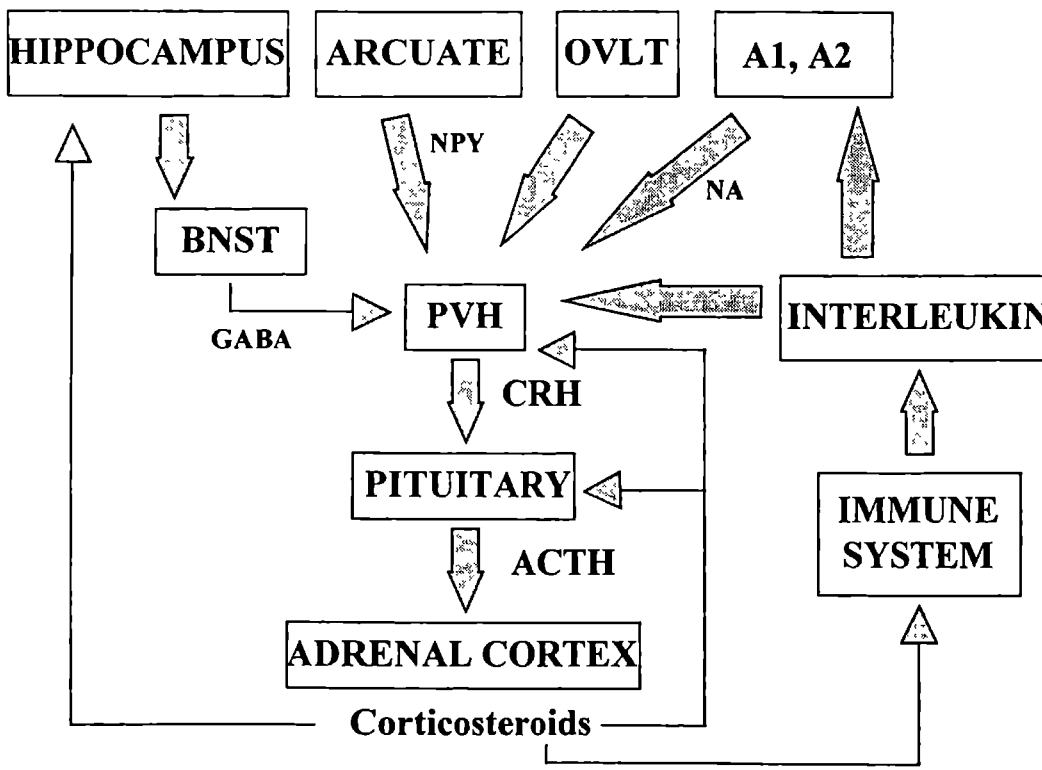

FIGURE 2 Schematic diagram illustrating the HPA-axis and some of its inputs. Thin lines with arrowheads represent inhibitory pathways, thick arrows represent stimulating pathways. A1, A2 = noradrenergic cell groups in ventrolateral medulla and nucleus of the solitary tract, ACTH = adrenocorticotropic hormone, arcuate = arcuate nucleus, BNST = bed nucleus of the stria terminalis, CRH = corticotropin-releasing hormone, NA = noradrenaline, NPY = neuropeptide Y, OVLT = organum vasculosum of the lamina terminalis, PVH = hypothalamic paraventricular nucleus

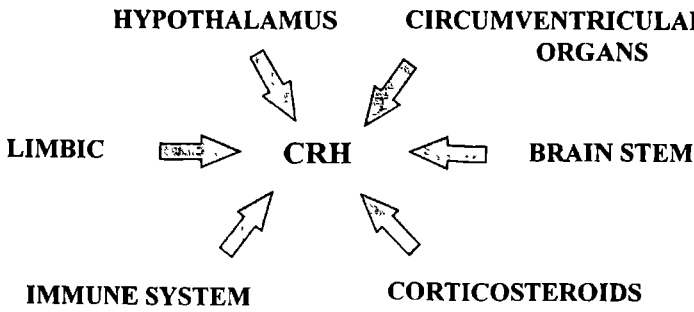

FIGURE 3
Schematic diagram illustrating the 6 main groups of input to the CRH neurons in the PVH.

LIMBIC STRUCTURES Several limbic structures such as the hippocampus, amygdala and septum have been shown to be of influence on the activity of the CRH neurons. The hippocampus is thought to play a major role in the corticosteroid feedback mechanism (Herman et al. 1989) (fig. 2), since it contains a large amount of corticosteroid receptors (Reul and De Kloet 1985). Hippocampal influence is thought to be inhibitory, since lesions of the hippocampus lead to an increased level of CRH mRNA (Herman et al. 1989, Herman et al. 1995), a rise in ACTH plasma levels (Feldman and Conforti 1980) and a hypersecretion of corticosteroids (Sapolsky et al. 1991). In addition, a corticosteroid receptor depletion in the hippocampus also evokes a corticosteroid hypersecretion (Sapolsky et al. 1984).

In contrast to the hippocampal input amygdaloid influence on the CRH neurons in the PVH is thought to be excitatory. Lesions of the central nucleus of the amygdala decrease the response of ACTH to stressful conditions (Beaulieu et al. 1986) and lesions of medial and central parts of the amygdala block the CRH release from the median eminence following photic or acoustic stimuli (Feldman et al. 1994). Furthermore, electrical stimulation in the central nucleus of the amygdala induces Fos immunoreactivity in the PVH (Petrov et al. 1994).

The lateral septum is also thought to be involved in the stress-response, since swim stress induces Fos immunoreactivity not only in the PVH and amygdala but also in the lateral septum (Duncan et al. 1993, Duncan et al. 1996). Electrical stimulation of the lateral septum induces inhibition of the PVH neurons (Saphier and Feldman 1987). Therefore, the septal influence on the PVH is thought to be inhibitory.

There is only limited evidence of direct axonal pathways from the above mentioned limbic structures to the PVH. Gray et al. (1989) found a small direct projection from the central nucleus of the amygdala to the PVH in the rat. In the cat Siegel and Tassoni (1971) found a limited projection from the dorsal hippocampus to the PVH. A small direct projection from the septal area to the PVH has also been shown (Oldfield et al. 1985). Therefore, it is thought that limbic structures exert their influence on the CRH neurons in the PVH via an indirect pathway, probably involving the bed nucleus of the stria terminalis (BNST) (fig. 2). Both the hippocampus and the amygdala have been shown to project to the BNST (Cullinan et al. 1993, Canteras et al. 1995). In its turn, the BNST has been shown to have a large projection towards the PVH (Silverman et al. 1981, Sawchenko and Swanson 1983), probably via a GABA-ergic pathway (Cullinan et al. 1993). Lesions of the BNST induce changes in the CRH mRNA expression in the PVH (Herman et al. 1994). In addition, the hippocampal projection to the BNST has been shown to direct innervate the BNST neurons projecting to the PVH (Cullinan et al. 1993) and lesion studies in the amygdala have shown that the effects of the amygdala on the activity of the HPA-axis are dependent on the BNST (Feldman et al. 1991).

BRAIN STEM The PVH receives a dense catecholaminergic input. Neuroanatomical studies indicate that the majority of this input finds its origin in several cell groups in the caudal medulla and brain stem (Weiss and Hatton 1990b). These cell groups are the A1 and C1 group in the ventrolateral medulla (VLM), the A2 and C2 groups in the nucleus of the solitary tract (NTS) and the A6 or locus coeruleus. The A1, A2 and A6 are primarily noradrenergic, while the C1 and C2 are primarily adrenergic. Sawchenko et al. (1985) showed that the catecholaminergic input to the PVH is often co-localized with neuropeptide Y. Electrical stimulation of the A1 and A2 groups evokes excitatory responses from the majority of the neurons in the PVH, which indicates that the noradrenergic input is probably excitatory (Saphier 1989) (fig. 2). This is confirmed by Orliaguet et al. (1995) who reported a rise in CRH release following a noradrenaline injection in an in vitro hypothalamus perifusion experiment. In addition an injection of noradrenaline in the PVH induces a rise in CRH mRNA levels (Itoi et al. 1994). The adrenergic input is likely to be inhibitory as shown by electrical stimulation in the C2 group, which leads to a majority of inhibitory responses of the neurons in the PVH (Saphier 1989). This is in agreement with observations made in an in vitro superfusion experiment, where adrenaline failed to have an effect on CRH secretion (Orliaguet et al. 1995).

Adrenergic as well as the noradrenergic fibers can be found for the largest part in the parvocellular region of the PVH (McKellar and Loewy 1981, Cunningham et al. 1990). However, the A1 group seems to innervate preferentially the vasopressin rich magnocellular region of the PVH (Cunningham and Sawchenko 1988). Electron microscopical investigation showed adrenergic synapses on CRH neurons (Liposits et al. 1986).

HYPOTHALAMIC AFFERENTS The PVH receives sparse to moderate projections from a variety of hypothalamic sites, such as the lateral and anterior hypothalamic area (Saper et al. 1978), the preoptic area, the ventromedial hypothalamic area, the supramammilary nucleus (Sawchenko and Swanson 1983), the arcuate nucleus (Bai et al. 1985) and the suprachiasmatic nucleus (Berk and Finkelstein 1981).

The different hypothalamic inputs each also have a different distribution over the PVH, which suggests that not all hypothalamic inputs are of influence to the CRH neurons. The lateral and anterior hypothalamic area project predominantly to the periventricular part of the PVH (Saper et al. 1978). The input of the periventricular part of the preoptic area is directed particularly at the parvocellular part of the PVH whereas the median preoptic nucleus has an equally distributed input over the whole PVH (Sawchenko and Swanson 1983). The ventromedial nucleus projects to the periventricular part of the PVH and the medial part of the parvocellular part. The arcuate nucleus does have a stimulatory effect on the activity of the HPA-axis (Wahlestedt et al. 1987) (fig. 2) and innervates directly the CRH neurons in the parvocellular part using neuropeptide Y as a neurotransmitter (Bai et al. 1985, Liposits et al. 1988). The suprachiasmatic nucleus, which regulates the circadian rhythm of the HPA-axis (Cascio et al. 1987) via an inhibitory input (Berk and Finkelstein 1981, Buijs et al. 1991) has a projection to the dorsomedial parvocellular part.

CIRCUMVENTRICULAR ORGANS Of the circumventricular organs, the subfornical organ and the organum vasculosum lamina terminalis (OVLT) both project to the PVH (Silverman et al. 1981, Sawchenko and Swanson 1983, Weiss and Hatton 1990a) (fig. 2).

The subfornical organ has been suggested to play a role in the response of the PVH to hemorrhage (Tanaka et al. 1993). The OVLT seems to be involved in activation of the HPA-axis by the immune system (Katsuura et al. 1990).

INTERACTIONS WITH THE IMMUNE SYSTEM

The HPA-axis interacts closely with the immune system. Corticosteroids have a negative effect on the immune system by, among others, inhibition of the release of interleukins (IL) from macrophages (Blalock 1994, Madden and Felten 1995). The other way around, IL seems to have a stimulatory effect on the HPA-axis, stimulating CRH release and increasing plasma ACTH and corticosteroid levels (Besedovsky et al. 1986, Berkenbosch et al. 1987, Rivest et al. 1992, Kovacs and Elenkov 1995) (figs. 2 and 3). However, the precise mechanism by which the immune system stimulates the HPA-axis remains unclear.

Some papers indicate that IL has a direct stimulatory effect on the adrenal glands, stimulating directly the release of corticosteroids (Andreis et al. 1991, Gwosdow et al. 1992). On the other hand, it has been shown that IL also stimulates the CRH neurons in the PVH (Veening et al. 1993, Lee and Rivier 1994) and the release of CRH (Berkenbosch et al. 1987, Sapolsky et al. 1987). There is some disagreement in literature whether this rise in CRH neuron activity and CRH release is necessary for the observed ACTH plasma level increase. Berkenbosch et al. (1987) reported that immuno neutralization of CRH prevented the ACTH surge after IL injection. However, Kovacs and Elenkov (1995) showed that IL could increase the ACTH plasma levels without an increase in CRH release. Thus, it might well be possible that IL stimulate the HPA-axis on different levels, in the hypothalamus, the pituitary and the adrenal glands.

The pathways through which IL stimulates the CRH neurons in the PVH remains to be elucidated. The organum vasculosum lamina terminalis has been thought to be involved in the stimulation of CRH in the PVH by IL (Katsuura 1990) but this is contradicted by other experiments, in which disruption of descending projections from circumventricular structures did not affect the IL-induced activation of the PVH (Ericsson et al. 1994). More likely candidates to serve as a relay for the activation mechanism of IL are the catecholaminergic groups in the medulla and brain stem (figs. 2 and 3). IL has been shown to induce Fos-like immunoreactivity in these cell groups, especially in the ventrolateral medulla and nucleus tractus solitarius, before the induction of Fos-like immunoreactivity in the PVH (Brady et al. 1994, Ericsson et al. 1994). In addition, disruption of the catecholaminergic pathways to the PVH modulate the IL effect on CRH release (Barbanel et al. 1993, Ericsson et al. 1994), which indicates involvement of these cell groups in the IL induced activation of CRH neurons.

ANIMAL MODELS

In the present thesis we used two different animal models (fig. 4). The first one was used in particular to visualize the CRH neurons immunohistochemically. In normal untreated rats it is very difficult to study the CRH neurons in the PVH with immunohistochemical techniques since the amount of CRH is too low to obtain a good staining. Therefore we used, next to untreated rats, adrenalectomized (ADX) rats. As explained earlier, removal of

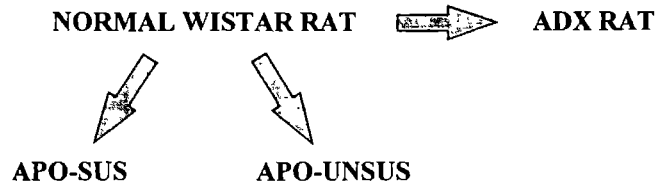

FIGURE 4
Schematic diagram illustrating the different animal models used in this thesis. ADX = adrenalectomized, APO-SUS = apomorphine-susceptible rats, APO-UNSUS = apomorphine-unsusceptible rats.

the adrenal glands leads to removal of the negative feedback of corticosteroids which stimulates the activity of the CRH neurons and increases the level of CRH mRNA as well as the neuropeptidergic content of these neurons (Sawchenko 1987). ADX also induces other phenomena in the brain, such as an increase in the content of co-localized vasopressin in the CRH neurons (Whittnall et al. 1985), hippocampal cell death (Sloviter et al. 1989) and receptor changes in the PVH (Jhanwar-Uniyal and Leibowitz 1986, Reul et al. 1987, Castrén and Saavedra 1989). Therefore, it has to be kept in mind that ADX will not only directly influence the CRH content in the PVH, but that ADX might well change the HPA-axis response to stress at other levels.

The second animal model we used consists of two lines of Wistar rats. These rats, which are thought to represent two extremes within a normal population, are pharmacogenetically selected on the basis of a gnawing test paradigm. After a similar subcutaneous injection with apomorphine one rat line shows a high gnawing response, the so-called apomorphine-susceptible rats (APO-SUS), whereas the other shows a low gnawing response, the so-called apomorphine-unsusceptible rats (APO-UNSUS) (Cools et al. 1990). These rats also show behavioral differences as illustrated in the open field test where the APO-UNSUS rats show less locomotor activity and faster habituation than the APO-SUS rats (Cools et al. 1990). In the defeat test, in which the rats are confronted with a much larger rat, the APO-SUS rats exhibit fleeing behavior whereas the APO-UNSUS exhibit freezing (Cools et al. 1990). In addition, these rat lines show differences at several levels of the HPA-axis. Under basal conditions the APO-SUS rats have a higher CRH mRNA level in the hypothalamus and higher plasma ACTH levels as compared to APO-UNSUS rats (Rots et al. 1995). In a novel environment and following a conditioned emotional stress the APO-SUS rats also show a higher and more prolonged ACTH and free corticosterone response than APO-UNSUS rats (Van Eekelen et al. 1992, Rots et al. 1995, Rots et al. 1996a). Furthermore, the hippocampal glucocorticoid receptor mRNA expression is higher in APO-SUS rats than in APO-UNSUS rats (Rots et al. 1996b). These differences in the stress response make the APO-SUS and APO-UNSUS rats an interesting model to study the neuroanatomical basis of the stress system.

GOALS AND OUTLINE OF THIS THESIS

In the preceding paragraphs the role of the CRH neurons in the PVH in the stress response has been discussed. It has been shown that CRH neurons constitute a major part of the HPA-axis, a complex system which is capable of processing and integrating multiple inputs in order to regulate the organism's response to stressful conditions. The question arises how the CRH neurons, which are located in the PVH in-between other neuropeptidergic neurons, are organized in order to be able to differentiate between the various inputs. Are there different

subpopulations of CRH neurons responsive to different sources of input and do they show a preferential localization within the PVH? Is there a strict organization in terms of synaptology i.e. do CRH neurons reveal a specific pattern of synaptic input arising from different input structures and clearly distinguishable from other PVH neurons?

To gain more insight in these issues dealing with the organization of the CRH neurons the present thesis investigated the neuroanatomical basis of the CRH mediated stress response. For this purpose emphasis was put on the following goals:

- characterization of the CRH neurons by determining morphometric parameters in the PVH and distribution of CRH neurons following different activation mechanisms;
- characterization of the input specific to the CRH neurons by investigation of the composition of the synaptic input as compared to other PVH neurons and the origin of this input;
- determination of the significance of the activity of the CRH neurons to the HPA-axis.

In chapter 2 the morphometric characteristics of the PVH are investigated. Both light microscopic (i.e. vascularity, neuronal size and neuronal density), as well as electron microscopic parameters (synaptic length and density) are quantified and compared in order to provide a general characterization of the PVH. These experiments are performed in APO-SUS and APO-UNSUS rats, to be able to determine whether there is a neuroanatomical basis in the PVH for the differences observed in the stress response between the two rat lines.

In chapter 3 the activation of the CRH neurons following a mild stressor is compared in APO-SUS and APO-UNSUS rats. For this purpose Fos-immunoreactivity is used as an marker of activity.

Chapter 4 describes the distribution and number of CRH neurons in normal and different treated rats, involving ADX, anti-CRH and colchicine injections and interleukin treatments. By comparison of the differences in number and distribution of the CRH neurons within the PVH it was investigated whether different activation pathways innervate different sub-populations of CRH neurons.

Chapter 5 describes the BNST projection to the PVH, which is thought to play a major role as a relay for limbic information. Normal and ADX rats were used to investigate whether there is a preferential input from the BNST to the CRH-rich part of the PVH and whether ADX influences this projection.

Chapter 6 describes the synaptic organization of the CRH neurons as compared to other PVH neurons. This is an electron microscopical quantitative study with particular emphasis on GABA as a neurotransmitter.

In the final chapter, summary and general discussion, the experiments are evaluated, the organization of the CRH neurons within the PVH is discussed in relation to the activity of the HPA-axis and some ideas for future experiments are presented.

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Chapter 2

The hypothalamic paraventricular nucleus in two types of Wistar rats with different stress responses:

I. Morphometric comparison

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SUMMARY

The present study evaluates the role of the hypothalamic paraventricular nucleus (PVH) in stress regulation by a morphometric comparison of the vascular, neuronal and synaptic properties of this nucleus in two lines of Wistar rats. It has been previously reported that these two lines of rats, indicated as APO-SUS (apomorphine-susceptible) and APO-UNSUS (apomorphine-unsusceptible) rats on the basis of their reactivity to a subcutaneous injection of apomorphine, display a variety of pharmacological and behavioral differences, including differences in their stress-coping mechanisms (Cools et al. 1993b). The results show a similar vascular and neuronal organization of the PVH in both lines, but distinct synaptic differences. The PVH (0.12 mm^3 volume with about 15,000 neurons on one side) has an overall vascular density of 5.6%, with significant differences between subdivisions (parvocellular central part: 8.3%, parvocellular dorsal/ventral/posterior part: 4.6-5.3%), which means that vascularity is a useful tool to delineate subdivisions in the parvocellular PVH. The neuronal density of 132 $\times$ $10^3/mm^3$ as found in the present study is two times higher than reported in a previous study (Kiss et al. 1991). Possible reasons for this discrepancy are extensively discussed. The most significant finding of the present study is the observation that APO-SUS rats have a significantly higher synaptic density (158 $\times$ $10^6/mm^3$) in the PVH than APO-UNSUS rats (108 $\times$ $10^6/mm^3$). It is discussed in which way this synaptic difference may be correlated with the different activity of the hypothalamo-pituitary-adrenal axis in both lines of Wistar rats.

INTRODUCTION

The hypothalamic paraventricular nucleus (PVH) plays an important role in the coordination of stress responses (Rivier et al. 1982, Bruhn et al. 1984). It contains a large number of corticotropin-releasing hormone (CRH) producing cells (Vale et al. 1981, Antoni et al. 1983, Swanson et al. 1983), which project to the neurohaemal zone of the median eminence (Lechan et al. 1980, Swanson and Kuypers 1980, Swanson et al. 1983, Whitnall et al. 1987), where CRH is released into the portal pituitary vessels. In the pituitary, CRH stimulates the secretion of ACTH, which in turn regulates the corticosteroid production in the adrenal cortex (Vale et al. 1981, Plotsky 1987). In this way the PVH stimulates the pituitary-adrenocortical activity in response to a variety of stressful situations (Moldow et al. 1987, Haas and George 1988, Van Oers et al. 1992).

A useful model to study the neuroanatomical basis of stress control is presented by two lines of Wistar rats with different stress responses (Sutanto et al. 1989, Cools et al. 1990, Cools et al. 1993a, Cools et al. 1993b). These two lines have been pharmacogenetically selected on the basis of their gnawing responses after a subcutaneous injection of apomorphine, and are indicated as APO-SUS (apomorphine-susceptible) and APO-UNSUS (apomorphine-unsusceptible) rats, showing a high and a low gnawing response, respectively, after an identical dose of apomorphine (Cools et al. 1990). Other differences can be observed in an open field test situation, in which APO-SUS rats show more locomotor activity, slower habituation and more edge-hugging behavior than APO-UNSUS rats. In the so-called defeat test, in which the rat is confronted with a much larger rat, APO-SUS rats show fleeing behavior, whereas APO-UNSUS rats exhibit freezing (Cools et al. 1990). These interline differences are probably induced by different levels of circulating plasmacorticosteroids during

the early postnatal period (Cools et al. 1990, Cools et al. 1993b), which might well cause differences in the PVH of both lines. The fact that the ACTH-response to exogenous CRH-administration is more pronounced in APO-SUS than in APO-UNSUS rats (Van Eekelen et al. 1992), also suggests differences between the PVH of both lines. The aim of the present study is to investigate whether quantitative morphological parameters in the PVH differ between APO-SUS and APO-UNSUS rats. Knowledge of such differences may contribute to a better understanding of the mechanisms giving rise to the line differences in responses to stress.

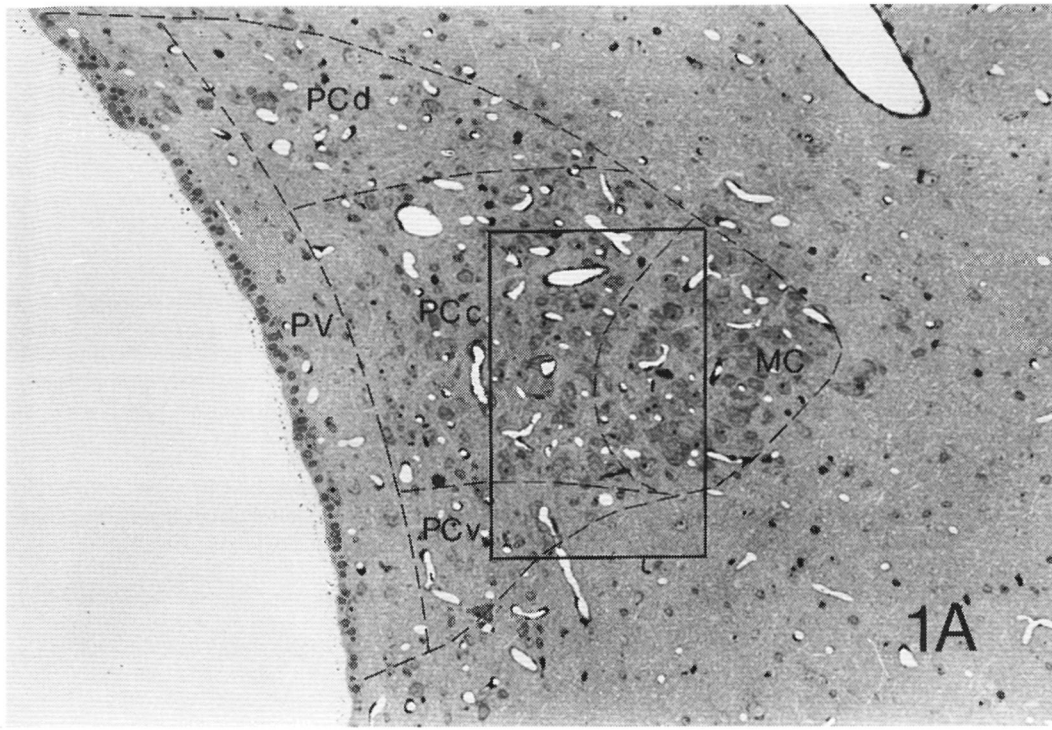
Previous morphometric studies of the PVH have been presented by Armstrong et al. (1980), Swanson and Kuypers (1980), Van den Pol (1982), Swanson et al. (1986) and Kiss et al. (1991). These authors have determined the numbers, sizes, shapes and densities of neurons in the different subdivisions of the PVH (Armstrong et al. 1980, Swanson and Kuypers 1980, Swanson et al. 1986, Kiss et al. 1991) or described the morphological and synaptic properties of PVH neurons qualitatively (Van den Pol 1982). Quantitative data on the synaptic organization of the different subdivisions are lacking, however. The present study extends the previous morphometric data to the synaptic level, and contributes to an evaluation of the significance of some of the morphometric characteristics of the PVH by comparing functionally different rat strains.

MATERIALS AND METHODS

ANIMALS The quantitative morphometric data presented in this study are based on three male APO-SUS (F8 generation) and three male APO-UNSUS rats (F9 generation) of 200-250 g, bred in our Animal Laboratory. In addition, five normal Wistar rats were used to estimate the degree of tissue shrinkage involved in the present study. All rats were originally housed in groups of 2-3 animals per cage (36 × 24 × 25 cm) in a room with a constant temperature (20 ± 2°C) and a 6.00 am to 6.00 pm light period. Food and water were given ad libitum. Three days before the open field test APO-SUS and APO-UNSUS rats were isolated in single cages (36 × 24 × 25 cm) in the same conditions as mentioned above.

TISSUE PROCESSING After characterization in the open field test, the rats were deeply anaesthetized with pentobarbital (6 mg/100 g bodyweight) and transcardially perfused with 100 ml saline (0.9% sodium chloride) followed by 450 ml of a 2% paraformaldehyde/2% glutardialdehyde mixture in a 0.1 M phosphate buffer (PB, pH 7.3). Immediately after perfusion, the dorsal part of the skull was removed and the rats were placed in a stereotactic device to make a precisely transverse incision. This allowed for sectioning of all brains in the same transverse plane.

After removal out of the skull, the brains were placed overnight in the perfusion fluid. Subsequently, sections of 100 or 75 μm were cut on a vibratome in PBS (0.1 M phosphate buffered saline pH 7.3). After rinsing in the same buffer, the sections were osmicated for one hour in 1% osmium tetroxide dissolved in 0.1 M PB, rinsed in PB, dehydrated in a graded series of ethanol, embedded in epon 812 via propylene oxide and mounted in epon 812 between a slide and coverslip coated with dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane). The latter allows for easy removal of slide and coverslip when necessary for



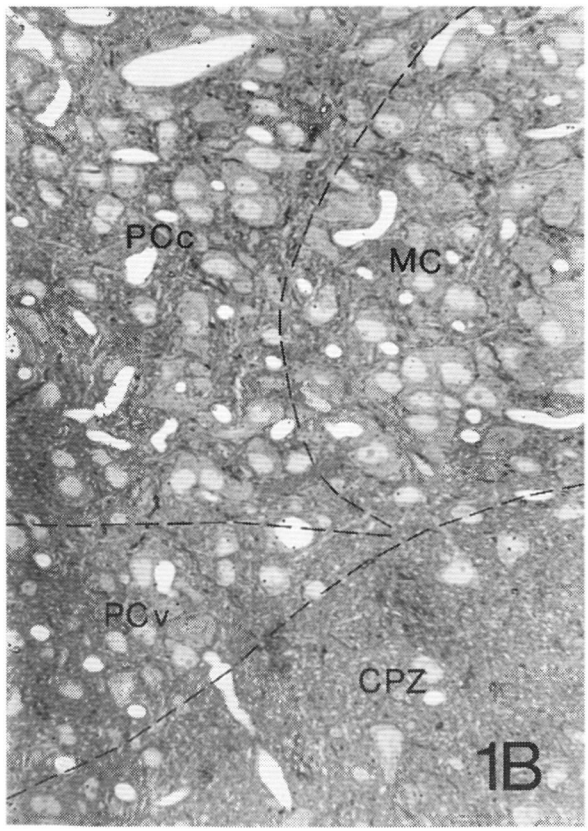


FIGURE 1 A: Photomicrograph of semithin (1 μm) section at the third level (see fig. 2), stained with toluidine blue, at a magnification of 135×, showing different subdivisions. B: Detailed photomicrograph of the indicated rectangular area in figure 1A, stained with paraphenylene-diamine, showing the somata with nuclei and nucleoli at a magnification of 340×. CPZ = cell poor zone. MC = magnocellular part. PCc = central parvocellular part. PCd = dorsal parvocellular part. PCp = posterior parvocellular part. PCv = ventral parvocellular part. PV = periventricular part.

further sectioning.

After polymerization for two days at 60°C, the sections containing the left PVH were remounted on epon blocks for semithin and ultrathin sectioning. At intervals of 50 or 37.5 μm (in 100 μm and 75 μm vibratome sections respectively), 1 μm thick semithin sections and adjacent 80 nm thick ultrathin sections were collected for morphometric analysis at the light and electron microscopic level, respectively. This procedure resulted in 10-12 PVH sample levels per animal. The semithin sections were stained with paraphenylene-diamine or toluidine blue and coverslipped with Entellan. The ultrathin sections were collected on 300 mesh copper grids and contrasted with uranylacetate and lead citrate.

LIGHT MICROSCOPIC ANALYSIS For quantitative analysis of blood vessels and neurons, the semithin sections were studied and drawn using a Zeiss light microscope and drawing tube. First, the contour of the PVH was determined on the basis of the surrounding cell-sparse zone at a magnification of 130× (fig. 1A). Next, the PVH was subdivided on the basis of differentiations in neuronal size and density as well as vascular density (see Results and figs. 1A, 1B and 2).

In each of the PVH subdivisions, blood vessels, nuclear profiles and somata containing a nucleus with a nucleolus were drawn at a magnification of 520× at each sample level, using the paraphenylene diamine sections (fig. 1B). The adjacent toluidine blue stained sections (fig. 1A) were used to verify that exclusively neuronal profiles and no glial cells were drawn. The drawings obtained were used to determine the following parameters with the aid of a Kontron-Videoplan equipment: 1) the total surface area of the PVH and its different subdivisions, 2) the number, surface area and ellipticity of the nuclei sampled, and 3) the ellipticity and surface area of somata containing a nucleus with a visible nucleolus in the plane of sectioning. The latter was done to obtain a reliable neuronal diameter estimation (Peduzzi and Crossland 1983, Born et al. 1987, Albers et al. 1988, Kiss et al. 1991). From these data the volume of all PVH subdivisions and their average neuronal sizes, densities and numbers were calculated in a way that has been previously described by our group and others (Floderus 1944, Abercrombie 1946, Albers et al. 1988, Royet 1991, Aalders and Meek 1993). This method implies the following steps:

The volume of each PVH subdivision (V) was calculated by means of the Cavalieri principle (Gundersen and Jensen 1987, Michel and Cruz-Orive 1988, Regeur and Pakkenberg 1989, Royet 1991), i.e. by multiplication of the mean surface area with the total length of each subdivision of the left PVH. At least 8 sample surfaces were measured for each subdivision, since this is a minimum for reliable estimations of V (Mayhew and Olsen 1991).

The mean neuronal diameter (D) was calculated from the surface area of somata containing a nucleus with a visible nucleolus in the plane of sectioning (Albers et al. 1988) as the D-circle. This is the diameter of a circle with the same surface area as the neuron traced.

Estimations of neuronal densities were based on nuclear tracings according to the formula presented by Floderus (1944) and Abercrombie (1946):

$$N_V = N_A / \bar{D} + t - 2h \quad \text{(see Royet 1991 for review)}$$

in which:

N_V = number of neurons per unit volume,

N_A = number of nuclei per unit test area = N_{sample} / A_{sample},

$\bar{D}$ = the mean nuclear diameter,

t = the section thickness,
h = the height of the smallest recognizable cap.
To determine $\bar{D}$ and h, the following formulae were used (Smolen et al. 1983):

$$\bar{D} = \bar{d} \cdot \left[1 - \left(\frac{(1-4/\pi)\,\bar{d}}{t + \bar{d}} \right) \right]$$

in which:
$\bar{d}$ = the mean profile diameter,
t = see above.

$h = R - \sqrt{(R_2 - r_o^2)}$ (Weibel 1979)

in which:
R = mean nucleus radius,
r_o = the radius of the smallest visible profile.
Neuronal numbers were calculated by multiplication of N_V and V per subdivision. The mean ellipticity index (smallest diameter divided by the largest diameter) of the nuclei was measured to be 0.62 ± 0.01, a value which allows for reliable estimations of neuronal densities with the formulae just explained (Bolender 1983, Albers et al. 1988).
Vascular densities were calculated by means of point counts (Weibel 1979, Royet 1991). A frame with points at 0.5 mm intervals was randomly positioned over the drawings made at a magnification of 520× and the points covering blood vessels as well as the total surface of each subdivision were counted. The volume fraction of blood vessels was calculated by dividing the number of points covering blood vessels by the number of points of the total area.

ELECTRON MICROSCOPIC ANALYSIS For analysis of the ultrathin sections of the PVH, quantitative synaptic parameters were systematic randomly sampled in a Philips EM 301 electron microscope by taking photographs in every corner and center of each 300 mesh grid square at a magnification of about 19,000×. A replica of 2,160 lines/mm was used to measure the precise magnification. This procedure yielded on average 18 photographs per subdivision per PVH, with a minimum of 12 photographs for the PCd. On average each electron micrograph showed 2 synaptic contacts. It was verified that this procedure yielded stable average values and standard errors of the synaptic parameters determined. The location of the electron micrographs in the PVH was determined by comparison of low magnification electron micrographs of the sections analysed with the adjacent semithin sections used for LM analysis. Each electron micrograph was covered by a square test frame and all synaptic contacts within this test frame and not touching the forbidden lines (Gundersen 1977, Royet 1991) were traced on the Kontron-Videoplan equipment (fig. 3).
From these measurements the mean synaptic contact trace length ($\bar{L}$) per subdivision was calculated and other parameters were determined as follows:
The synaptic contact diameter was estimated on the basis of the formula: $\bar{D} = (4/\pi) \times \bar{L}$ (Mayhew 1979, Albers et al. 1990, Aalders and Meek 1993).
The synaptic density (N_V) was determined with the formula: $N_V = N_A/\bar{L}$ (Collonier and Beaulieu 1985, Calverley et al. 1988, Albers et al. 1990), in which N_A is the number of profiles per area.
The synapse to neuron (S/N) ratio was determined as the ratio of synaptic and neuronal density: N_V (synapse)/N_V (neuron).

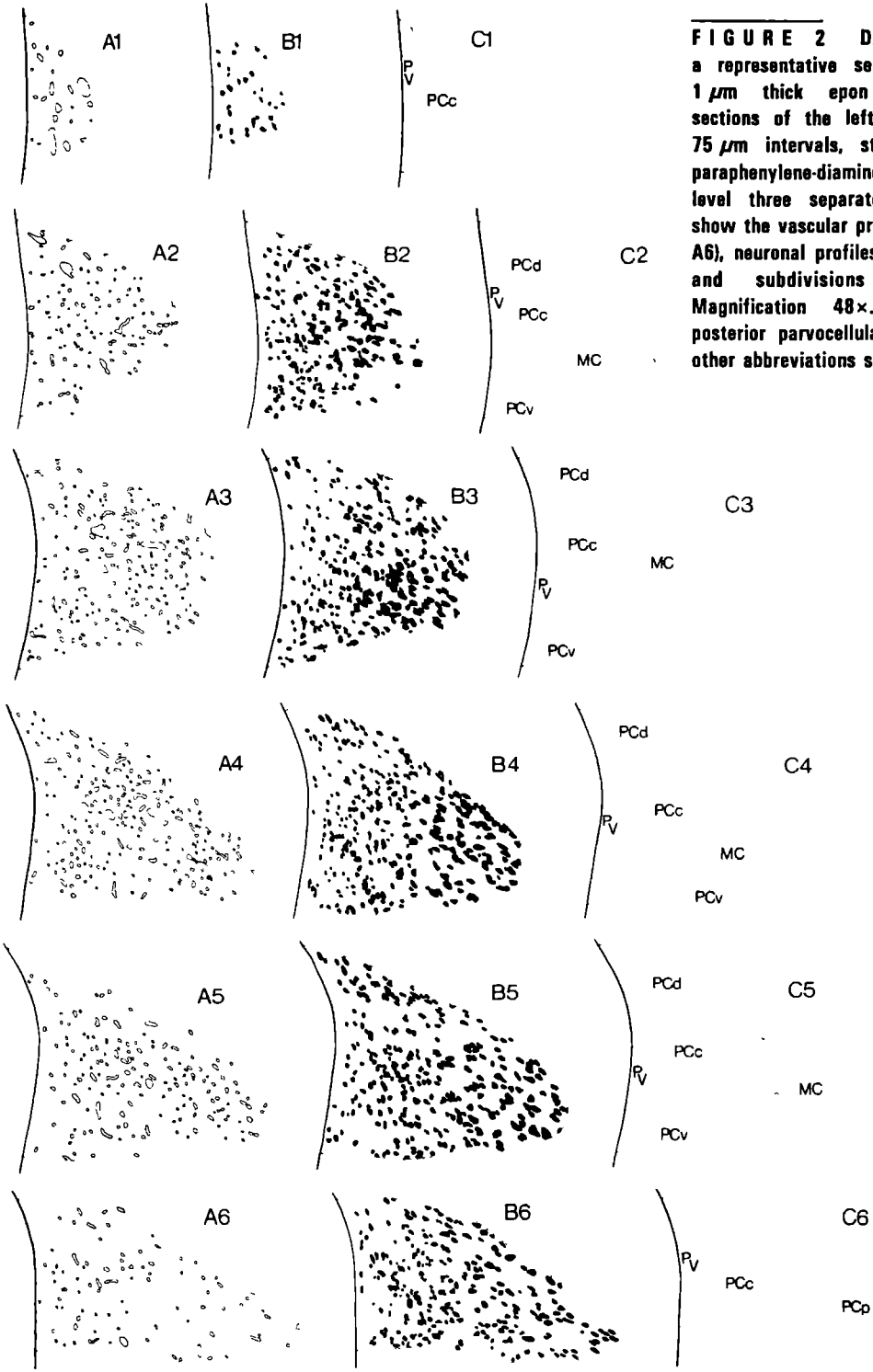


FIGURE 2 Drawings of a representative series of six 1 μm thick epon embedded sections of the left PVH with 75 μm intervals, stained with paraphenylene-diamine. At each level three separate drawings show the vascular profiles (A1 ... A6), neuronal profiles (B1 ... B6) and subdivisions (C1...C6). Magnification 48×. PCp = posterior parvocellular part. For other abbreviations see figure 1.

SHRINKAGE The tissue volume as well as neuronal and synaptic densities are influenced by shrinkage of the PVH, caused by fixation and other histological procedures. To be able to make corrections for shrinkage, this parameter was estimated as follows: Five male Wistar rats (200-250 g) were deeply anaesthetized with pentobarbital and placed in a stereotactic

device where four small dorso-ventral holes were made at a transverse and sagittal distance of 5 mm. Subsequently, these rats were perfused and fixed overnight in the same way as described above. After removal of the brain out off the skull and fixation overnight, the distance between the four holes made was measured to estimate the shrinkage caused by perfusion and postfixation. This appeared to be 8.5 ± 1.0% linearly. After vibratomy the dimensions of the vibratome sections were measured before and after the histological procedure applied as described above. The histological procedure caused a linear shrinkage of 2.6 ± 0.3%. Thus, the total linear shrinkage in our material is 10.8 ± 1.1%, resulting in a three-dimensional shrinkage of 29 ± 2.8%.

28 **STATISTICS** Statistical analysis of differences between the two lines (APO-SUS and APO-UNSUS rats) was performed with the Mann-Whitney U test. For statistical comparison of subdivisions, the Wilcoxon Matched-Pairs Signed-ranks test was used (Siegel 1956).

RESULTS

The results as obtained for the left PVH are summarized in table I, which presents values that are uncorrected for shrinkage. The effect of shrinkage will be discussed in the discussion section (table II). A visualization of some of the light microscopic parameters determined is presented in figure 2. In this figure the blood vessels, neurons and subdivisions delineated in series of six representative semithin sections have been drawn separately.

SUBDIVISIONS OF THE PVH In the semithin series of sections used for the present study the PVH could unequivocally be subdivided in six subdivisions on the basis of neuronal sizes and densities as well as vascular densities, as follows (see also figure 2):

- The periventricular part (PV) is a cell-sparse zone with fusiform neurons and a minor vascularization, situated along the third ventricle.
- The magnocellular part (MC) contains clearly larger neurons than the remaining part of the PVH. This subdivision extends laterally towards the fornix and is bordered on its lateral and ventral edges by the cell-sparse zone. The latter can easily be distinguished from the MC because of obvious difference in neuronal density and cytoarchitecture.
- The parvocellular part (PC), lying between the PV and MC, can be subdivided into four subdivisions on the basis of differences in vascular density, to be quantified below. The central parvocellular part (PCc) is as richly vascularized as the MC. It is bordered at the dorsal side by the dorsal parvocellular part (PCd) and ventrally by the ventral parvocellular part (PCv), both less vascularized. Posterior to the PCc, the PCd and the PCv fuse into the sparsely vascularized posterior parvocellular part (PCp).

VASCULARITY The overall vascularity in the PVH, expressed as the percentage of volume occupied by blood vessels, is 5.6% (fig. 4). The PV is the least vascularized subdivision (3.2%, $p < 0.05$). The MC is significantly more vascularized than the other subdivisions (7.8%, $p < 0.05$) except for the PCc, which has an equally rich vascular density (8.3%). The intermediate vascular densities of the PCd (4.6%), the PCv (4.6%) and the PCp

TABLE I Morphometric parameters as determined for the left PVH of 3 APO-UNSUS and 3 APO-SUS rats (mean ± SEM)

Subdivisions	Vascularity (%)	Volume (mm^3)	Soma diameter (μm)	Neuronal density ($\times 10^3/mm^3$)	Ellipticity index somata	Synaptic contact length (nm)	Synapse density ($\times 10^6/mm^3$)	S/N ratio
Overall								
PVH	5.6 ± .14	.083 ± .0037	11.754 ± .177	185.4 ± 8.8	.582 ± .004	368 ± 6	187 ± 17	1009 ± 83
PV	3.2 ± .35	.014 ± .0004	9.581 ± .182	131.8 ± 10.7	.528 ± .010	360 ± 12	184 ± 16	1454 ± 212
MC	7.8 ± .57	.014 ± .0011	14.385 ± .264	176.7 ± 11.5	.612 ± .008	385 ± 12	157 ± 22	899 ± 134
PCc	8.3 ± .22	.022 ± .0011	10.679 ± .304	230.0 ± 19.4	.605 ± .005	364 ± 4	203 ± 21	913 ± 134
PCd	4.6 ± .20	.008 ± .0008	11.579 ± .203	185.8 ± 12.8	.553 ± .011	400 ± 29	169 ± 33	871 ± 157
PCv	4.6 ± .28	.009 ± .0007	10.979 ± .196	206.1 ± 16.4	.623 ± .005	341 ± 11	216 ± 18	1069 ± 115
PCp	5.3 ± .38	.016 ± .0025	11.212 ± .197	180.6 ± 12.3	.569 ± .014	373 ± 10	185 ± 19	1025 ± 102
Cell-sparse zone						351 ± 13	234 ± 13	
APO-UNSUS								
PVH	5.8 ± .09	.086 ± .0016	11.954 ± .229	172.0 ± 13.2	.586 ± .008	379 ± 5	152 ± 9	893 ± 83
PV	4.0 ± .18	.014 ± .0005	9.424 ± .178	137.4 ± 19.4	.530 ± .009	367 ± 20	154 ± 11	1155 ± 158
MC	7.1 ± .28	.014 ± .0007	14.675 ± .204	157.2 ± 12.6	.613 ± .008	405 ± 11	129 ± 24	851 ± 227
PCc	8.2 ± .35	.022 ± .0016	11.068 ± .419	220.2 ± 35.2	.613 ± .008	369 ± 3	162 ± 23	770 ± 185
PCd	4.9 ± .21	.007 ± .0015	11.787 ± .351	164.1 ± 9.2	.564 ± .018	431 ± 48	121 ± 43	710 ± 279
PCv	5.0 ± .26	.010 ± .0007	11.085 ± .323	181.3 ± 14.7	.630 ± .004	345 ± 20	187 ± 21	1072 ± 237
PCp	5.7 ± .63	.019 ± .0029	11.461 ± .232	170.4 ± 9.4	.568 ± .013	393 ± 9	147 ± 17	875 ± 162
APO-SUS								
PVH	5.4 ± .37	.076 ± .0059	11.575 ± .227	198.8 ± 5.4	.577 ± .003	357 ± 7	222 ± 15	1124 ± 120
PV	2.5 ± .33	.013 ± .0005	9.737 ± .290	126.3 ± 13.0	.525 ± .018	352 ± 12	214 ± 19	1753 ± 332
MC	8.4 ± .97	.013 ± .0021	14.096 ± .426	196.2 ± 11.0	.610 ± .013	365 ± 14	185 ± 29	946 ± 189
PCc	8.3 ± .27	.021 ± .0014	10.290 ± .304	239.8 ± 23.4	.597 ± .001	359 ± 5	244 ± 13	1056 ± 188
PCd	4.3 ± .25	.008 ± .0000	11.371 ± .113	207.4 ± 16.1	.541 ± .011	369 ± 21	216 ± 33	1031 ± 142
PCv	4.2 ± .37	.008 ± .0010	10.874 ± .205	230.9 ± 22.5	.616 ± .008	337 ± 8	244 ± 19	1066 ± 100
PCp	4.9 ± .28	.012 ± .0029	11.010 ± .245	190.7 ± 23.7	.571 ± .024	353 ± 8	223 ± 16	1175 ± 53

TABLE II Morphometric parameters of the left PVH as corrected for 10.8% linear shrinkage

Subdivisions	Volume (mm^3)	Neuronal density ($\times 10^3/mm^3$)	Synaptic density ($\times 10^6/mm^3$)
Overall			
PVH	.117	131.6	133
PV	.020	93.6	131
MC	.020	125.5	111
PCc	.031	163.3	144
PCd	.011	131.9	120
PCv	.013	146.3	153
PCp	.023	128.2	131
APO-UNSUS			
PVH	.121	122.1	108
PV	.020	97.6	109
MC	.020	111.6	92
PCc	.031	156.3	115
PCd	.010	116.5	86
PCv	.014	128.7	133
PCp	.027	121.0	104
APO-SUS			
PVH	.107	141.1	158
PV	.018	89.7	152
MC	.018	139.3	131
PCc	.030	170.3	173
PCd	.011	147.3	153
PCv	.011	163.9	173
PCp	.017	135.4	158
Cell-sparse zone			166

(5.3%) show no significant mutual differences, but are all significantly higher than the vascular density of the PV ($p < 0.05$) and lower than the vascular densities of the MC and PCc ($p < 0.05$).

APO-SUS and APO-UNSUS rats showed no statistically significant differences in PVH vascularity except for the PV. This subdivision was slightly but significantly higher vascularized in APO-UNSUS rats (4.0%) than in APO-SUS rats (2.5%, $p < 0.05$, table I).

NEURONAL SIZE AND SHAPE The mean soma diameter in the PVH is 11.8 μm, with the following differentiation between its subdivisions (table I): The PV contains on average the smallest neurons (9.6 μm, $p < 0.05$). These have a fusiform shape as indicated by an ellipticity index (ell.ind.) of 0.53 (table I). The MC contains obviously the largest neurons of the PVH (14.4 μm, $p < 0.05$), which are more rounded (ell.ind. 0.61, table I). The mean soma diameter of the fusiform neurons of the PCd (11.6 μm, ell.ind. 0.55, table I) is slightly but significantly larger that of the PCc (10.7 μm, $p < 0.05$) and the PCv (11.0 μm, $p < 0.05$). The neuronal sizes observed in the PCc, PCv and PCp show no significant mutual differences. There are no statistically significant differences in neuronal size or shape between APO-SUS and APO-UNSUS rats.

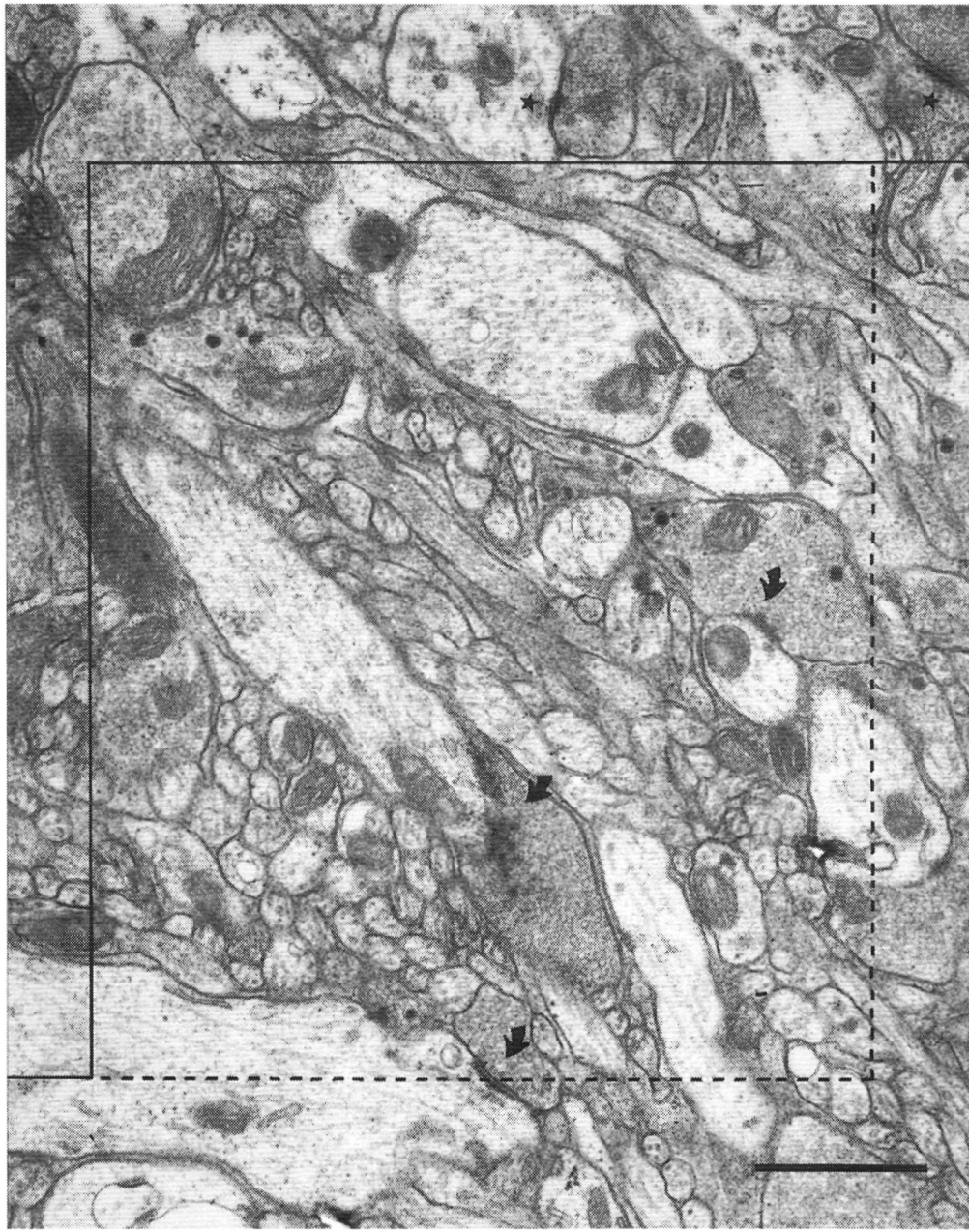


FIGURE 3 Electron micrograph of a representative part of the PVH, showing synaptic contacts within (arrows) and outside (stars) the forbidden lines. Bar = 1 μm.

NEURONAL DENSITIES AND NUMBERS The overall neuronal density in the PVH is about 185 × 10^3 cells/mm^3 (fig. 4). The neuronal density of the PV (132 × 10^3/mm^3) is significantly smaller ($p < 0.05$) than the neuronal density of the MC (177 × 10^3/mm^3), PCc (230 × 10^3/mm^3), PCd (186 × 10^3/mm^3) and PCv (206 × 10^3/mm^3). The neuronal

density of the PCp ($181 \times 10^3/mm^3$) is, although larger, not statistically different from that of the PV. However, the difference in neuronal density between the MC and the PCc is statistically significant ($p < 0.05$). Statistical comparison between APO-UNSUS and APO-SUS rats yielded no significant differences in this respect.

The number of neurons in the PVH and its subdivisions was estimated by multiplication of the neuronal densities with the tissue volumes calculated. On average, the left PVH occupies a volume of 0.083 mm^3 and contains about 15,400 cells (table I). The PCc contains the largest amount of cells, about 5,000, which is 33% of the total population. The other subdivisions contribute between 12% and 19% to the total population in both APO-SUS and APO-UNSUS rats.

SYNAPTIC CONTACT LENGTH The average synaptic contact trace length in the PVH is 368 nm (table I), which corresponds to an average synaptic contact diameter of 469 nm. The synaptic contact size is basically similar in all subdivisions as well as in the cell-sparse zone around the PVH (table I). The only significant difference found between subdivisions is a slightly smaller contact length in the PCv (341 nm) compared with the MC (385 nm, $p < 0.05$). Comparison of APO-SUS and APO-UNSUS rats showed that the synaptic contact length in the PCp of APO-UNSUS rats (393 nm) is significantly larger than that of APO-SUS rats (353 nm, $p < 0.05$).

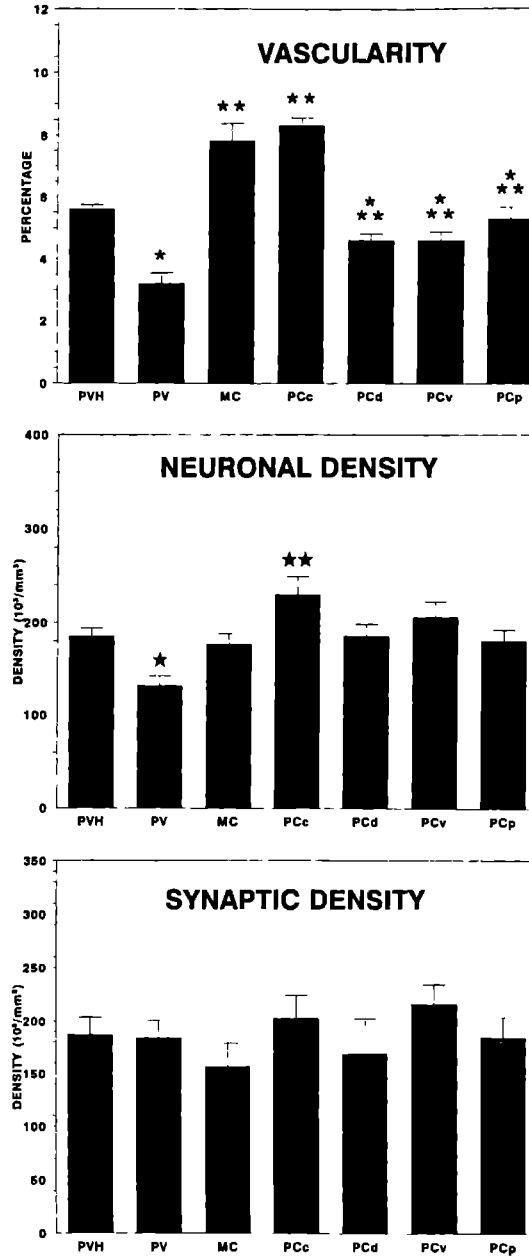


FIGURE 4 Histograms of the vascular densities, neuronal densities and synaptic densities in the left PVH and its six subdivisions (mean ± SEM, n = 6). • = significantly different from MC, PCc, PCd, PCv and PCp ($p < 0.05$). •• = significantly different from PV, PCd, PCv and PCp ($p < 0.05$). ••• = significantly different from PV, MC and PCc ($p < 0.05$). ★ = significantly different from MC, PCc, PCd and PCV ($p < 0.05$). ★★ = significantly different from MC ($p < 0.05$). For abbreviations see figure 1.

SYNAPTIC DENSITIES AND NUMBERS The mean synaptic density in the PVH is 187 × 10^6/mm^3 without statistically significant differences between its subdivisions (fig. 4). In the cell-sparse zone surrounding the PVH a similar synaptic density was observed as well. It is somewhat higher than the synaptic density in the PVH (table I) but this difference is not statistically significant. However, the PVH of APO-SUS and APO-UNSUS rats showed a marked difference (fig. 5). APO-UNSUS rats have a significantly smaller overall synaptic density (152 × 10^6/mm^3) than APO-SUS rats (252 × 10^6/mm^3, $p < 0.05$). This difference is present in all subdivisions of the PVH (fig. 5) and was statistically significant in our sample for the PV, PCc and PCp ($p < 0.05$). No interline difference was found in the cell-sparse zone surrounding the PVH.

Synapse numbers were estimated by multiplication of the volume of the PVH subdivisions with their synaptic density, resulting in a number of about 16 × 10^6 synapses in the total left PVH. The PCc contains about 29% of these synapses, the PV 17%, the MC 14%, the PCd 9%, the PCv 12% and the PCp 19%. The total number of synapses in the left PVH of APO-SUS rats (17 × 10^6) is considerably larger than in APO-UNSUS rats (13 × 10^6). However, this difference was not statistically significant in our sample, because of the large individual variations.

SYNAPSE-TO-NEURON RATIOS The overall synapse-to-neuron (S/N) ratio in the PVH is 1009. This means that PVH neurons have around 1,000 synaptic contacts on their receptive surface, provided that they do not have dendrites outside the PVH, and that neurons outside the PVH do not have extensive dendrites within the PVH. The S/N ratio in the PV appears to be significantly higher (1454) than that in the MC (899) and PCc (913, table I). APO-SUS and APO-UNSUS rats are similar in this respect.

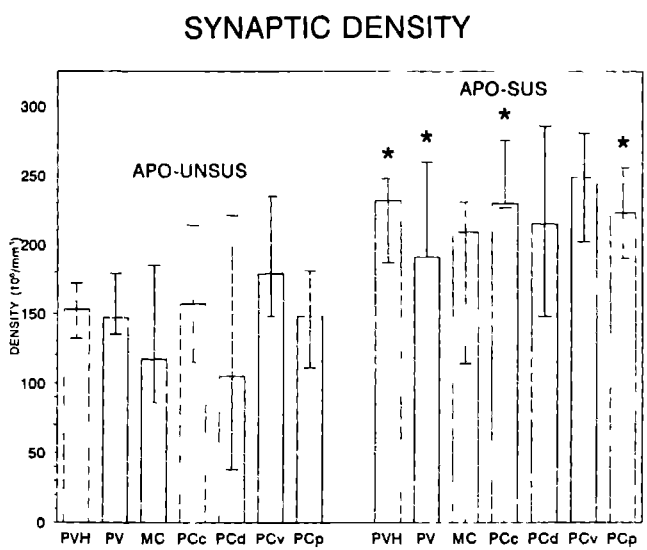


FIGURE 5
Synaptic densities in APO-UNSUS (n = 3) and APO-SUS (n = 3) rats in the left PVH and its six subdivisions (median value ± highest and lowest value). • = significantly different ($p < 0.05$) from the corresponding subdivision in the APO-UNSUS rats. For abbreviations see figure 1.

DISCUSSION

The goal of the present study is to compare the histological differentiation of the PVH of APO-SUS and APO-UNSUS rats, and to extend previous morphometric data with a quantitative characterization of the synaptic organization. In particular the latter has yielded new data showing marked differences between both rat lines. Before these differences can be evaluated in detail, a discussion on PVH subdivisions and neuronal morphometry is necessary, however, since for these aspects some striking differences with previous reports are present.

DELINEATION OF PVH SUBDIVISIONS In the present study the PVH has been subdivided into six parts on the basis of neuronal sizes and densities as well as vascular densities. Although the latter criterion has not been used in previous studies, the resulting subdivisions correspond well to previously described ones.

All previous studies agree in the distinction of a parvocellular and a magnocellular PVH on the basis of average cell size. The laterally located magnocellular part has been further subdivided into three parts by Armstrong et al. (1980): an anterior commissural nucleus (ACN), a medial (PVM) and a lateral part (PVL). Swanson and Kuypers (1980) and Swanson et al. (1986) distinguished four magnocellular parts, only slightly different from the subdivisions made by Armstrong et al. (1980), i.e. an anterior (AM), a medial (MP) and a posterior part (PM) that is further subdivided in a medial oxytocinergic region and a lateral vasopressinergic region. In contrast, Kiss et al. (1991) considered the magnocellular section as a single entity because of the observed homogeneity in neuronal size and density. Our data are in line with the latter view (table I).

In the parvocellular PVH, Armstrong et al. (1980) distinguished three regions: 1) the large anterior parvocellular portion (PVPA), starting immediately rostral to the PVM and projecting mainly to the median eminence, 2) the somewhat smaller dorsomedial cap (PVDC), which borders the dorsal surface of the PVH, and 3) the posterior subnucleus (PVPO), which extends dorsolaterally. In contrast to the PVPA, the latter two subdivisions contain large amounts of neurons that project to the brainstem and spinal cord (Armstrong et al. 1980). Swanson and Kuypers (1980) and Swanson et al. (1986) distinguished six parts in the parvocellular PVH on the basis of differential projections and cytoarchitectonical characteristics: an anterior (AP), dorsal (DP), lateral (LP), periventricular (PV) and a medial (MP) part, of which the latter is further subdivided in a dorsal and medial region. Kiss et al. (1991) have subdivided the parvocellular PVH on the basis of quantitative histological characteristics in a periventricular (pv) and a medial subdivision (mp), corresponding with the PV and MP of Swanson and Kuypers (1980) and Swanson et al. (1986) (table III). Within the medial subdivision they have delineated an anterior (map), a lateral (mlp), a medial (mmp) and a caudal part (mcp) on the basis of inhomogeneities in cell density. In addition, Kiss et al. (1991) have distinguished a mediocellular PVH subdivision with neurons of intermediate size, subdivided into a dorsal (d) and a posterior subdivision (p), the former corresponding with the PVDC of Armstrong et al. (1980) and DP of Swanson and Kuypers (1980) and Swanson et al. (1986), and the latter corresponding with the PVPO of Armstrong et al. (1980) and LP of Swanson and Kuypers (1980) and Swanson et al. (1986) (table III).

The five parvocellular subdivisions that we distinguish in the present study, predominantly on the basis of differences in vascularity, correspond largely with the subdivisions just

surveyed (table III). Only the PCv has never been described as a distinct subdivision, but has always been regarded as a part of another subdivision. This means that the differentiation in vascular density in the PVH offers a well defined and reproducible basis for delineations within the parvocellular PVH, resulting in similar subdivisions as distinguished previously on the basis of the histochemical, cytoarchitectonic and connectional differentiation of the PVH (Armstrong et al. 1980, Swanson and Kuypers 1980, Swanson et al. 1986, Kiss et al. 1991).

VASCULARITY OF THE PVH Previous studies have shown that the blood supply to the PVH originates from the retrochiasmatic artery (Ambach and Palkovits 1974) and that the PVH is more richly vascularized than the surrounding hypothalamic region (Ambach and Palkovits 1974, Van den Pol 1982) with an overall vascular density of 3.0% (Van den Pol 1982). In the present study we find an overall vascular density of 5.6%. This is about twice the value reported by Van den Pol (1982), who excluded the periventricular part in his analysis and used only transverse sections at the level of the magnocellular subdivision. A close comparison shows that the different values reported are not caused by the mean number of vessel profiles per section surface (Van den Pol (1982): approx. 8 per 10,000 μm^2, present study: approx. 9 per 10,000 μm^2). This means that in particular different surface areas per vessel profile are the source of the difference between both studies, in spite of the fact that both studies used similar fixation and semithin section techniques to determine vascular densities.

The vascularity of different subdivisions has never been quantified before. Only a few contradictory remarks on this subject may be found in the literature. According to Ambach and Palkovitz (1974), the magnocellular part is more densely vascularized than the parvocellular part, but Van den Pol (1982) claims that the magnocellular and parvocellular part are equally vascularized. Our results show that the PCc is equally vascularized as the MC, which is in accordance with Van den Pol (1982). The discrepancy with the study of Ambach and Palkovitz (1974) may be caused by the fact that the parvocellular part described by Ambach and Palkovitz (1974) is comparable with our combined PCc, PCd, PCv and PCp, which together indeed have a lower vascular density than the MC or the PCc separately. The low vascularity of the PV as reported in the present study, is in accordance with the observations

TABLE III Subdivisions of the present study and the corresponding subdivisions of previous studies

Present study	Armstrong et al. (1980)	Swanson and Kuypers (1980) Swanson et al. (1986)	Kiss et al. (1991)
PV		PV	pv
MC	PVM, PVL	PM	m
PCc	PVPA	AP, MP	map, mlp, mmp, mcp
PCd	PVDC	DP	d
PCv	PVPA	MP	p
PCp	PVPO	LP	p

ABBREVIATIONS AP = anterior parvocellular part, d = dorsal subdivision, DP = dorsal parvocellular part, LP = lateral parvocellular part, m = magnocellular subdivision, map = medial subdivision anterior part, mcp = medial subdivision caudal part, mlp = medial subdivision lateral part, mmp = medial subdivision medial part, MP = medial parvocellular part, p = posterior subdivision, PM = posterior magnocellular part, PV/pv = periventricular part, PVDC = dorsomedial cap of the paraventricular nucleus, PVL = lateral paraventricular nucleus, PVM = medial paraventricular nucleus, PVPA = parvocellular portion of the paraventricular nucleus, PVPO = posterior portion of the paraventricular nucleus.

of Van den Pol (1982).

The functional relevance of the higher vascularity of the PCc and MC is uncertain. It has been suggested by Van den Pol (1982) that cells in highly vascularized areas have a higher metabolic rate, correlated with the high quantities of peptides they produce and thus their higher demand for nutrients and oxygen. This would mean that the MC and PCc are the most active subdivisions of the PVH.

NEURONAL PROPERTIES The present results confirm that the MC contains the largest neurons of the PVH (14.4 μm) and the PV the smallest ones (9.5 μm), while the other subdivisions consist of medium-sized neurons of about 11-12 μm. This is largely in agreement with previous studies (Armstrong et al. 1980, Swanson and Kuypers 1980, Swanson et al. 1986, Kiss et al. 1991). Apart from a magnocellular and a parvocellular part, Kiss et al. (1991) also distinguished a mediocellular region, comparable with our PCd, PCv and PCp. The cells in the PCd are indeed somewhat larger (11.6 μm, $p < 0.05$) than in the other parvocellular subdivisions. However, the cells in the PCp and PCv, also included by Kiss et al. (1991) in the mediocellular subdivision, are not larger than those in other parvocellular subdivisions.

The present data on neuronal densities show in fixed tissue an overall value of 185,000 cells/mm^3, with a highest neuronal density in the PCc (230,000/mm^3) and a lowest neuronal density in the PV (132,000/mm^3, table I). For comparison with previous results, the present data on neuronal densities in the different subdivisions have to be corrected for shrinkage (estimated to be 10.8% linearly in our study). The results of such a correction are shown in table II. Compared with Kiss et al. (1991), who reported a neuronal density in all regions of the PVH of around 60,000 cells/mm^3, with the exception of the pv (40,000/mm^3) and the mlp (86,000/mm^3), the presently calculated densities as corrected for shrinkage are about twice as high (PVH: 132,000/mm^3, PV: 94,000/mm^3, PCc: 163,000/mm^3, table II). Several factors may be involved in this discrepancy. Kiss et al. (1991) quantified neuronal nuclei containing at least one nucleolus in 11 μm thick sections, whereas we quantified neuronal nuclei in semithin (1 μm) sections. However, both methods are based on the formulae of Floderus (1944) and Abercrombie (1946) and should yield reliable and comparable data (Bolender 1983, Smolen et al. 1983, Albers et al. 1988), unless a substantial portion of the PVH neurons would lack a distinct nucleolus. Such neurons would be incorporated in the present study, but would not have been counted by Kiss et al. (1991). We excluded the incorporation of glial cells in our counts by comparing every drawing of a paraphenylene-diamine stained section with an adjacent toluidine blue stained section.

A factor that might be correlated with the different neuronal densities calculated is the different PVH volume reported in both studies. Kiss et al. (1991) calculated an average volume of 0.18 mm^3, whereas we calculated a volume 0.12 mm^3. This might partly be the result of a different delineation of the PVH, resulting in inclusion of parts of the cell-sparse zone by Kiss et al. (1991). This would enlarge the PVH volume and reduce the overall neuronal density. However, this would also enlarge the number of neurons calculated in the PVH, which is not the case: Kiss et al. (1991) reported a substantially lower number of PVH neurons than the present study. Another factor influencing the determination of the volume as well as the neuronal density of the PVH is shrinkage. The shrinkage factor used by Kiss et al. (1991) (17% linearly, i.e. 43% three-dimensionally) is different from the presently used one (10.8% linearly, i.e. 29% three-dimensionally) but this relatively small difference cannot account for the large differences in calculated volumes and densities. Moreover, when the

shrinkage factors applied would not represent the true values, such a deviation would not influence the neuronal numbers calculated in the PVH, which still are different in both studies. Nevertheless, delineation and shrinkage differences may still partially have their influence, since the neuronal density in the present study is two times higher, but the neuronal number is only 1.5 times higher compared with the study of Kiss et al. (1991). Differences in rat lines used might also be involved in the different neuronal densities and PVH volumes, since Kiss et al. (1991) used CFY rats, whereas we used Wistar rats. It is presently uncertain to what extent any of the factors enumerated are involved in the discrepancies just discussed.

In spite of the differences in the estimated absolute values of the neuronal densities, the mutual differences between subdivisions as presented by Kiss et al. (1991) and the present study are similar. The PV has the lowest neuronal density, while the PCc, largely corresponding with the mlp of Kiss et al. (1991), has the highest neuronal density. The other subdivisions have intermediate neuronal densities.

As is already mentioned above, previous estimates of the total number of cells in the PVH are considerably lower than the present results, due to the higher neuronal density observed in our study (Kiss et al. (1983): 9,000 cells, Swanson and Sawchenko (1983): 10,000 cells, Kiss et al. (1991): 10,750 cells, present study: 15,400 cells, one side). According to our results, the PV contains 12% of the total cell population, the MC 15.5%, the PCc 33% and the PCd 9.5%. This percentive distribution is largely comparable with the results of Kiss et al. (1991), except for the PCv and PCp. These subdivisions together represent the posterior subdivision (p, table III) of Kiss et al. (1991) and contain 30% of the total cell population in our study, opposite to the 16% reported by Kiss et al. (1991). In the PCc, which contains an estimated total number of 5,000 cells, about 2,000 CRH cells have been observed (Swanson et al. 1983, Sawchenko and Swanson 1985).

SYNAPTIC CHARACTERISTICS The present study is the first one giving quantitative data on the synaptic organization of the PVH. Previously reported quantitative EM data exclusively concerned the surface and number of presynaptic boutons (Kiss et al. 1983). The synaptic trace length observed in the PVH (368 ± 6 nm) is relatively large compared with other brain regions, such as the colliculus superior of rabbits (Vrensen and De Groot 1977) (241-257 nm) and rats (Albers et al. 1990) (268 nm), the visual cortex (Sirevaagh and Greenough 1985) (276-310 nm), and the hypothalamic aggression region (Aalders and Meek 1993) (267-355 nm) of rats, except for the cerebellum (Hillman and Chen 1985) (373 nm).

Similar to the neuronal density, the synaptic density has to be corrected for shrinkage to have an estimate of the situation in normal, unfixed, living tissue (cf. tables I and II). Our data show a similar overall synaptic density in the PVH ($133 \times 10^6/mm^3$) and the surrounding cell-sparse zone ($166 \times 10^6/mm^3$), without significant differences between PVH subdivisions. However, the synaptic density differs significantly between APO-SUS and APO-UNSUS rats, as will be discussed below. The overall synapse-to-neuron (S/N) ratio of 1009 is in agreement with the results of Kiss et al. (1983) who reported the presence of about 1,000 synaptic boutons per cell within the PVH.

APO-SUS VERSUS APO-UNSUS RATS The most significant finding of the present study is the difference in synaptic density between APO-SUS ($158 \times 10^6/mm^3$) and

APO-UNSUS rats (108 x 10^6/mm^3) (table II). This is a quite specific result, since the synaptic density in the cell-sparse region surrounding the PVH is similar in both lines, as is the neuronal density in the PVH. The higher synaptic density in the PVH of APO-SUS rats compared to APO-UNSUS rats suggests the presence of a more elaborate micro-circuit in the PVH of APO-SUS rats compared to APO-UNSUS rats. This differentiation might be correlated with differences in circulating plasmacorticoid levels during early postnatal life, since it has been shown that treatment with corticosteroids retards synaptic genesis (De Kloet et al. 1988). This suggests, as is hypothesized before (Cools et al. 1990, Cools et al. 1993b), that APO-SUS rats are exposed to lower levels of corticosteroids than APO-UNSUS rats during early development.

It is not clear whether the higher synaptic density in the PVH of APO-SUS rats is the result of a general enhancement of synaptic development or an enhancement of one or a few specific inputs to the PVH. It is not very likely that interneurons or recurrent collaterals are involved, since these only infrequently occur in the PVH (Van den Pol 1982, Rho and Swanson 1989). Previous experiments suggest a more active hypothalamo-pituitary-adrenal axis in adult APO-SUS rats compared to adult APO-UNSUS rats (Cools et al. 1993b), the latter showing a significantly lower basal plasma ACTH level and a lower mineralocorticoid receptor capacity in the pituitary than the APO-SUS rats. Furthermore, it has been found that a conditioned emotional stimulus induces a higher plasma ACTH level in APO-SUS rats compared with APO-UNSUS rats (Van Eekelen 1992). Consequently, the higher synaptic density in APO-SUS rats correlates well with a presumed higher activity of the PVH during stress regulation in APO-SUS rats. However, recent work in our laboratory showed that the PVH of APO-SUS rats contains significantly less Fos-immunoreactive cells than the PVH of APO-UNSUS rats (Mulders et al. 1993) after a stressful stimulus. This suggests a reduced activity of the PVH of APO-SUS rats compared to APO-UNSUS rats, which would mean that the increased synaptic density in the PVH evokes an increased inhibitory effect on the PVH. To evaluate these possibilities further, it is necessary to incorporate our Fos experiments in detail in the discussion, which will be done in the following paper (Mulders et al. 1995).

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Chapter 3

The hypothalamic paraventricular nucleus in two types of Wistar rats with different stress responses:

II. Differential Fos-expression

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SUMMARY

The present study investigates the role of corticotropin-releasing hormone (CRH) neurons in stress regulation by a comparison of stress induced Fos-immunoreactivity and CRH-immunoreactivity in the hypothalamic paraventricular nucleus (PVH) of APO-SUS (apomorphine-susceptible), APO-UNSUS (apomorphine-unsusceptible), normal Wistar and adrenalectomized Wistar (ADX) rats. The first two types represent a good model to study the role of the PVH in stress regulation, since they show different stress responses and a differential synaptic organization of the PVH. After placement on an open field for 15 minutes all rats showed an increase in the number of Fos-immunoreactive nuclei compared to control handling. Interestingly, open field stress, but not control handling, induces significantly fewer Fos-immunoreactive nuclei in the PVH of APO-SUS rats (1255 ± 49) compared to APO-UNSUS rats (1832 ± 201). Experiments with ADX rats revealed that 93% of the CRH-immunoreactive neurons contained a Fos-immunoreactive nucleus, which suggests that the differential Fos-expression in APO-SUS and APO-UNSUS rats represents a differential activation of the CRH neurons. This hypothesis is discussed in relation to reported differences in stress responses, stress-induced ACTH levels and synaptic organization of the PVH.

INTRODUCTION

The hypothalamic paraventricular nucleus (PVH) plays an important role in the regulation of stress responses. It contains corticotropin-releasing hormone (CRH) producing parvocellular neurons, which induce adrenocorticotropic hormone (ACTH) release from the corticotrope cells in the anterior pituitary (Antoni 1989). ACTH subsequently regulates corticosteroid production in the adrenal cortex (Whitnall 1993). Corticosteroids are involved in several stress reactions and exert a negative feedback on CRH and ACTH release (Keller-Wood and Dallman 1984). Consequently, removal of the circulating corticosteroids by adrenalectomy (ADX) induces an increase of CRH mRNA on the short term and an increase of CRH on the long term (Sawchenko 1987, Almeida et al. 1992).

Several stimuli evoke stress responses, and for some of these the expression of c-fos mRNA or Fos-like immunoreactivity (Fos-IR) has been used to demonstrate the involvement of the PVH. C-fos mRNA or Fos are often used as activation markers (Sagar et al. 1988, Sheng and Greenberg 1990, Morgan and Curran 1991), since c-fos is responsible for the synthesis of the nuclear protein Fos, which, together with other factors, makes complexes with DNA at AP-1 binding sites (Curran and Franza 1988) to regulate transcription (Morgan and Curran 1991, Schilling et al. 1991). Accordingly, the expression of c-fos and the accumulation of the protein Fos are associated with cell activation in response to a variety of stimuli (Doucet et al. 1990). Stressful stimuli that have been used to induce c-fos or Fos-IR in the PVH include intraperitoneal hypertonic saline injections (Sharp et al. 1991), immobilization stress (Ceccatelli et al. 1989, Imaki et al. 1992), pain (Senba et al. 1993) and swim stress (Duncan et al. 1993).

A new model to study stress responses is presented by two lines of Wistar rats, which have been pharmacogenetically selected on the basis of their gnawing responses after an identical, subcutaneous dose of apomorphine (Cools et al. 1990, Cools et al. 1993a, Cools et al. 1993b). They are indicated as APO-SUS (apomorphine susceptible) and APO-UNSUS (apomorphine unsusceptible) rats, showing a high and low gnawing response, respectively (Cools et al.

1990). Other interline differences can be observed on an open field, where APO-SUS rats show more locomotor activity and edge-hugging behavior than APO-UNSUS rats. In the so-called defeat test, in which the rat is confronted with a much larger rat, APO-SUS rats show fleeing behavior, whereas APO-UNSUS rats exhibit freezing (Cools et al. 1990, Cools et al. 1993b). Furthermore, a conditioned emotional stress stimulus evokes higher plasma ACTH levels in APO-SUS rats than in APO-UNSUS rats (Van Eekelen et al. 1992, Rots et al. 1995). The present paper investigates the stress induced expression of Fos-IR in the PVH of APO-SUS and APO-UNSUS rats. For this purpose, we quantified Fos-IR in the PVH after a mild novelty stress in both APO-SUS and APO-UNSUS rats and compared this with Fos-IR in the PVH of normal Wistar rats after the same stress. Novelty-induced stress is known to modulate differentially the hippocampal content of dynorphine (Cools et al. 1993a) as well to affect differentially the release of ACTH and corticosteroids in APO-SUS and APO-UNSUS rats (Rots et al. 1995). We investigated whether the novelty-induced Fos-IR is expressed by the CRH-cell population using ADX rats, since untreated rats do not show distinct CRH-IR in the PVH. The differences in Fos-expression between APO-SUS and APO-UNSUS rats will be correlated with the reported differences in synaptic densities in the PVH (Mulders et al. 1995) and differential stress induced ACTH levels in both types of rats (Van Eekelen et al. 1992).

MATERIALS AND METHODS

ANIMALS The present study is based on 44 male Wistar rats (weighing 200-330 g), including 6 APO-SUS and 8 APO-UNSUS rats. All rats were bred in our Animal Laboratory and originally housed in groups of 2-3 animals per cage (36 × 24 × 25 cm) in a room with a constant temperature (20 ± 2°C) and a 6.00 am to 6.00 pm light period. Food and water were given ad libitum. All rats (except for 3 rats used for CRH-immunocytochemistry) were isolated in separate cages three days before the experimental procedure. Bilateral adrenalectomy (ADX) was performed in a number of rats under ether anaesthesia 4 weeks before the experimental procedure (rats weighing 170 ± 10 g at time of ADX). The ADX rats were given saline (0.9% sodium chloride in aqua dest) instead of water.

EXPERIMENTAL PROCEDURES In order to investigate whether Fos-immunoreactivity (Fos-IR) in the PVH shows interline variation between APO-SUS and APO-UNSUS rats and whether this Fos-IR is expressed by the CRH-cell population, we applied different experimental procedures to 5 groups of rats. We investigated Fos-IR after open field stress in the PVH of normal Wistar rats (group A), as well as in the PVH of APO-SUS and APO-UNSUS rats (group B). The relation between Fos-IR and CRH-IR was studied in ADX rats, since untreated rats do not show distinct CRH-IR in the PVH. The PVH of ADX Wistar rats was stained for either Fos (group C) or CRH (group D), or double-stained for Fos and CRH (group E). In more detail the experimental groups were treated as follows:

Group A: Three Wistar rats were placed for 15 minutes on an open field (open field stress; see Cools et al. 1990) and then returned to their home cages. 60 minutes later these experimental rats were perfused and processed for Fos-IR (see below). Three control Wistar

rats were handled for 15 seconds, returned to their home cages for 15 minutes, handled again for 15 seconds and left in their home cages for the next 60 minutes. Subsequently, they were perfused and processed in the same way as group A.

Group B: Three APO-SUS (F14 generation, 240-330 g) and five APO-UNSUS rats (F14 generation, 200-330 g) were treated similarly as the experimental rats in group A. Three APO-SUS (F18 generation, 190-230 g) and three APO-UNSUS rats (F18 generation, 220-250 g) control rats were treated similarly as the controls of group A.

Group C: Three ADX rats were treated similarly as the experimental rats in group A, and three ADX rats were treated similarly as the control rats of group A.

Group D: Three ADX rats were perfused and processed for CRH-IR (see below), without open field stress.

Group E: Three ADX rats were treated similar as group C, but the sections obtained from the PVH were double-stained for Fos and CRH (see below).

TISSUE PROCESSING For perfusion, rats were deeply anaesthetized with pentobarbital (6 mg/100 g bodyweight) and transcardially perfused with 100 ml saline (0.9% sodium chloride) followed by 450 ml 4% paraformaldehyde (PF, group D and E) or 2% PF (group A, B, and C) in 0.1 M phosphate buffered saline (PBS, pH 7.3). Immediately after perfusion, they were decapitated, and the dorsal part of the skull was removed. The heads were placed in a stereotactic device, and a transverse incision was made to allow sectioning of all brains in the same transversal plane. After removal of the brains out of the skull, they were placed overnight in the same fixative as used for perfusion.

The procedure just described resulted from comparing different fixation protocols after intraperitoneal injections of a hypertonic salt solution (NaCl, 1.5 M, 1 ml/100 g bodyweight), a stimulus inducing intense Fos-IR in the PVH (Veening et al. 1993). For this purpose rats were perfused transcardially with 2% or 4% PF in 0.1 M PBS (pH 7.3), followed by a postfixation overnight (18 hours) or for 42 hours. Quantitative analysis showed that 2% PF with 18 hours postfixation yields 1876 ± 693 Fos-IR nuclei in the PVH, 2% PF with 42 hours postfixation 1766 ± 292 Fos-IR nuclei, 4% PF with 18 hours postfixation 1057 ± 143 Fos-IR nuclei and 4% PF with 42 hours postfixation only 735 ± 204 Fos-IR nuclei. Consequently, we decided to use 2% PF with a postfixation overnight, since a further increase of PF concentration as well as fixation time results in a reduction of Fos-IR.

For visualization of Fos-IR, sections of 75 μm were cut on a vibratome in PBS (pH 7.3). After rinsing (one hour in PBS), sections were pre-incubated with 5% normal horse serum, 0.5% Triton X-100 and 0.1% bovine serum albumin (BSA) in PBS for one hour. Subsequently, the sections were incubated overnight at room temperature with a sheep polyclonal Fos antiserum, diluted 1:2,000 (Cambridge Research Biochemicals, Inc., Wilmington). After rinsing, sections were incubated for 90 minutes with a horse anti-sheep antibody (1:100 in PBS) and rinsed again. Next, the sections were treated for 90 minutes with sheep-peroxidase-anti-peroxidase (sh-PAP; Nordic) diluted 1:600 in PBS. After additional rinsing, sections were pre-incubated for 10 minutes in 0.02% 3,3'-diaminobensidine-4HCl (DAB, Sigma), 0.3% nickel ammonium sulphate in a 0.05 M Tris(hydroxymethyl)-aminomethane solution (pH 7.6), followed by an incubation for 10 minutes in the same solution containing 25 μl H_2O_2 (DAB reaction). Subsequently, the sections were mounted on gelatine coated slides, dried overnight in a stove of 37°C, dehydrated and coverslipped with Entellan.

For visualization of CRH-IR, vibratome sections (75 μm) were pre-incubated with normal goat serum, 0.5% Triton X-100 and 0.1% BSA in PBS for 1 hour and subsequently incubated overnight at room temperature with a rabbit polyclonal CRH antiserum diluted 1:1,000 (for characterization see below). After rinsing, sections were incubated for 90 minutes with a goat anti-rabbit antibody (1:100 in PBS), rinsed again and treated for 90 minutes with rabbit-PAP diluted 1:600 in PBS. After rinsing, sections were treated for a DAB reaction. When double-staining (Fos followed by CRH) was performed, the DAB solution for the CRH-staining did not contain nickel ammonium sulphate, which resulted in black nuclei and brown cytoplasm. After the DAB reaction, sections were mounted as described above.

CHARACTERIZATION OF THE CRH-ANTISERUM The antiserum (8Bo) was raised in a rabbit against $rCRH_{1-41}$ conjugated to thyroglobulin. For immunization 25 μg equivalent CRH (approx. 160 μl CRH-conjugate) plus 340 μl PBS was mixed with 500 μl Freunds complete adjuvant (FCA) and injected intramuscularly and subcutaneously. After 1 month the rabbit received a boost injection with CRH-conjugate plus FCA as above. The antiserum was characterized by 'immunospotting' and immunocytochemistry.

Immunospotting: $r/hCRH_{1-41}$ solutions of 10^{-9} to 10^{-4} M in distilled water were spotted (1 μl) on nitrocellulose filter (pore size 0.45 μm, Schleicher and Schuell). After drying (5 minutes) the filters were fixed in freshly prepared 4% PF in 0.1 M PB (pH 7.6). After washing (3×) in 0.01 M PBS (pH 7.6) the filters were incubated for 1h with 8Bo diluted 1/100 or 1/500 in PBS containing 0.5% BSA and 0.1% Tween 20 (incubation buffer). After washing (3×) in PBS with 0.1% Tween 20 (PBS-Tween), the filters were incubated for 30 minutes with goat anti-rabbit antiserum (Nordic) in incubation buffer. The filters were washed (3× PBS-Tween) and incubated for 30 minutes with rabbit PAP-complex (DAKO) in incubation buffer, washed (2× PBS-Tween and 1× 0.1 M Tris/HCl buffer) and subsequently stained with 0.1 M DAB in Tris/HCl containing 0.05% H_2O_2. All washes and incubations were performed at 21°C ± 1). 8Bo showed an antibody-concentration dependent staining of CRH. Spots of CRH showed a concentration dependent staining from 10^{-8} to 10^{-5} M CRH; staining of 10^{-6} M CRH was completely abolished by preincubation of 8Bo with 10^{-6} M CRH (2h, 37°C). No staining was observed with vasopressin, oxytocin or αMSH spots (up to 10^{-3} M).

Immunocytochemistry: vibratome sections (50 μm) of immersion or perfusion fixed tissues showed an excellent signal to background ratio with 8Bo dilutions of 1/400 to 1/800 (incubations overnight at 4°C in a 0.1 M Tris buffer, pH 7.6, containing 0.2% BSA, 0.2% NGS, 0.1% NaN_3 and 0.1% Triton X-100) and subsequent PAP/DAB procedures. After colchicine treatment (50 μg colchicine in 10 μl saline, injected i.c.v., survival 24h) strong CRH immunostaining was found in the PVH, the external layer of the median eminence (ZEME), the stria terminalis (ST) and the central amygdala (CA). Weak to moderate staining was observed in neurons of the nucleus supraopticus (SON), the periventricular nucleus (NP), the bed nucleus of the stria terminalis (BNST) and in some scattered cells in the lateral hypothalamic area (LHA). The observed staining patterns are similar to those reported for several other CRH antibodies (Cummings et al. 1983, Swanson et al. 1983, Sawchenko et al. 1993), and correlate well with reported distribution of CRH mRNA in hypothalamic nuclei (Beyer et al. 1988). All immunostaining is completely blocked by pre-incubation of 8Bo with 10^{-5} $r/hCRH_{1-41}$ (2h, 37°C), whereas no inhibition was seen after pre-incubation with up to 10^{-3} M vasopressin or αMSH. No immunostaining was found in control sections in which the first antibody was omitted.

QUANTITATIVE AND STATISTIC ANALYSIS All Fos-IR nuclei and CRH-IR neurons within the left PVH of the experimental and control animals were drawn with the aid of a Zeiss light microscope and drawing tube (magnification used for single stained sections: 125×; for double stained sections: 312.5×). To determine their number and distribution within the PVH, the immunopositive cells were plotted in an atlas containing 8 levels of the PVH (fig. 2). Subdivisions of the PVH were delineated on the basis of our previous morphometric results (Mulders et al. 1995). Since darkly as well as lightly stained Fos-IR nuclei and/or CRH-IR cells were plotted without distinction, the quantitative results reflect only the numbers of neurons and not the intensity of staining. Statistical analysis was performed using the Mann-Whitney U test.

RESULTS

The numbers of Fos-IR nuclei and CRH-IR neurons observed in the left PVH of the different experimental and control groups are summarized in table I. Details on their distribution and intensity of staining are described in detail in the following paragraphs and are visualized in figures 1, 2 and 3.

FOS-IR AFTER OPEN FIELD STRESS In normal Wistar rats that were exposed to open field stress, 1360 ± 21 Fos-IR nuclei were counted in the PVH (group A), whereas in the control group only 261 ± 74 Fos-IR nuclei were found. This statistically significant difference ($p < 0.05$) occurs in all subdivisions of the PVH with the exception of the ventral parvocellular part (PCv) (table I, fig. 1C; for delineation of PVH subdivisions, see Mulders et al. 1995). The Fos-IR nuclei are not homogeneously distributed over the different subdivisions in the PVH. The central parvocellular part (PCc) contains about 63% of the total number of Fos-IR nuclei, the periventricular part (PV) 10%, the magnocellular part (MC) 9%, the parvocellular dorsal part (PCd) 4%, the PCv 5% and the parvocellular posterior part (PCp) 9% (fig. 1C). The control group shows a somewhat different distribution: the PCc contains 42%, the PV and MC 17%, the PCd 6% and the PCv and PCp, respectively 5% and 13% of the total number of Fos-IR nuclei (fig. 1C).

Similar to normal Wistar rats, APO-SUS and APO-UNSUS rats show a statistically significant ($p < 0.05$) increase in the number of Fos-IR nuclei after open field stress (APO-SUS: 1255 ± 49 versus 249 ± 62 in controls, APO-UNSUS: 1832 ± 201 versus 227 ± 39 in controls). This statistically significant difference is present in all subdivisions of the PVH (table I, figs. 1A and 1B). Moreover, the PVH of APO-UNSUS rats contains significantly more Fos-IR nuclei after open field stress than the PVH of APO-SUS rats ($p < 0.05$; figs. 1A, 1B and 2), but not after control handling. The numerical difference of Fos-IR after open field stress is corroborated by the higher intensity of the Fos-IR in APO-UNSUS rats compared to APO-SUS rats (fig. 3). The difference in Fos-IR between APO-SUS and APO-UNSUS rats finds its origin predominantly in the PCc (figs. 1A, 1B, 2 and 3), the only subdivision of the PVH that shows a statistically significant difference in the number of Fos-IR nuclei between the two rat lines: It contains 1157 ± 152 Fos-IR nuclei in APO-UNSUS rats but only 695 ± 37 Fos-IR nuclei in APO-SUS rats ($p < 0.05$), numbers representing 63% and 56% of the total number of Fos-IR nuclei in the PVH, respectively (figs. 1A and 1B).

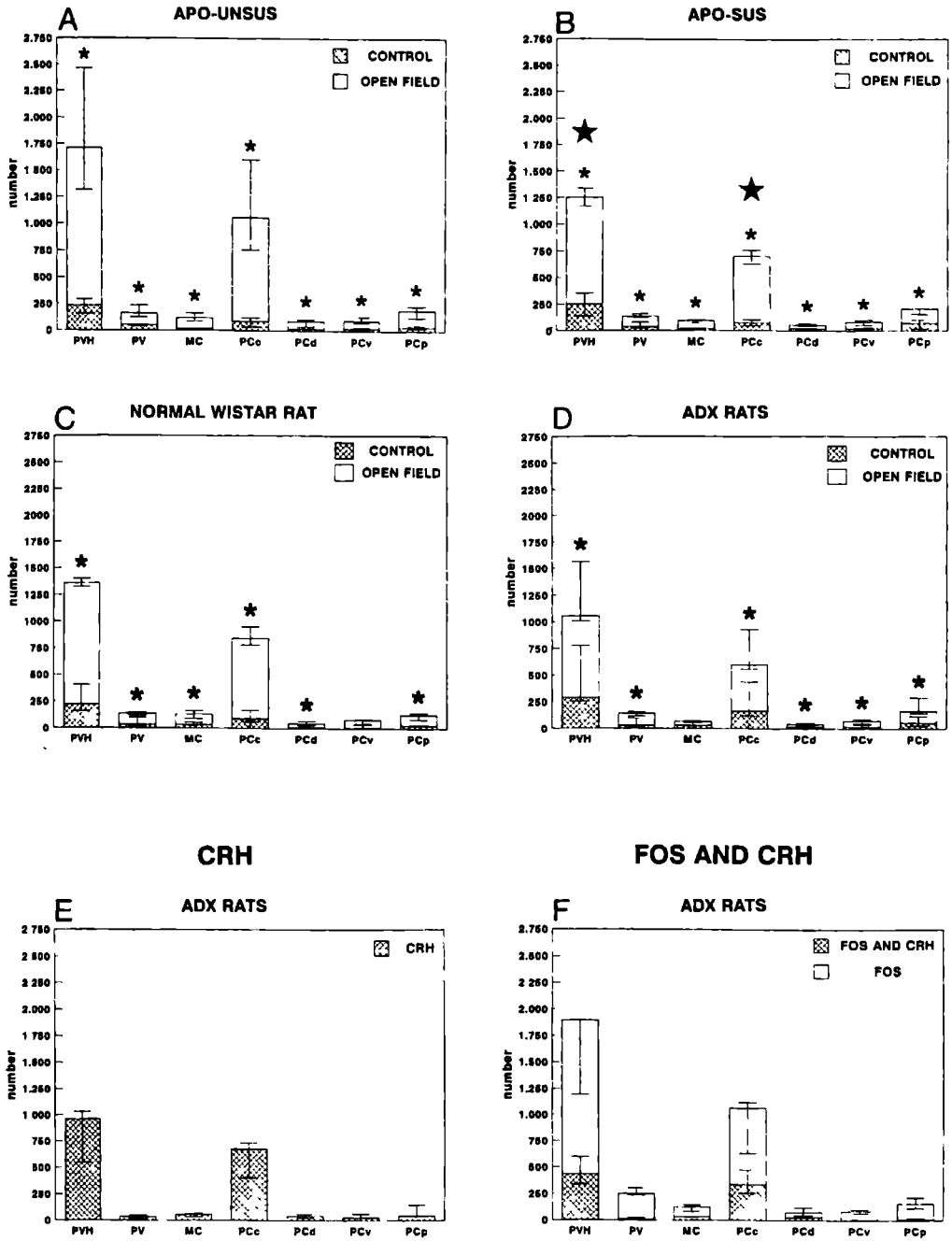


FIGURE 1 Histograms of the number of Fos-IR nuclei in the left PVH and its six subdivisions after different experimental procedures: Fos-IR after open field stress in APO-UNSUS rats (A), APO-SUS rats (B), normal Wistar rats (C) and ADX rats (D); E: CRH-IR after ADX and F: Fos-IR nuclei and double stained (CRH and Fos) neurons after an open field stress (median value ± highest and lowest value, n = 3 except for the APO-UNSUS group: n = 5). * = significantly different ($p < 0.05$) from the corresponding control group. ★ = significantly different from the corresponding APO-UNSUS open field group. PVH = hypothalamic paraventricular nucleus. PV = periventricular part. MC = magnocellular part. PCc = central parvocellular part. PCd = dorsal parvocellular part. PCv = ventral parvocellular part. PCp = posterior parvocellular part.

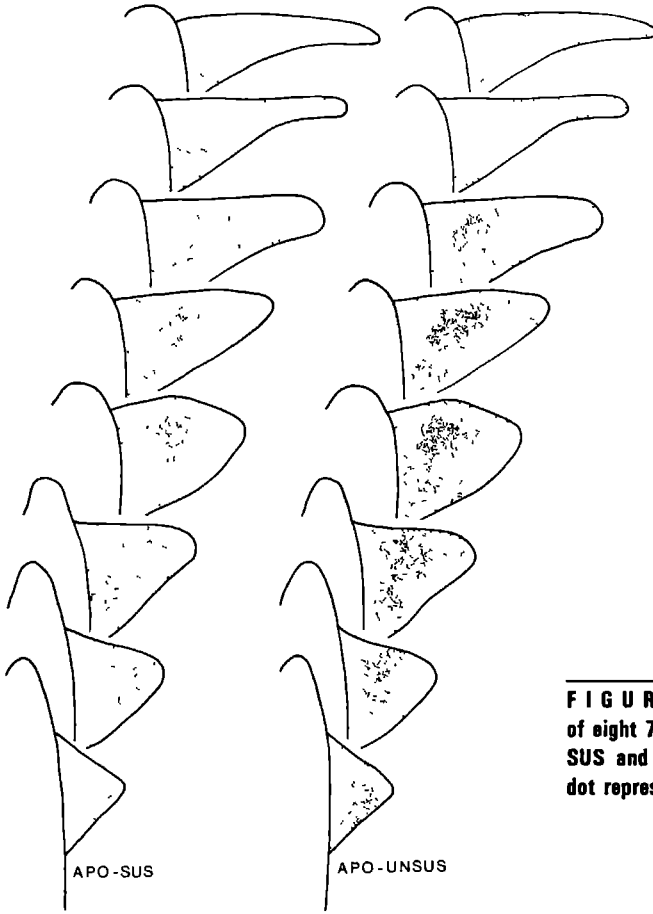


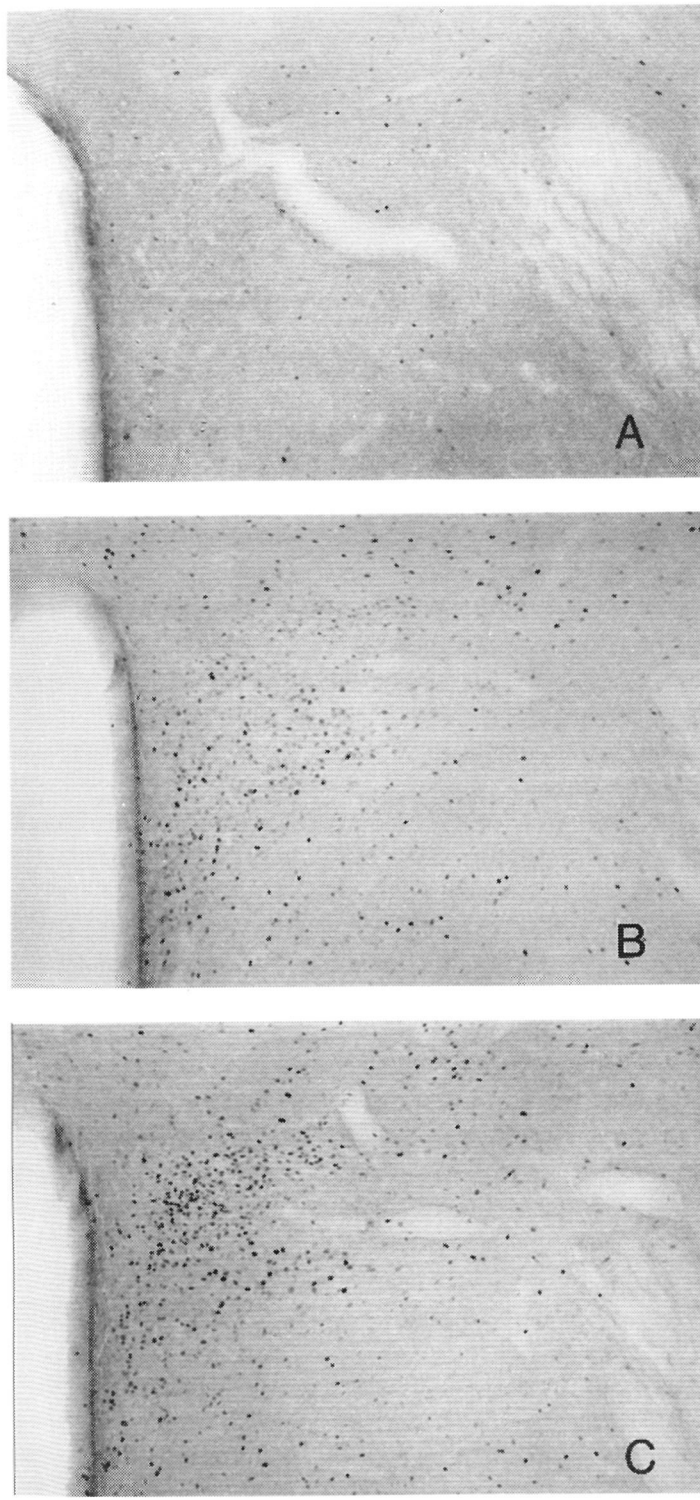
FIGURE 2 **Drawings of two representative series of eight 75 μm thick sections of the left PVH of an APO-SUS and an APO-UNSUS rats, stained for Fos-IR. Each dot represents one Fos-IR nucleus. Magnification 52×.**

The total number of Fos-IR nuclei in the PVH after open field stress and control handling of both APO-SUS as well as APO-UNSUS rats is not statistically significant from that of the overall population of Wistar rats (table I). Likewise, the distribution of Fos-IR nuclei over the different subdivisions of the PVH after open field stress and control handling is basically similar in normal Wistar rats and APO-UNSUS rats (cf. figs. 1A and 1C). In contrast, APO-SUS rats show some statistically significant differences in the number of Fos-IR nuclei after open field stress compared to normal Wistar rats: The number of Fos-IR nuclei in their PCc is slightly but significantly smaller (APO-SUS: 695 ± 37; normal Wistar: 858 ± 50; $p < 0.05$) and in their PCp slightly but significantly larger (APO-SUS: 194 ± 18; normal Wistar: 119 ± 18; $p < 0.05$).

Control ADX rats have a similar number and distribution of the Fos-IR nuclei in the PVH as control Wistar rats (ADX Wistar: 440 ± 169; intact Wistar: 261 ± 74). Likewise, ADX rats that have been subjected to open field stress (group C) show a similar Fos-IR in the PVH (1209 ± 178) as the normal Wistar rats (1360 ± 21) after open field stress. Apparently, the increase of Fos-IR in the PVH of ADX rats induced by open field stress (from 440 ± 169 to 1209 ± 178), is similar to that induced in normal Wistar rats (cf. figs. 1C and 1D).

TABLE I Number of CRH-IR cells, Fos-IR nuclei or double-stained cells in the 5 different experimental groups (mean ± SEM)

Exp. group				PVH	PV	MC	PCc	PCd	PCv	PCp
Fos-IR										
A:	Normal Wistar	open field	n = 3	1360 ± 21	135 ± 8	128 ± 21	858 ± 50	51 ± 9	68 ± 15	119 ± 18
	Normal Wistar	control	n = 3	261 ± 74	50 ± 25	39 ± 8	109 ± 31	15 ± 8	20 ± 13	29 ± 1
B:	APO-SUS	open field	n = 3	1255 ± 49	140 ± 10	89 ± 8	695 ± 37	56 ± 5	80 ± 14	195 ± 19
	APO-SUS	control	n = 3	249 ± 62	49 ± 16	18 ± 1	75 ± 16	22 ± 3	22 ± 5	63 ± 25
	APO-UNSUS	open field	n = 5	1832 ± 201	170 ± 21	140 ± 15	1157 ± 152	82 ± 11	106 ± 12	194 ± 18
	APO-UNSUS	control	n = 3	227 ± 39	48 ± 4	18 ± 2	82 ± 25	18 ± 2	22 ± 7	39 ± 7
C:	ADX	open field	n = 3	1209 ± 178	137 ± 11	64 ± 4	696 ± 119	41 ± 6	70 ± 12	200 ± 45
	ADX	control	n = 3	440 ± 169	49 ± 25	37 ± 10	242 ± 100	20 ± 5	23 ± 7	68 ± 16
CRH-IR										
D:	ADX	control	n = 3	846 ± 152	32 ± 9	52 ± 9	605 ± 102	42 ± 10	36 ± 12	79 ± 35
Fos-IR + CRH-IR										
E:	ADX	open field	n = 3	457 ± 131	15 ± 5	33 ± 1	359 ± 64	32 ± 6	6 ± 2	13 ± 5
	Single Fos-IR	open field	n = 3	1661 ± 234	266 ± 20	120 ± 19	942 ± 154	82 ± 21	87 ± 11	164 ± 29
	Single CRH-IR	open field	n = 3	489 ± 70	15 ± 6	38 ± 2	380 ± 60	37 ± 6	6 ± 2	13 ± 5



CRH-IR AND FOS-IR AFTER ADX Four weeks after ADX a large number of darkly stained CRH-IR neurons can be observed in the PVH (846 ± 152; table I; group D). Most of these CRH-IR neurons (72%) are located in the PCc (605 ± 102; fig. 1E). The PV, MC, PCd, PCv and PCp contain 32 ± 9 (4%), 52 ± 9 (6%), 42 ± 10 (5%), 36 ± 12 (4%) and 79 ± 34 (9%) CRH-IR neurons, respectively. The total number of CRH-IR neurons in the PVH after ADX is significantly lower than the number of Fos-IR nuclei in ADX rats after an open field test (1209 ± 178; $p < 0.05$). However, their distribution over the different subdivisions is similar (cf. figs. 1D and 1E). Double staining for Fos and CRH in the PVH of ADX rats after exposure to open field stress (group E), revealed 1661 ± 234 Fos-IR nuclei and 489 ± 70 CRH-IR neurons of which 457 ± 131 were double stained (table I, fig. 1F). So, 93% of the CRH-IR neurons contained a Fos-IR nucleus after double-staining, but only 28% of the total number of Fos-IR nuclei was located in a

FIGURE 3 **Photomicrographs of vibratome (75 μm) sections of the left PVH showing Fos-IR of a control rat (A), and an APO-SUS (B) as well as an APO-UNSUS rat (C) after open field stress. Magnification 89×.**

CRH neuron. The most extensive double labeling was found in the PCc (38%) and PCd (38%). In the double-staining experimental group the total number of Fos-IR nuclei is substantially higher than in single stained ADX rats after open field stress, and the number of CRH-IR neurons is considerably lower than in the control ADX animals, although both not statistically significant. Most likely, technical aspects of the double-staining procedure are involved in these differences.

DISCUSSION

The present study investigates the role of CRH neurons in stress regulation by a comparison of stress-induced Fos-IR and CRH-IR in the PVH of normal Wistar, APO-SUS, APO-UNSUS and ADX rats. To evaluate the functional significance of the results, they will first be compared with previous studies that determined Fos-IR in the PVH after stressful stimuli and secondly with previous studies on the number and distribution of CRH cells in the PVH. Finally, the significance of the differences in Fos-IR between APO-SUS and APO-UNSUS rats will be discussed in relation to the reported differences in stress responses, stress-induced ACTH levels and the synaptic organization of the PVH.

STRESS-INDUCED FOS-IR Open field stress proves to be an adequate stimulus to induce Fos-IR in the PVH of normal Wistar, APO-SUS and APO-UNSUS rats since a five-to-eight-fold increase in Fos-IR nuclei was found compared to controls, yielding 1200-1800 Fos-IR nuclei per PVH (table I). This response is stronger than observed previously after different stressful stimuli. Duncan et al. (1993) counted 840 Fos-IR cells in rats subjected to swim stress and 36 Fos-IR cells in controls. Other stimuli that have been applied to induce Fos-IR in the PVH are immobilization and painful stress, inducing 1093 and 958 Fos-IR cells, respectively, in the PVH (Senba et al. 1993). These numbers are lower than counted in the present study, which is surprising since an open field is considered to be a mild stressor compared to immobilization and painful stress. However, the differences with other studies are most probably due to histotechnical factors such as the use of different perfusion fluids and postfixation periods, which are known to influence immunohistochemical staining. We have shown that higher concentrations of paraformaldehyde and increased postfixation periods decrease the number of Fos-IR nuclei (see Materials and Methods).

In all rats used in the present paper the majority of Fos-IR nuclei in the PVH is situated in the PCc, both after open field stress (63%) and after control handling (43%, fig. 1C). This agrees with previous studies, which report a majority of Fos-IR neurons in the dorsal medial parvocellular part after immobilization or pain stimulation (Ceccatelli et al. 1989, Senba et al. 1993). This subdivision is comparable with our PCc (Mulders et al. 1995).

To study the colocalization of Fos-IR and CRH-IR we used ADX rats, since untreated rats show little or no visible CRH-IR in the PVH. ADX rats show a similar response as normal Wistar rats with respect to the distribution of Fos-IR nuclei in the PVH both after control handling and after open field stress. (cf. figs. 1C and 1D). This is in agreement with the study of Wintrip et al. (1993), who found induction of Fos-IR in the parvocellular part of the PVH 4 hours after ADX, but little or no Fos-IR 24 hours after ADX. Jacobson et al. (1990) report that Fos-IR in the PVH is enlarged up to 7 days after ADX.

COMPARISON OF FOS-IR AND CRH-IR Four weeks after ADX we found a similar number of CRH-IR neurons in the PVH (846 ± 152) as Swanson et al. (1983), who counted approximately 750 CRH neurons in the PVH of ADX rats. In agreement with previous studies (Swanson et al. 1983, Sawchenko 1987), we observed that the majority of CRH-IR neurons, about 72%, is localized in the PCc (table I; fig. 1E).

The present study shows that the distribution of CRH-IR neurons after ADX is similar to that of Fos-IR nuclei after an open field stress (cf. figs. 1C and 1D). Most Fos-IR nuclei as well as CRH-IR neurons are observed in the PCc (63% and 72%, respectively) and only a minor portion in the other subdivisions. This suggests that the Fos-IR evoked in the PVH after open field stress is predominantly localized in the CRH-IR neurons, since other types of neurons (e.g. vasopressinergic, oxytocinergic etc.) have different distributions in the PVH (Lightman and Young 1987, Kiss 1988, Dohanics et al. 1990). Double-staining corroborated a relationship between Fos and CRH, since 93% of the CRH-IR neurons contained a Fos-IR nucleus (table I), which implies that almost all neurons that display CRH-IR after ADX are activated by open field stress. This agrees with previous studies showing colocalization of Fos and CRH, or c-fos mRNA and CRH mRNA after immobilization stress (Beyer et al. 1988, Imaki et al. 1992).

In addition to the CRH neurons that are detectable after ADX, other neurons appear to be activated as well by open field stress, since after double-labeling only 28% of all Fos-IR nuclei in the PVH (and 38% in the PCc) is localized in a CRH-IR neuron and consequently 72% of all Fos-IR was observed in other neurons (fig. 1F). It is presently uncertain whether these latter neurons represent CRH neurons not responding to ADX, or belong to other types of peptidergic or non-peptidergic PVH neurons.

APO-SUS VERSUS APO-UNSUS RATS The most remarkable finding of present study is that open field stress yields substantially fewer Fos-IR nuclei in the PVH of APO-SUS rats than in the PVH of APO-UNSUS rats (table I, figs. 2 and 3). This difference originates mainly from the PCc, the only subdivision that shows a significant difference ($p < 0.05$) between the APO-SUS (695 ± 37) and the APO-UNSUS rats (1157 ± 152) (figs. 1A and 1B).

The reduced Fos activation in the PVH of APO-SUS rats is correlated with an increased synaptic density (Mulders et al. 1995), which suggests that this increased synaptic density has an increased (direct or indirect) inhibitory effect on CRH neurons. An important source of (indirect) inhibitory influences on the PVH is the hippocampus (Herman et al. 1989, Sapolsky et al. 1991, Cullinan et al. 1993). These influences are probably exerted via the bed nucleus of the stria terminalis (BNST), since the hippocampus projects to the GABA-ergic neurons in the BNST, which in turn are known to project to the PVH (Cullinan et al. 1993). So, the reduced Fos-IR in APO-SUS rats could point to an increased (inhibitory) hippocampal-BNST influence on the PVH in APO-SUS rats, compared with APO-UNSUS rats. The observation that the hippocampus of APO-SUS rats contains 50% more mineralocorticosteroid receptors than the hippocampus of APO-UNSUS rats (De Kloet et al. 1987, Sutanto et al. 1989, Cools et al. 1990, Cools et al. 1993b) is in line with this suggestion, but other structures and neurotransmitters may be involved as well. The PVH is known to receive an inhibitory input from serotonin-containing neurons in the midbrain raphe nuclei (Sawchenko et al. 1983, Saphier and Zhang 1993) and an important noradrenergic input (Swanson et al. 1981, Ginsberg et al. 1994), arising mainly from the brain stem (Saphier 1989).

The influence of noradrenaline on the PVH may also be inhibitory (Suda et al. 1987), although stimulatory effects on the hypothalamo-pituitary-adrenal axis have also been described (Assenmacher et al. 1989).

Remarkably, the reduced Fos-IR in the PVH of APO-SUS rats after open field stress is at variance with the observation that a conditioned emotional stimulus induces a higher plasma ACTH level in APO-SUS rats compared with APO-UNSUS rats (Van Eekelen et al. 1992, Rots et al. 1995), and with the higher responsiveness of the hypothalamo-pituitary-adrenal axis to stress in the APO-SUS rats (Cools et al. 1990, Cools et al. 1993b, Rots et al. 1995). So, stressed APO-SUS rats seem to combine a reduced activity of CRH neurons with an increased ACTH release compared with APO-UNSUS rats, which is surprising in view of the stimulatory effect of CRH on ACTH release. Several mechanisms may be involved in this discrepancy. E.g., in APO-SUS rats the negative feedback of corticosteroids on the ACTH release may be weaker than in APO-UNSUS rats, as may be due to differences in number and/or properties of hypophyseal corticosteroid receptors (Rots et al. 1995). Likewise, there may be a differential regulation of synthesis and release of CRH in APO-SUS and APO-UNSUS rats. Increased synthesis, which is visualized by Fos-IR (Morgan and Curran 1991, Schilling et al. 1991), does not necessarily indicate a simultaneously increased release, as has been shown for several peptides (Hanley and Wellings 1985, Van Strien et al. 1993). Further research has to be carried out to investigate the differential stress regulation in APO-SUS and APO-UNSUS rats in more detail.

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Chapter 4

The number and distribution of activated corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus under different experimental conditions: A qualitative and quantitative light microscopical study

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SUMMARY

Corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVH) play an important role in the stress response. They stimulate the release of adrenocorticotropic hormone (ACTH) from the pituitary and consequently regulate the production of corticosteroids in the adrenal glands. The activation of the CRH neurons is regulated by a variety of factors, including corticosteroid feedback, the immunesystem and afferent input from several brain structures. The present study compares the number and distribution of activated CRH neurons in the PVH following different experimental procedures likely to stimulate the CRH neurons. These procedures include adrenalectomy (ADX), colchicine injections, interleukin treatment and anti-CRH injection. In this way we tried to determine whether different regulation mechanisms activate different CRH neuron subpopulations. We showed that ADX results in a two-fold increase in the number of activated CRH neurons as compared to untreated rats. This CRH neuronal activation was particularly observed in subdivisions which contain large amounts of neurons projecting to the median eminence. It appeared that anti-CRH mimics the effect of ADX. Colchicine treatment results in the visualization of again twice as much CRH neurons as ADX, probably staining all CRH neurons also that ones which are not involved in the stress regulation. We failed to show an effect of intracerebroventricular interleukin injection, but, remarkably, revealed a long-term inhibitory effect of intravenous interleukin injection on the activation of the CRH neurons. This inhibitory effect of IL seems to affect only part of the CRH neurons projecting to the median eminence. The mechanism of this effect remains to be elucidated. In conclusion, in our experiments we could detect at least two subpopulations of differentially regulated CRH neurons, one which projects to the median eminence, and is thus activated by ADX and anti-CRH, and one which is not. In addition, our results suggest a third subpopulation under the influence of the immunesystem which is part of the CRH subpopulation projecting to the median eminence, since the long-term treatment of intravenous IL seems to inhibit the activity of only a part of the population of CRH neurons which can be activated by ADX and anti-CRH.

INTRODUCTION

The highest density of corticotropin-releasing hormone (CRH) neurons within the brain is found in the hypothalamic paraventricular nucleus (PVH) (Swanson et al. 1983). The largest portion of these neurons projects to the neurohemal zone of the median eminence (Lechan et al. 1980) whereas a small percentage projects to the brain stem (Swanson and Kuypers 1980). In the median eminence CRH is released into the portal pituitary vessels from where it reaches the anterior pituitary (Lennard et al. 1993). Within the pituitary gland CRH is the main regulator of the release of adrenocorticotropic hormone (ACTH) (Vale et al. 1981, Rivier et al. 1982), which in its turn regulates the production of corticosteroids in the adrenals (Van Oers et al. 1992). This cascade in the hypothalamo-pituitary-adrenal (HPA) axis plays a major role in the organism's adaptation to stressful conditions (Moldow et al. 1987, Haas and George 1988).

Corticosteroids have a negative feedback effect on the activity of the CRH neurons in the PVH (Sawchenko 1987b), but the HPA-axis is by no means a closed loop system, since several other brain mechanisms have been suggested to be involved in the regulation of CRH

neurons as well. These include limbic structures (Allen and Allen 1975, Dunn and Orr 1984, Gray et al. 1989, Herman et al. 1989) and catecholaminergic cell groups in the brain stem (McKellar and Loewy 1981, Cunningham and Sawchenko 1988, Cunningham et al. 1990). In addition, the HPA-axis interacts closely with the immunesystem. One of the actions of corticosteroids is the suppression of immunefunctions, by, among others, the decrease of the interleukin (IL) production by macrophages (Madden and Felten 1995). The other way around, IL has a potent release effect on ACTH and corticosteroids (Besedovsky et al. 1986), which may well be mediated by an activation of the CRH neurons in the PVH (Berkenbosch et al. 1987, Sapolsky et al. 1987, Veening et al. 1993).

The study of CRH neurons is complicated by their low basal activity, which makes immunohistochemical staining of these neurons difficult. Therefore, several methods have been used to increase the immunoreactivity (IR) of CRH neurons, such as injections with colchicine, which non-specifically interrupts axonal transport (Sawchenko 1987a, Rho and Swanson, 1989) and adrenalectomy (ADX), which removes the negative feedback of corticosteroids and in this way stimulates the production of CRH (Sawchenko 1987b). The latter results in an intense staining of the CRH neurons (Mulders et. al 1995, Mulders et al. submitted). Intravenous and intracerebroventricular injections with IL enhance the activity of CRH neurons as well (Berkenbosch et al. 1987, Sapolsky et al. 1987, Rivest et al. 1992, Veening et al. 1993). The question arises whether the CRH populations as made visible with these different techniques are similar, since they all interfere with different activation mechanisms. For instance, it is likely that ADX will not activate the CRH neurons which project to the brain stem, whereas colchicine will most likely affect all CRH neurons. Furthermore, it has previously been suggested that ADX visualizes two different subpopulations, one, which co-releases vasopressin (VP) and one, which does not (Whittnall 1988, Mulders et al. submitted). This observation also suggests that there are several different CRH populations present in the PVH.

In the present study we investigated the distribution and number of activated CRH neurons following different experimental procedures, in order to determine what part of the CRH population is influenced as well as the potency of the different methods used in comparison to controls. In this way we want to gain more insight in the existence of different subpopulations of CRH neurons in the PVH and their role in the activation of the HPA-axis.

MATERIALS AND METHODS

ANIMALS The present study is based on 33 male Wistar rats (weighing at time of perfusion 200-250 g). All rats were bred in our Animal Laboratory and originally housed in groups of 2-3 animals per cage (36 × 24 × 25 cm) in a room with a constant temperature (20 ± 2°C) and a 6.00 am to 6.00 pm light period. Food and water were given ad libitum. In 6 rats bilateral adrenalectomy (ADX) was performed under ether anaesthesia 4 weeks before the perfusion (rats weighing 170 ± 10 g at time of ADX). These ADX rats were given saline (0.9% sodium chloride in aqua dest) instead of water.

EXPERIMENTAL GROUPS To investigate the effects of 5 different treatments, colchicine, ADX, IL icv and anti-CRH after 2 survival times, and 2 different CRH antibodies

(5Bo and 8Bo, gifts of Drs. Tilders and Schmidt, Amsterdam, The Netherlands, for characterization of these antibodies see: Schmidt et al. 1995 and Mulders et al. 1995, respectively) with respect to the visualization of CRH neurons we formed 6 different experimental groups.

Group 1 consisted of nine control rats which were perfused and processed for CRH immunohistochemistry with either 8BO (group 1A, n = 6) or the 5Bo antibody (group 1B, n = 3), respectively.

Group 2 consisted of two groups of three rats that were adrenalectomized (ADX) and perfused four weeks later and processed with either 8Bo (group 2A) or the 5Bo antibody (group 2B).

Group 3 consisted of three rats which received an intracerebroventricular (icv) injection of 5 μl of colchicine solution (10 mg/ml), perfused 24 hours later and processed for CRH immunohistochemistry with the 8Bo antibody.

Group 4 consisted of three rats which received an icv injection with a solution of 100 ng interleukin-1ß in 10 μl saline (provided by Dr. D. Boraschi, Sclavo, Siena, Italy) and were perfused 4 hours later and processed in the same way as the groups above.

Group 5 consisted of six rats which were cannulated in the jugular vein. Seven days later (at t = -90) three of these rats were injected intravenously with a monoclonal antibody against rat CRH (anti-CRH; PFU 83, batch 9102-A) (Van Oers et al. 1989) in a volume of 1.0 ml/rat (30 nmol/ml). The other three rats were injected with normal rat IgG (NRI) in the same volume. Subsequently, (t = 0) all rats received an iv injection with IL-1β (0.5 μg in 300 μl saline). Blood samples were taken at t = 0, t = 15, t = 30, t = 60, t = 120 and t = 240 for measurement of plasma corticosterone (B) levels. (For a more extensive description of this procedure and results of the physiological measurements, see Van der Meer et al. 1996). Three days later these rats were perfused and processed in the same way as the groups above with the 8Bo antibody.

Group 6 consisted of six rats which were treated similar to group 5, but were perfused ten days after the physiological experiments and processed in the same way as the groups above.

TISSUE PROCESSING For perfusion rats were deeply anaesthetized with pentobarbital (6 mg/100 g bodyweight) and transcardially perfused with 100 ml saline followed by 450 ml 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.3). Following perfusion rats were decapitated, and the dorsal part of the skull was removed. The heads were placed in a stereotactic device, and a transverse incision was made which allowed sectioning of all brains in the same transverse plane. Subsequently, the brains were removed from the skull and placed overnight in the same fixative as used for perfusion.

For visualization of CRH-immunoreactivity (CRH-IR) 75 μm sections were cut on a Bio-Rad vibratome and rinsed in PBS. Rats were stained with either a PAP method or an ABC method. Comparison showed that both methods yield similar results with respect to number and distribution of activated CRH neurons, albeit with different antibody concentrations (as follows). For the PAP method sections were pre-incubated with normal goat serum, 0.5% Triton X-100 and 0.1% BSA in PBS for 1 hour and subsequently incubated overnight at room temperature with a rabbit polyclonal CRH antiserum diluted 1:1000 (8BO). After rinsing, sections were incubated for 90 minutes with a goat anti-rabbit antibody (1:100 in PBS), rinsed again and treated for 90 minutes with rabbit-PAP diluted 1:600 in PBS. After rinsing sections were preincubated for 10 mins. in 0.02% 3,3'-diaminobensidine.4HCl (DAB, Sigma), followed by an incubation for 10 mins. in the same solution containing 25 μl of H_2O_2 (DAB reaction).

After rinsing sections were mounted on gelatin coated slides, dried overnight in a stove of 37°C, dehydrated in a graded series of ethanol and coverslipped with Entellan.

For the ABC method, sections were incubated overnight with a rabbit polyclonal CRH antiserum diluted 1:5000 (8Bo) or 1:1000 (5Bo) in PBS containing 0.5% Triton-X and 0.1% BSA. Then sections were rinsed in PBS and incubated for 60 mins. with a biotin-conjugated donkey-anti-rabbit antibody 1:400 in PBS (Jackson Immunoresearch). After rinsing sections were incubated for two hours with the ABC complex (Vectastain ABC elite kit, Vector Laboratories) and rinsed again. Subsequently sections were treated with a DAB reaction and mounted on slides as described above.

QUANTITATIVE AND STATISTIC ANALYSIS All CRH-IR neurons within the left PVH of each animal were plotted with the aid of a Zeiss light microscope and drawing tube (magnification 125×) in an atlas containing 8 levels of the PVH (Mulders et al. 1995) (fig. 1). On the basis of these plots we determined the number of neurons in the different subdivisions of the PVH in all experimental groups. Darkly as well as lightly stained CRH neurons were plotted without distinction, which means that our quantitative results present only the numbers of neurons, but not the intensity of staining. Our global impression is, however, that numbers and intensity are rather well correlated. Statistical comparison of CRH neuron number was performed using the Mann-Whitney U test.

RESULTS

GROUP 1: CONTROL ANIMALS In the control rats stained with the 8Bo antibody (group 1A) the sections show a smudged appearance resulting from a limited staining of the cell bodies. Dendrites are only vaguely visible and axons can hardly be traced (fig. 2). However, quantification of the cell bodies is possible due to the low background staining. The use of the 5Bo antibody in control rats (group 1B) results in far more intense staining, including clearly identifiable dendrites and axons. The CRH somata are more clearly distinguishable than in group 1A (fig. 2) and can be counted easily.

We counted 414 ± 33 neurons in the whole PVH of group 1A, and twice as much neurons (864 ± 136) in the PVH of group 1B (table I). Statistical comparison

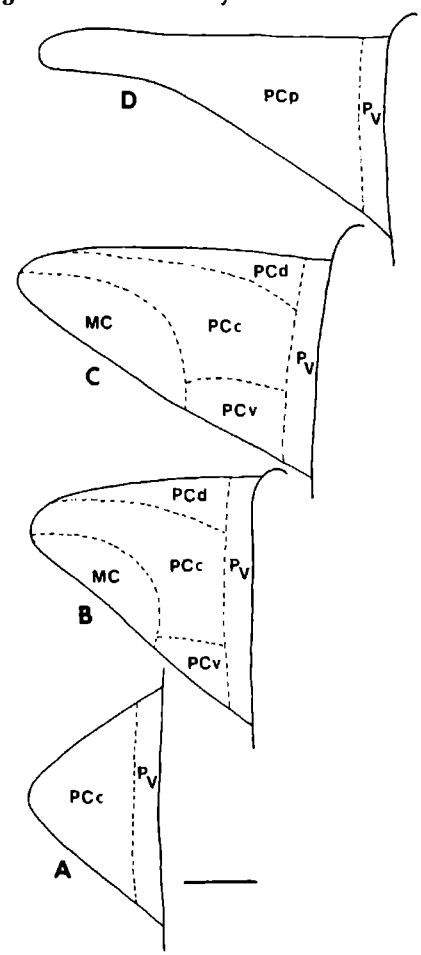

FIGURE 1 Drawings of level 1 (A), 3 (B), 5 (C) and 7 (D) of the standardized atlas of the PVH (Mulders et al. 1995) used for plotting of the CRH neurons. Level 5 (C) is the level as illustrated in the photographs in figure 2. MC = magnocellular part, PCc = central parvocellular part, PCd = dorsal parvocellular part, PCv = ventral parvocellular part. PCp = posterior parvocellular part, PV = periventricular part. Bar indicates 175 μm.

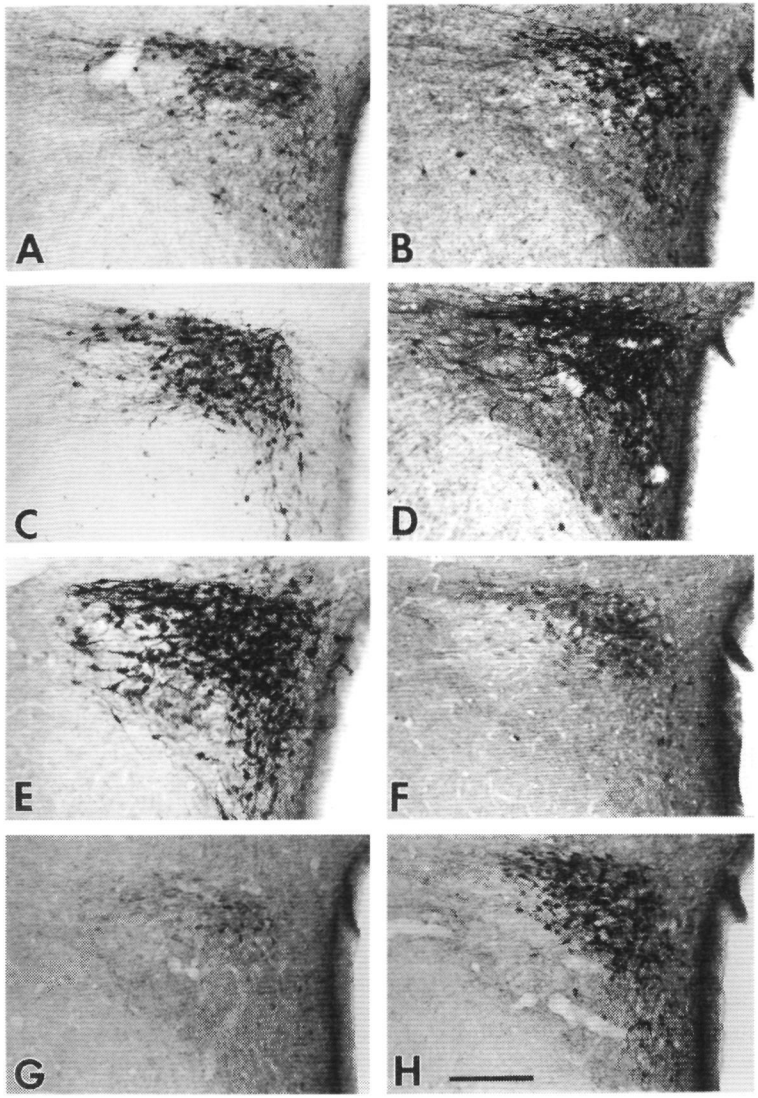

FIGURE 2 Photomicrographs of 75 μm vibratome sections of the PVH of untreated rats (A and C), ADX rats (B and D), colchicine treated rats (E), icv IL treated rats (F), NRI/IL 10d (G) and anti-CRH/IL 10d (H) either stained with a 8Bo (A,B,E,F,G,H) or 5Bo (C,D) antibody against CRH. All photomicrographs are taken at the same level of the PVH (level 5 see figure 1). Bar indicates 175 μm.

of the 2 groups shows a significant increase in the number of visible CRH neurons after treatment with the 5Bo antibody (group 1B) as compared to 8Bo (group 1A) in the overall PVH ($p < 0.01$), as well as in all subdivisions except for the posterior parvocellular part (PCp) (table I).

GROUP 2: ADRENALECTOMIZED RATS Activated CRH neurons in ADX rats can be sharply delineated, both following staining with the 8Bo (group 2A) as well as the 5Bo antibody (group 2B). These neurons typically display two or three dendrites, while their axon can be followed over a long distance coursing in the direction of the fornix, with a substantial number of varicosities. Comparison of the treatments with either the 8Bo or 5Bo antibody after ADX show a similar difference as observed in controls (fig. 2), the use of 8Bo resulting in 846 ± 152 activated CRH neurons in the whole PVH as compared to 1383 ± 78 after treatment with 5Bo ($p < 0.05$). This statistically significant difference ($p < 0.05$) is also present in the PV (32 ± 9 versus 193 ± 24), PCc (605 ± 102 versus 935 ± 42) and PCv (36 ± 12 versus 69 ± 1) (table I).

TABLE I Number and percentages of activated CRH-IR neurons in controls, ADX rats, rats injected icv with colchicine or IL, and rats treated with anti-CRH/IL or NRI/IL

Exp. group	number	PVH	PV	MC	PCc	Pcd	PCv	PCp
Group 1								
Controls: 8BO	n = 6	414 ± 33	19 ± 3	22 ± 5	318 ± 26	22 ± 5	15 ± 2	13 ± 4
			4.6	5.3	76.8	5.3	3.6	3.1
Controls: 5Bo	n = 3	864 ± 136	78 ± 21	45 ± 9	599 ± 74	61 ± 11	40 ± 8	42 ± 16
			9.0	5.2	69.3	7.1	4.6	4.9
Group 2								
ADX: 8BO	n = 3	864 ± 152	32 ± 9	52 ± 9	605 ± 102	42 ± 10	36 ± 12	79 ± 35
			3.8	6.1	71.5	5.0	4.3	9.3
ADX: 5Bo	n = 3	1383 ± 78	193 ± 24	57 ± 12	935 ± 42	69 ± 9	69 ± 1	59 ± 11
			14.0	4.1	67.6	5.0	5.0	4.3
Group 3								
Colchicine: 8Bo	n = 3	1722 ± 143	196 ± 6	104 ± 48	1013 ± 83	180 ± 8	72 ± 11	157 ± 25
			11.4	6.0	58.8	10.5	4.2	9.1
Group 4								
IL icv: 8Bo	n = 3	521 ± 117	17 ± 3	23 ± 11	422 ± 160	27 ± 10	16 ± 3	17 ± 2
			3.3	4.4	81.0	5.2	3.1	3.1
Group 5								
anti-CRH/IL: 8Bo (3d)	n = 3	542 ± 18	22 ± 2	27 ± 7	379 ± 18	39 ± 4	18 ± 3	56 ± 17
			4.1	5.0	69.9	7.2	3.3	10.3
NRI/IL: 8Bo (3d)	n = 3	296 ± 68	10 ± 4	16 ± 1	224 ± 52	20 ± 4	7 ± 4	17 ± 6
			3.4	5.4	75.9	6.8	2.4	5.8
Group 6								
anti-CRH/IL: 8Bo (10d)	n = 3	609 ± 10	11 ± 4	22 ± 5	474 ± 13	54 ± 1	15 ± 2	33 ± 6
			1.8	3.6	77.8	8.9	2.5	5.4
NRI/IL: 8Bo (10d)	n = 3	280 ± 37	11 ± 2	14 ± 1	215 ± 35	19 ± 3	5 ± 1	17 ± 2
			3.8	5.1	76.3	6.7	1.7	6.3

Comparison of the ADX treated animals with the appropriate controls (group 1A versus group 2A and group 1B versus group 2B) shows that the number of activated CRH neurons is statistically significantly increased after ADX. After treatment with 8Bo this holds true for the overall PVH ($p < 0.01$) as well as all subdivisions except for the PV and the PCd, which show no mutual significant differences (table I, fig. 3). In the groups stained with the 5Bo antibody a significant increase in the number of activated CRH neurons after ADX can be observed in the overall PVH as well as in the PV, PCc and PCv ($p < 0.05$) (table I, fig. 3).

GROUP 3: COLCHICINE TREATMENT The colchicine treated rats typically show a dilated third ventricle in comparison to the other experimental groups. The CRH neurons also appear more swollen than in the other groups and show frayed dendrites. Axons are only partly or not at all visible. Very darkly as well as lightly stained CRH neurons are observed, with a characteristic distribution over the PVH. Darkly stained neurons are observed especially in the PCc, whereas more lightly stained neurons show a preferential location in the MC, PCd and PCv (fig. 2).

Quantitative comparison with the appropriate controls (group 1A) shows a significant increase in the number of activated CRH neurons in colchicine treated rats in the overall PVH (from 414 ± 33 to 1722 ± 143, $p < 0.01$), as well as in all subdivisions. In comparison to ADX rats (group 2A) colchicine treatment results in a significantly higher number of activated CRH neurons in the overall PVH, as well as in the PV, the PCc and the PCd ($p < 0.05$) (table I, fig. 3).

GROUP 4: INTRACEREBROVENTRICULAR INTERLEUKIN (ICV IL) INJECTION The overall view of the PVH sections obtained from the rats which received an icv IL injection shows a similar result as the untreated control rats (group 1A). The PVH has a smudged appearance and cells can not be sharply outlined, whereas dendrites and axons are only faintly visible (fig. 2). In line with these qualitative observations the numbers of CRH neurons counted are similar to the ones for controls without statistical differences (table I, fig. 3).

GROUP 5: INTRAVENOUS IL INJECTION WITH OR WITHOUT ANTI-CRH (3 DAYS) The PVH of rats treated with normal rat IgG in combination with interleukin and which were perfused 3 days later (NRI/IL 3d), shows an image similar to the untreated rats (group 1A), whereas the PVH of the rats injected with anti-CRH and interleukin perfused after the same period (anti-CRH/IL 3d) shows more pronounced CRH staining with more apparent neurons. In agreement with these observations, we found a statistically significant increase in the number of activated CRH neurons in the anti-CRH/IL 3d (542 ± 18) compared to the NRI/IL 3d (295 ± 68), both with regard to the overall PVH as well as to the PV, PCc and PCd ($p < 0.05$) (table I, fig. 3). Quantitative comparison of the controls (group 1A) with the NRI/IL 3d rats shows a significant decrease in the latter group for the overall PVH ($p < 0.02$), as well as in the PV, PCc and PCv. On the other hand, the anti-CRH/IL 3d rats show a significant increase as compared to controls in the overall number of CRH neurons ($p < 0.01$), and in the PCc, PCd and the posterior parvocellular part (PCp) ($p < 0.02$).

Comparison with the ADX rats (group 2A) shows a higher number of activated CRH neurons following ADX than after NRI/IL 3d both in the overall PVH as well as in all subdivisions ($p < 0.05$), with an exception of the PV. Comparison of ADX rats with anti-CRH/IL 3d rats shows that both treatments result in a similar high amount of activated CRH neurons. The only significant difference was observed in the PCc ($p < 0.05$) (table I).

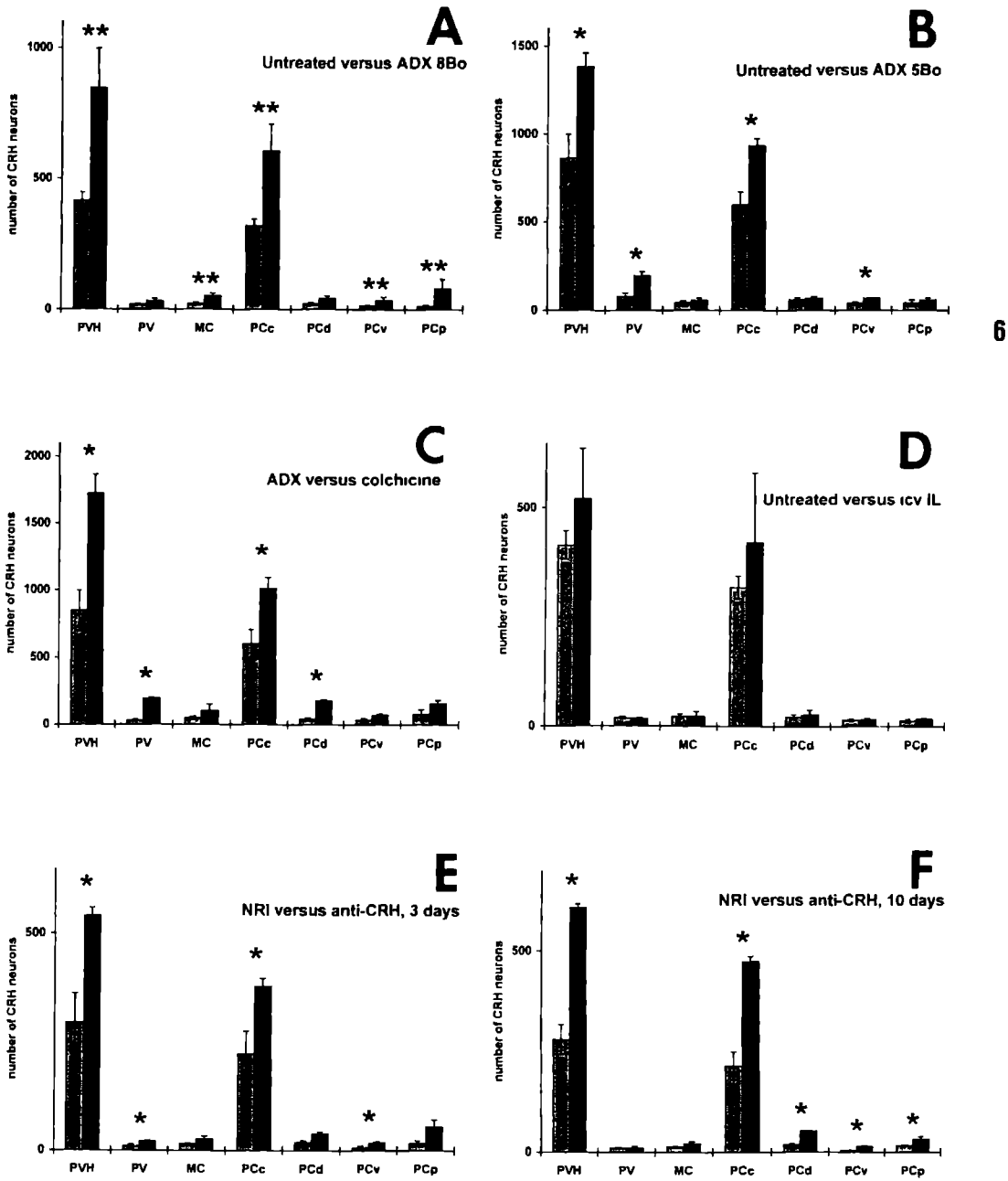

FIGURE 3 Histograms of the number of CRH neurons and SEM in the left PVH and its subdivisions after different experimental procedures: untreated rats versus ADX rats stained with the 8Bo antibody (A), untreated rats versus ADX rats stained with the 5Bo antibody (B), ADX rats versus colchicine treated rats (C), untreated versus icv IL treated rats (D), NRI/IL 3d rats versus anti-CRH/IL 3d rats (E) and NRI/IL 10d rats versus anti-CRH/IL 10d rats (F). Grey bars represent first mentioned group, black bars represent secondly mentioned group in histograms. * = significantly different ($p < 0.05$). ** = significantly different ($p < 0.01$). For abbreviations see figure 1.

GROUP 6: ANTI-CRH/IL AND NRI/IL (10 DAYS) TREATMENT Perfusion 10 days after NRI/IL or anti-CRH/IL (NRI/IL 10d and anti-CRH/IL 10d) treatment yields similar results as perfusion 3 days after similar treatment as described above (figs. 2 and 3). The overall number of activated CRH neurons is substantially larger in the anti-CRH/IL 10d (10 days) (609 ± 10) as compared to the NRI/IL 10d (280 ± 37), an increase which can also be observed in the PCc, PCd, PCv and PCp ($p < 0.05$) (table I, fig. 3). Quantitative comparison with the controls (group 1A) shows a decrease in the overall number of activated CRH neurons in the NRI/IL 10d rats ($p < 0.01$) as well as in the PV ($p < 0.02$), PCc ($p < 0.01$) and PCv ($p < 0.01$), similar to group 5 (NRI/IL, 3 days). The anti-CRH/IL 10d rats, however, show an increased number of activated CRH neurons compared to the controls in the overall PVH ($p < 0.01$) as well as in the PV ($p < 0.05$), PCc ($p < 0.01$), PCd ($p < 0.01$) and PCp ($p < 0.02$).

The ADX rats (group 2A) show a higher number of activated CRH neurons in the PVH compared to the NRI/IL 10d rats in the overall PVH and all subdivisions ($p < 0.05$). Compared to the anti-CRH/IL 10d rats ADX rats have an increased number of activated CRH neurons in the MC, PCv and PCp ($p < 0.05$), but not in the PV, PCc and PCd.

DISCUSSION

In the present study we investigated the effects of different experimental procedures, known to stimulate the CRH neurons in the PVH, as well as the effects of two different CRH antibodies, by quantitative comparison of the number and distribution of activated CRH neurons in the PVH. In the first paragraph we will discuss the observed difference between the two antibodies used. In the following paragraphs we will discuss the effect of the different experimental conditions.

EFFECTS OF DIFFERENT CRH ANTIBODIES

Since the number of CRH neurons is significantly increased in untreated rats with the use of the 5Bo antibody as compared to the use of the 8Bo antibody (group 1A versus group 1B), the 5Bo antibody seems to have a greater potency than the 8Bo antibody in staining CRH neurons. In addition, staining of the activated CRH neurons in ADX rats with the 5Bo antibody (group 2B) also shows an increase in the overall PVH as well as in the PV, PCc and PCv, in comparison with 8Bo treatment after ADX (group 2A). This is remarkable in view of the fact that the number of activated CRH neurons as reported previously by us in the PVH and PCc with the use of the 8Bo antibody is similar to that reported previously by other authors (Swanson et al. 1983, Sawchenko 1987a). This suggests that the difference between the two antibodies is not due to the lack of potency of the 8Bo antibody to stain all CRH neurons activated following ADX. It might be possible that the 5Bo antibody is not exclusively binding to CRH but also to other antigens in the PVH. This would be in agreement with our observation that 5Bo also stains more neurons outside the PVH. However, it is contradicted by the observation that the distribution of activated CRH neurons in 5Bo and 8Bo treated rats is similar and by the fact that this antibody does not show cross-reactivity with oxytocin, αMSH or vasopressin and by the fact that immunostaining can be prevented by pre-incubation with r/hCRH41 (Schmidt et al. 1995, Schmidt et al. 1996). Most likely, since the increase in the number of activated CRH neurons after ADX as compared to untreated rats is similar following 5Bo and 8Bo staining, 5Bo is a more sensitive antibody capable of staining

not only the CRH neurons following ADX which are involved in the functioning of the HPA-axis, but also other CRH neurons. This would explain both the higher number of CRH neurons stained in untreated as well as ADX rats with the use of the 5Bo antibody and a similar increase following ADX. However, it should be noted that the use of the 5Bo antibody in the PVH of ADX rats did not result in a similar staining as 8Bo in the PVH of colchicine treated animals and therefore, it is not likely that 5Bo stains all CRH neurons present in the PVH.

EFFECTS OF ADRENALECTOMY In this and the following paragraphs only the results obtained with the use of the 8Bo antibody will be discussed and compared. Four weeks of ADX induces a two-fold increase in the number of activated CRH neurons in the PVH as compared to untreated rats, resulting in approximately 850 neurons (group 2A, see also Mulders et al. 1995). This observation is in agreement with previous studies showing an increase in CRH-IR following ADX (Swanson et al. 1983, Sawchenko 1987b). In addition, the intensity of staining is largely enhanced in ADX rats as compared to untreated rats. As we have reported in a previous paper (Mulders et al. 1995) approximately 70% of the CRH neurons in the PVH is located in the PCc after ADX. This subdivision is also known as the major source of hypophysiotropic CRH neurons, which project to the median eminence (Rho and Swanson 1989, Lennard et al. 1993). However, the results suggest that this subdivision is not the only source of CRH neurons which respond to changes in corticosteroid levels and are thus likely to be hypophysiotropic, since also in the MC, PCv and PCp the number of CRH neurons is significantly increased after ADX. On the other hand, the number of CRH neurons in the PV and PCd is unchanged after ADX, which suggests that the neurons in these subdivisions are not involved in the regulation of the HPA-axis.

EFFECTS OF COLCHICINE Immunohistochemical staining clearly shows the destroying neuronal effect of colchicine, causing swelling of the somata, frayed dendrites and loss of axons. Nonetheless, the number of activated CRH neurons counted is similar to previous papers, resulting in about 2000 CRH neurons in one side of the brain (group 3 of the present results, Swanson and Sawchenko 1980, Sawchenko and Swanson 1985) and about 1000 in the PCc (Sawchenko 1987b). Since colchicine is an unspecific inhibitor of axonal transport, these activated CRH neurons represent most likely the total population of CRH neurons in the PVH.

In comparison to ADX colchicine induces an increase in the number of activated CRH neurons in the overall PVH as well as the PV, PCc and PCd. It is likely that the neurons activated by colchicine but not by ADX are not involved in the regulation of the HPA-axis and do not project to the median eminence. This means that about 50% of the total population of about 2000 CRH neurons does not project to the median eminence, but to other targets in the brain, since the increase after colchicine treatment is two-fold as compared to ADX. Our results indicate that the CRH neurons that do not project to the median eminence, originate mainly in the PV, PCc and PCd, since only in these subdivisions colchicine significantly increases the number of CRH neurons compared with ADX. Target structures of the CRH neurons besides the median eminence include nuclei in the dorsolateral medulla, such as the nucleus of the solitary tract, and in the spinal cord, as is shown previously by tracing experiments (Swanson and Kuypers 1980, Sawchenko 1987a).

However, according to Sawchenko (1987a) only 5.5% of the CRH neurons is involved in these descending projections, so other targets of the CRH neurons must be present as well. CRH is also present in a subset of the oxytocin neurons, which project to the posterior pituitary (Sawchenko et al. 1984). These neurons are probably also visualized by the treatment with colchicine, although our results do not show a significant increase following colchicine treatment in the MC where the oxytocin neurons are predominantly located.

EFFECTS OF INTRACEREBROVENTRICULAR INTERLEUKIN INJECTION (ICV IL) Our results show neither an increase in the number of CRH neurons, nor in the intensity of staining in the PVH following icv IL. This seems to be in conflict with other studies showing an increase in c-fos immunoreactivity in CRH neurons in the PVH following iv (Rivest et al. 1992, Veening et al. 1993, Ericsson et al. 1994) or icv (Rivest et al. 1992) IL injection, which suggests an activation of the CRH neurons in response to IL. In addition, IL has been shown to stimulate release of CRH from superfused rat hypothalamo-neurohypophyseal complexes (Ohgo et al. 1991) and a similar icv dose of IL as used in the present study significantly increased CRH mRNA levels in the PVH (Lee and Rivier 1994). These studies all indicate an activation of the CRH neurons following IL treatment.

The discrepancy between these studies and the present results may have several reasons. First of all, the time between the icv IL injection and perfusion (4 hours in the present study) might have been too limited to reveal significant differences. Lee and Rivier (1994) used a waiting period of 5 hours between the icv injection of IL and perfusion, but these authors do not mention whether this is a critical timespan. Secondly, the dose used in the present study may have been too low. However, a similar dose has been shown to be sufficient to increase plasma ACTH levels (Rivest et al. 1992) and to evoke an increase in the CRH mRNA in the PVH (Lee and Rivier 1994). Thirdly, when IL induces high synthesis as well as release of CRH, it might well be possible that immunohistochemical CRH staining is not enhanced in the PVH. In our opinion, this latter option is the most likely reason for the observed lack of an increased number of CRH neurons in this study, thus that the synthesis and release of CRH is equally enhanced after IL treatment which hampers immunohistochemical staining.

EFFECTS OF INTRAVENOUS IL INJECTION WITH OR WITHOUT ANTI-CRH (ANTI-CRH/IL AND NRI/IL) In the rats injected with NRI/IL (group 5 and 6) Van der Meer et al. (1996) report a rise in plasma corticosteroids which declined again after 240 mins. This indicates a stimulatory effect of IL on the plasma corticosteroid level, which disappears after 240 mins. The treatment with anti-CRH/IL resulted in a similar rise of plasma corticosteroids except for the first 15 mins. (Van der Meer et al. 1996), which suggests that the effect of IL on the corticosteroid level is probably only initially mediated via the PVH, since anti-CRH blocked the rise in corticosteroids only for the first 15 mins. After these first 15 mins. the IL effect must be mediated by extrahypothalamic sites, such as a direct effect on the pituitary or adrenals, as has been suggested previously by different authors (Andreis et al. 1991, Gwosdow et al. 1992, Kovac and Elenkov 1995).

Remarkably, we found a decrease in the number of activated CRH neurons in NRI/IL treated rats as compared to untreated rats both 3 and 10 days after treatment. This is surprising in view of the fact that the corticosteroid level already returned to normal after

240 mins. and that the plasma corticosteroid level increasing effect of IL is probably only initially mediated via the PVH (Van der Meer et al. 1996). The present results suggest a long-term downregulating effect of IL on the CRH neurons in the PVH. The mechanism of such an effect remains to be elucidated. It should be noted that two of the subdivisions (PCc and PCv) in which the decreased number of activated CRH neurons is found are similar to the subdivisions influenced by ADX. This suggests that at least part of the CRH neurons projecting to the median eminence is affected by long-term IL treatment. Different structures within the brain have been suggested to play a role in the regulation of CRH neurons by IL, such as the organum vasculosum of the lamina terminalis and ascending catecholaminergic projections (Weidenfeld et al. 1989, Katsuura et al. 1990). The catecholaminergic projections seem to be particularly interesting in this respect, since Barbanel et al. (1993) have shown that lesions of the dorsal part of the ventral noradrenergic ascending bundle (VNAB), arising mainly from the A2/C2 medullary nuclei, inhibits the increase of ACTH after intraarterial IL injection, whereas the ventral part of the VNAB, arising from the A1/C1 medullary nuclei stimulates this increase in ACTH. This result shows a biphasic influence of the noradrenergic medullary nuclei, which might be involved in short-term and long-term effects of IL on the CRH neurons in the PVH.

The anti-CRH/IL treated rats showed an increase in the number of activated CRH neurons as compared both to NRI/IL treated rats and untreated rats, showing a lasting effect of anti-CRH treatment, which indicates a low clearance rate of the CRH antibody from the circulation. An intravenously injection with a CRH antibody, which removes CRH from the circulation and subsequently prevents ACTH release and corticosteroid responses, and removes the corticosteroid feedback effect, results in a comparable overall number of activated CRH neurons as ADX, with only small differences in some subdivisions. These small differences might be induced by a shorter survival time of 3 or 10 days for the anti-CRH/IL treated rats as compared to the four weeks survival of ADX rats. It is likely that four weeks of CRH blockade with a CRH antibody would result in a similar number of CRH neurons as in ADX rats and our results suggest that both ADX and anti-CRH treatment interfere with the same mechanism of CRH activation.

CONCLUSIONS In conclusion, we have shown an increase in the number of CRH neurons following ADX, resulting from a decreased inhibitory effect of corticosteroids. In addition, we showed that the use of a CRH antibody is a good alternative for ADX, resulting in a significant increase in the number of CRH neurons from three days post-treatment on. Colchicine visualizes twice as much CRH neurons by unspecific inhibition of axonal transport, but has large destroying effects on the neuronal morphology. We failed to show an effect of icv IL, which is most likely due to the fact that IL stimulates synthesis as well as release of CRH. We also found that IL might have a long-term inhibitory effect on the activation of part of the CRH neurons. In particular, our results suggest that part of the CRH neurons responsive to ADX and thus most likely projecting to the median eminence, is under the influence of the immunesystem. Therefore, our experimental set-up revealed two subpopulations within the PVH, one group of CRH neurons responsive to circulating corticosteroids and thus probably projecting to the median eminence, as illustrated by ADX and anti-CRH treatment and one unresponsive, as illustrated by colchicine treatment. The subpopulation of CRH neurons projecting to the median eminence is found to be located in the MC, PCc, PCv and PCp. In addition, our results suggest that there might be a third sub-

population which exists of part of the CRH neurons responsive to circulating corticosteroids and which is under the influence of the immunesystem as indicated by the inhibitory effect observed after long-term IL treatment.

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Chapter 5

Plasticity in the stress-regulating circuit: Decreased input from the bed nucleus of the stria terminalis to the hypothalamic paraventricular nucleus following adrenalectomy

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SUMMARY

The bed nucleus of the stria terminalis (BNST) is involved in the stress-regulating circuit by funneling limbic information to the hypothalamic paraventricular nucleus (PVH). Since adrenalectomy (ADX) influences both limbic structures (by inducing cell death in the hippocampus) and the PVH (by increased corticotropin-releasing hormone (CRH) synthesis), we investigated whether the BNST is also influenced by ADX. For this purpose, we analysed and compared the projection from the BNST to the PVH in normal and ADX rats by anterograde tracer injections in the BNST. Quantitative analysis of the fiber pattern in the PVH of normal rats revealed a homogeneous distribution of BNST fibers over the different subdivisions of the PVH. In ADX rats the absolute fiber density was significantly lower in all subdivisions of the PVH ($1.17 \pm 0.27 \times 10^{-3}$ $\mu m/\mu m^3$ in ADX rats versus $2.59 \pm 0.24 \times 10^{-3}$ $\mu m/\mu m^3$ in normal rats; $p < 0.01$). The largest decrease of fiber density was found in the CRH-rich part of the PVH (ADX rats: 0.602 ± 0.106, versus 1.095 ± 0.019 in normal rats, $p < 0.01$). These results show a loss of input from the BNST to the PVH, and particularly to the CRH neurons, following ADX. The data suggest that this part of the stress-regulating circuit is marked by a substantial plasticity of the synaptic organization in adult rats and may imply that human disorders associated with corticosteroid disbalance are allied to a changed synaptic circuitry in the brain.

INTRODUCTION

Parvocellular neurons in the hypothalamic paraventricular nucleus (PVH) produce corticotropin-releasing hormone (CRH), which is involved in stress-regulation (Antoni et al. 1983, Swanson et al. 1983). After a stressful stimulus CRH is delivered to the portal vascular system of the median eminence (Antoni et al. 1983) and induces adrenocorticotropic hormone (ACTH) release from the anterior pituitary (Rivier et al. 1982, Rivier and Plotsky 1986, Antoni 1989), which, in turn, is responsible for the regulation of the corticosteroid production in the adrenal cortex (Whitnall 1993). Corticosteroids influence their own production by a negative feedback effect on CRH as well as ACTH release (Keller-Wood and Dallman 1984, Jingami et al. 1985, Spinedi et al. 1991). Consequently, removal of the adrenals by adrenalectomy (ADX) induces an increase of CRH mRNA as well as CRH levels (Sawchenko 1987).

The CRH neurons of the PVH are influenced by several limbic structures, including the hippocampus (Feldman and Conforti 1980, Dunn and Orr 1984, Herman et al. 1989, Sapolsky et al. 1991b, Feldman and Weidenfeld 1993) and the amygdala (Liang et al. 1992, Swiergiel et al. 1993). Hippocampal influence is thought to be inhibitory, since hippocampal lesions increase the expression of CRH mRNA in the PVH (Herman et al. 1989), cause hypersecretion of glucocorticoids (Sapolsky et al. 1991b) and reduce the feedback effects of dexamethasone, a type II glucocorticoid receptor agonist, on ACTH responses (Feldman and Conforti 1980). In addition, the hippocampus shows a large sensitivity to the level of circulating corticosteroids, resulting in granule cell death following ADX (Sloviter et al. 1989, Sapolsky et al. 1991a, Sloviter et al. 1993). Amygdaloid lesions block the effect of intracerebroventricular CRH (Liang et al. 1992) and cause a decrease of stress-induced plasma ACTH and corticosterone levels (Allen and Allen 1975, Beaulieu et al. 1986, Feldman et al. 1994), indicating an excitatory role for the amygdala. However, inhibitory effects of the

amygdala have been reported as well (Dunn and Whitener 1986).

Axonal tracing studies have shown no or very limited direct projections from the hippocampus and amygdala to the PVH (Tribollet and Dreifuss 1981, Oldfield et al. 1985, Oldfield and Silverman 1985, Gray et al. 1989). Consequently, these limbic structures have to exert their influence on the PVH via indirect connections. The bed nucleus of the stria terminalis (BNST) is thought to be of crucial importance in this respect. Tracing studies have shown that both the hippocampus and amygdala project to the BNST, which in turn projects to the PVH (Silverman et al. 1981, Sawchenko and Swanson 1983, McDonald 1988, Sun et al. 1991, Cullinan et al. 1993, Thellier et al. 1994). Lesion studies have shown that the stimulatory effect of the amygdala is dependent on the BNST (Feldman et al. 1990), and tracer studies have shown that GABA-ergic neurons in the BNST, which are innervated by the ventral subiculum, project to the PVH (Cullinan et al. 1993).

Since ADX influences both the hippocampus, by inducing granule cell death, as well as the PVH, by increasing the activity of CRH neurons, we were interested in the question whether other parts of the stress-regulating circuit are also influenced by ADX. We decided to investigate especially the BNST-PVH connection, since the BNST is an important relay for limbic information to the PVH. For this reason, we compared qualitatively and quantitatively the results of anterograde tracer experiments in the BNST of normal and ADX rats and combined tracer visualization with CRH immunocytochemistry.

MATERIALS AND METHODS

ANIMALS The present study is based on experiments with 41 male Wistar rats, weighing between 220 and 280 g. All rats were bred in our animal laboratory and housed two-three animals per cage (36 × 24 × 25 cm) in a room with a constant temperature (20 ± 2°C) and a 6.00 am to 6.00 pm light period. Food and water were given ad libitum. Three-four weeks before neuroanatomical tracing 28 rats (at that moment weighing 170 ± 10 g) were bilaterally adrenalectomized under ether anaesthesia and were given saline (0.9% sodium chloride in aqua dest) instead of normal drinking water after recovery.

EXPERIMENTAL PROCEDURE For neuroanatomical tracing we used 5% 3,000 MW or 10,000 MW biotinylated dextran amine (BDA, Mol. Probes, Inc.), dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.3). For tracer injection, the rats were anaesthetized intraperitoneally with pentobarbital (5 mg/100 g bodyweight) and placed in a stereotactic device. Iontophoretic injections (5 μA, 7 seconds on/off) were made for 5 to 10 minutes with glass micropipettes with a diameter between 32 and 65 μm. Following injection, the pipette was left in place for 5 minutes and then removed in small steps (0.5 mm/30 secs.) to minimize leakage of the tracer along the pipette track.

After three or seven days survival time (after injection with BDA 3,000 or 10,000 MW, respectively), the rats were deeply anaesthetized with pentobarbital (6 mg/100 g bodyweight) and transcardially perfused with 100 ml saline followed by 400 ml fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picrinic acid in 0.1 M phosphate buffer, pH 7.3. After perfusion the rats were decapitated and the dorsal part of the skull was removed. The heads were placed in a stereotactic device and a transverse incision was made to

allow sectioning of all brains in the same transverse plane. Subsequently, the brains were removed from the skull and postfixed overnight at 4°C in the same fixative.

TISSUE PROCESSING 75 μm Sections were cut with a vibratome (Bio-Rad) and rinsed in phosphate buffered saline (PBS, pH 7.4). Alternate sections were used for light microscopical analysis, which resulted in 4 sections per PVH per rat to be processed. These sections were preincubated overnight in PBS containing 0.5% Triton X-100 and 0.1% bovine serum albumin (BSA).

For visualization of BDA, a Vectastain ABC Elite kit (Vector laboratories) was used in a dilution of one drop of avidin and one drop of biotinylated horseradish peroxidase in 40 ml

0.1 M PBS (pH 7.4). Sections were incubated for two hours in the ABC complex and rinsed three times in 0.1 M PBS. Subsequently, sections were pre-incubated for 10 minutes in 0.02% 3,3'-diaminobenzidine-4HCl (DAB, Sigma), 0.3% nickel ammonium sulphate in a 0.05 M Tris (hydroxymethyl)-aminomethane solution (pH 7.6), followed by a 10 minutes incubation period in the same solution containing 20 μl H_2O_2 (DAB reaction). After rinsing in PBS, sections not to be stained for CRH were mounted on gelatine coated slides, dried overnight in a stove at 37°C, dehydrated and coverslipped with Entellan.

For visualization of CRH (after tracer visualization), sections of ADX rats were incubated overnight with a rabbit polyclonal CRH antiserum (Mulders et al. 1995b) diluted 1:10,000 in PBS containing 0.5% Triton X-100 and 0.1% BSA. After rinsing three times in PBS, sections were incubated for 60 minutes with a biotin-conjugated donkey-anti-rabbit antibody diluted 1:400 in PBS (Jackson ImmunoResearch) and rinsed again. This was followed by incubation for two hours with the ABC complex (see above). After rinsing, sections were treated with a DAB reaction as described above but without nickel ammonium sulphate, resulting in brown CRH neurons among black BDA fibers. After final rinsing, the sections were mounted as described above.

MORPHOLOGICAL ANALYSIS The precise location of each injection was determined by plotting its distribution in the atlas of Swanson (1992). The distribution of BDA fibers and CRH neurons in the PVH was drawn (magnification 126×) in the atlas of the PVH presented by Mulders et al. (1995a,b), in which the central parvocellular part (PCc) was further subdivided into a CRH-rich part containing 50% of all CRH neurons of the PVH, and a remaining CRH-poor part, which appears to contain only about 21% of all CRH neurons in the PVH. The PVH can easily be distinguished from the surrounding hypothalamus by the higher neuronal and vascular density, which enables localization of this nucleus, even in unstained sections.

For quantitative analysis we used 6 normal and 7 ADX rats with injection sites in the central part of the BNST. Using a curvilinear test system (Merz 1967, Weibel 1979), we calculated the length density, i.e. the length of fibers (μm) per volume unit (μm^3), of labeled BNST fibers in the different subdivisions of the PVH. For this purpose, we superimposed the standardized atlas of the PVH on an image of hemicircles (Weibel 1979), put it under the drawing tube of our microscope (magnification 126×) and counted all crossings of the hemicircles with labeled fibers in the different subdidivions of the PVH. Subsequently, we used the formula in Weibel (1979) to determine the absolute fiber length density in the PVH and the different subdivisions. From these values the relative fiber density per PVH

subdivision was determined by dividing the length density per subdivision by the average PVH fiber density per animal. So, this relative density relates the density of any subdivision to the overall PVH fiber density. Statistical analysis of the differences in fiber length density in the PVH of normal and ADX rats was performed by means of a two-tailed Student's t-test.

RESULTS

THE ORIGIN OF BNST PROJECTIONS TO THE PVH The 41 iontophoretic tracer injections made in and around the BNST in normal and ADX rats are of small to large size, varying from 300 μm to 800 μm in their transverse diameter, and from 150 μm to 1 mm in rostral-caudal direction. Seventeen injections resulted in labeled fibers in the PVH and are therefore characterized as positive (fig. 1, A1-D1). The remaining 24 injections did not result in any fiber labeling in the PVH and are used as controls (fig. 1, A2-D2). The location of the control injections was subtracted from that of the positive ones to exclude regions that contain BDA deposit in positive injections but do not contribute to labeled fibers in the PVH. In this way we determined which subdivisions of the BNST really project to the PVH (fig. 1, A3-D3). This method showed that subdivisions located in the anterior division of the BNST and dorsal to the anterior commissure (i.e. the anterodorsal, the anterolateral, the oval and the juxtacapsular nuclei and the central core, see Swanson 1992) do not project to the PVH (fig. 1, A3-D3). In contrast, the anteroventral, the dorsomedial, the fusiform and the rhomboid nuclei in the anterior division, which are all located ventral to the anterior commissure, project to the PVH (fig. 1, A3-D3). Our data in the posterior division of the BNST do not allow definite conclusions, since these are restricted to 1 control and 2 positive injections. The interfascicular and transverse nuclei in the posterior division of the BNST probably project to the PVH (fig. 1, D3), but other areas, such as the ventral nucleus and the medial parts of the principal and interfascicular nucleus, have remained uncharacterized by our experiments, since there were neither positive nor negative injectinos located in these nuclei (fig. 1, D1 and D2). The results as obtained from substraction of control injections from positive ones in the anterior BNST are corroborated by several small injections: we never find positive injections exclusively dorsal to the anterior commissure, whereas small injections restricted to the region ventral to the anterior commissure are always positive.

THE DISTRIBUTION OF BNST FIBERS IN THE PVH BNST injections result both in normal and ADX rats in almost exclusively ipsilateral hypothalamic labeling. Labeled BNST fibers in the PVH are either very delicate and thin or thick up to 1 μm (figs. 2 en 4). All fibers show a large number of varicosities. The distribution of labeled fibers depends on the localization of injection in the BNST: injections in the anterior part of the BNST (fig. 1, B1-C1) result in labeled fibers that are almost exclusively restricted to the PVH, without labeling of the hypothalamic area surrounding the PVH (fig. 2B). However, injections in posterior divisions of the BNST, which also include parts of the substantia innominata and the lateral hypothalamic area (fig. 1, D1), result in labeled fibers in the PVH as well as in the hypothalamic area surrounding the PVH (fig. 2A).

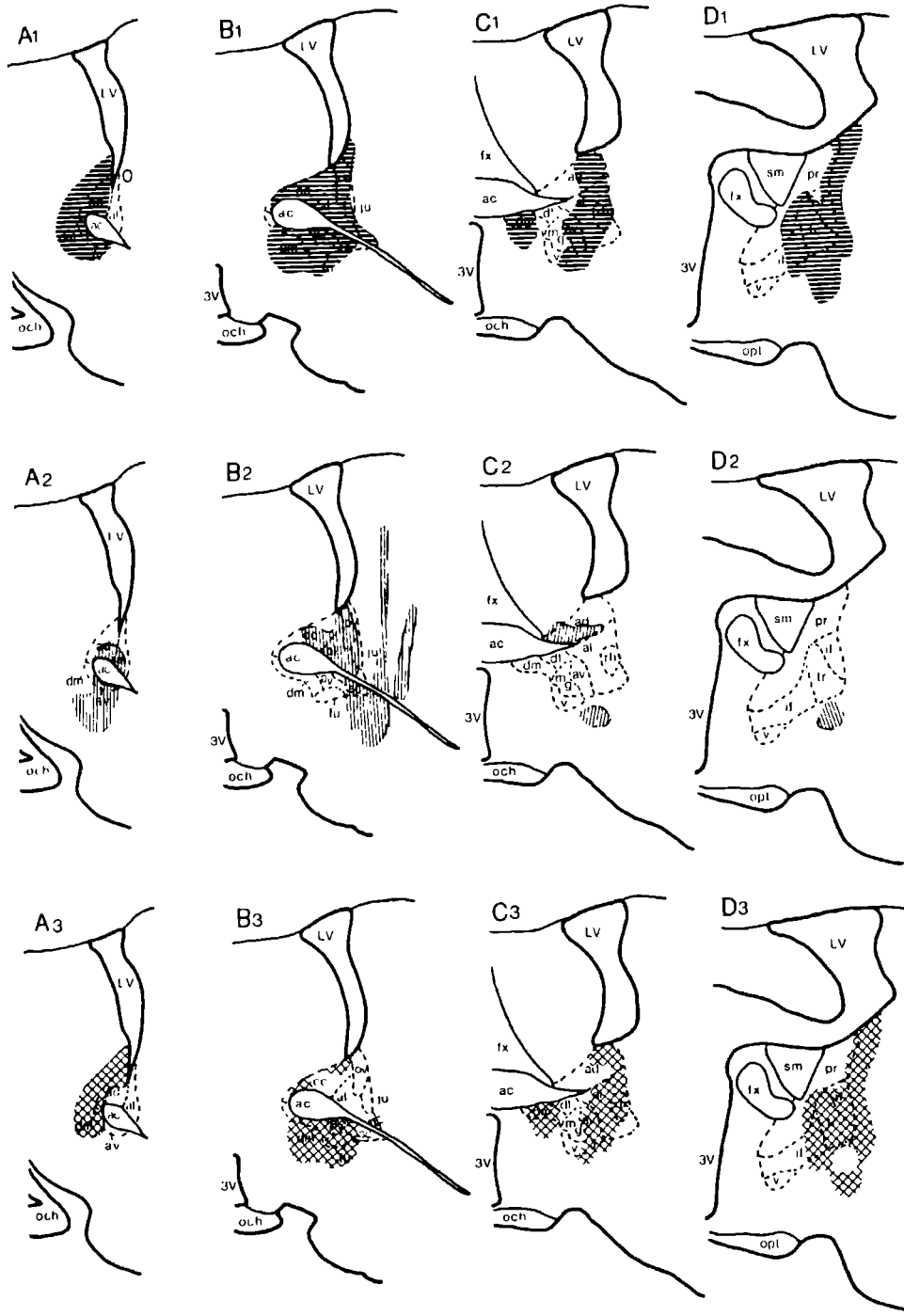


FIGURE 1 Rostrocaudal series of drawings of transverse sections through the BNST at four levels (A-D) with a summation of BDA injection-sites that resulted in labeled fibers in the PVH (positive injections, A1-D1); a summation of injection-sites that did not result in any labeled fibers in the PVH (negative injections, A2-D2), and a subtraction of the negative from the positive injection-sites showing the BNST regions that contribute to the PVH projections (A3-D3). (BNST subdivisions are according to the atlas of Swanson, 1992.) Magnification = 11×.

Most of the labeled BNST fibers in the PVH are horizontally orientated, as is particularly obvious in the posterior PVH (fig. 2B). In normal rats the parvocellular and magnocellular part of the PVH are about equally innervated by the BNST (figs. 4 and 5), but in ADX rats the central parvocellular part, which contains the largest number of CRH neurons, shows relatively less labeling after positive injections in the BNST in comparison to the other subdivisions, as quantified in the next section. Double labeling of BDA and CRH in ADX rats seldomly shows varicosities in close apposition to CRH neurons.

COMPARISON OF NORMAL AND ADX RATS 6 Normal and 7 ADX rats with comparable positive injections in the central part of the anterior division of the BNST were used for quantitative analysis of the fiber length density in the PVH and its subdivisions. All injections in these rats are of small to moderate size, except for two injections in ADX rats, which are substantially larger and extend into the posterior BNST (fig. 3).

In ADX rats an overall length density of BNST fibers in the PVH of $1.17 \times 10^{-3} \pm 0.27 \times 10^{-3}$ $\mu m/\mu m^3$ was found, which is statistically significantly lower than the overall length density in the PVH of normal rats ($2.59 \times 10^{-3} \pm 0.24 \times 10^{-3}$ $\mu m/\mu m^3$, $p < 0.01$). This difference holds for all subdivisions (table I, figs. 4 and 5). However, the relative fiber density was only significantly reduced in the CRH-rich part of the PVH of ADX rats, i.e. from 1.095 ± 0.019 to 0.602 ± 0.106 ($p < 0.01$, table II). The other subdivisions of normal and ADX rats show no mutual statistically significant differences in relative fiber length density. The length density of BNST fibers in the PVH after injections situated in the posterior BNST were not quantitatively analyzed, because of the limited number of experiments available.

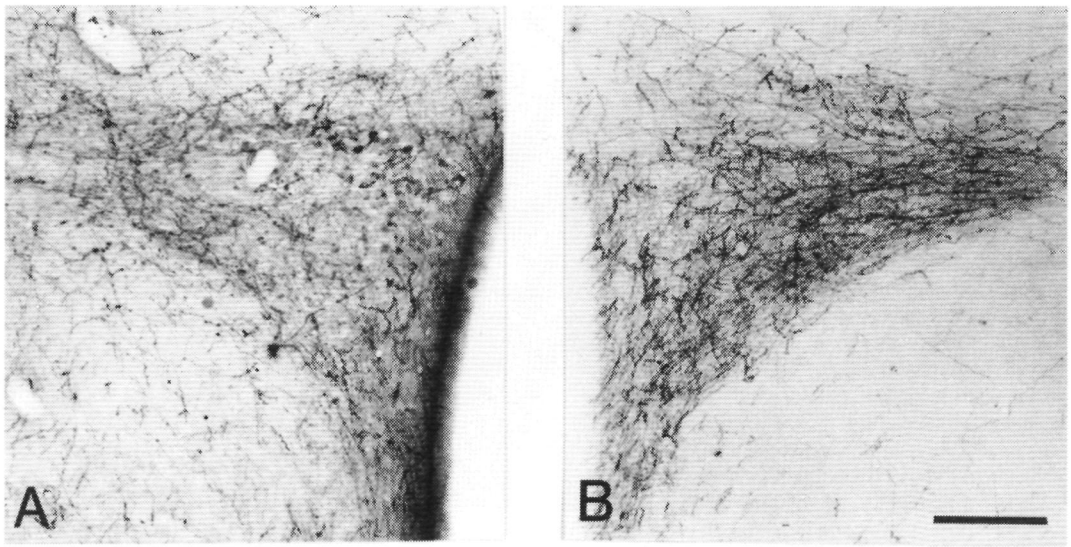


FIGURE 2 Photomicrographs illustrating BNST fiber patterns in and/or around the PVH following an injection in the BNSTp (A, ADX rat), and an injection in the BNSTa (B, normal rat). Scale bar = 170 μm.

DISCUSSION

The present study shows a significant change in the projection from the BNST to the PVH after ADX, which suggests an important effect of the corticosteroid plasma level on the synaptic organization of the stress-regulating circuit in adult rats. To evaluate our results in detail we will first compare our data with previous tracer studies and discuss possible technical artefacts involved in the differences between normal and ADX rats. Next, we will discuss possible mechanisms involved in the presently reported plasticity. The last part of the discussion will focus on the functional significance and possible clinical relevance of the present findings.

THE BNST-PVH PROJECTION Previous studies dealing with the projections from the BNST to the PVH predominantly used retrograde tracing techniques (Silverman et al. 1981, Sawchenko and Swanson 1983, Cullinan et al. 1993, Moga and Saper 1994, Thellier et al. 1994). All these studies have shown that the subdivisions ventral to the anterior commissure in the anterior division of the BNST (BNSTa) project to the PVH, since large amounts of retrogradely labeled neurons are found in this region following tracer injection in

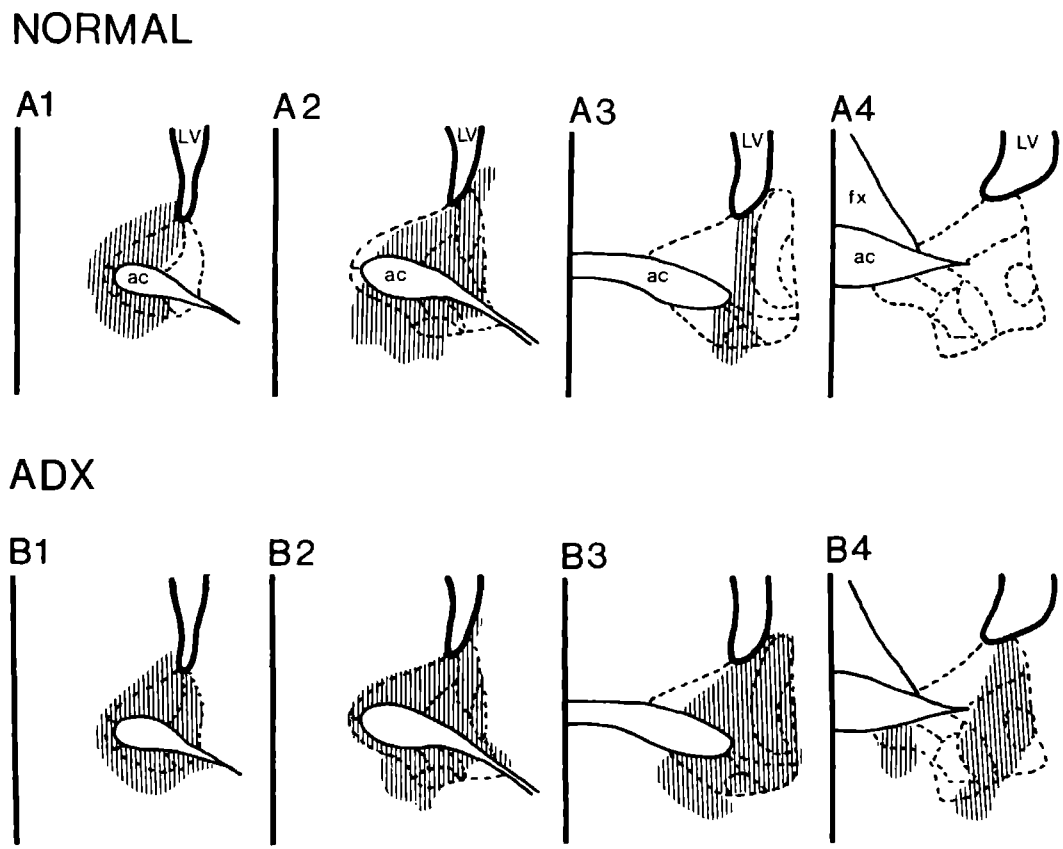


FIGURE 3 **Rostrocaudal series of drawings of transverse sections of the central BNST (1-4) illustrating a summation of BDA injections used for quantitative analysis of BNST projections to the PVH in normal (A1-A4) and ADX rats (B1-B4). Magnification = 17×.**

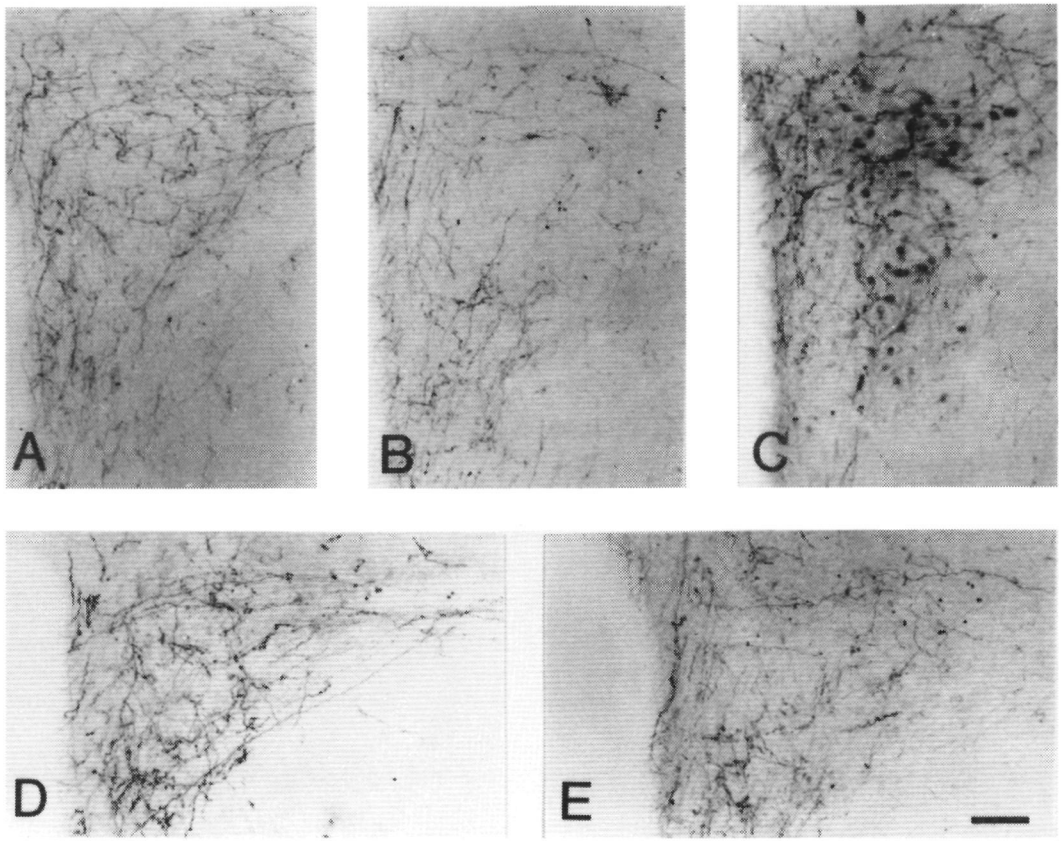


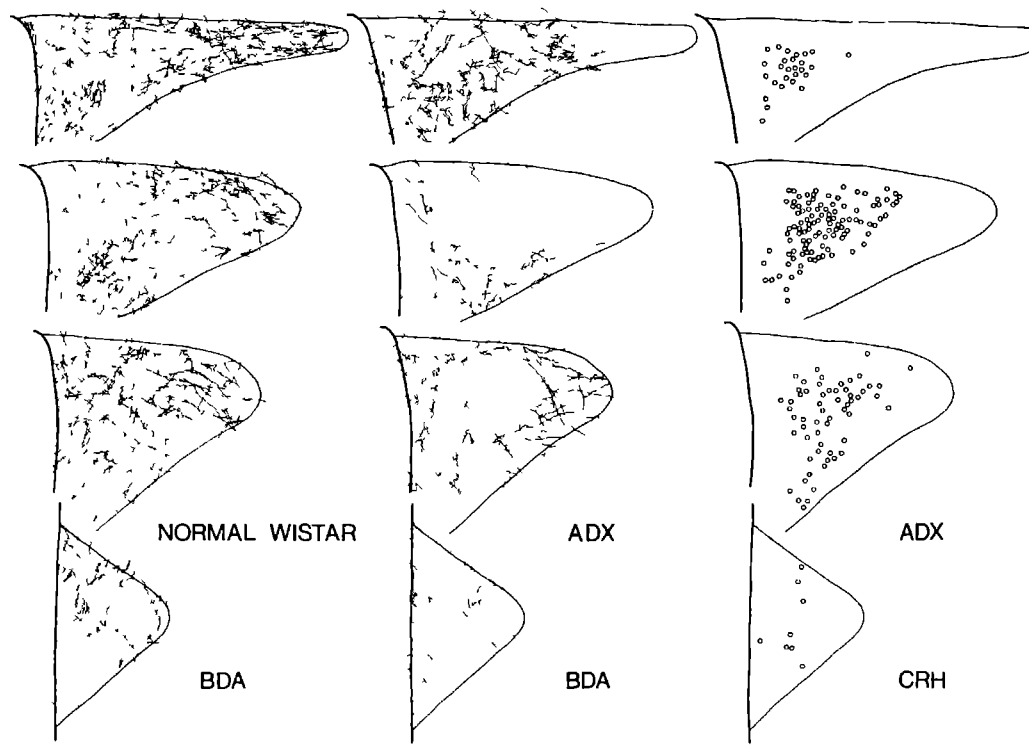
FIGURE 4 Photomicrographs of BDA-labeled BNST fiber patterns (A-E) and CRH-neuron distribution (C) in the PVH (A-C: rostral PVH; D,E: caudal PVH). A and D: normal rats. B, C and E: ADX rats. Scale bar = 85 μm.

the PVH. Likewise, most studies agree that the region dorsal to the anterior commissure in the BNSTa is not involved in the projection to the PVH, since no or only few scattered neurons are detected following identical injections. However, Cullinan et al. (1993) found a considerable amount of labeled neurons in this dorsal region. Our results do not confirm the latter, but are in agreement with the above mentioned studies showing that there is a rather specific projection to the PVH from the ventral part of the BNSTa, without an important contribution of the dorsal part.

With respect to the PVH projection from the posterior BNST (BNSTp) there are some discrepancies in the literature. Silverman et al. (1981) have found no labeled cell bodies in the BNSTp after retrograde tracing injection in the PVH. However, Sawchenko and Swanson (1983), Moga and Saper (1994) and Thellier et al. (1994) describe some labeling in this region, whereas Cullinan et al. (1993) have found a large amount of labeled neurons in the posterior subdivision. Given the limited number of BNSTp injections it can not be definitely concluded that the BNSTp or certain BNSTp subdivisions project to the PVH and areas surrounding the PVH.

Our study shows a more or less random distribution of labeled BNSTa fibers in the different subdivisions of the PVH in normal rats, without preference for the parvocellular or magnocellular part. Therefore, our results do not suggest a specific relation between the BNST and the CRH neurons, which are particularly situated in the central parvocellular part (PCc) of

FIGURE 5 Series of representative drawings of four levels of the PVH (distance about 150 μm; rostral to caudal level from bottom to top) illustrating BNST fiber patterns in a normal rat (left row) and an ADX rat (middle row), and the CRH-neuron distribution in the same ADX rat (right row). Magnification = 63×.



the PVH (Mulders et al. 1995a,b). This is corroborated by the fact that in all subdivisions fibers show a large amount of varicosities which probably represent axon terminals (Wouterlood and Groenewegen 1985). However, other tracer studies suggest a higher innervation of the parvocellular part compared with the magnocellular part of the PVH (Sawchenko and Swanson 1983, Thellier et al. 1994) which is in contrast with the present results. The reason for this discrepancy is not clear.

COMPARISON OF NORMAL AND ADX RATS An interesting result of the present study is the statistically significant decrease in BNST fiber density in the PVH of ADX rats (1.17×10^{-3} $\mu m/\mu m^3$) compared to normal rats (2.59×10^{-3} $\mu m/\mu m^3$, table I). This difference holds for all subdivisions of the PVH. However, the relative length density, which relates the density of any subdivision to the overall PVH fiber density, is particularly reduced in the CRH-rich part of the PCc of ADX rats (0.602 versus 1.095 in controls, table II). This means that the innervation of the CRH-rich part of the PVH by BNST fibers is far more reduced after ADX than the innervation of the other subdivisions, which suggests a specific effect of ADX and corticosteroids on the relationship between BNST and CRH neurons.

A first aspect to be discussed is whether the reduced BNST fiber density after ADX represents a technical artefact or a real changed input. Several technical factors might cause a

TABLE 1 Mean fiber length density (10^{-1} $\mu m/\mu m^3$ ± SEM) in the PVH and its subdivisions in normal (n = 6) and ADX rats (n = 7). * = statistically significantly different ($p < 0.05$) compared to normal Wistar rat. ** = statistically significantly different ($p < 0.01$) compared to normal Wistar rat

	PVH	PV	MC	PCc	CRH+	CRH−	PCd	PCv	PCp
Normal Wistar									
Mean	2.59	2.14	2.51	2.74	2.85	2.67	2.18	2.19	3.13
SEM	0.24	0.29	0.25	0.22	0.78	0.19	0.29	0.22	0.47
ADX									
Mean	1.17**	1.12*	1.27*	1.05**	0.75**	1.25**	1.18*	1.16*	1.34*
SEM	0.27	0.22	0.38	0.25	0.21	0.30	0.32	0.25	0.41

differential labeling, such as the site and size of injections, different tracer uptake by BNST cell bodies after ADX, or a decreasing speed or total absence of axonal tracer transport in the BNST neurons following ADX. We selected injections of normal and ADX rats which were largely comparable with regard to their size and location. The injections of ADX rats were in some cases even larger than that of normal rats but still resulted in a reduced labeling of the PVH with a particular 'empty' CRH-rich part (figs. 4 and 5). So, injection site and size are probably not involved in the observed differences in fiber density. To our knowledge, mechanisms such as reduced tracer uptake or transport after ADX have never been described, and even when they would play a role in our observation, they only might influence the absolute, overall decrease in density of labeled fibers, but not the specific reduction in innervation of the CRH-rich subdivision of the PVH. Therefore, we conclude that the difference in fiber density reflects a reduced BNST innervation of the PVH, especially of its CRH-rich part, after ADX. This means that ADX causes a loss of input from the BNST to the PVH and in particular to the CRH neurons.

At present it is not known whether the loss of input is a reversible process. To solve this question we have planned additional experiments involving glucocorticoid replacement after ADX. Nonetheless, our results indicate that hormonal disbalances can induce substantial synaptic changes in the adult rat brain. A comparable phenomenon has been described in female rats where the preovulatory estrogen surge causes a 30-50% decrease in the number of synapses in the arcuate nucleus (Garcia-Segura et al. 1986, Leedom et al. 1994). Likewise, in the brain of adult canaries the changing of seasons is accompanied by a alternating loss and formation of dendritic segments and synapses in song control nuclei (Nottebohm 1981, Alvarez-Buylla and Nottebohm 1988).

MECHANISMS INVOLVED IN SYNAPTIC CHANGES The presently reported loss of input might be evoked by anterograde or retrograde mechanisms. Anterograde mechanisms could start in a limbic structure such as the hippocampus, where ADX is known to induce granule cell death (Sloviter et al. 1989, Sapolsky et al. 1991a, Sloviter et al. 1993). In turn, this cell loss might influence the BNST by inducing cell death or axon retraction, thus changing the hippocampal influence on the PVH. Another possible structure involved is the amygdala, since electrophysiological experiments have shown that ADX changes the reponse of BNST neurons to amygdala stimulation (Sánchez et al. 1995), which suggests an altered amygdala-BNST pathway. To result in a specific reduction of input to CRH cells such anterograde mechanisms would require a very specific anatomical pathway

TABLE II Relative fiber length density (± SEM) per subdivision of the PVH in normal (n = 6) and ADX rats (n = 7). ** = statistically significantly different ($p < 0.01$) compared to normal Wistar rat

	PV	MC	PCc	CRH+	CRH-	PCd	PCv	PCp
Normal Wistar								
Mean	0.822	0.973	1.062	1.095	1.042	0.835	0.846	1.194
SEM	0.095	0.070	0.018	0.019	0.037	0.093	0.031	0.103
ADX								
Mean	1.033	1.029	0.893	0.602*	1.085	0.878	1.174	1.164
SEM	0.104	0.103	0.116	0.106	0.128	0.150	0.207	0.179

from these limbic structures via the BNST to the parvocellular PVH. However, tracing experiments have shown the opposite: The hippocampus has a rather widespread projection to the BNST via the ventral subiculum, and also projects to parts which do not project to the PVH (Cullinan et al. 1993, present results). Likewise, the medial amygdala has an abundant projection to all parts of the BNST as well (Canteras et al. 1995), which renders it rather unlikely that anterograde mechanisms are involved in the reduction of BNST input to the PVH.

In our opinion, the loss of BNST input after ADX is most likely evoked by a retrograde mechanism, such as presynaptic feedback (O'Dell et al. 1991), since loss of input is especially present in the CRH-rich region of the PVH, where CRH neurons might induce this plastic phenomenon. Apart from an increased CRH content in the PVH ADX causes an increase in binding capacity of type II glucocorticoid receptors in the PVH (Reul et al. 1987), a decreased number of angiotensin receptors on CRH neurons (Castrén and Saavedra 1989, Aguilera et al. 1995) and a decrease in the number of α_2-noradrenergic receptors in the PVH (Jhanwar-Uniyal and Leibowitz 1986). These receptor changes – and possibly other effects of ADX – might well induce withdrawal of BNST fibers by presynaptic feedback of CRH neurons via a retrograde messenger to their pre-synaptic elements.

FUNCTIONAL SIGNIFICANCE In general, it is thought that hormonal balance is very important for the development of the synaptic organization of brain but that it does not influence the structure of the adult brain (Mugila et al. 1995, Zhou et al. 1995). However, the presently reported reduction of input from the BNST to the PVH following ADX implies that hormones are capable to induce synaptic changes in the adult mammalian brain. To evaluate the function of this plasticity one has to consider that ADX increases the activity of CRH neurons (Kiss et al. 1984, Sawchenko et al. 1984, Whitnall et al. 1985, Sawchenko 1987) and that BNST input evoked by the hippocampus is probably partly GABA-ergic and thus inhibitory (Cullinan et al. 1993). Therefore, loss of this input would implicate a reduction of inhibition, which would be helpful to keep the CRH neurons in a highly activated state after ADX. This would imply that the withdrawal of BNST fibers is most likely a mechanism to decrease the inhibitory influence of the hippocampal input to the CRH neurons. However, several lesion and stimulation studies have shown that the BNST input does not only have inhibitory effects on the PVH but sometimes also excitatory effects (Dunn 1987, Feldman et al. 1990, Herman et al. 1994). Thus, the precise functional significance of the reduced BNST input to the CRH neurons has to be elucidated by future results.

It is clear that ADX is not a physiological condition. In normal circumstances the opposite situation, i.e. a high level of corticosteroids during chronic stress is more likely to occur. In view of the present results, it is tempting to hypothesize that the plasticity of the BNST-PVH projection is a reversible mechanism counteracting disturbances in the corticosteroid plasma level. This might well have implications for disorders which are associated with a hypo- or hyperactivity of the hypothalamo-pituitary-adrenal axis. Several examples of such disorders are known, such as Cushing disease, Addison disease, depression, anorexia and multiple sclerosis (Gold et al. 1987, Reus 1987, Owens and Nemeroff 1993, Von Werder and Müller 1993, Erkut et al. 1995). The present results indicate that these disorders might well be allied to a changed synaptic circuitry in the brain compared to the normal situation.

LIST OF ABBREVIATIONS

ac	anterior commissure
ACTH	adrenocorticotropic hormone
ad	anterodorsal area, BNST
ADX	adrenalectomy
al	anterolateral nucleus, BNST
av	anteroventral area, BNST
BNST	bed nucleus of stria terminalis
cc	central core, BNST
CRH	corticotropin-releasing hormone
BDA	biotinylated dextran amine
dl	dorsal lateral division, BNST
dm	dorsomedial nucleus, BNST
fu	fusiform nucleus, BNST
fx	fornix
if	interfascicular nucleus, BNST
ju	juxtacapsular nucleus, BNST
LV	lateral ventricle
MC	magnocellular part, PVH
mg	magnocellular nucleus, BNST
och	optic chiasm
opt	optic tract
ov	oval nucleus, BNST
PCc	central parvocellular part, PVH
PCd	dorsal parvocellular part, PVH
PCp	posterior parvocellular part, PVH
PCv	ventral parvocellular part, PVH
pr	principal nucleus, BNST
PV	periventricular part, PVH
PVH	hypothalamic paraventricular nucleus
rh	rhomboid nucleus, BNST
sm	stria medullaris
tr	transverse nucleus, BNST
v	ventral nucleus, BNST
3V	third ventricle

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Chapter 6

Synaptic organization of the corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus of the rat: A quantitative immuno-electron microscopical study

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SUMMARY

The corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVH) play an important role in the stress-regulating circuit. To elucidate their synaptic organization we studied electron microscopical sections of adrenalectomized rats, since these allow for a reliable identification of CRH neurons. By means of pre-embedding immunohistochemistry and serial section reconstructions we determined that the synaptic density of CRH somata varies from 0.067 to 0.314 per μm^2 with an average of 0.150 per μm^2 and that their synaptic number varies from 34 to 109 with an average of 63. By means of post-embedding immunohistochemistry and quantitative analysis of serial sections we determined that approximately 62% of the synaptic input of CRH neurons is GABA-ergic,

without differences between the CRH neurons with or without co-localized vasopressin. CRH immunonegative parvocellular neurons receive a similar degree of GABA-ergic input (68%). However, magnocellular vasopressinergic as well as non-vasopressinergic neurons receive a statistically significantly lower percentage of GABA-ergic input (43% and 42%, respectively). Our results suggest that the synaptic organization of PVH neurons is not very strictly regulated and that the location of neurons within a certain subdivision of the PVH is more important in the determination of their synaptic input than their neurochemical properties.

INTRODUCTION

The hypothalamic paraventricular nucleus (PVH) plays an important role in behavioral and autonomic functions, such as maternal (Numan and Corodimas 1985, Insel and Harbaugh 1989), sexual (Hughes et al. 1987, Pfaus et al. 1993) and eating behavior (Flanagan et al. 1992, Atrens and Menéndez 1993), the regulation of body temperature (De Luca et al. 1989, Horn et al. 1994) and cardiovascular regulation (Kannan and Yamashita 1985, Gelsema et al. 1989, Harland et al. 1989). These multiple functions are predominantly subserved by large populations of oxytocin, vasopressin and corticotropin-releasing hormone (CRH) neurons (Rhodes et al. 1981, Swanson et al. 1983), which are densely innervated by adrenergic (Cunningham et al. 1990), noradrenergic (Cunningham and Sawchenko 1988), neuropeptide Y (Liposits et al. 1988), GABA (Decavel et al. 1989) and galanin immunoreactive fibers (Sawchenko and Pfeiffer 1988). Inputs to the PVH arise mainly from catecholaminergic cell groups in the medulla (Weiss and Hatton 1990), from the limbic system, via the bed nucleus of the stria terminalis (Cullinan et al. 1993, Mulders et al. submitted) and from different hypothalamic cell groups (Simerly and Swanson 1988, Vrang et al. 1995). Efferents of the PVH project predominantly to the median eminence (Lechan et al. 1980) and to preganglionic neurons of the autonomic system in the brain stem and spinal cord (Swanson and Kuypers 1980). It is obvious that the PVH needs a refined structural organization with specific input-output relations to subserve its different functions. However, the precise morphological and synaptic basis of this organization is only partly known.

At the light microscopical level the morphology of the PVH is well documented. It consists of a parvocellular and a magnocellular part, which can be further subdivided in 5 to 10 subdivisions on the basis of cytoarchitectonic or neurochemical criteria (Armstrong et al. 1980, Swanson and Kuypers 1980, Kiss et al. 1991, Mulders et al. 1995a). Remarkably, the distribution of immunocytochemical characterized cell groups does not show a strict

correlation with cytoarchitectonic subdivisions. For example, CRH neurons are predominantly located (about 70%) in the central parvocellular part, but occur in lower densities in all other subdivisions as well (Swanson et al. 1983, Mulders et al. 1995b). The same holds for oxytocin and vasopressin, which are predominantly located in the magnocellular division but can be found in lower amounts in the parvocellular part as well (Rhodes et al. 1981). A similar 'mismatch' is observed with respect to the distribution of afferents over different subdivisions. For example, brain stem and medullary afferents terminate mainly in the central parvocellular part, but also contact magnocellular neurons (McKellar and Loewy 1981, Cunningham et al. 1990). So, the PVH does not seem to be very strictly organized with respect to the compartmentalization of distinct sets of neurons and afferents.

At the synaptic level, the PVH does equally not show much differentiation. The overall synaptic density is about 200 x $10^6/mm^3$ and the synaptic contact length about 370 nm, without significant differences between the various subdivisions (Kiss et al. 1983, Mulders et al. 1995a). In addition, Decavel et al. (1987) describe a homogeneous dopaminergic innervation of the parvocellular and magnocellular PVH. The GABA-ergic innervation seems to be homogeneous throughout the PVH as well, since in both the parvo- and magnocellular part 50% of the synaptic input is GABA-ergic (Decavel et al. 1989, Decavel and Van den Pol 1990). Some differentiation can be found in the distribution of noradrenergic input, which seems to innervate mainly the parvocellular part and the vasopressinergic area of the magnocellular part (Decavel et al. 1987). In spite of this preference, noradrenergic terminals in the magnocellular part account for only 1-2% of the total input of vasopressin neurons (Nakada and Nakai 1985, Silverman et al. 1985). Other neurotransmitters showing a non-homogeneous distribution in the PVH are neuropeptide Y and galanin, which both occur in higher densities in the parvocellular part than in the magnocellular part (Sawchenko and Pfeiffer 1988).

To evaluate to which degree specific synaptic contact patterns between afferents and neurons are involved in the complex multifunctional organization of the PVH, quantitative immuno-EM studies are necessary. The present study is our first approach in this respect, focussing on a quantitative characterization of the synaptic input of CRH neurons. These are of specific interest to us (Mulders et al. 1995a,b, Mulders et al. submitted), since they play an important role in the stress-regulating circuit (Rivier et al. 1982, Antoni et al. 1983, Swanson et al. 1983). For visualization of CRH we used adrenalectomized (ADX) rats since it is hardly possible to show CRH in normal rats with current immunohistochemical techniques. By means of quantitative analysis of the synaptic input of CRH neurons and comparison with other PVH neurons we will try to elucidate 1) to which degree the synaptic input of individual CRH neurons is specific or variable with respect to number, distribution and immunohistochemical characterization; 2) to which degree the synaptic input of CRH neurons that contain co-localized vasopressin or not following ADX is different; and 3) to which degree the synaptic input of CRH neurons differs from other parvocellular neurons that are co-distributed in the parvocellular region as well as from magnocellular neurons, which might indicate whether the immunohistochemical properties or the location of neurons primarily determines their synaptic input in the PVH. For these purposes, we quantified the synaptic density on reconstructed CRH neurons and compared the percentage of GABA-ergic and non-GABA-ergic input on reconstructed CRH and/or vasopressin immunoreactive neurons.

MATERIALS AND METHODS

ANIMALS For the present study we used 6 adrenalectomized (ADX) Wistar rats to optimize the immunohistochemical procedures to be described. Brain slices of two of these rats were selected for serial section reconstruction and quantification. All rats were bred in our Animal Laboratory. ADX was performed under ether anaesthesia four weeks before perfusion (rats at that time weighing 170 ± 10 g). Rats were housed two-three animals per cage in a room with a constant temperature (20 ± 2°C) and a 12 hours light period. Food and saline (0.9% sodium chloride in distilled water), which replaced the drinking water, were available continuously. The rat used for quantification of the results obtained by pre-

embedding immunohistochemistry received three days before perfusion an anterograde tracer injection of biotinylated dextran amine in the bed nucleus of the stria terminalis. This injection did not result in biotinylated dextran amine labeled fibers among the CRH neurons (Mulders et al. submitted) and consequently does not interfere with the present analysis.

TISSUE FIXATION The rats were deeply anaesthetized with pentobarbital (6 mg/100 g bodyweight) and transcardially perfused with 100 ml saline which was followed by 400 ml of a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picrinic acid in 0.1 M phosphate buffer (pH 7.4, PB) for pre-embedding immunohistochemistry or by a mixture of 2% paraformaldehyde and 2% glutaraldehyde in PB for post-embedding immunohistochemistry. Subsequently, the dorsal part of the skull was removed and the rats were placed in a stereotactic device to make a transverse incision which allowed sectioning of all brains in the same transverse plane. Next, the brains were removed from the skull and post-fixed at 4°C in the same fixative as used for perfusion. The next day a vibratome (Bio-Rad) was used to cut 75 μm sections, which were collected and rinsed in phosphate buffered saline (PBS, pH 7.4).

IMMUNOHISTOCHEMISTRY

PRE-EMBEDDING IMMUNOHISTOCHEMISTRY For electron microscopical demonstration of CRH by means of pre-embedding immunohistochemistry selected sections were incubated overnight with rabbit polyclonal CRH antiserum (8Bo, gift from Dr. Tilders, Amsterdam, The Netherlands) diluted 1:10,000 in PBS containing 0.1% bovine serum albumin (for characterization and specification of this antibody, see: Mulders et al. 1995b). After rinsing in PBS, sections were incubated for 60 minutes in a biotin-conjugated donkey-anti-rabbit antibody (Jackson ImmunoResearch), diluted 1:400 in PBS and rinsed again in PBS. Subsequently, they were incubated for two hours in a solution of one drop avidin and one drop biotinylated horseradish peroxidase in 40 ml PBS (pH 7.4, Vectastain ABC Elite kit: Vector Laboratories). After rinsing sections were pre-incubated for 10 minutes in 0.02% 3,3'-diaminobenzidine-4HCl (DAB; Sigma) and 0.3% nickel ammonium sulphate in a 0.05 M Tris(hydroxymethyl)-aminomethane solution (pH 7.6), followed by a 10 minutes incubation in the same solution containing 20 μl H_2O_2.

After immunostaining sections were rinsed in 0.1 M PB, osmicated for one hour in 1% osmium tetroxide dissolved in distilled water and rinsed again in PB. Next, sections were dehydrated in a graded series of ethanol, embedded in Epon 812 via propylene oxide and mounted in Epon 812 between a slide and coverslip coated with dimethyldichlorosilane

solution (2% in 1,1,1-trichloroethane). The latter ensures easy removal of the sections from the slide and coverslip. After polymerization for two days in a stove at 60°C one section of the central PVH was selected (fig. 1) and remounted on an Epon block for ultrathin sectioning. A Reichert Ultracut-E was used to cut a continuous series of about 100 thin sections (85 nm), which were mounted on formvar coated one-hole copper grids.

POST-EMBEDDING IMMUNOHISTOCHEMISTRY For preparation of ultrathin sections suitable for post-embedding immunohistochemistry selected vibratome sections of the PVH were cryo-protected in increasing concentrations of glycerol (10-20-30%) in PB, 30 minutes for each concentration. Subsequently, the parts containing the PVH were cut out, oriented on pieces of Thermanox (LAB-TEK DIV., Miles Laboratories Inc., Naperville) and frozen rapidly by plunging in liquid propane (-190°C) with the use of a rapid freezing apparatus (KF 80, Reichert-Jung, Germany). The propane was cooled with liquid nitrogen. After freezing the pieces were transferred to the precooled chamber (-90°C) of a CS auto freeze substitution apparatus (Reichert-Jung, Germany), in which freeze substitution was performed as described by Müller et al. (1980). The tissue was immersed overnight at -90°C in anhydrous methanol containing 0.5% uranyl acetate as fixing agent. The temperature was raised in small steps with 4°C per hour to -45°C. The samples were washed several times with anhydrous methanol to remove water and excessive uranyl acetate, prior to infiltration with Lowicryl HM20 resin (Bio-Rad Richmond, California). The embedding process was carried out at -45°C in three stages, with a progressively increasing ratio of resin to methanol. Diffuse UV-radiation (360 nm) was used to catalyze polymerization first at -45°C overnight and then at room temperature for one day. One section of the central PVH (fig. 2) was selected to cut a series of about 150 ultrathin sections (85 nm) on a Reichert Ultracut-E which were mounted on formvar coated one-hole copper grids.

For visualization of CRH, vasopressin and GABA by immunogold labeling ultrathin sections were washed for one hour in PBS containing 0.1% sodium borohydrate and 50 mM glycine (this step was omitted for GABA) followed by 10 minutes in PBS containing 0.5% BSA and 0.5% cold fish skin gelatine (PBG) and then incubated overnight at 4°C in drops of PBG containing a CRH antibody (8Bo, similar to pre-embedding immunohistochemical procedure) diluted 1:100, a vasopressin antibody (a gift from Dr. Van Leeuwen, Amsterdam, The Netherlands) diluted 1:100 or a GABA antibody (a gift from Dr. Buijs, Amsterdam, The Netherlands) diluted 1:6000. The sections were washed for 20 mins. in PBG and incubated in gold-labelled goat anti-rabbit immunoglobulin G (IgG) (10 nm, Ammersham, Den Bosch, The Netherlands). After the incubation sections were washed 3 × 10 minutes in PBS and post-fixed with 2.5% glutaraldehyde in PBS for 5 mins. to minimize loss of gold label during subsequent washing with distilled water and contrasting with a saturated solution of uranyl acetate. The way in which sections were selected for either CRH, vasopressin or GABA immunohistochemistry will be described below.

ELECTRON MICROSCOPIC ANALYSIS For the analysis of the sections prepared according to the pre-embedding immunohistochemical procedure described above we photographed all detectable CRH neurons at a magnification of 5,000× in every fifth section of our series using a Jeol 1010 electron microscope. For the recognition of individual CRH neurons in subsequent sections we used their location with respect to each other and to the blood vessels in the PVH (fig. 1). A replica of 2,160 lines/mm was used to determine the

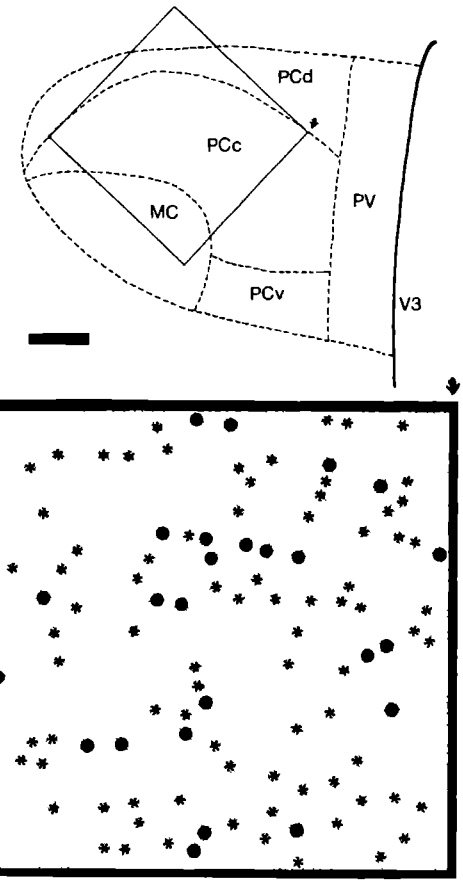

FIGURE 1 Schematic drawings of the location of the section (A) and neurons (B) used for quantitative EM analysis of the PVH after a pre-embedding immunohistochemical procedure to visualize CRH. Subdivisions were distinguished on the basis of previously described criteria (Mulders et al. 1995a). Scale bar represents 75 μm. Arrows indicate corresponding corners. Black dots indicate CRH+ neurons and asterisks indicate CRH− neurons. White areas indicate blood vessels. Abbreviations: MC = magnocellular part, PCc = central parvocellular part, Pcd = dorsal parvocellular part, PCv = ventral parvocellular part, PV = periventricular part, V3 = third ventricle.

precise magnification. Subsequently, the total length of the perimeter of every CRH profile was determined with the help of a Kontron-Videoplan equipment and all synapses on these profiles were counted. With these data and the average synaptic contact length in the central parvocellular part as previously determined (Mulders et al. 1995a) we calculated the synaptic density per neuronal surface area (N_s) for every analyzed neuron, by use of the formula of Colonnier and Beaulieu (1985), as adapted by Albers et al. (1990), reading:

$$N_s = \frac{N_a}{\bar{L} \cdot \Sigma B \cdot (4/\pi)}$$

in which:

N_a = number of synapses counted on an identified cell membrane in a certain reference volume

$\bar{L}$ = mean trace length of synaptic contacts (364 nm, Mulders et al. 1995a)

ΣB = the trace length of total measured boundary of a certain cell.

To gain more insight in the distribution of synapses all CRH neurons analyzed as described above were reconstructed. Some of the resulting reconstructions are shown in figure 3. For a caudal view of the reconstructed cells the contours and synaptic contacts of subsequent photographs of each neuron were superimposed, starting from the most caudal photograph, with omission of contours hidden by more caudal ones. For a dorsal view the width of each neuron and the position of the synaptic contacts were plotted. After addition of reconstructed height lines, which depict changes of 0.85 μm in height, the resulting dorsal view of the reconstructed neurons was obtained. From 11 neurons which were most completely present in the reconstructions we calculated the diameter (2.r) and perimeter ($2\pi r^2$), in order to estimate the approximate total number of synapses on these cell bodies ($4\pi r^2 \times N_s$).

For the electron microscopical analysis of the series of 150 lowicryl embedded ultrathin sections we selected an area in the PVH for detailed analysis that included the central parvo-

cellular part (PCc) almost completely and adjacent parts of the magnocellular part (MC), the dorsal parvocellular part (PCd) and the ventral parvocellular part (PCv) (fig. 2). Subsequently, we stained every 15th section for CRH, yielding 10 stained sections, and investigated the CRH immunoreactivity of all neurons and terminals in the selected area. For quantification, we selected 61 neurons which showed a nucleus in the central section of the series, since these neurons were most likely to continue throughout the whole series of sections. In these neurons, granules were classified as CRH-immunoreactive when they contained at least three gold particles. Neurons were classified as CRH-positive (CRH+) when they contained two or more immunoreactive granules in the cytoplasm in at least 9 from the 10 sections and as non-CRH neurons (CRH-) when they did not show any CRH immunoreactive granules in at least 9 out of the 10 sections analyzed. This method resulted in the distinction of 20 CRH+ neurons and 20 CRH- neurons. The remaining 21 neurons were treated as unidentified, since these neurons did not meet the criteria as mentioned above.

On 10 ultrathin sections adjacent to the sections stained for CRH we applied an immunogold vasopressin (VP) labeling and investigated which of the CRH+ and CRH- neurons could be classified as vasopressinergic (VP+) or non-vasopressinergic (VP-). We also checked whether there were VP-positive synapses present on the CRH+ and CRH- neurons. This was done in a similar way as described for CRH classification: granules were considered VP+ if they contained three or more gold particles and CRH- neurons were considered VP+ when they contained two or more immunoreactive granules in at least 9 out of 10 sections. However, we developed different criteria for classification of CRH+ as VP+ or VP-, since these neurons showed a different degree of VP labeling. CRH+ neurons were considered VP+ when they contained at least two immunoreactive granules in at least two sections.

On 10 other ultrathin sections adjacent to the sections stained for CRH we applied an immunogold GABA labeling. These sections were used to count all synapses on the selected neurons and to classify these synapses as either GABA-positive (GABA+) or GABA-negative (GABA-).

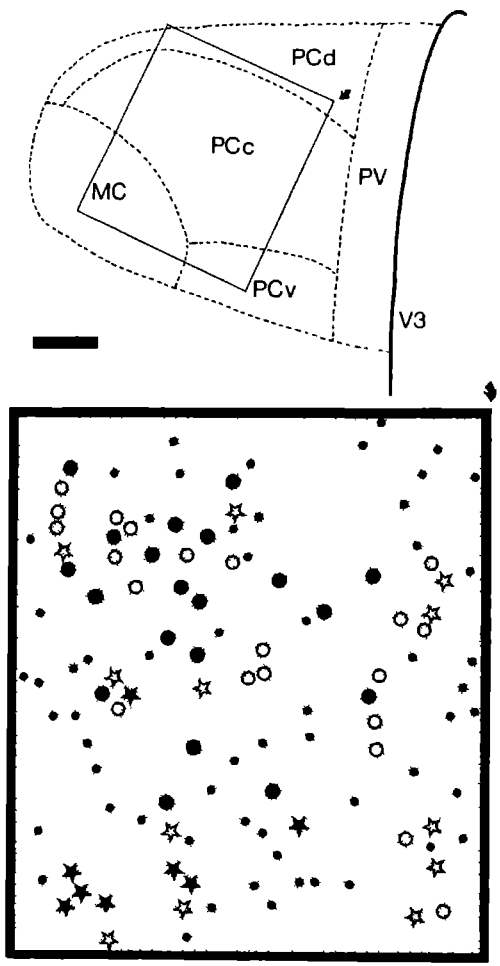

FIGURE 2 Schematic drawings of the location of the section (A) and neurons (B) used for quantitative EM analysis of the PVH after a post-embedding immunohistochemical procedure to visualize CRH, VP and GABA. For abbreviations of subdivisions: see figure 1. Scale bar represents 75 μm. Arrow indicates corresponding corner. Large black dots indicate CRH+ neurons. Black stars indicate VP+ neurons. Open stars indicate CRH-/VP- neurons. Open circles indicate investigated but unidentified neurons. Small black dots = non-investigated neurons. White areas = blood vessels.

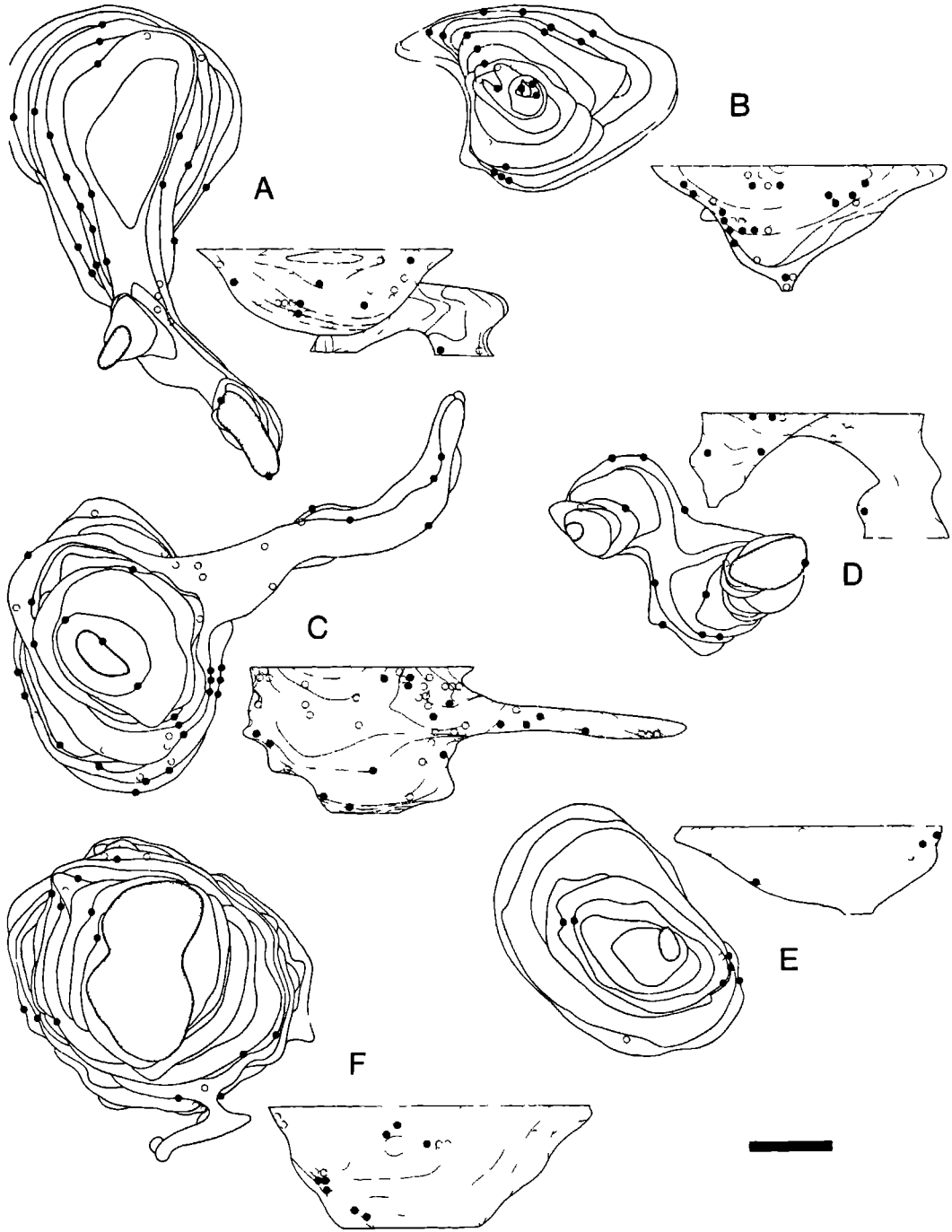

FIGURE 3 Drawings of 6 reconstructed CRH neurons and their synaptic contacts. The left drawing of each pair shows a caudal view and the right drawing shows a dorsal view of the reconstructions. The midline of the brain is at the left side of the drawings. In the caudal views lines represent the cellular contours as present in the different transverse sections used. In the dorsal views lines represent reconstructed heights lines. Black dots indicate synapses on the viewed surface and open circles indicate synapses on the backside of the reconstructed cells. Shaded areas indicate the end of the series of sections investigated. Scale bar represents 4 μm.

STATISTICS Statistical analysis of the differences in GABA+/GABA- ratio between CRH+ and CRH- and other populations of neurons was performed by means of a two-tailed Student's t-test.

RESULTS

THE OVERALL SYNAPTIC ORGANIZATION OF CRH NEURONS

In the series of ultrathin sections treated according to the pre-embedding histochemical procedure for CRH we identified 22 CRH neurons (fig. 1). In these neurons the DAB nickel precipitate is distributed throughout the cytoplasm. The CRH neurons are round to oval shaped and often have an indented nucleus (fig. 4). They have a moderate to low density of synaptic contacts on their somatic and proximal dendritic surface, and symmetrical as well as asymmetrical synapses contact the CRH neurons (fig. 4). Synapses contain clear vesicles and, occasionally, dense core vesicles.

A number of CRH neuron reconstructions are shown in figure 3. From these reconstructions it appears that the synaptic density varies largely between the CRH neurons; some are rather richly innervated (e.g. 3A, 3B and 3C) but others moderately to poorly (e.g. 3D, 3E and 3F). The synapses are not equally distributed over the cell body, since clustering of synaptic contacts occurs to a greater or less extend on all neurons. The synapse clusters do not show a preference for specific areas such as the origin of dendrites. None of the synapses contacting the CRH neurons contain DAB precipitate, so none of them is probably CRH immunoreactive.

The synaptic density (N_s) per CRH cell body varies from 0.067 to 0.314 per μm^2 with an average of 0.150 $\pm$ 0.014 per μm^2 (table I). The 11 neurons that were most completely present in the reconstructed sections have diameters between 10 and 13 μm, which implies a surface varying from 328 to 508 μm^2. Multiplication of these surfaces with the synaptic densities reveals that the estimated number of synapses per cell body varies from 34 to 109 with an average of 63 $\pm$ 7.8 synapses per cell body (table I).

THE LOCALIZATION OF CRH AND VP In sections treated by means of post-embedding immunohistochemistry, the immunogold labeling indicating the presence of CRH or VP is confined to neurosecretory granules. CRH immunogold labeling was only detected in rather electron lucent granules in the cytoplasm which are generally located close to the cell membrane. By far not all granules within a CRH immunopositive neuron contain gold particles. On average we could detect 3 to 5 immunoreactive granules per neuron per section between a large number of unlabeled granules (fig. 5). In contrast, VP immunohistochemistry revealed that magnocellular neurons contain large amounts of heavily labeled electron-dense VP+ granules. However, smaller VP+ neurons contain only small amounts of VP+ granules, i.e. only 2 to 3 VP+ granules per section, similar to the CRH distribution. These small VP+ neurons were always CRH+ as well.

After electron microscopical analysis of a total of 61 neurons in the CRH and VP immunogold labeled sections we were able to distinguish the following 5 different neuropeptidergic groups of neurons; CRH immunopositive but VP immunonegative neurons (CRH+/VP-, n = 8), CRH as well as VP immunopositive neurons (CRH+/VP+, n = 12), CRH as well as VP immunonegative neurons (CRH-/VP-, n = 12), VP immunopositive but CRH immuno-

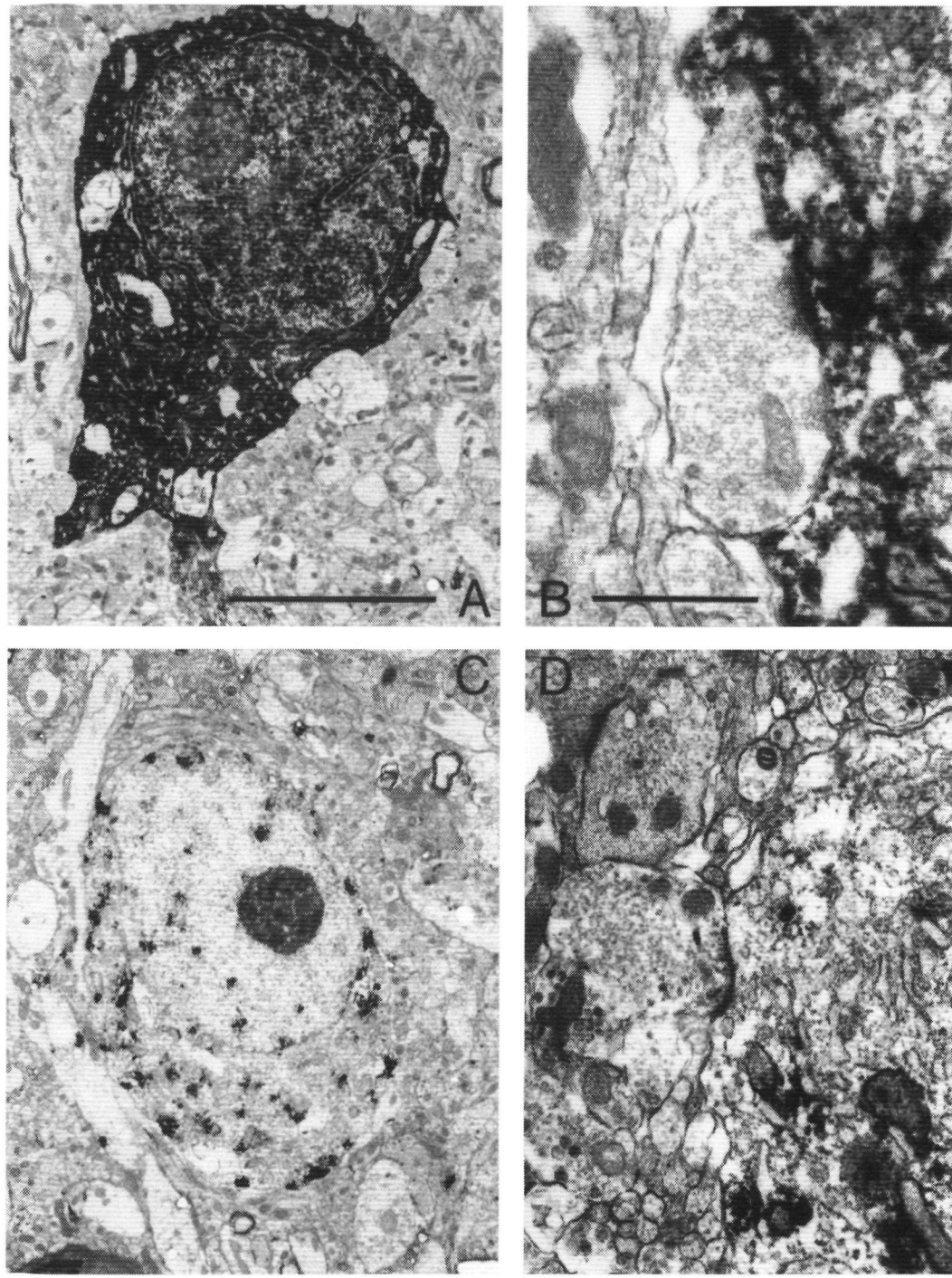

FIGURE 4 Electron micrographs of two PVH neurons treated with pre-embedding CRH-immunohistochemistry, illustrating the difference of staining intensity at a similar depth from the vibratome section surface (A and C). B and D show synaptic contacts on a darkely and a lightly stained CRH neuron, respectively. Calibration bars: 5 μm (A, also applies to C) and 1 μm (B, also applies to D).

TABLE I The synaptic density (per μm^3) and estimated number of synapses as determined for the reconstructed CRH cell bodies in the PVH

Neurons	Synaptic density (N_s)	Estimated number of synapses
01	.081	
02	.216	84
04	.132	
05	.150	
07	.203	
08	.067	
09	.133	
10	.126	64
11	.089	40
12	.132	64
13	.177	
14	.153	
16	.154	55
18	.268	109
20	.077	37
21	.221	
22	.075	
23	.123	51
24	.096	34
25	.169	
26	.139	48
27	.314	103
Mean	.150	63
SD	.064	25.8
SEM	.014	7.8

negative neurons (VP+/CRH-, n = 8) and a group of not clearly immunohistochemically identifiable neurons (n = 21) (fig. 2). Of the 8 CRH+/VP- neurons 6 were located in the PCc, 1 in the MC and 1 in the PCd. The CRH+/VP+ neurons were all located in the PCc where they were intermingled with the CRH+/VP- neurons. Of the 8 VP+/CRH- neurons 6 were located in the MC and 2 in the PCc. The 12 CRH-/VP- neurons were distributed over the MC (3), the PCc (6) and the PCv (3). The CRH-/VP- neurons in the MC seem to be larger than the CRH-/VP- neurons observed in the other subdivisions and also larger than the CRH+ neurons, regardless whether the latter are VP+ or VP-.

In line with the results of the pre-embedding immunohistochemical procedure CRH+ synapses or terminals were not observed on the CRH+ neurons. Equally, we did not find CRH+ synapses or VP+ terminals on any other type of neuron.

THE DISTRIBUTION OF GABA In sections treated for post-embedding GABA immunogold labeling gold particles appear to be distributed throughout the terminals and not confined to their clear vesicles. To compare the distribution of the GABA positive (GABA+) and GABA negative (GABA-) synapses of the different neurons as characterized above we counted the number of GABA+ and GABA- synapses per profile and summated these numbers per neuron, after which the GABA+/GABA- synapse ratio per neuron was calculated. The results are summarized in table II. In line with the results of the pre-

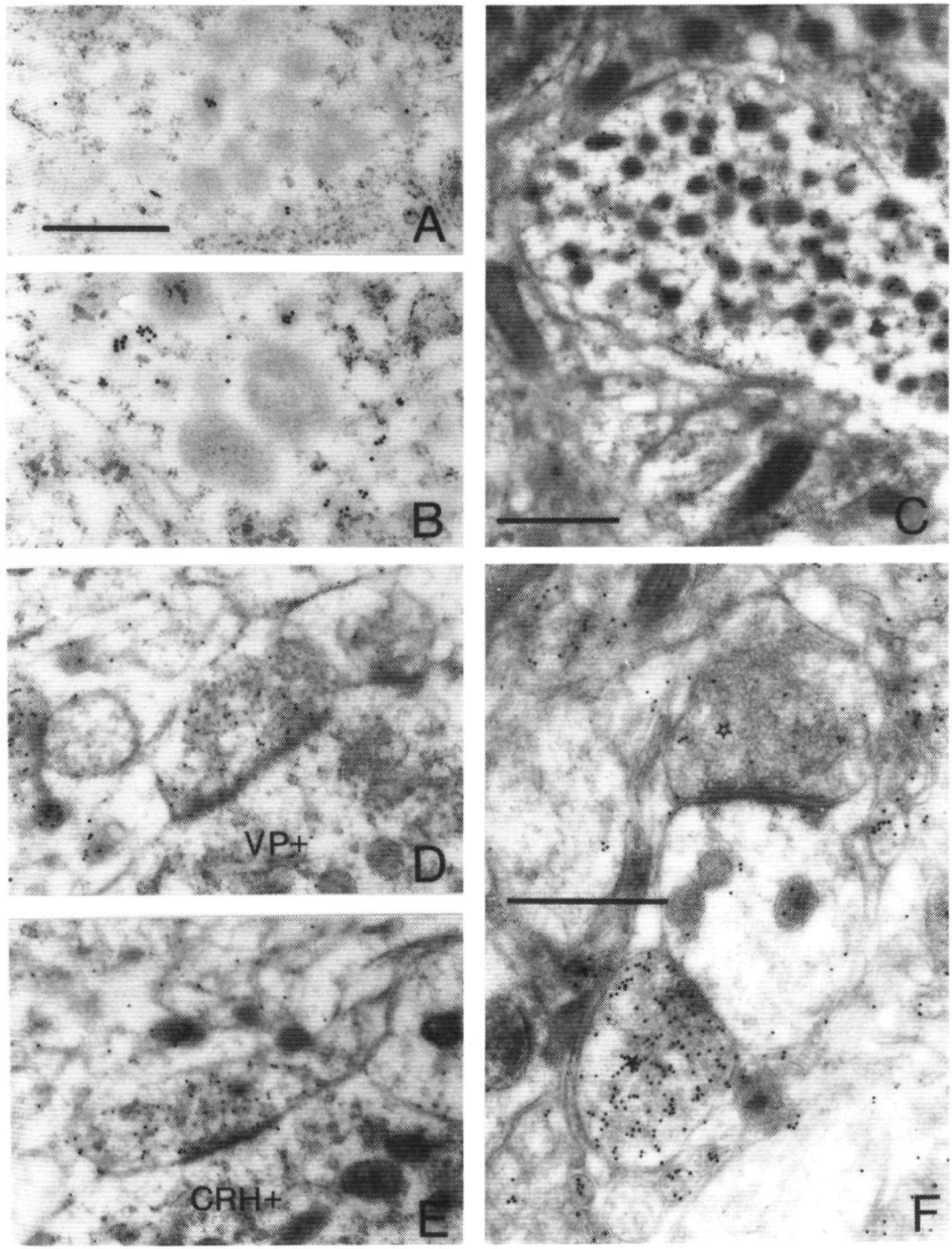

FIGURE 5 Electron micrographs of PVH sections treated for post-embedding immunohistochemistry, illustrating the presence of goldparticles on electron lucent CRH-immunoreactive granules (A and B) and on electron dense VP-immunoreactive granules (C). D and E show GABA positive synapses on a VP+/CRH– neuron and a CRH+/VP– neuron, respectively. F shows the convergence of a GABA positive (black star) and a GABA negative (empty star) synapse on an unidentified dendritic profile. Calibration bars: 0.5 μm (A, also applies to B) and 1 μm (C and F, the latter also applies to D and E).

TABLE II Number of GABA+ and GABA– synapses and the GABA+/GABA– ratio on different types of PVH neurons (mean ± SEM)

Parvocellular CRH+/VP– neurons			Parvocellular CRH+/VP+ neurons			Parvocellular CRH–/VP– neurons			Magnocellular CRH–/VP– neurons			Magnocellular VP+/CRH– neurons		
GABA+	GABA–	ratio	GABA+	GABA–	ratio	GABA+	GABA–	ratio	GABA+	GABA–	ratio	GABA+	GABA–	ratio
14	9	1.56	17	11	1.54	9	5	1.80	7	12	0.58	20	22	0.91
19	12	1.58	8	9	0.89	5	9	0.56	16	18	0.89	13	19	0.68
17	9	1.89	10	9	1.11	6	6	1.00	19	27	0.70	7	13	0.54
20	14	1.43	16	11	1.45	9	12	0.75				10	15	0.67
17	7	2.43	15	11	1.36	20	15	1.33				9	17	0.53
17	16	1.06	15	11	1.36	12	4	3.00				18	15	1.20
9	10	0.90	13	6	2.17	9	2	4.50				15	17	0.88
24	9	2.67	13	6	2.17	13	3	4.33				11	16	0.69
			3	2	1.50									
			9	9	1.00									
			19	10	1.90									
			7	5	1.40									
Mean 17	11	1.69	13	9	1.57	9	7	2.09	14	19	0.72	13	17	0.76
SEM 1.56	1.06	0.22	1.13	0.64	0.14	1.84	1.34	0.50	3.61	4.36	0.09	1.60	0.98	0.08

embedding histochemical procedure we found a large interneuronal variation in the number of synapses. On CRH+/VP– neurons the number of synapses observed on the 10 profiles analysed per cell varies from 19 to 34 with an average number of 28 and on CRH+/VP+ neurons this synapse number varies from 12 to 29 with an average of 22. The GABA+/GABA– ratio is 1.62 ± 0.12 for the CRH+ neurons without significant differences between the CRH+/VP– (1.69 ± 0.22) and CRH+/VP+ neurons (1.57 ± 0.14). On magnocellular VP+/CRH– neurons the number of synapses as counted on 10 profiles per cell varies from 20 to 42 with an average of 30, which is comparable with the synapse numbers on the CRH+ neurons. However, the GABA+/GABA– ratio of these VP+/CRH– neurons is statistically significantly lower than that of the other neurons (0.76 ± 0.08, $p < 0.04$).

The CRH–/VP– neurons show a larger variation in the number of synapses than the CRH+ neurons since on these neurons the synapse number per 10 profiles varies from 5 to 46 with an average number of 21. Nonetheless, these neurons show a similar GABA+/GABA– synapse ratio (1.75 ± 0.41) as the CRH+ neurons. They can be further subdivided in two subgroups on the basis of location in the PVH, i.e. a parvocellular group (n = 9) with a GABA+/GABA– ratio of 2.09 ± 0.50 and a magnocellular group (n = 3) with a GABA+/GABA– ratio of 0.72 ± 0.09. Statistical comparison shows that

the magnocellular CRH-/VP- group is statistically significantly different from the parvocellular CRH+/VP- ($p < 0.01$) and CRH+/VP+ neurons ($p < 0.02$), but not from the magnocellular VP+/CRH- neurons. In contrast, the parvocellular group of CRH-/VP- neurons is statistically significantly different from the magnocellular VP+/CRH- neurons ($p < 0.01$) but not from the parvocellular CRH+ neurons.

DISCUSSION

In the present study we have established that the number of synapses contacting the somata of CRH neurons in the PVH may vary from 34 to 109, with an average of 63. Approximately 62% of these synapses is GABA-ergic without differences between CRH+ neurons with or without co-localized VP-immunoreactivity (-IR). In contrast, magnocellular VP+ as well as VP- neurons have a statistically significantly lower GABA+/GABA- synapse ratio (only 42% is GABA-ergic) than the parvocellular CRH+ as well as CRH-/VP- neurons (68% is GABA-ergic). To evaluate these results we will first discuss the consequences of ADX, and then compare our results with previous light and electron microscopical data concerning input and function of CRH+ and CRH- neurons.

CONSEQUENCES OF ADX In normal rats the neurochemical content of CRH in the parvocellular neurons of the PVH is too low to visualize these neurons with current immunohistochemical techniques, especially in the electron microscope. To overcome this problem we used adrenalectomized (ADX) rats to have a tool to differentiate between neurochemically and thus probably also functionally different PVH neurons. Removal of the negative feedback of plasma corticosteroids by ADX increases the CRH content in the parvocellular neurons substantially (Sawchenko 1987). However, ADX does not only introduce a higher CRH content in CRH neurons, but also a number of other changes in the central nervous system, including hippocampal cell death (Sloviter et al. 1989, Sapolsky et al. 1991), increased production of vasopressin by the CRH neurons (Whitnall et al. 1985) and receptor changes in the PVH (Jhanwar-Uniyal and Leibowitz 1986, Reul et al. 1987, Castrén and Saavedra 1989). In addition, ADX induces a reduction of input from the bed nucleus of the stria terminalis (BNST) to the CRH neurons in the PVH (Mulders et al. submitted). The latter is important in view of the present results, since it indicates that ADX might induce changes in the synaptic organization of the PVH and especially in the synaptic input of its CRH neurons. Therefore, one should be careful extrapolating the present results to the normal situation since input to CRH neurons in ADX rats might differ from similar data in untreated rats, both with respect to the synaptic density and the immunohistochemical composition. In spite of these possible changes, ADX rats are still well suited to reveal constraints and variability in the synaptic organization of distinct populations of neurons in the PVH and the use of ADX rats is presently the only way to study the questions put forward in the introduction. To investigate the precise impact of ADX on the synaptic organization of the PVH, more sensitive immunohistochemical methods, allowing for the reliable distinction of CRH neurons in electron microscopical sections of normal rats, have to become available.

This study is the first one describing the number of synapses on CRH somata, which is found to be 63 ± 7.8. In a previous morphometric study we calculated the overall synapse-neuron ratio of different PVH subdivisions, which appeared to be approximately 900 in the central parvocellular part (PCc) (Mulders et al. 1995a). Since the majority of the CRH neurons is situated in the PCc (Swanson et al. 1983, Mulders et al. 1995b) and most dendrites of PVH neurons are confined to the subdivision in which the parent cell bodies are situated (Armstrong et al. 1980, Van den Pol 1982), this means that about 7% of the synapses on CRH neurons is axo-somatic. This percentage is comparable with the results of Decavel and Van den Pol (1990) who found 4% of all synapses in the parvocellular part of the PVH to be axo-somatic. As is visible in table I the interneuronal variability in synaptic density per neuronal surface (N_s) and the number of synapses between CRH neurons is large. Both the N_s and synapse number show a large variation, varying from 0.067 to 0.314 per μm^2 and from 34 to 109, respectively. These data suggest that the synaptic input to CRH neurons is not very strictly organized or hard-wired, but instead rather variable. The large interneuronal variation might well point to a plasticity at the synaptic level allowing for adaptation to physical and/or environmental changes. This is in line with our previous experiments which indicated plasticity of the input from the BNST to the CRH neurons (Mulders et al. submitted).

We did not find any CRH-IR synapses on CRH+ neurons, neither in the sections treated according to the pre-embedding immunohistochemical procedure nor in the sections treated according to the post-embedding immunohistochemical procedure. The latter procedure also revealed that CRH-IR synapses did not contact CRH- neurons. This suggests that CRH is not involved as a neurotransmitter in the regulation of PVH neurons. However, previous EM studies indicated that CRH axon collaterals establish synaptic contacts with CRH+ as well as CRH- neurons, suggesting a local influence of CRH neurons (Liposits et al. 1985, Silverman et al. 1989, Hisano et al. 1992). The discrepancy with the present result might partly be due to methodological differences and partly to the use of different rat strains. Liposits et al. (1985) studied ADX Wistar rats using a silver intensification method and describe that CRH-IR synaptic contacts on CRH somata occur occasionally, without a further quantification. Our results do not exclude the occasional occurrence of such contacts, but suggest that these are very infrequent.

Silverman et al. (1989) used Fischer 344 rats in which CRH staining can be obtained without ADX. They found numerous CRH synapses, particularly in the periventricular zone (PV). The largest portion of these synapses is axo-dendritic, but axo-somatic contacts were also observed: 10% of the synapses investigated contacted CRH+ neuronal cell bodies and 17% CRH- neuronal cell bodies. Fischer 344 rats seem to have a different mechanism for activation of the CRH neurons, enabling visualization even in untreated rats. Thus, it is well possible that these rats have a different synaptic organization of the CRH neurons than Wistar rats, which might explain the differences with the present results. Moreover, Silverman et al. (1989) describe in particular the synaptic organization in the PV which was not included in the present study. Hisano et al. (1992) used Sprague-Dawley rats treated with colchicine and combined DAB and silver-gold particle methods. They investigated especially the oxytocin-rich area of the magnocellular PVH. In this subdivision they occasionally observed CRH synapses on magnocellular as well as on smaller neurons. However, they did not investigate the parvocellular part of the PVH, as was done in the present study.

We could not detect any VP+ synapses on the neurons analyzed, suggesting a limited role for

VP as a neurotransmitter in the MC, PCc, PCd and PCv of the PVH. In literature there is some disagreement on the presence of VP axon collaterals and VP terminals in the PVH. According to a morphological study of Van de Pol (1982) axon collaterals of magnocellular neurons do not occur in the PVH, whereas axon collaterals of parvocellular neurons do occur. In contrast, Choudhury and Ray (1990) and Ray and Choudhury (1990) did find VP-IR axon collaterals in the PVH, although their number was limited. These authors and others also describe VP-IR terminals in contact with VP+ somata (Nakada and Nakai 1985, Choudhury and Ray 1990, Ray and Choudhury 1990). This is in contrast with the results of the present study as well as of Piekut (1983) who describes an absence of VP-IR terminals on VP+ neurons. The lack of VP synapses on VP- neurons as found in the present study is in agreement with the results of Nakada and Nakai (1985) who equally did not find VP synapses on VP- neurons.

DISTRIBUTION OF GABA-ERGIC INPUT We investigated the GABA-ergic innervation of the somata of the different types of neurons, i.e. parvocellular CRH-IR but not VP-IR neurons (CRH+/VP-), parvocellular CRH-IR as well as VP-IR neurons (CRH+/VP+), magnocellular VP-IR but not CRH-IR neurons (VP+/CRH-), and finally the magnocellular as well as parvocellular neither CRH-IR nor VP-IR neurons (CRH-/VP-), by comparison of the GABA+/GABA- ratio. The predominant location of the VP+/CRH- neurons in the MC is in agreement with previous studies describing the distribution of magnocellular VP neurons (Rhodes et al. 1981). The VP-IR granules observed in a portion of the CRH+ neurons were never present in the same amount as observed in magnocellular CRH- neurons. Co-localization of VP and CRH is likely to be a result of ADX, since it has been shown that ADX enhances VP-IR in a subgroup of the CRH+ neurons (Whitnall et al. 1985, Whitnall 1990), which co-release VP together with CRH to potentiate the release of ACTH (Gillies et al. 1982, Whitnall 1988).

Interestingly, immunohistochemically distinct groups of parvocellular neurons show no mutual differences in GABA-ergic innervation. The same holds for magnocellular neurons, whether they are VP+ or VP-. However, the parvocellular types all differ significantly from the magnocellular types with respect to their GABA-ergic synaptic input. These data strongly suggest that the distribution of GABA-ergic input in the PVH is primarily determined by the location of neurons within the PVH and not by the neurochemical content of these neurons. This idea is in line with the results of Meister et al. (1988) who describe a dense network of glutamic acid decarboxylase-fibers (GAD, the GABA-synthesizing enzyme) in the parvocellular part and a less dense network in the magnocellular part, which also suggests a specificity of input for distinct subdivisions. Since GABA-ergic input is inhibitory (Calogero et al. 1988), our results suggest that inhibitory input is less important in the magnocellular PVH than in the parvocellular PVH.

The large GABA-ergic input to the CRH neurons is somewhat unexpected in view of our previous tracer studies in which we found a reduced innervation of the CRH-rich part of the PVH from the BNST after ADX (Mulders et al. submitted). Since the input from the BNST to the PVH is probably GABA-ergic (Cullinan et al. 1993), we hypothesized that the GABA-ergic input to the CRH neurons following ADX would be significantly diminished compared with controls. This suggests that the GABA-ergic innervation of CRH neurons in normal rats may even higher than that observed in the present study for ADX rats. However, the relative contribution of GABA-ergic input from the BNST might be relatively small compared to the

GABA-ergic input from hypothalamic GABA-ergic cell groups (Tappaz 1982, Tappaz et al. 1983, Tasker and Dudek 1993) and thus a decrease in input from the BNST might induce only a minor change in synaptic organization.

The transmitters used by the 40-60% GABA- synapses on PVH neurons have not yet been characterized, but many other neurotransmitters than GABA have been shown to be involved in the synaptic organization of the PVH. One major type of input is catecholaminergic, both in the parvocellular as well as the magnocellular PVH (Nakada and Nakai 1985, Silverman et al. 1985, Decavel et al. 1987). Also neuropeptide Y, co-localized with catecholamines, seems to play a major role in the PVH (Kagotani et al. 1989a) making a large amount of synaptic contacts in the parvocellular (Sawchenko and Pfeiffer 1988) as well as in the magnocellular PVH (Kagotani et al. 1989b). Galanin (Sawchenko and Pfeiffer 1988), ACTH and substance P (Heike et al. 1986, Liposits et al. 1988) also seem to play a role. The precise distribution of these neurotransmitters on distinct cell types of the PVH neurons remains to be determined with additional quantitative electron microscopical analyses in the future.

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Chapter 7

General discussion and summary

PREFACE

In this thesis we investigated morphological and functional characteristics of the CRH neurons in the PVH, in order to gain more insight in the organization of these neurons. The PVH contains besides CRH a variety of other neuropeptides which all subserve different functions. Therefore, it seems logical that all these peptides must be differentially regulated. This implies that the PVH receives large amounts of different inputs which must all be organized in such a way that they can innervate a particular type of neuron and thus affect a particular function. Consequently, we expected a rather rigid organization of the PVH and its CRH neurons, which means an univocal and predetermined correlation between different morphological aspects, as distribution of neurons, immunohistochemistry and synaptology, as well as inputs and also function of the different neurons.

However, the different experimental set ups, as used in this thesis, show that several characteristics of the CRH population are considerably variable between rat lines and under different hormonal conditions. In addition, the synaptic arrangement does not seem to be strictly organized according to neuropeptidergic contents of neurons, e.g. according to function, but more according to location of neurons within the PVH. Finally, the pathway of activation of the CRH neurons does not seem to make much difference as to which CRH neurons are activated.

In the following paragraphs we will summarize our results from chapter 2 till 6 and discuss the different observations in view of CRH function and argue that the observed lack of a strict organization is important in maintaining plasticity and adaptation possibilities in the HPA-axis. At the end, we will present some general ideas for future experiments designed to shed more light on questions raised following the experiments performed in this thesis.

SUMMARY OF RESULTS

In chapter 2 we compared the morphometric characteristics of the PVH in two Wistar rat lines which are pharmacogenetically selected on the basis of their susceptibility for apomorphine, the APO-SUS and APO-UNSUS rats. These rats, which represent two extremes of a normal Wistar population and have been shown to display different stress responses (Cools et al. 1990, Van Eekelen et al. 1992, Rots et al. 1995, Rots et al. 1996), show a similar organization of the PVH within different subdivisions as appears from light microscopical criteria such as neuron size and neuronal density (chapter 2). However, these rat lines do show a different organization at the electron microscopical level as appears from the statistically significant different synaptic densities in the PVH of both rat lines. The APO-SUS rats have a higher synaptic density in the overall PVH as well as in the PV, PCc and PCp as compared to the APO-UNSUS rats. This observation implicates that even within one strain of rats the micro-organization of the PVH is variable. The question arises whether this observed synaptic density difference correlates in any way with the results obtained in chapter 3.

In chapter 3 we found, after a mild stressful stimulus, an open field paradigm, less activated CRH neurons in the PVH of APO-SUS rats as compared to APO-UNSUS rats. Since the differences in synaptic density between the two rat lines were found in the PV, PCc and PCp and since these subdivisions are also the major source of the hypophysiotropic CRH neurons, projecting to the median eminence (Swanson and Kuypers 1980, chapter 4), we think that

this difference in the number of activated CRH neurons might well be associated with the different synaptic densities in both rats. If the lower number of activated CRH neurons following a mild stressor is correlated with the higher synaptic density in APO-SUS rats, while the higher number of activated CRH neurons after the same stressor is correlated with a lower synaptic density in APO-UNSUS rats, then this would indicate a larger inhibitory input to CRH neurons in APO-SUS rats.

A major inhibitory neurotransmitter in the PVH is GABA, which is found in large amounts in all subdivisions of the PVH and also in direct contact with CRH neurons (Decavel et al. 1989, Decavel and Van den Pol 1990). One source of GABA in the PVH is the bed nucleus of the stria terminalis (BNST). The BNST is thought to play an important role in funneling limbic information from the hippocampus and amygdala to the PVH (Feldman et al. 1991, Cullinan et al. 1993, Canteras et al. 1995). With respect to the results obtained in chapter 2 and 3 the hippocampus seems to be of major importance since this structure is thought to play a role in the feedback mechanism of corticosteroids. Our results suggest an increased activity of the hippocampal-BNST-PVH pathway in APO-SUS rats. Rots and coworkers showed a higher corticosteroid response in APO-SUS rats after stressful stimulation (Rots et al. 1995, Rots et al. 1996). The corticosteroids then activate the hippocampus, which, in its turn, inhibits the activity of the CRH neurons via the BNST. An increased GABA-ergic pathway from the BNST to the PVH in APO-SUS rats, as suggested by the results of chapter 2 and 3 might enhance this inhibition, leading to a low number of activated CRH neurons in the PVH following a mild stressor in APO-SUS rats. An increased influence from the hippocampus is in agreement with the work of Sutanto et al. (1989) who found a higher in vivo binding of corticosteroids to the mineralocorticoid receptor in the hippocampus of APO-SUS rats than in APO-UNSUS rats. A higher binding of corticosteroids in the hippocampus of APO-SUS rats would also enhance the inhibiting capacity of the hippocampus on the activity of the HPA-axis. The lower number of activated CRH neurons seems to be in conflict with the higher ACTH and corticosteroid response as reported by Rots et al. (1995, 1996). However, these authors also found a higher basal level of CRH mRNA in APO-SUS rats (Rots et al. 1995). It is possible that this basal CRH mRNA is used after a stress stimulus to increase the ACTH levels which makes immediate activation of the CRH neurons unnecessary. On the other hand, it could be possible that the pituitary in APO-SUS rats has a higher sensitivity to CRH, which would explain the higher ACTH and subsequent corticosteroid response even after a low release of CRH. Since the pituitary contains a large amount of CRH receptors (Grigoriadis et al. 1993), the number and/or binding capacities might well be enhanced in APO-SUS rats as compared to APO-UNSUS rats, leading to a higher sensitivity for CRH. In conclusion, our results and the work of Rots et al. (1995, 1996) clearly show that the APO-SUS and APO-UNSUS rats show differences at several levels of the HPA-axis. Further studies will be necessary to elucidate the precise functioning of this system in the two rat lines.

In chapter 4 we compared the distribution of activated CRH neurons under different experimental conditions. Comparison between colchicine treated and ADX rats illustrated that only a part of the CRH neurons is involved in the regulation of the HPA-axis and is under the control of circulating corticosteroids, confirming earlier studies (Swanson and Kuypers 1980, Kawano et al. 1988, Rho and Swanson 1989). This observation was confirmed by treatment with anti-CRH, which resulted in a similar distribution of activated CRH neurons as ADX. Anti-CRH removes CRH from the circulation, preventing ACTH release and in this way keeps the level of circulating corticosteroids low. Therefore, the use of anti-

CRH would be a good alternative to ADX. The most remarkable finding in this chapter is the observed effect of IL. Although we failed to show an excitatory effect of IL on the short term by icv injection, as we expected on the basis of previous studies (Berkenbosch et al. 1987, Sapolsky et al. 1987, Veening et al. 1993, Lee and Rivier 1994), we did observe an inhibitory effect on the long term. Three and ten days after a single iv injection with IL we counted less activated CRH neurons in the PVH as compared to control rats. To our knowledge such an inhibitory effect of IL has not been shown previously and further studies are necessary to gain more insight in the mechanism underlying this phenomenon.

Since chapter 2 and 3 suggested that inhibitory pathways to the PVH, and possibly in particular the input from the hippocampus via the BNST, play an important role in the CRH regulation, we investigated the BNST-PVH pathway in more detail in chapter 5. With the use of an anterograde tracer we observed a large projection from the BNST to all subdivisions of the PVH, in agreement with earlier studies (Silverman et al. 1981, Sawchenko and Swanson 1983). Remarkably, in ADX rats, after similar injections in the BNST as in normal rats, we observed a significantly decreased input to all subdivisions of the PVH and specifically to the CRH-rich part. This result indicates that ADX evokes changes in the pathway from the BNST to the PVH and especially to its CRH neurons and thus changes in the synaptic organization. When evaluating this result several aspects must be considered and different questions arise. First of all, what is the function of this loss of input? Input from the BNST is thought to be GABA-ergic (Cullinan et al. 1993) and thus inhibitory. ADX evokes a constant activation of the CRH neurons, which leaves inhibitory input useless and even unwanted. Removal of the inhibitory GABA-ergic input might be a mechanism to facilitate the chronic activated state of the CRH neurons following ADX. Secondly, and closely correlated with the functional aspect of the decreased input, one must note that ADX is not a physiological condition. This means that under normal circumstances a loss of corticosteroids is unlikely to occur. However, the opposite, an elevated corticosteroid level, will be present during chronic stressful conditions. The question arises whether a long-term rise of corticosteroid levels will induce the opposite of the observed phenomenon, i.e. an increased input from the BNST to the PVH? Thirdly, how does this phenomenon of decreased input occur? Is the loss of axons evoked by cell death in the BNST or do the axons actively retract from their target neurons? This question is closely associated with the following question. What is the underlying mechanism of this decreased input from the BNST to the PVH? In our view there are two possibilities, an anterograde or retrograde mechanism. An anterograde mechanism might finds its origin in the hippocampus. ADX is known to induce cell death in the hippocampus (Sloviter et al. 1989, Sapolsky et al. 1991), which might lead to reduced input from the hippocampus to the BNST which, in its turn, might be responsible for the reduced input to the PVH by cell death in the BNST. A retrograde effect might be evoked in the PVH itself. ADX is known to induce receptor changes in the PVH (Jhanwar-Uniyal and Leibowitz 1986, Reul et al. 1987, Castrén and Saavedra 1989), which might evoke retraction of certain axonal inputs by a presynaptic feedback mechanism. Finally, it would be interesting to find out whether this decreased input from the BNST to the PVH is a reversible phenomenon. When this is the case then plasticity of this BNST-PVH pathway and thus changes in the synaptic organization might well be a mechanism for the organism to cope with extreme changes in corticosteroid levels.

In relation to the apparent plasticity in synaptic organization observed in chapter 5 we investigated in more detail the synaptic organization of the PVH and in particular the GABA-ergic innervation of CRH and non-CRH neurons in chapter 6. We performed this

experiment on ADX rats only to obtain a reliable CRH staining. We found that about 7% of the synapses in the PVH is axo-somatic. In CRH neurons, with or without co-localized vasopressin, about 65% of these axo-somatic synapses is GABA-ergic. The CRH immunonegative parvocellular neurons receive a similar degree of GABA-ergic input, whereas magnocellular neurons, vasopressinergic and non-vasopressinergic, receive a statistically significantly lower percentage of GABA-ergic input of about 42%. These results suggest that the synaptic organization of the GABA-ergic input to the PVH is not very strict and that the position of neurons within the PVH is more important than their immunohistochemical content. It was surprising that we found such a high GABA-ergic input to CRH neurons in these ADX rats. We expected a lower input of GABA in agreement with the results of chapter 5. However, since also hypothalamic GABA-ergic groups are known to project to the PVH (Tappaz et al. 1982, Tappaz et al. 1983, Tasker and Dudek 1993), it might well be that the relative contribution of BNST GABA-ergic fibers is small as compared to these hypothalamic GABA-ergic inputs.

CONCLUSIONS

MORPHOLOGICAL AND MORPHOMETRIC OBSERVATIONS In this thesis we investigated the CRH neurons in the PVH by looking into morphological and functional aspects of these neurons in order to elucidate their organization structure. The experiments of chapter 2 and 4 were designed to gain more insight in this organization at different levels by determination of morphometric and immunohistochemical characteristics. Looking into the morphometric characteristics of the PVH reveals 6 subdivisions on the basis of neuronal size, density and vascularity (chapter 2), which are partly in agreement with the subdivisions made by other authors (Armstrong et al. 1980, Swanson and Kuypers 1980, Kiss et al. 1991). However, there is no rigid separation between these subdivision in the sense that magnocellular neurons do occur in the parvocellular part and, also the other way around, that parvocellular neurons do occur in the magnocellular part. A similar lack of a strict organization is illustrated by the electron microscopical morphometric characteristics of the PVH such as synaptic density (chapter 2). All subdivisions in the PVH show a similar synaptic density, which indicates that all subdivisions receive a similar degree of input. Although the composition and origin of this synaptic input is not determined in this experiment and might well differ between the subdivisions, these electron microscopical results, similar to the light microscopical observations, fail to show an obvious organization of the PVH and its subdivisions. In this view the results of chapter 5 are noteworthy, showing an uniform distribution of BNST fibers in the PVH, supporting the morphometric observations in chapter 2.

Investigations after the distribution of the CRH neurons in the PVH over the 6 subdivisions showed a preferential location of CRH neurons in the PCc (chapter 3 and 4). However, these neurons were all found in the other subdivisions as well. Using different treatments to activate the CRH neurons (chapter 4) we tried to gain more insight in to how the different CRH neurons are distributed over the PVH. We did find a subdivision of the CRH neurons in two major groups, one responsive to ADX and thus responsive to disruptions of the HPA-axis and one not responsive to ADX. These two subgroups seem to correlate to the CRH neurons shown to project to the median eminence and the ones projecting to the spinal cord,

as reported in previous studies (chapter 4, Swanson and Kuypers 1980, Rho and Swanson 1989). Although these functional subgroups have a preferential location within the PVH, the different CRH neurons are also intermingled within the different morphometrically determined subdivisions involved (chapter 4, Swanson and Kuypers 1980). In agreement, the same is shown to hold true for other neuropeptidergic neurons in the PVH, such as vasopressin and oxytocin, as is shown by other authors (Rhodes et al. 1981, Coolen et al. submitted). These latter two types of neurons are predominantly found in the magnocellular part but can be found in the parvocellular part as well.

When we summarize the results of the chapters discussed above we can conclude that none of the experiments revealed a very strict or rigid organization of the PVH. Not as far as general morphometric parameters are concerned, nor in the distribution of the CRH neurons. The question thus still remains how the paraventricular nucleus is regulated, since it is involved in a variety of functions, involving different neuropeptides.

ACTIVATION OF CRH NEURONS In chapter 3 and 4 we used different activation mechanisms to stimulate the CRH neurons and different detection methods to determine neuronal activation. In chapter 3 we used an open field paradigm as a mild stressful stimulus for two Wistar rat lines, APO-SUS and APO-UNSUS rats and used a Fos immunostaining to measure activation of neurons in the PVH. Since the distribution of Fos positive nuclei was similar to the distribution of CRH neurons following ADX and double staining revealed large amounts of double stained Fos/CRH neurons, we hypothesized that the Fos nuclei visualized in the PVH following an open field paradigm represent activated CRH neurons. This is in line with previous observations showing co-localization of Fos and CRH and c-fos and CRH mRNA after different stressors (Beyer et al. 1988, Imaki et al. 1992).

In chapter 4 we used CRH immunohistochemistry to reveal activated CRH neurons. Control rats show a very low intensity of CRH immunostaining as well as a low number of visible CRH neurons and CRH immunostaining is thought to be a useful tool to visualize activated CRH neurons. From these experiments we confirmed earlier studies that ADX and anti-CRH treatment and thus disruption of the HPA-axis, only activates a part of the CRH neurons in the PVH (Swanson et al. 1983, Sawchenko 1987).

Since we failed to show an effect of icv IL on the CRH neurons (chapter 4) we cannot exclude the possibility that IL activates a specific subgroup of CRH neurons. It has been shown previously that ADX induces co-localization of vasopressin in a subgroup of the CRH neurons (Whitnall et al. 1985) and it is possible that IL influences specifically these CRH neurons or an other yet to be determined subgroup. Nonetheless, similar to the morphometric experiments in chapter 2 we could again not detect a strict organization within the PVH of the CRH neurons with respect to their activation mechanism and localization.

The fact that we could not find an increase in the number of CRH neurons as compared to controls after icv IL seems to be in conflict with earlier studies investigating the effects of icv IL (Rivest et al. 1992), since these studies did report a rise in Fos immunostaining after IL icv. Does this mean that there is no correlation between the Fos staining and CRH neuron activation as hypothesized in chapter 3? In my opinion this is not the case. Several studies have shown a correlation between Fos and CRH (Beyer et al. 1988, Imaki et al. 1992). The discrepancy might be that there is a high release rate after icv IL which prevents stacking of CRH in the PVH neurons and thus might prevent CRH immunostaining. This would also explain a measurable Fos and CRH mRNA level in the PVH following IL treatment (Rivest

et al. 1992, Lee and Rivier 1994), but an absence of detectable CRH peptide staining since measurement of mRNA is more sensitive than immunostaining of the CRH peptide.
The most remarkable observation in chapter 4 was the apparent inhibition of CRH neurons after long-term IL treatment. Since this inhibition was notably only in the PCc and PCv, two of the subdivisions influenced by ADX or anti-CRH treatment, this means that only part of the CRH neurons involved in the HPA-axis is responsive to long-term IL treatment. This may indicate the existence of a third subgroup of CRH neurons.

INPUTS AND SYNAPTOLOGY In chapter 2, 5 and 6 we looked deeper into the synaptic organization and input of the PVH and its subdivisions. As already discussed above the synaptic density in the PVH did not reveal a strict organization between subdivisions. All subdivisions received a similar amount of input. A similar result was observed in chapter 5 where tracer experiments in the BNST showed that all subdivisions received a similar amount of fibers from the BNST. It must be noted that other authors observed a larger innervation of the parvocellular part of the PVH as compared to the magnocellular part from the BNST (Sawchenko and Swanson 1983, Thellier et al. 1994). Nonetheless, our experiments failed again to show a strict organization of the PVH in the sense that we did not find a univocal correlation between subdivisions and synaptic input.

The immunocytochemical study at the electron microscopical level (chapter 6) also failed to reveal a strict organization of the CRH neurons in the PVH as compared to other neurons. We showed that the GABA-ergic innervation of the PVH neurons does not depend on the neurochemical identity of the paraventricular neurons but on their localization within the PVH. Magnocellular neurons, whether vasopressinergic or not, receive a similar degree of GABA-ergic input. The same holds true for the parvocellular CRH or non-CRH neurons, whether they co-localize vasopressin or not. It cannot be excluded that other neurotransmitters do show a more specific organization by a preference for certain neuropeptidergic neurons in the PVH. Previous studies have shown that noradrenergic input innervates mainly the magnocellular part (Decavel et al. 1987), whereas neurotransmitters as neuropeptide Y and galanin are found predominantly in the parvocellular part (Sawchenko and Pfeiffer 1988). Further research has to be carried out to elucidate whether these inputs are distributed according to the anatomical subdivisions within the PVH or according to the neuropeptidergic content of paraventricular neurons. As far as our results stand we can state that the synaptic GABA-ergic innervation of the PVH and its CRH neurons does not show a very strict organization. Apparently, the efferent connections of the PVH must be organized in such a way that they can innervate a particular group of neuropeptidergic neurons in the PVH but in our experiments we could not find any sign of such a organization at the level of the PVH.

VARIABILITY OF THE CRH NEURONS IN THE PVH As follows from the obtained knowledge on the organization of the CRH neurons in the PVH or rather the lack of organization, the present thesis provides information on the variability and plasticity of the CRH neurons in the PVH. The results of chapter 2 and 3 illustrate that even within one strain of Wistar rats variability in neuroanatomical characteristics can be observed. The two rat lines investigated, the APO-SUS and APO-UNSUS rats, show differences at the level of synaptic organization as well as on the functioning of the HPA-axis by a differential

responsiveness of CRH neurons to a mild stressor. The differences between both rat lines are thought to be evoked by a higher level of circulating corticosteroids in the early postnatal period (Cools et al. 1990). Remarkably, the results of chapter 6 indicate that circulating corticosteroids are also capable of inducing large changes in the organization of the brain in adult rats. Loss of corticosteroids by ADX evokes a loss of interconnectivity and thus information transfer between the BNST and the PVH in mature rats. We hypothesized that this loss of information serves as a mechanism to cope with changes in the organism's environment. We were not the first ones describing anatomical changes following hormonal disturbances, since ADX has also been shown to evoke receptor changes in the PVH (Jhanwar-Uniyal and Leibowitz 1986, Reul et al. 1987, Castrén and Saavedra 1989).

As a conclusion, our results suggest that the HPA-axis is a very plastic system capable of adaptation to changes in the hormonal balance of an organism in young and adult stages, at least at the level of the PVH. It is important to note that although we use the term plasticity we have not actually shown that the changes observed are reversible. Further research has to be carried out to determine whether these changes are reversible or are a form of unrepairable damage as holds for the apoptotic processes in the hippocampus following ADX (Sloviter et al. 1989, Sapolsky et al. 1991).

In our view, the lack of organization in the PVH, thus the lack of an univocal relation between morphological criteria and function, and the apparent ability of the PVH to adapt to environmental or hormonal changes might well be correlated. To be precise, the capability of the PVH to adjust so rigorously to hormonal changes might well find its origin in the lack of organization in this nucleus. A loose, not univocal and not predetermined organization is likely to result in more flexibility of the system, in comparison to a rigid organization. In a very strict and rigid organized arrangement changes will disrupt the whole system whereas in a loosely organized system changes can be more easily compensated for.

The apparent plastic and variable characteristics of the PVH should lead to caution in interpretation of results when studying this hypothalamic nucleus and the functioning of the HPA-axis. Since disruption of hormonal balance seems to be able to induce large changes in neuroanatomical criteria, using ADX animals or stressing the laboratory animals while using them for long-term experiments might well influence not only neuroanatomical observations but also biochemical, functional and behavioral data.

As a final remark in this concluding paragraphs we want to compare the data on ADX rats with the data on APO-SUS and APO-UNSUS rats. When looking at the PVH and the input from the hippocampus via the BNST, which part of the stress system has most prominently come to our attention in this thesis, it seems that this connection is particularly sensitive to hormonal changes. We hypothesized that the BNST-PVH pathway is different in the APO-SUS and APO-UNSUS rats due to hormonal changes in the early postnatal period. Since we then showed a change in this pathway following ADX, we can conclude that the input from the BNST to the PVH is indeed under the influence of corticosteroids. This conclusion adds strong arguments to our hypothesis regarding the observations made in two rat lines in that the PVH of APO-SUS rats receives an increased GABA-ergic input from the BNST as compared to APO-UNSUS rats. Looking at the different data, our results suggest that the situation in APO-SUS rats is the opposite of the situation observed in ADX rats, thus showing an increased input from the BNST to the PVH.

The experiments in this thesis raised several questions and in this way create opportunities for further research. Although it is beyond the scope of these final paragraphs to describe in detail all the possible relevant experiments we want to describe in a general way some new ideas for future experiments.

With respect to the APO-SUS and APO-UNSUS rats the first question to be solved is how the low number of activated CRH neurons after a mild stressor in APO-SUS rats correlates with the high level of ACTH and corticosteroids in this rat line observed by other authors. We hypothesized that this discrepancy may be due to a higher sensitivity of the pituitary in APO-SUS rats. Therefore, a CRH receptor binding study in the pituitary of both APO-SUS and APO-UNSUS rats would be useful. Secondly, with regard to the higher synaptic density in APO-SUS rats it would be interesting to find out which neurotransmitter(s) is(are) more abundantly present in these rats as compared to the APO-UNSUS rats. In our opinion, GABA would be a likely candidate, but others may be involved as well. Closely related to this experiment would be to answer the question where this input comes from. The BNST seems to be a good choice to start this investigation with, especially in view of the results in chapter 5.

In view of the results of chapter 4, it would be necessary to study the unexpected long-term effect of IL on the activation of the CRH neurons with in situ hybridization techniques. These techniques are more sensitive than immunohistochemical techniques and a confirmation of our results with these techniques will exclude the possibility of an artifact in our results and will be helpful by determining the timespan over which this phenomenon takes place.

The results of chapter 5 raised most questions and therefore open up a whole line of new research. The observed loss of input from the BNST to the PVH caused by corticosteroids in adult rats may be an advanced mechanism of the PVH to cope with disturbances at the hormonal level but further research is necessary to confirm this hypothesis. Two sets of experiments would elucidate many of our questions raised. First of all the reversibility of this phenomenon has to be investigated. This can be done by corticosteroid replacement studies in ADX rats in combination with a tracer study. Such an experiment would also shed more light on the question whether the loss of input is evoked by cell death in the BNST or retraction of axons, since only the latter option would be reversible. Secondly, which structures provide input to the BNST subdivisions projecting to the PVH? Is the hippocampus, and especially that parts of this structure sensitive to corticosteroids, involved? These question would be solved accurately by using a combination of retrograde and anterograde tracers in the BNST.

The electron microscopical experiment in chapter 6 did not reveal a very strict organization of GABA-ergic input in the PVH of ADX rats. In view of the large effects of ADX we think it is necessary to repeat this experiment in normal rats. This approach needs the availability of in situ hybridization techniques, which enables visualization of CRH even in normal rats or a very sensitive CRH antibody. In this respect the 5Bo antibody, as used in chapter 4, would be a good candidate. Unfortunately, this antibody became available only at the end of the Ph.D. period spend on this thesis. Finally, also the organization of other neurotransmitters, such as catecholamines, neuropeptide Y, galanin, serotonin and others should be investigated with a similar quantitative electron microscopical study to gain more insight in the until now apparently loosely organized PVH and its CRH neurons.

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SAMENVATTING

De parvocellulaire corticotropin-releasing hormoon (CRH) neuronen in de nucleus paraventricularis van de hypothalamus (PVH) spelen een grote rol in de stress reactie. Na een stressvolle gebeurtenis stimuleert CRH, via de afgifte van adrenocorticotrope hormoon (ACTH) uit de hypofyse, de afgifte van corticosteroiden uit de bijnierschors. Deze cascade wordt ook wel hypothalamus-hypofyse-bijnier-as (HHB-as) genoemd. Corticosteroiden zijn onder meer verantwoordelijk voor het vergroten van de beschikbaarheid van glucose uit het bloed en het remmen van immuunfuncties. Corticosteroiden hebben ook een inhiberend effect op de activiteit van de CRH neuronen.

In dit proefschrift zijn morfologische en functionele aspecten van de CRH neuronen in de PVH bestudeerd om meer inzicht te krijgen in de organisatie van deze neuronen. De PVH bevat, naast CRH, ook nog vele andere neuropeptiden, welke allemaal betrokken zijn bij verschillende functies. Het lijkt daarom logisch te veronderstellen dat al deze neuronen verschillend gereguleerd worden. De PVH ontvangt informatie vanuit een verscheidenheid van andere hersengebieden via vezelstromen en deze zogenoemde input in de PVH moet zo georganiseerd zijn dat een bepaald type neuron kan worden geinnerveerd en dus ook een bepaalde functie kan worden beïnvloed. Dus werd een strakke organisatie van de PVH en de CRH neuronen verwacht, in de zin van rechtlijnige en vastgelegde verbanden tussen de distributie van neuronen, peptiderge inhoud, synaptologie, input vanuit de verschillende hersengebieden en functie van neuronen.

Teneinde meer inzicht te krijgen in de daadwerkelijke organisatie van de CRH neuronen zijn in dit proefschrift de volgende doelen gesteld: karakterisatie van de CRH neuronen via het bepalen van morfometrische parameters in de PVH en de distributie van de geactiveerde CRH neuronen in de PVH na verschillende manipulaties; karakterisatie van de input welke specifiek is voor de CRH neuronen door het bestuderen van de samenstelling alsmede de oorsprong van de synaptische input; bepaling van het belang van de activatie van de CRH neuronen voor de stress reactie.

In hoofdstuk 2 zijn een aantal morfometrische waarden in de PVH bepaald in twee Wistar rattenlijnen welke genetisch zijn geselecteerd op basis van hun gevoeligheid voor apomorfine. Dit zijn de zogenoemde APO-SUS (apomorphine-susceptible/apomorfine-gevoelige) en APO-UNSUS (apomorphine-unsusceptible/apomorfine-ongevoelige) ratten. Deze ratten komen beide voor in een normale populatie maar vertonen onder meer verschillende stress reacties. Op de basis van morfologische criteria (vascularisatie, neuron grootte en neuron dichtheid) werd de PVH onderverdeeld in 6 subgebieden. De lichtmicroscopische waarden, zoals neuron dichtheid en neuron grootte, vertonen geen verschillen tussen beide rattenlijnen. Echter, de APO-SUS ratten hebben een significant hogere synaptische dichtheid (een electronmicroscopisch bepaalde waarde) in vergelijking met de APO-UNSUS ratten, zowel in de totale PVH als in enkele van de subgebieden. Deze observatie impliceert dat zelfs binnen één rattenpopulatie de organisatie van de PVH variabel is.

In hoofdstuk 3 is een stress experiment beschreven dat is uitgevoerd met de APO-SUS en APO-UNSUS ratten. Na een milde stress stimulus, een open veld test, werden minder geactiveerde CRH neuronen gevonden in de PVH van APO-SUS ratten dan in de PVH van APO-UNSUS ratten. Daar de distributie van deze geactiveerde neuronen overeenkomt met de distributie van de CRH neuronen welke betrokken zijn bij de activatie van de HHB-as (hoofdstuk 3 en 4) en met de distributie van subgebieden waar de verschillen in synaptische dichtheid werden gevonden (hoofdstuk 2), lijkt het zeer wel mogelijk dat het verschil in

activatie van CRH neuronen gecorreleerd is met verschillen in de synaptische dichtheid. Dus, als inderdaad het lage aantal geactiveerde CRH neuronen na een milde stress gecorreleerd is met een hoge synaptische dichtheid in APO-SUS ratten, en omgekeerd, d.w.z. het hoge aantal geactiveerde CRH neuronen is na eenzelfde stress stimulus gecorreleerd met een lage synaptische dichtheid in APO-UNSUS ratten, dan betekent dit een grotere inhiberende input voor de CRH neuronen in APO-SUS ratten.

Een belangrijke inhiberende neurotransmitter in de PVH is GABA, welke een directe synaptische verbinding met de CRH neuronen vertoont. Een bron van GABA in de PVH is de bed nucleus van de stria terminalis (BNST). De BNST wordt gedacht een rol te spelen bij het doorsluizen van limbische informatie (van amygdala en hippocampus) naar de PVH. Met het oog op de resultaten van hoofdstuk 2 en 3 lijkt de hippocampus belangrijk te zijn, daar deze struktuur een rol speelt in de inhiberende invloed van corticosteroiden op de CRH neuronen. Onze resultaten suggereren een verhoogde activiteit van het hippocampus-BNST-PVH traject in APO-SUS ratten; dus een grotere inhibitie van de CRH neuronen na stress.

In hoofdstuk 4 is de distributie van geactiveerde CRH neuronen onderzocht na verschillende manipulaties. De totale populatie van CRH neuronen werd zichtbaar gemaakt door een injectie met colchicine, een aspecifieke axonale transport blokker. Door het weghalen van de bijnieren (adrenalectomy = ADX) en daardoor het verwijderen van corticosteroiden uit de bloedcirculatie, werd een gedeelte van de CRH neuronen geactiveerd en dus zichtbaar gemaakt. Een zelfde resultaat werd bereikt door het injecteren van een antilichaam tegen CRH in de circulatie. Een onverwacht resultaat werd behaald met interleukine. Dit eiwit wordt geproduceerd door het immuunsysteem en wordt verondersteld de CRH neuronen te stimuleren. Echter, met een acute intracerebroventriculaire injectie met interleukine werd geen activatie van de CRH neuronen geobserveerd. Een interesssante observatie werd gedaan 3 en 10 dagen na een intraveneuze injectie met interleukine: er werden minder geactiveerde CRH neuronen aangetroffen dan in de controle ratten. Dit wijst op een inhiberend effect van interleukine dat lange tijd aanwezig blijft. Het mechanisme dat aan dit fenomeen ten grondslag ligt, moet nog opgehelderd worden.

Daar hoofdstuk 2 en 3 suggereren dat de invloed van de hippocampus via de BNST een belangrijke rol speelt bij de regulatie van de CRH neuronen, werd het BNST-PVH traject nader onderzocht in hoofdstuk 5. Met gebruik van een anterograde tracer injectie werd een grote projectie van de BNST naar alle subgebieden van de PVH gevonden. Opmerkelijk was dat in ADX ratten, na overeenkomstige injecties, een significant verminderde projectie werd gevonden naar het CRH-rijke deel van de PVH. Dit resultaat impliceert dat ADX veranderingen veroorzaakt in het BNST-PVH traject en in het bijzonder in de projectie naar de CRH neuronen en dus veranderingen in de synaptische organisatie. Deze observatie levert een grote hoeveelheid vragen op zoals: Wat is de functie van dit verlies van projectie? Is de inhiberende invloed van de hippocampus-BNST na ADX overbodig? En wat gebeurt er bij een omgekeerde situatie, dus bij een verhoogd niveau van corticosteroiden? Is deze verandering plastisch, d.w.z. is dit een omkeerbaar proces? En ook, wat veroorzaakt deze verandering? Zijn er veranderingen in de hippocampus die dit fenomeen oproepen of vinden er veranderingen plaats in de PVH die hiervoor verantwoordelijk zijn? Nader onderzoek is vereist om een antwoord op deze boeiende vragen te geven.

In verband met de geobserveerde plasticiteit in synaptische organisatie (hoofdstuk 5), is in hoofdstuk 6 de organisatie van de GABA-erge input van CRH en niet-CRH neuronen onderzocht. Dit experiment werd uitgevoerd bij ADX ratten, omdat een immunokleuring van CRH op electronmicroscopisch niveau bij normale ratten moeilijk is. Zeven procent van

de synapsen in de PVH is axo-somatisch. Bij CRH neurons, met en zonder gecolokaliseerd vasopressine, is 65 % van deze synapsen GABA positief. De parvocellulaire niet-CRH neuronen vertonen eenzelfde percentage GABA-positieve input. Magnocellulaire neuronen, vasopressine positief of negatief, hebben een GABA-positief percentage van 42%. Deze resultaten impliceren dat de synaptische organisatie van GABA niet is gereguleerd volgens peptiderge inhoud en dus functie van neuronen, maar volgens locatie in de PVH. Het hoge percentage GABA-positieve synapsen op de CRH neuronen in de ADX ratten was verrassend in het licht van de resultaten van hoofdstuk 5. Dit kan betekenen dat in normale ratten het percentage GABA-positieve synapsen nog hoger is of dat er ook nog andere GABA-erge input is, of dat de daling GABA-erge synapsen na ADX alleen zichtbaar is bij de axo-dendritische synapsen.

In dit proefschrift zijn verschillende aanwijzingen gevonden voor variabiliteit en plasticiteit in de PVH. De experimenten met de APO-SUS en APO-UNSUS ratten, die beschreven zijn in hoofdstuk 2 en 3 hebben laten zien dat zelfs binnen één rattenpopulatie verschillen in synaptische organizatie en een daarmee gepaard gaande verschillende stress respons kunnen worden gevonden. Verder is duidelijk geworden dat corticosteroiden grote anatomische veranderingen kunnen veroorzaken in het volwassen brein (hoofdstuk 6). Als conclusie kan gesteld worden dat de resultaten in dit proefschrift suggereren dat de HHB-as een plastisch systeem is, hetgeen het mogelijk maakt zich aan te passen aan veranderingen in de omgeving en hormonale schommelingen. Er zijn geen aanwijzingen gevonden voor een strakke organisatie van de PVH en de CRH neuronen, dus geen rechtlijnige correlatie tussen de verschillende morfologische karakteristieken en functie. Het lijkt zeer wel mogelijk dat dit gebrek aan een strakke organisatie ten grondslag ligt aan de plasticiteit en flexibiliteit van dit systeem.

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CURRICULUM VITAE

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