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SUDOMOTOR ACTIVITY AND CEREBROVASCULAR CHOLINERGIC AND ADRENERGIC BINDING SITES IN AGING AND ALZHEIMER'S DEMENTIA.

By

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I would like to dedicate this thesis to my parents who will be absent during my graduation. It is in their love that my motivation has found its roots and by their encouragement that my dedication to this work has sprouted.

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I am especially thankful to Mrs. Lyne Grégoire our lab technician for the kindness and extreme patience she showed while teaching me the different techniques I have used to carry on my work in Dr. Hamel's lab.

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Acetyl CoA = Acetyl coenzyme A.

ACh = Acetylcholine.

AChE = Acetylcholine esterase.

AD = Alzheimer's disease.

AF-DX-116 = 11-[[2-[(dimethylamino)-methyl]-1-piperidinyl] acetyl]-5-11-dihydro-6H-pyrido [2,3-b][1,4] benzodiazepine-6-one.

ANP = Atrial natriuretic peptide.

Bmax = maximal binding capacity.

cAMP = Cyclic adenosine monophosphate.

CBF = Cerebral blood flow.

CGRP = Cacitonin gene related peptide.

CNS = Central nervous system.

Ca^{++} = Cacium ion.

ChAT = Choline acetyl transferase.

4-DAMP = 4-diphenylacetoxy-N-methylpiperidine methiodide.

D.R. = Direct response.

E = Elderly.

EBC/TPB = Ethyl Butyl Cetone/Tetra Phenyl Borate.

EDRF = Endothlium-dependent relaxing factor.

F = Female.

[^{3}H]-CGP 12177 = ((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H] benzimidazol-2-ONE)

[^{3}H]-DHA = [^{3}H]-Dihydroalprenolol.

[^{125}I]-HEAT =(±)-ß-([^{125}I]iodo-4-hydroxyphenyl)-ethyl-aminomethyl-tetralone.

[^{3}H]-NMS = N-methyl-[^{3}H]-scopolamine methyl chloride.

[^{3}H]-RX 781094 = (1,4-[6,7- ^{3}H]benzodioxan-2-yl)-2-imidazoline hydrochloride.

K_D = Dissociation constant.

M = Male.

MAO = Mono amine oxidase.

MGH = Montreal General Hospital.

min. = minute.

ml = milliliter.

mmole = millimole.

nM = nanomolar.

P.A.R.S. = Pure axon reflex sweating.

PET = Positron emission tomography.

PI = Phosphoinositide.

PNS = Peripheral nervous system.

QNB = Quinuclidinyl benzilate.

QSART = Quantitative sudomotor axon reflex test.

rCBF = Regional cerebral blood flow.

S.Q.R. = Standard QSART response.

sec = seconds.

SP = Substance P.

μg = Microgram.

μl = Microliter.

VIP = Vasoactive intestinal polypeptide.

Y = Young.

Abstract

Alzheimer's dementia (AD) is a degenerative neurological disorder involving predominantly the central cholinergic neurons but also other neurotransmitter systems. Over the last decade, several investigators have reported the presence of AD-related peripheral manifestations many of which involving cholinergic and adrenergic markers in non-neuronal tissues such as platelets, fibroblasts, lymphocytes and others. However, since these are unrelated to structures innervated by the peripheral nervous system, it is difficult to comment on their possible pathological as well as functional implications in the process of the disease.

The aim of the present study was to evaluate possible AD-related dysfunctions in peripheral tissues known to be innervated by anatomical structures located outside the CNS. Cholinergic as well as adrenergic receptors density and affinity were evaluated in pial vessels obtained post-mortem from 8 confirmed cases of AD and 8 elderly non-demented controls. In addition, direct and axonal reflex sweating responses elicited by local iontophoretic application of acetylcholine were evaluated in 30 probable AD patients, 30 elderly and 30 young controls.

There was a significant ($p < 0.05$) 43% reduction in total muscarinic binding sites in AD pial vessels as compared to control vessels. When the binding assays were performed in the presence of 75 nM pirenzepine (in order to block muscarinic binding sites with high affinity to pirenzepine), there was still a significant ($p < 0.05$) 40% diminution in the labelled sites compared to control vessels. The population of α1-adrenergic binding sites was also significantly

reduced (35%; $p < 0.05$) in AD vessels. In all cases, affinity of the cerebrovascular receptors was comparable in AD and control vessels. On the other hand, ChAT activity (used as an index of cerebrovascular cholinergic innervation) determined in human pial vessels showed a non-significant 25% reduction in AD compared to control pial membranes. Similarly, ß-adrenergic binding site density was unaltered in AD vessels. Functional evaluation of the sweating response in AD patients and related controls showed that the nicotinic receptor mediated axonal reflex sweating response was significantly reduced in AD patients as compared to young controls and elderly controls (44% and 40% respectively, $p < 0.05$). In contrast, the slight diminution observed in direct sweat response (mediated by muscarinic receptors) between AD patients, elderly and young controls was not statistically significant.

In view of the reported distribution of muscarinic and adrenergic receptors in the pial vessel wall, the reduction in total and subtype specific muscarinic binding sites as well as the drop in α1-adrenoceptors may argue in favour of a degenerative process at the level of the blood vessel wall itself. Such conclusion would agree with the relatively preserved cholinergic innervation suggesting spared cerebrovascular innervation. In contrast, the significant reduction in axonal reflex sweat response observed in AD, is strongly suggestive of a peripheral degenerative process involving post-ganglionic sympathetic neurons to sweat glands.

The detected changes are suggestive of a further decrement of autonomic control in AD different from what have been observed in normal aging. These, changes may contribute to the deterioration of the brain perfusion and autonomic functions regulation in AD.

Résumé

La maladie d'Alzheimer est une affection neurologique qui touche en premier lieu les neurones cholinergiques centraux, mais qui implique également d'autres systèmes neurotransmetteurs. Au cours de la dernière décennie, plusieurs chercheurs ont signalé la présence de manifestations périphériques reliées à la maladie d'Alzheimer dont un grand nombre impliquent les marqueurs cholinergiques et adrénergiques dans des tissus non neuronaux tels que les thrombocytes, les fibrocytes, les lymphocytes et autres. Toutefois, étant donné que ces derniers ne sont pas apparentés aux structures innervées par le système nerveux périphérique, on peut difficilement évoquer la possibilité d'implications pathologiques et fonctionnelles dans le processus de la maladie.

La présente recherche a eu pour but d'évaluer la possibilité de dérèglements associés à la maladie d'Alzheimer au niveau des tissus périphériques innervés par des structures anatomiques situées à l'extérieur du système nerveux central. Nous avons évalué la densité et l'affinité de récepteurs cholinergiques et adrénergiques provenant de vaisseaux pie-mériens prélevés post-mortem chez huit cas de maladie d'Alzheimer confirmés et huit personnes âgées non atteintes de démence et servant de cas témoins. De plus, les réactions sudorifiques dues aux réflexes directs et axonaux induits par iontophorèse locale à l'acétylcholine ont été évaluées chez trente personnes vraisemblablement atteintes de maladie d'Alzheimer, trente personnes âgées et trente cas témoins jeunes.

En comparaison avec des vaisseaux provenant de cas témoins, on a noté une réduction significative de 43% ($p < 0.05$) de la densité des sites de liaison muscariniques dans les vaisseaux pie-mèriens prélevés des cas d'Alzheimer. Lorsque l'on a procédé aux tests de liaison en présence de 75 nM de pirenzépine (afin de bloquer les sites de liaison muscariniques démontrant une forte affinité avec la pirenzepine), on a tout de même noté une importante réduction des sites marqués par rapport aux vaisseaux témoins. La population des sites de liaison α1-adrenergiques s'est également avérée réduite de manière significative ($35\%; p \leq 0.05$) dans les vaisseaux des cas d'Alzheimer. Dans tous les cas, l'affinité des récepteurs cérébrovasculaires

s'est avérée comparable dans les cas d'Alzheimer et dans les vaisseaux témoins. Par contre, l'activité ChAT (utilisée comme indice de l'innervation cholinergique cérébrovasculaire) déterminée dans les vaisseaux humains pie-mériens a démontré une réduction non-significative de 25% dans les cas d'Alzheimer comparée avec celle des membranes cérébrovasculaires témoins. De même, la densité des sites de liaison ß-adrénergiques est demeurée inchangée pour les vaisseaux des cas d'Alzheimer. L'évaluation fonctionnelle de la réaction sudorifique chez les patients atteints d'Alzheimer et chez les témoins a démontré que le réflexe axonal de réaction sudorifique provoqué par le récepteur nicotinique était réduit de manière significative chez les patients atteints d'Alzheimer par rapport aux témoins jeunes et aux témoins âgés (44% et 40% respectivement, $p \leq 0.05$). Par contre, la légère baisse de réaction sudorifique directe (induite par les récepteurs muscariniques) observée entre les patients atteints d'Alzheimer, les témoins âgés et les témoins jeunes ne s'est pas avérée significative sur le plan statistique.

Etant donné la distribution des récepteurs muscariniques et adrénergiques dans les parois des vaisseaux pie-mériens, la réduction des sites de liaison muscariniques, ainsi que la baisse d'α1-adrénocepteurs pourraient indiquer un processus dégénératif au niveau de la paroi des vaisseaux sanguins. Cette conclusion viendrait corroborer le fait que l'innervation cholinergique se trouve relativement préservée, ce qui suggère que l'innervation cérébro-vasculaire n'aurait pas été impliquée. Par contre, la baisse significative de la réaction sudorifique du réflexe axonal observée chez les personnes atteintes d'Alzheimer suggère fortement un processus dégénératif périphérique impliquant les neurones post-ganglionnaires sympathiques des glandes sudoripares.

Les changements décelés suggèrent une diminution plus marquée du contrôle du système neuro-végétatif chez les personnes atteintes de la maladie d'Alzheimer, comparativement aux sujets âgés normaux. Ces changements pourraient contribuer à la détérioration de l'irrigation sanguine cérébrale et de la régulation des fonctions du système neuro-végétatif chez les personnes atteintes d'Alzheimer.

Introduction

Due to increasing prevalence with age, Alzheimer's dementia (AD) constitutes a significant medical and social problem in the western communities. Clinically, AD is characterized by a progressive deterioration of multiple cognitive abilities including memory, language and visuospatial organization (Price 1976). Its incidence has been estimated to range between 3 - 8% of the population over the age of 65 years old (Jörm et al 1987).

Extensive pathological studies have suggested a number of factors that may contribute to the development of AD including transmissible agents, toxins and lack of trophic factors (for a review see Price 1986). Neverthless the true etiology of AD remains unclear.

Despite the general agreement that degeneration of central cholinergic pathways is a major contributor to the cognitive deficits in AD (Whitehouse et al 1982), dysfunctions in other neurotransmitter systems have been also described (Bondareff et al 1982, Jennie Eirmann etal 1984, Palmer et al 1987, Minthon et al 1990). In addition to the brain neuronal deficits, the literature now carries a flow of reports suggesting the contribution of a cerebrovascular component in the pathology of AD (Lechner et al 1991, De La Torre et al 1992). Indeed cerebral blood flow measurements in AD patients have shown a tendency towards a reduction in the fronto-temporo-parietal regions (Simmard et al 1971, Yamaguchi et al 1980, Frackowiack et al 1981). These blood flow changes may, in fact, be secondary to a diminished cerebral metabolic demand due to neuronal degeneration thereby obfuscating whether these changes are causal or an effect of the aging process. This causal relationship has been questioned in the recent years following the detection of AD-related selective cerebrovascular biochemical, structural and functional abnormalities (see sections 1.4).

In addition, numerous deficits in neurotransmitter-related functions have also been reported in AD in several tissues located outside the brain. These include changes in skin vessels reactivity (Hörnqvist et al 1987), reduced sweat response (Lamb et al 1984) as well as a diminished binding capacity of adrenergic (Adunsky et al 1989) and cholinergic binding sites (Rabey et al 1986) in blood platelets. The similarity between the central and the peripheral neurotransmitter impairments may point to a generalized nature of AD. However there have been few studies aimed at investigating the possible interrelation between the central and peripheral changes in AD. In particular, the possible existence of a generalized decline of neurotransmitter systems both in the peripheral nervous system (PNS) and the central nervous system (CNS) of AD patients, has not been addressed extensively.

Based on the lack of understanding of the relationship between the central and peripheral manifestations in AD, this research project attempted to answer the following points:

1- <u>Is there any specific deficit in the neurogenic control of brain extra-cerebral blood vessels in AD ?</u>

Pial vessels located at the surface of the brain cortical mantle receive a rich adrenergic and cholinergic innervation originating from sympathetic and parasympathetic ganglia (Edvinsson et al 1977, Suzuki et al 1990). These systems have been implicated in cerebral vasoconstriction and vasodilation, respectively. Both vasomotor functions are mediated by specific receptors located on the blood vessel walls. We suggest that if there is a general decline in peripheral neurotransmitter systems in AD, alterations in the neurogenic control of brain pial vessels should be apparent. Thus, we have evaluated cerebrovascular innervation and receptor densities in pial vessels obtained from histopathologically confirmed cases of AD and age-matched neurologically free controls.

2- Is there a functional impairment of peripheral neurotransmission in AD ?

Unlike other sympathetic actions, sweat secretion is exclusively a function of sympathetic post-ganglionic cholinergic neurons. In addition, direct stimulation of these glands by administration of cholinomimetic agents (Coon and Rothman 1939, Low et al 1983), has also been shown to elicit sweat production centrifugally by a process refered to as axonal reflex sweating (see section 1.2.b. for more details). We suggest that if a functional peripheral cholinergic decline is present in AD, it can be detected by assessing sweat responses to adequate cholinergic stimulation. In this study, we have measured the two components of sweat production namely, direct sweating and axonal reflex sweating, in young and elderly controls as well as in clinically diagnosed AD patients. An alteration (if any) of the direct response would point to a dysfunction of the sweat glands themselves whereas a diminished sweat production through axonal reflex stimulation would suggest an impairement of the post-ganglionic autonomic cholinergic neurons.

1. Review of the literature:

1.1 Central and peripheral changes in Alzheimer's dementia:

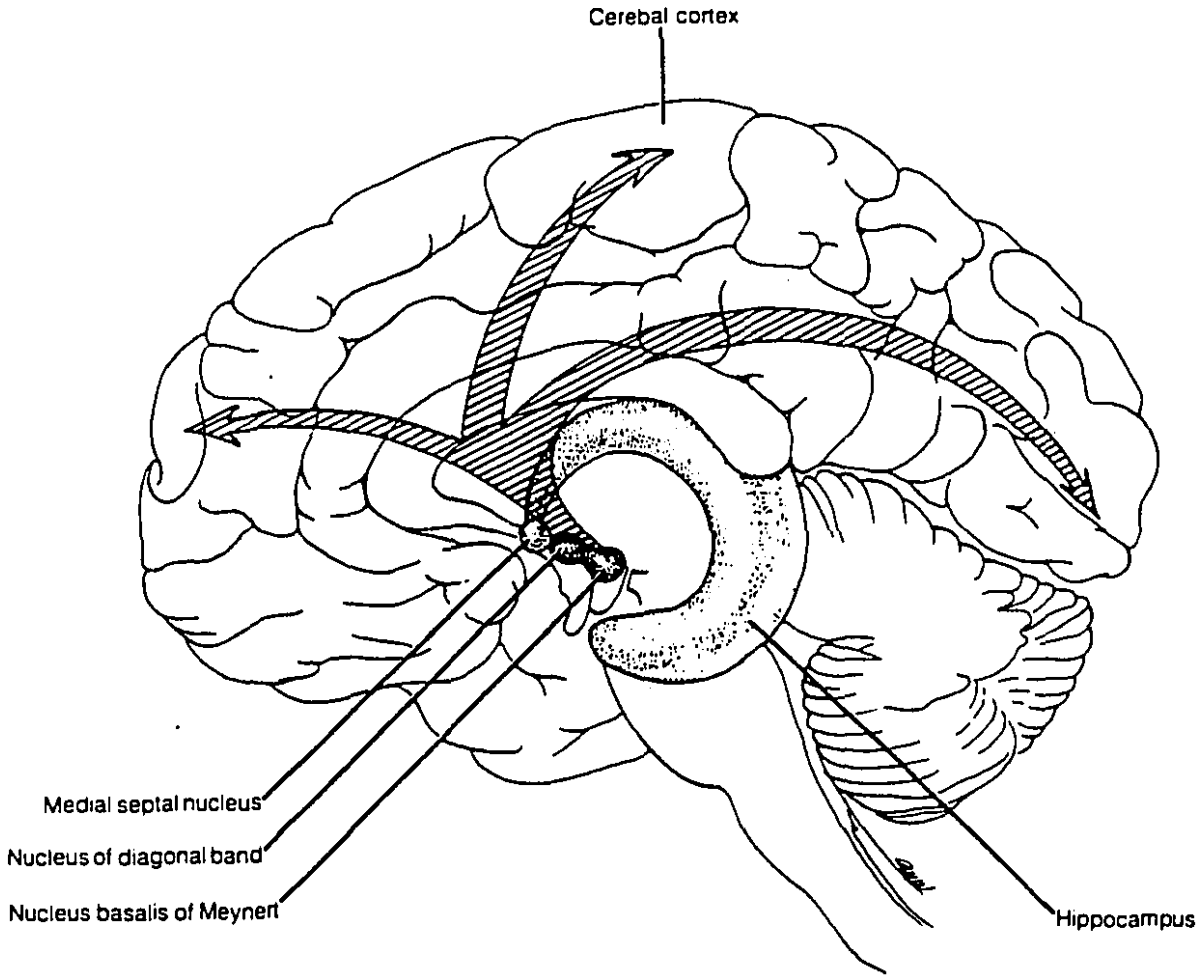
1.1.a. Alzheimer's dementia and central neurotransmission:

Abnormalities in several neurotransmitter systems characteristically occur in AD. Beside the well recognized cholinergic and adrenergic alterations (see below), dysfunctions in a number of other neurotransmitter systems have been frequently reported. Measurement of the brain serotonin content (Palmor et al 1986) and determination of cortical serotonergic binding sites (Sparks 1989, Jennie Eirmann et al 1984) demonstrated a selective reduction in AD. Similarly, failures of peptidergic (Minthon et al 1990) and dopaminergic (Jennie Eirmann et al 1984) systems as well as a loss of opiate receptors (Jansen et al 1990, Jennie Eirmann et al 1984) have all been described. Neverthless, since the detection of possible peripheral alterations in most of these systems is beyond the scope of this study, the following sections will briefly underline most currently accepted changes in central cholinergic and adrenergic systems in AD.

1.1.b The cholinergic system:

Dysfunctions of the central cholinergic systems constitute the most constant and generally recognized changes involved in AD. Severe degeneration of the cholinergic neurons projecting from medial septal nucleus to the hippocampus as well as those projecting from the basal forebrain nuclei (including the nucleus basalis of Meynert, NBM) to multiple cortical areas (Whitehouse et al 1982) (figure 1) has been evidenced by the marked depletion of the acetylcholine synthesizing enzyme choline acetyltransferase (ChAT) in the cerebral cortex and the hippocampus of AD patients (Quirion et al 1988, Rinne et al 1989). The role played by

Fig. 1: Cholinergic pathways implicated in the pathogenesis of Alzheimer's dementia


Cerebal cortex
Medial septal nucleus
Nucleus of diagonal band
Nucleus basalis of Meynert
Hippocampus

central cholinergic neurons in learning and memory was evidenced by Drachman & Leavitt (1974) who showed cognitive and memory disturbances in human subjects following blockade of muscarinic cholinergic receptors with scopolamine. Memory impairments were also observed in nucleus basalis magnocellularis (the rodent equivalent of Meynert's nucleus) lesioned animals (Hepler & Olton 1985). Subsequent investigations on the central cholinergic alterations in AD reported controversial results; However, recent studies tend to agree on the selective reduction in presynaptic cholinergic markers including (ChAT) activity , M2 muscarinic as well as nicotinic receptors in the frontal cortex and the hippocampus. Meanwhile, total muscarinic and M1 binding sites appear to remain unaltered in most brain areas (Whitehouse et al 1986, Kellar et al 1987, Araujo et al 1988, Quirion et al 1988, Rinne et al 1989). A modest and selective increase in M1 binding sites has been reported in the hippocampus (Araujo 1988), Such alteration was interpreted as a compensatory upregulation of these post-synaptic receptors. The reduction in cortical ChAT activity and specific binding sites seem to relate to the degeneration of the basal forebrain nuclei (Rogers et al 1985, McGeer et al 1984) projecting to the cerebral cortex. Interestingly, neurons of this brain region have also been involved in the regulation of intracortical microcirculation (Arneric 1988, Biesold et al 1989, Lacombe et al 1989).

1.1.c. The adrenergic system:

In addition to the cholinergic deficits mentioned above, a concomitant involvement of the central noradrenergic system constitutes a well recognizable feature of AD. Dramatic loss of cells in the locus ceruleus, which constitutes the origin of the cortical noradrenergic projections, has been well documented (Bondareff et al 1982, Mann et al 1982). Also, noradrenaline concentrations in brain of demented patients have been found to be significantly

reduced (Adolfsson et al 1979). Other reported changes in catecholamines in the brain of AD patients include a reduced dopamine-ß-hydroxylase activity (Cross et al 1981) and decreased levels of homovanillic acid and dopamine in specific brain regions such as the nucleus caudatus, putamen, thalamus, hippocampus, frontal cortex and pons (Adolfsson et al 1979). Radioligand binding assays also reported changes in the density of various types of adrenergic binding sites within the cerebral cortex of AD patients. For instance, significant reductions in both $\alpha 1$ (Shimohama et al 1986) and $\alpha 2$ (Shimohama et al 1986, Kalaria & Anderson 1991) adrenoceptors have been documented in brain areas such as the hippocampus, the NBM and the thalamus in AD. Similarly, ß1 and ß2-adrenergic receptor subtypes were both significantly reduced in selected brain regions of patients with AD (Shimohama et al 1987).

1.1.d. Detection of peripheral alterations in AD:

The general belief that the pathological features of AD are exclusively confined to the CNS has recently been challenged by a large number of studies. Abnormalities accompanying AD have been seen in a variety of peripheral tissues suggesting that AD expression is not restricted to the central nervous system (CNS) but could also involve tissues located outside the brain (Blass & Zemcov 1984).

Platelets, which have been used by various authors as a model for nerve terminals (Briely et al 1980), exhibited increased binding to $\alpha 2$ receptors in 75 cases of probable AD as compared to age- and sex-matched controls (Adunsky et al 1989). Furthermore, an increase in patelet mono-amine oxidase (MAO) activity (the enzyme responsible for catecholamine breakdown) was observed, together with a parallel increase in brain MAO activity, in the same AD patients (Adolfson et al 1980). Other blood constituents have also been found to be abnormal in AD. For instance, acetylcholinesterase (the degrading enzyme for acetylcholine) activity was consistently

diminished in red blood cells obtained from AD patients (Smith et al 1981). In lymphocytes, diminished muscarinic binding site capacity (Rabey et al 1988) and reduced production of interleukin 1 (Khansari et al 1985) have been reported in demented patients. On the other hand, controversial results were obtained with respect to choline metabolic changes in red blood cells. Miller et al (1985) found an abnormally low influx of choline (thought to be due to a defect in the transport systems across the erythrocyte membrane) while such finding could not be confirmed by two other groups (Kanof et al 1985, Sherman et al 1986).

Owing to their easy accessibility, skin fibroblasts were also extensively studied and, like blood components, fibroblasts derived from patients with AD demonstrated a wide spectrum of metabolic variations. These include a slower inwards transport of choline (Mokrash 1989), reduced protein Kinase-C concentrations and altered phosphorylation (Twan Van Huynh 1989) as well as decreased activity of thiamine dependent enzymes (Gibson et al 1988). In addition, a significant reduction in ChAT activity was observed selectively in sympathetic neurons grown in culture with a medium conditioned by exposure to skin fibroblasts obtained from AD patients (Kessler 1987).

Altogether, these previous studies seem to point to the presence of a variety of peripheral AD-related alterations some of which being specifically involved with the cholinergic systems .

1.2. Sudomotor function and axon reflex sweating:

1.2.a. CNS-related control of sweat production:

Eccrine sweat glands play an important role in heat regulation. Thermal as well as electrical stimulation studies in animal have demonstrated that the control of sweat production lies in the preoptic/anterior hypothalamic region. This area acts through specialized warm-sensitive neurons

(warm thermoreceptors) involved in heat regulation (Wit & Wang 1968, Andersson & Persson 1957). The stimulus triggering the activation of heat loss mechanisms by the preoptic anterior hypothlamic area has been mainly attributed to change in the temperature of blood reaching the hypothalamus. This was reported to be nine times more effective than skin temperature in driving sweat production (Nadel et al 1971), a finding recently confirmed in the monkey (Gisolfi et al 1988). However, there is ample evidence to suggest that skin temperature plays a significant role in sweat production by acting as a modifier of the central drive for sweating (Smiler et al 1976, Nadel et al 1971).

Fibers from the preoptic anterior hypothalamic area descend in the brain stem and spinal cord to supply sympathetic preganglionic vasomotor and sudomotor neurons. In an attempt to find the exact location of these fibers in human spinal cord, Nathan and Smith (1986, 1987) used clinical data obtained from a number of patients who were subjected to cordotomies at different levels. They concluded that descending fibers from the hypothalamus lie close to the posterior angle of the anterior horn (in the cervical cord) and at the base of the posterior horn and lateral horn (in the thoracic cord). They also reported that fibers supplying the head and neck originate in the upper cervical cord and run entirely unilaterally whereas those taking origin at a more caudal level run bilaterally, with the partial decussation happening at the level of the T1 segment.

Beside the central stimulation of sudomotor activity, sweat can be produced locally through a CNS independent pathway called axon reflex sweating.

1.2.b. Axon reflex sweating:

The notion of "axon reflex" defines a local response in which impulses are conveyed from the point of stimulation (the receptor point) centripetally through branches of ramifying axons (the receptor limb) and then centrifugally (the effector limb) to the effector organ (fig 2). Axon reflex

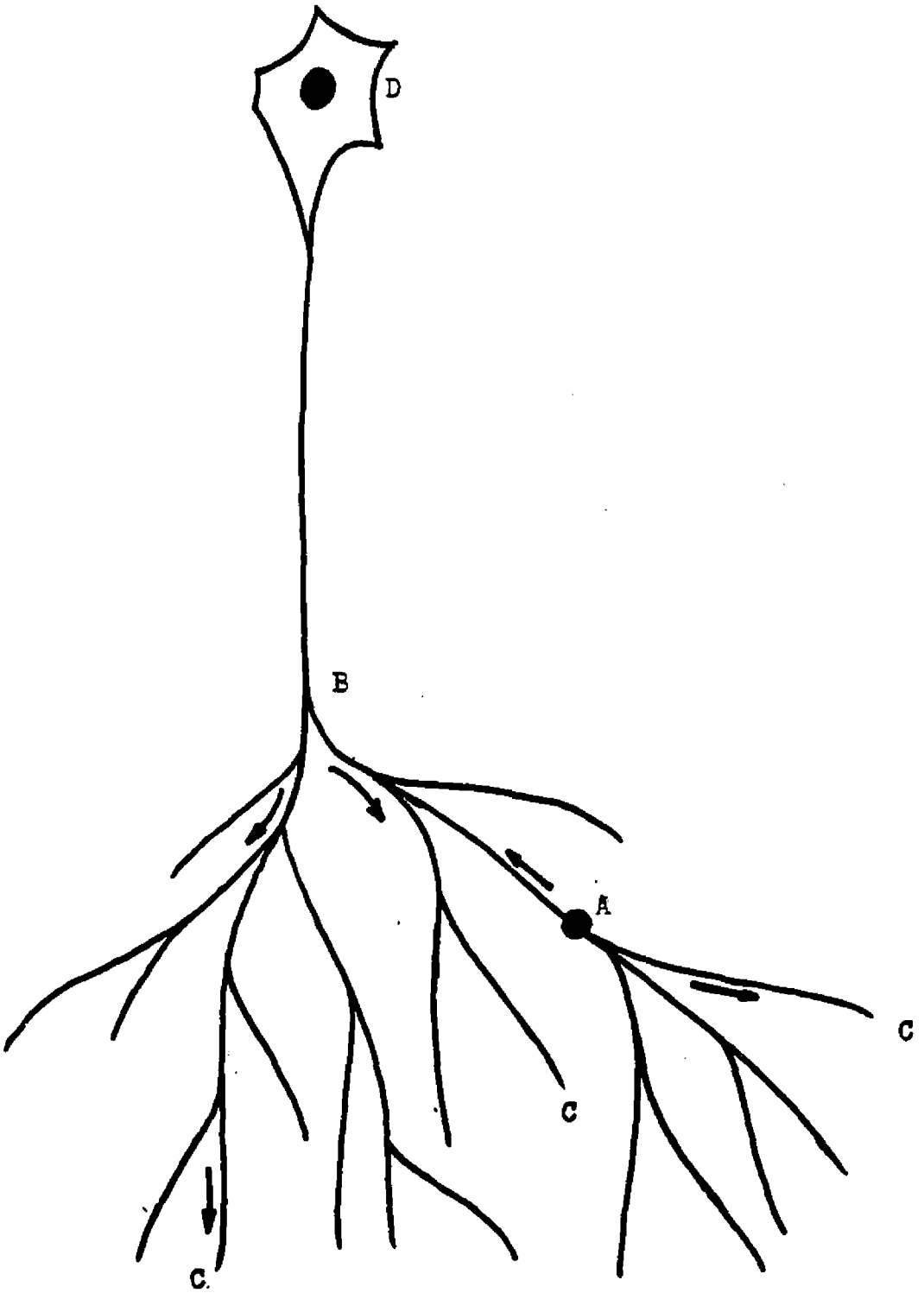
Fig. 2: Scheme of the axon reflex sweating (after Rothman and Coon 1939):

A = Point of reception.

B = Highest point of ramification.

C = Nerve ending.

D = Ganglion cell.


D
B
A
C
C
C

sweating was first described by Bickford (1938) and Wilkins, Newman and Doupe (1938) who found independently a local sweating response produced by faradic stimulation of human skin. Subsequently, a similar response was obtained with intradermal injection of nicotine, acetylcholine (Coon and Rothman 1939) or high concentrations of sodium chloride (Wada et al 1952). The pathway of this axonal reflex was originally thought to involve the post-ganglionic cholinergic neurons to the sweat glands (Bickford 1938, Wilkins et al 1938, Coon and Rothman 1939, 1941, Rothman and Coon 1940) as confirmed later by Wada et al (1955) who similarly failed to elicit the axon reflex sweating response in the skin clinically deprived of postganglionic sympathetic input. The cholinergic nature of the response was suggested based on the ability of drugs with nicotine-like action to elicit the reflex (Coon and Rothman 1939, Wada et al 1952) while it was weakened or abolished by the muscarinic blocker atropine (Bickford 1938, Coon and Rothman 1939, 1941, Rothman and Coon 1940). A more detailed investigation of the two distinct effects of acetylcholine on sweat secretion suggested: 1) a direct effect on the sweat glands through a post-synaptic muscarinic receptors, and 2) an indirect effect by stimulating the pre-synaptic receptors through nicotinic receptors. The clear physiological significance of the axon reflex sweating is not clearly understood but its involvement in normal thermogenic responses appears to be very unlikely (Collins and Weiner 1961). Neverthless, methods for evaluation of axon reflex sudomotor response were introduced (Mac Millan etal 1969, Low et al 1983) in the aim of developing a tool by which the clinical evaluation of the threatened integrity of postganglionic sympathetic neurons, encountered in dysautonomias or as a part of generalized peripheral neuropathies, can be monitored.

1.2.c. Post-ganglionic cholinergic innervation:

Sweat glands have the peculiar characteristic of being innervated by sympathetic post-ganglionic cholinergic neurons. This was originally demonstrated in cats by Dale and Feldberg (1934) and subsequently confirmed in human (Rechardt et al 1976). In this later study, a dense plexus of unmyelinated acetylcholinesterase positive fibers was found to form an intricate network around the secretory coil of eccrine and apocrine sweat glands. Electron microscopic visualization of nerve endings, however, revealed no penetration or synaptic contact with the basement membrane. This morphological characteristic suggested that depolarization of the secretory cell membrane is secondary to neuronal release of acetylcholine in the vicinity of the sweat glands. Once released from the nerve terminals, acetylcholine seems to exert dual functions via paracrine mechanisms: 1) stimulation of sweat production by the secretory portion of the sweat gland and 2) contraction of the myoepithelial cells surrounding the secretory and excretory portions, thus providing a structural support to the secretory epithelium during the process of sweat secretion. The independent control of secretory epithelial and contractile myoepithelial functions was first suggested in the monkey (Sato 1977) and later documented in human (Sato 1980).

1.2.d. Possible adrenergic innervation of sweat glands:

The presence of an adrenergic mechanism in the control of sweating has long been suggested by various studies after detecting a positive sudorific response following either intradermal injection (Foster et al 1970, Wolf and Maibach 1974) or systemic administration of adrenaline (Gordon & Maibach 1965). The previous data were strongly indicative of the possible existence of an adrenergic innervation to the sweat glands but the effort of many investigators (Falck & Rorsman 1963, Fuxe & Nilsson 1965, Rechardt et al 1976) failed to demonstrate such innervation. Recently, however, the presence of adrenergic nerves around eccrine sweat glands

was reported in dogs (Morishima 1970), monkey (Uno & Montagna 1975) and human skin (Uno 1977). This observation revived the longstanding theory of a dual adrenergic and cholinergic innervation of sweat glands. From an anatomical point of view, the widespread distribution of adrenergic fibers throughout the skin surface is a strong proof against the previous belief that adrenergic sweating is limited to the palms and soles.

1.2.e. Regulatory peptides and sweat gland innervation:

To complicate even more the picture of the post-ganglionic innervation to the sweat glands, the literature recently reports several studies which indicate the presence of a number of biologically active peptides in the skin of experimental animals and man. Perhaps the most frequently detected is the 28 amino acids long peptide, vasoactive intestinal peptide (VIP) which is found around sweat glands in a distribution that closely matches that of acetylcholinesterase positive fibers (Lundberg et al 1979, Vaalasti et al 1985, Kummer et al 1990). This raises the possibility that the two substances coexist in the same fibers as has been reported to occur in other tissues (e.g. blood vessels, Suzuki et al 1990). VIP is also colocalized with calcitonin gene related peptide (CGRP) (Landis & Fredien 1986, Lindh et al 1988), substance P (Lindh et al 1988) and peptide histidine metionine (Eedy et al 1990) in fibers that supplied innervation to sweat glands. Other neuropeptides known to be present in these post-ganglionic fibers are galanin and atrial natriuretic peptide (ANP) (Tainio et al 1987). The significance of the presence of several peptides in the innervation around the sweat glands is still to be ellucidated.

1.2.f. Sudomotor activity in relation to aging and dementia:

A reduction in the number of active sweat glands has been reported in old age (Mac Kinnon 1954). More detailed investigations were, however, not as conclusive about age-related decline

in the population of functional sweat glands. The sweating capacity of elderly subjects was found to be significantly reduced compared to young controls. This was attributed to a diminution in sweat gland output rather than recruitment of fewer sweat glands (Silver et al 1964, Foster et al 1976, Shoenfeld et al 1978, Anderson et al 1987, Inoue et al 1991). The precise mechanism behind the reduced sweating capacity in old age is still unclear. Part of the phenomenon may be explained by a diminished sensitivity of the effector organ (sweat gland) as part of the senile changes occuring in the skin as suggested by Foster et al (1976). Further support of this hypothesis was more recently obtained by Kenney and Fowler (1988) who failed to induce a larger sweat gland output with increasing concentrations of acetyl-ß-methylcholine. Atrophy of the sweat glands has also been shown with increasing age (Sato 1977). Another factor might be the age-related decline in physical activity and maximal aerobic capacity (VO_2max) which could also influence the sweating responsiveness to cholinergic agonists (Buono 1988). In studies comparing sweat production in trained and untrained individuals of young and old ages (Buono 1991, Tankersley 1991) an improved sweating capacity close to that found in young subjects was observed in the older trained individuals. Other explanations of age-related reduced sweating capacity could possibly be due to disturbances in the function of the autonomic nervous system commonly encountered in the aged (Collins et al 1977).

On the other hand, based on the assumption of a systemic component of cholinergic innervation in AD, Lamb et al (1983) found a significantly reduced response in AD patients compared to non-demented controls, following intradermal administration of cholinomimetics. They, however, did not conclude about the possible mechanisms (as discussed above) which can be directly involved in AD. Because of the higher incidence of AD with increasing age, it will be important to define whether the difference in sudorific responses observed are age- or disease- (AD) related.

1.3. Neurotransmitter systems and cerebrovascular innervation

1.3.a. Subdivisions of the cerebrovascular circulation:

Blood vessels supplying the brain are subdivided anatomically into two distinct compartments. The first comprises the extra-cerebral blood vessels which include the major cerebral arteries at the base of the brain as well as their cortical ramifications, most commonly referred to as the pial vessels. The cerebral arteries originate from the vertebral and the two internal carotid arteries whose major branches coalesce medially to form the circle of Willis. The internal carotid arteries, which give rise to the anterior circulation, divide into four major branches: the anterior cerebral, the middle cerebral, the anterior choroidal and the posterior communicating arteries. The posterior circulation originates from the two vertebral arteries that fuse to form the basilar artery which then divides into the two posterior cerebral arteries (figure 3). In this process, a series of three paired cerebellar arteries has emerged. The pial arteries are the small vessels at the surface of the brain mainly derived from the middle and posterior cerebral arteries (figure 4). They extend to all regions of the cerebral cortex and, eventually will give rise to the penetrating arteries from which the second cerebrovascular compartment is derived. This component consists of the intra-cerebral blood vessels which corespond to the arterioles and capillaries present within the brain parenchyma (figure 5). These two distinct cerebrovascular compartments are beleived to respectively regulate global and local cerebral blood flow. The diversity of the neurogenic control of extra-cerebral blood vessels is evidenced by the presence of perivascular sympathetic, parasympathetic and sensory nerve fibers which have been well characterized over the past years (see section 1.3.b and 1.3.d.). In adition, multiple neuropeptides have also been identified within these perivascular fibers such as VIP, substance P (SP), neuropeptide Y (NPY) and others (Uddman 1987). The innervation of the intra-cerebral microvessels, on the other hand, has been

Fig. 3: Anterior and posterior cerebral circulation: The anterior circulation comprises the internal carotid artery and its branches (anterior and middle cerebral artery). The posterior circulation consists of the vertebral artery, the basilar artery and their branches (posterior inferior cerebellar, anterior inferior cerebellar, superior cerebellar and posterior cerebral arteries)

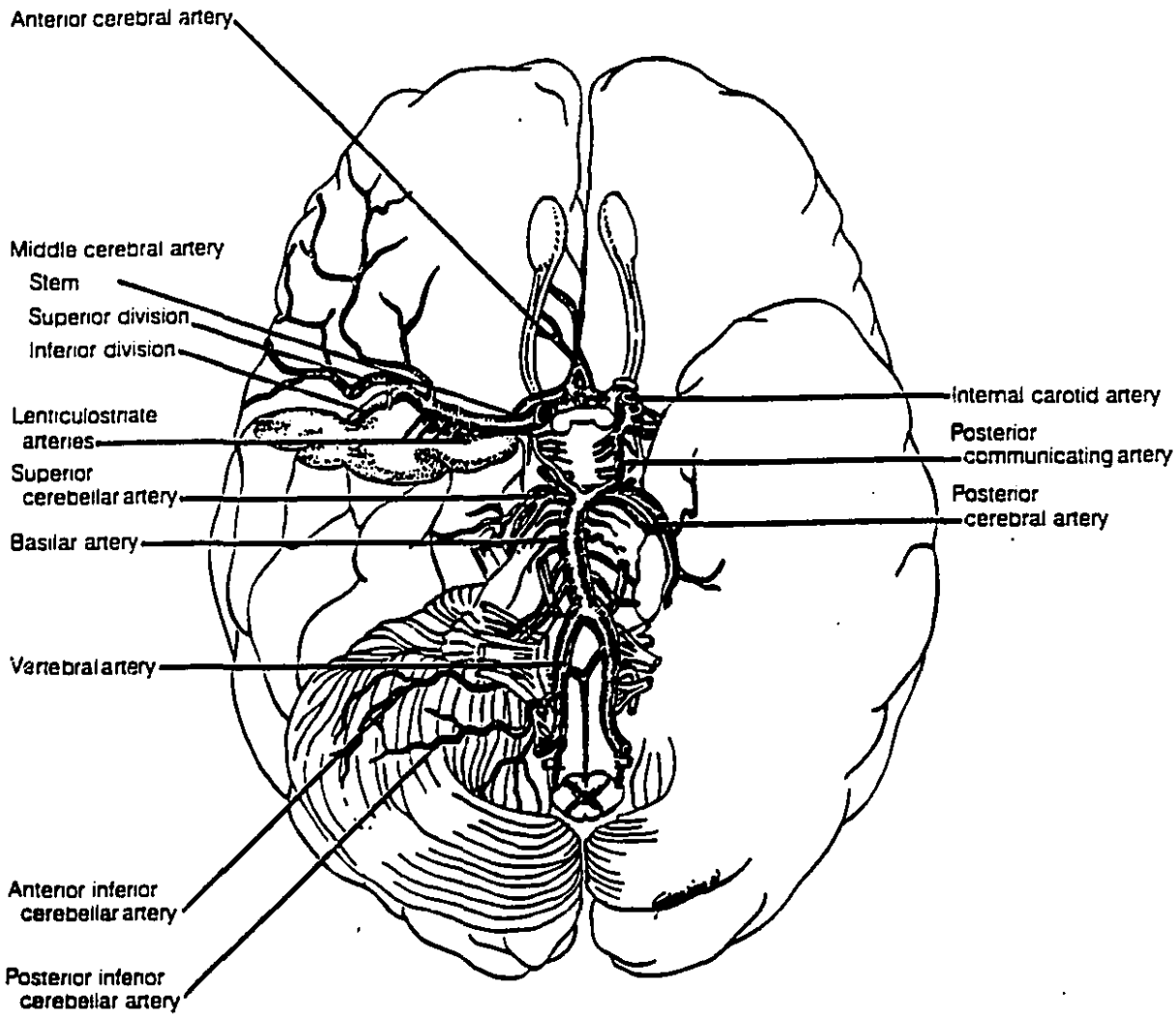
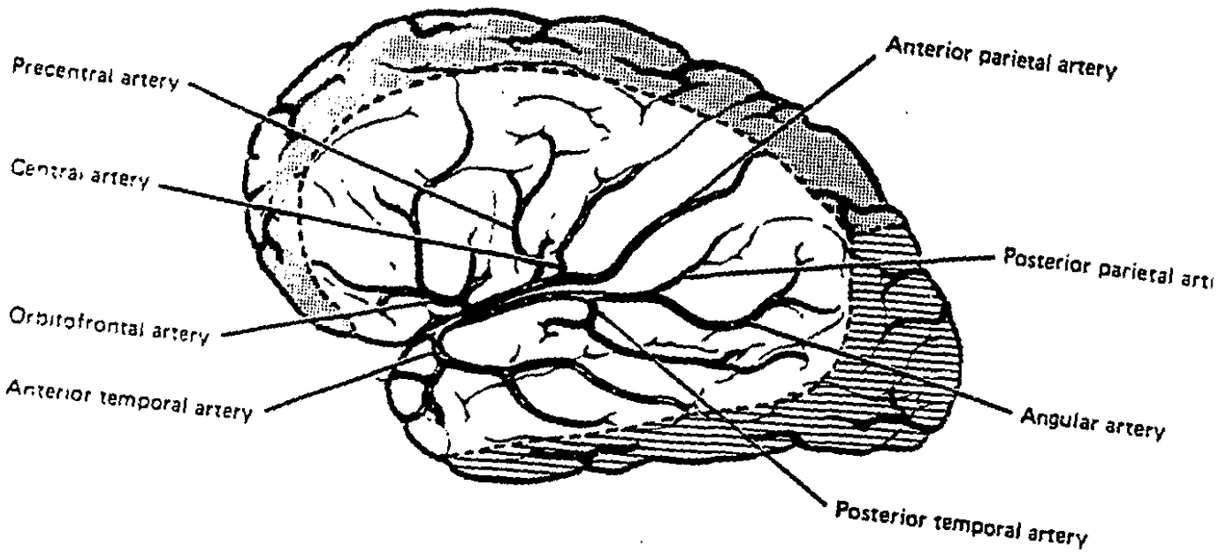

Anterior cerebral artery
Middle cerebral artery
Stem
Superior division
Inferior division
Lenticulostriate arteries
Superior cerebellar artery
Basilar artery
Vertebral artery
Anterior inferior cerebellar artery
Posterior inferior cerebellar artery
Internal carotid artery
Posterior communicating artery
Posterior cerebral artery

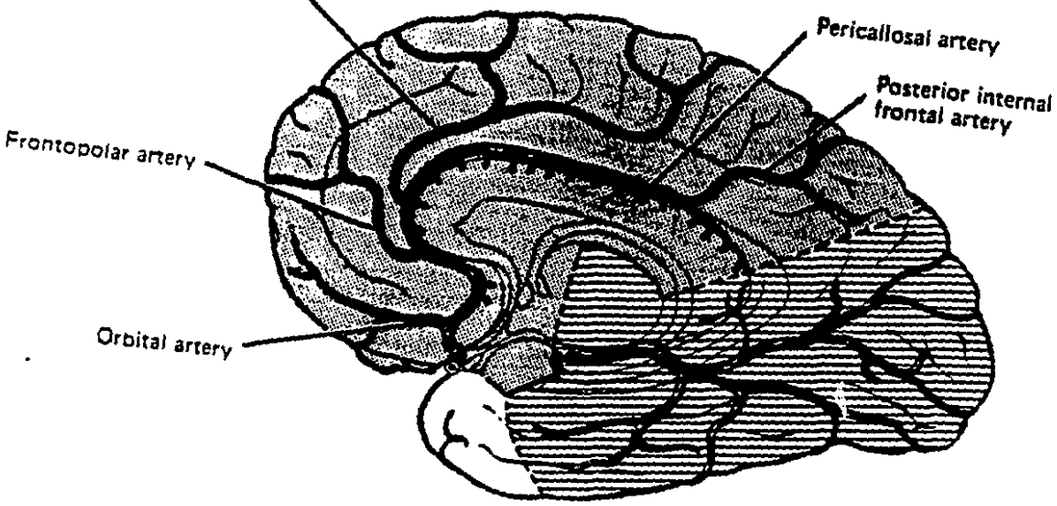
Fig. 4: Distribution of anterior, middle and posterior cerebral arteries

on the lateral and median surfaces of the brain and subdivisions of the cortical mantle they irrigate.

LATERAL SURFACE



MEDIAN SURFACE

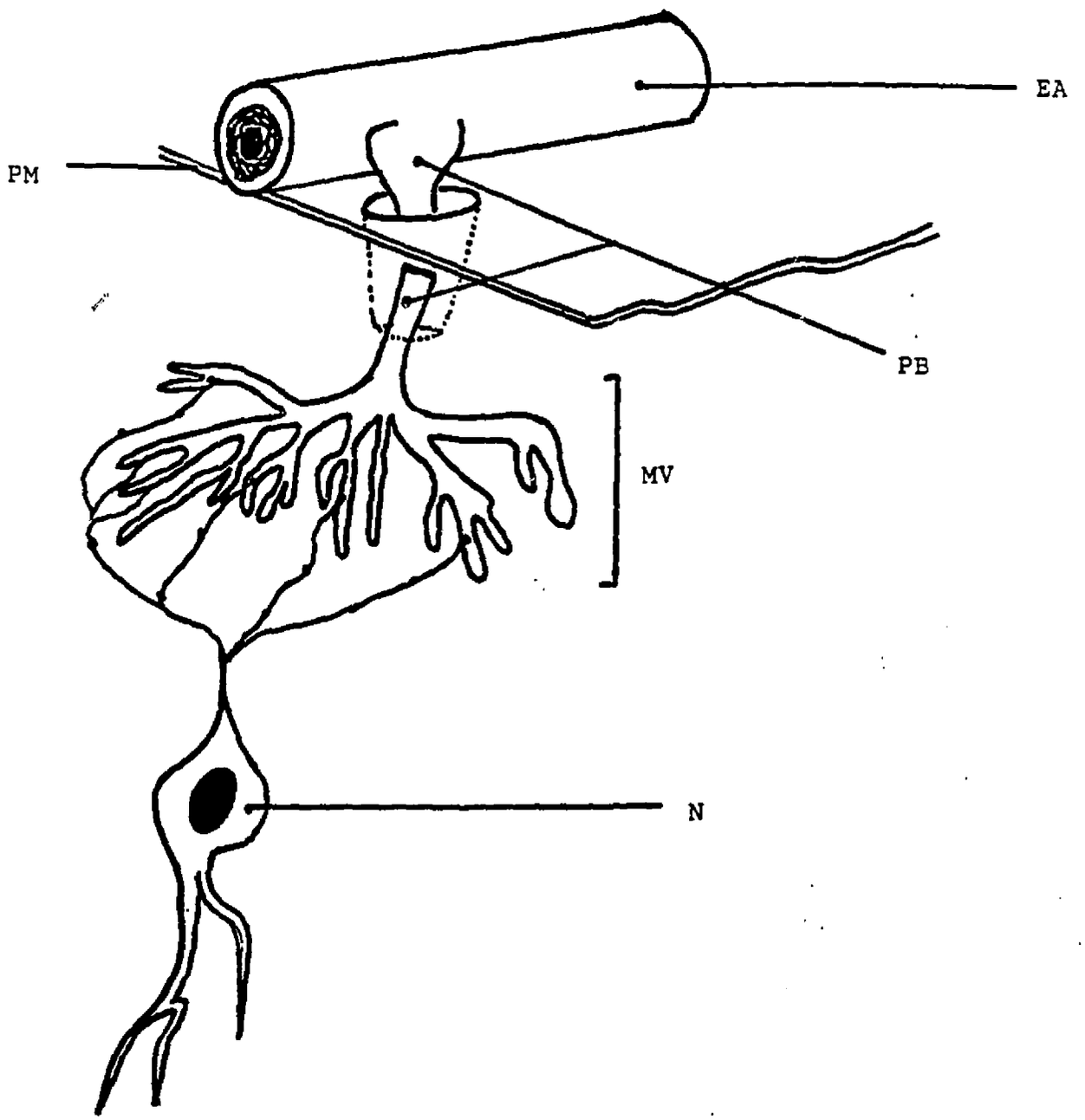


Anterior cerebral artery

Middle cerebral artery

Posterior cerebral artery

Fig. 5: Schematic representation of the cerebrovascular system showing a segment of an extra-cerebral artery (EA) sending a penetrating branch (PB) through the pia matter (PM). This branch ends by dividing into intra-parenchymal microvessels (MV) innervated by neurons (N) originating from central nuclei.


EA
PM
PB
MV
N

related to specific intrinsic brain nuclei such as the adenergic neurons of the locus ceruleus (Kalaria and Harik 1989), the cholinergic ones in the basal forebrain (Lacombe et al 1989, Linville et al 1993) as well as the serotonergic raphe nuclei (Di Carlo 1984). For the purpose of the present study, only the adrenergic and cholinergic cerebrovascular innervations will be discussed in details below.

1.3.b. Adrenergic innervation of cerebral blood vessels:

Extra-cerebral as well as intra-cerebral blood vessels appear to be regulated by noradrenaline containing nerve fibers. Histofluorescence and immunocytochemical studies have demonstrated the existence of a dense plexus of noradrenaline containing fibers around major cerebral arteries and pial vessels of the brain (Edvinsson and Mackenzie 1976). The perivascular plexus of sympathetic nerves could be followed from the basilar arteries to the convexities where arteriole as small as 15 to 20 μm in diameter are accompanied by adrenergic fibers (Edvinsson 1982).

Biochemical studies have further confirmed the presence of noradrenaline in the major pial vessels by measuring the content of noradrenaline and of the enzymes involved in its biosynthesis and degradation (Hardebo et al 1977, Edvinsson et al 1984, Bonvento et al 1990). Denervation experiments, in combination with histofluorescent detection of noradrenaline as well as the determination of noradrenaline concentration, uptake and release processes have unequivocally established that the vast majority of the noradrenergic nerves that invest the extracerebral blood vessels originate in the sympathetic superior cervical ganglia (MacKenzie and Scatton 1987) with a relatively sparse contribution from the stellate ganglion to the vertebrobasilar trunk (Nielsen and Owman 1967).

At the level of the intracerebral microcirculation, noradrenaline as well as the enzymes involved in its synthesis and degradation have been detected at significant levels in isolated brain

intraparenchymal blood vessels (Hardebo et al 1977). Noradrenergic terminals running in proximity to intracerebral microvessels have been visualized both at the light and electron microscopic levels (Swanson 1977) and it has been suggested that these axons constitute a functional neurovascular innervation of intraparenchymal microvessels by brain intrinsic neurons, most likely originating in the locus ceruleus.

1.3.c. Cerebrovascular adrenergic receptors:

Pharmacological studies with selective adrenergic antagonists have established the presence of α- and ß-adrenoceptors in cerebral blood vessels. The sympathomimetic-induced contractions of feline and human pial arterial segments are blocked by phentolamine, piperoxane, dibenamine and phenoxybenzamine indicating the presence of α-adrenoceptors. Subsequent studies have shown that the contraction elicited by noradrenaline in feline and canine cerebral arteries is blocked by yohimbin and rauwolscine (Sakakibara 1982) thus arguing in favour of the presence of α2-adrenoceptor in these species. In fact, interspecies variations appear evident since man, guinea pig and rat cerebrovascular adrenoceptors exhibit an α1-pharmacology (Skärby et al 1984, Nakai et al 1986) while those in cat, dog and rabbit have been identified as α2-adrenoceptors (Shärky et al 1981, Edvinsson et al 1981).

Noradrenaline may also interact with ß-adrenoceptors in cerebral vessels and, as such, dilatation may be induced by sympathomimetic agents. This response can be shifted towards higher concentrations by propranolol and practolol, suggesting the presence of a ß1-adrenoceptor. in feline cerebral arteries (Edvinsson & Owman 1974).

Even though there is a paucity of information regarding the functional effects of neurally-released noradrenaline onto brain microarterioles and capillaries, both vasomotor and blood brain barrier functions have been suggested. Interestingly, radioligand binding studies have revealed

the presence of α1 , α2 and ß-adrenoceptors in human brain microvessels (Ferrari-DiLeo & Potter 1985, Harik et al 1988) as well as in other species (Peroutka et al 1980, Harik et al 1981).

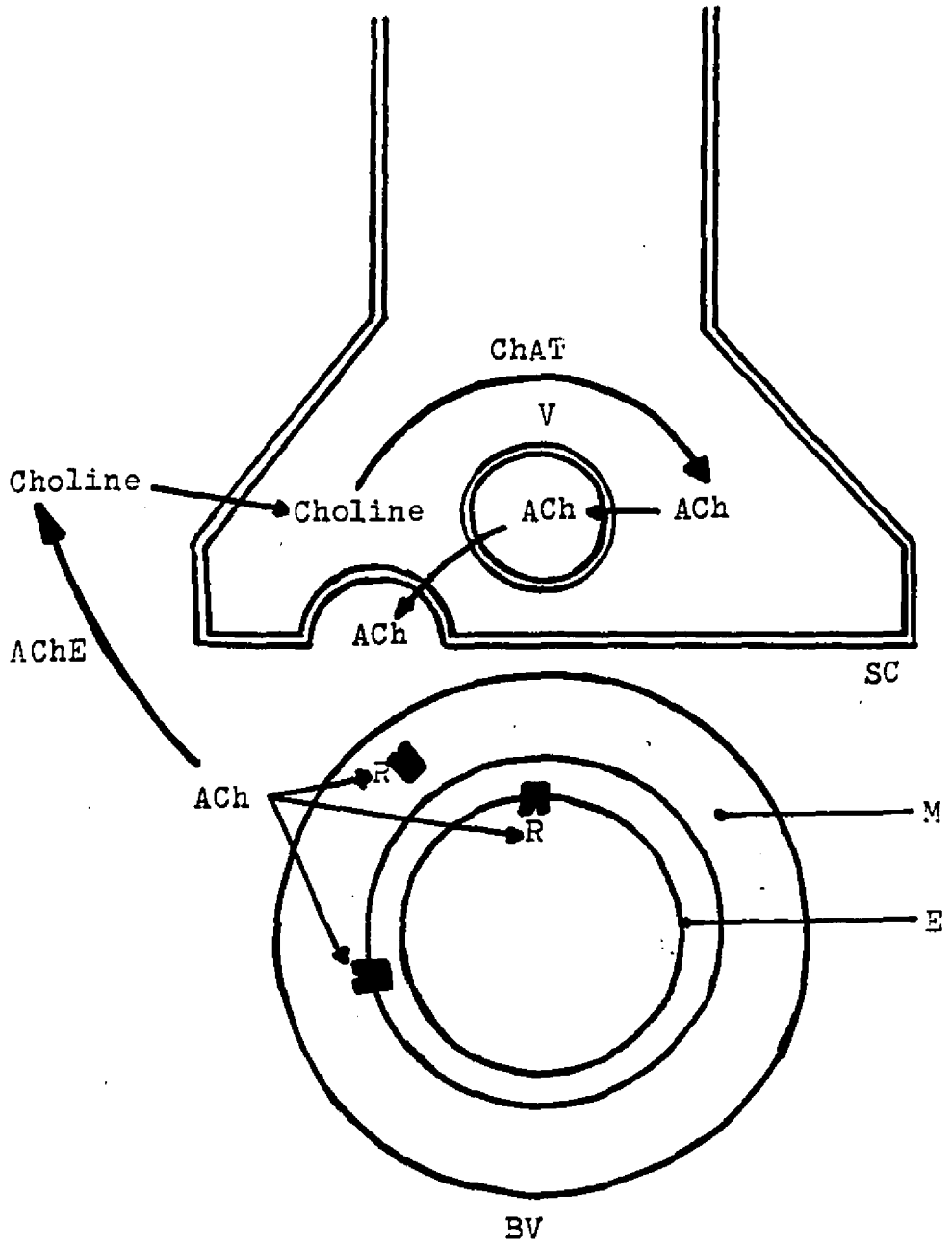
1.3.d. Cerebrovascular cholinergic innervation:

As in the CNS, acetylcholine (ACh) in cerebrovascular nerves is synthesized by the acetylation of choline, a reaction catalyzed by the enzyme Choline acetyltransferase (ChAT) which uses acetyl CoA as a source of the acetyl group. The activity of ChAT in cerebral tissues has been shown to be a reliable index of cholinergic innervation (Hamel et al 1987).

ACh is packed in vesicles and released through a Ca^{++}-dependent mechanism onto the vascular wall upon nerve depolarization (Hamel et al 1987). Then, ACh can act on specific cerebrovascular receptors to induce vasomotor responses (see below) or, alternatively, be degraded by the enzyme acetylcholinesterases (AChE) back into choline and free acetyl group. Through a selective reuptake system, choline reenters the nerve terminals where it can be reused in the biosynthesis of ACh (figure 6).

Unlike the well characterized biochemistry of cholinergic cerebrovascular nerves (for a review, see Hamel & Estrada 1989), morphological evidence for the presence of cholinergic fibers around extra- cerebral blood vessels have been difficullt and long to obtain. Fibers histochemically stained for AChE, the degrading enzyme for ACh, have been observed on major cerebral arteries (Edvinsson et al 1976, Kobayashi et al 1981, Ando 1981) as well as small pial vessels (Hara and Weir 1986, Edvinsson et al 1976, Saito et al 1985). They were found to largely colocalize with VIP (Hara et al 1985) and to originate primarily from the sphenopalatine and otic ganglia (Hara et al 1985). However, AChE is not a selective marker for cholinergic nerves since it has also been found in other neuronal systems. Subsequent studies have immunocytochemically

Fig. 6: Schematic representation of acetylcholine synthesis, release and degradation in a perivascular nerve ending: Choline obtained from dietary sources or from the degradation of acetylcholine (ACh) by the enyme acetylcholinesterase (AChE) is methylated by choline acetyltransferase (ChAT) to form acetylcholine. The neurotransmitter is stored in vesicles (V) to be released upon depolarization into the synaptic cleft (SC) where it binds to acetylcholine muscarinic receptors (R) distributed on the endothelial (E) and /or smooth muscle (M) layer of blood vessels (BV).


ChAT
V
Choline
Choline
ACh
ACh
ACh
AChE
SC
ACh
R
R
M
E
BV

detected fibers containing ChAT, the most selective anatomical marker for cholinergic neurons and fibers, and thus have confirmed the presence of cerebrovascular cholinergic nerves (Saito et al 1985, Suzuki et al 1990). Using this approach, however, a low degree of co-localization with VIP was found in cerebrovascular fibers and ganglionic neurons (Suzuki et al 1990). The incomplete disappearance of the cholinergic fibers (Hara et al 1989) and the minor decrease in ChAT activity and ACh levels (Dauphin et al 1992) following lesions to the sphenopalatine ganglion suggested other origins for these cerebrovascular fibers. Such statement was further substantiated by the identification of ChAT positive fibers projecting to large cerebral arteries and originating from the otic and the miniganglion of the internal carotid artery (Suzuki et al 1990).

With respect to the microcirculation, it has been shown that cortical vasodilatation and concomittant increase in local cerebral blood flow (CBF) can be obtained following stimulation of the basal forebrain cholinergic neurons (Lacombe et al 1989, Arneric 1989, Biesold et al 1989). Other sources of cholinergic neurons intrinsic to the brain may also participate in the neurogenic control of cortical microcirculation as suggested by physiological (Scremin et al 1991), biochemical (Galea et al 1991) and anatomical (Chédotal et al 1994) studies.

1.3.e. Cerebrovascular cholinergic receptors:

The biological action of ACh onto the cerebral blood vessel wall has been relatively well studied. At low doses (up to 10^{-6}M) Ach induces an endothelial dependent relaxation mediated by an endothelial dependent releasing factor (EDRF) which corresponds probably to nitric oxide (NO) (Palmer et al 1987). On the other hand, a constriction is observed at higher doses (Edvinsson et al 1977, Lee 1980). This dual effect was originally explained by the presence of smooth muscle muscarinic receptors (for the contraction) and of an endothelial population of

muscarinic receptors (for the relaxation). Meanwhile, pharmacological as well as molecular biology procedures have both revealed the heterogeneous nature of vascular muscarinic receptors. Molecular biology has identified five genes encoding different muscarinic receptors (M1 - M5: Bonner et al 1988, Palmer et al 1987) while pharmacological tools allow to distinguish clearly between three subclasses of muscarinic receptors (M1, M2 and M3: Doods et al 1987) which correspond to the cloned m1 - m3 subtypes (Buckley et al 1989). The M1 subtype has high affinity for pirenzepine and 4-Diphenylacetoxy-N-methylpiperidine (4-DAMP), the M2 exhibits high affinity for 11-[[2-[(diethylamino) methyl]-1-piperidinyl] acetyl]-5, 11-dihydro-6H-pyrido [2,3-b] [1,4] benzdiazepine-6-one (AF-DX 116) and a low affinity for both pirenzepine and 4-diphenyl acetoxy-N-nmethylpiperidine methiodide (4-DAMP), whereas the M3 subtype is characterized by high affinity for 4-DAMP, intermediate affinity for pirenzepine and low affinity for AF-DX 116 (Doods et al 1987). The functional equivalent of the m4 and m5 subclasses are not clearly distinguished. Furthermore, the heterogeneity of the muscarinic subtypes seem to be also expressed at the level of signal transduction mechanisms. In a general manner, M1, M3 and m5 subtypes has been reported to stimulate phosphoinositol turnover (Peralta et al 1987) while M2 and M4 receptors induce an inhibition of adenylate cyclase activity (Ayikama et al 1986). Although there has been several reports of cerebrovascular muscarinic receptors in various species (Peroutka et al 1980, Tsukahara et al 1986, Dauphin and Hamel 1992), the presence of specific muscarinic binding sites in human cerebral blood vessels has been reported by few groups (Tsukahara et al 1986, Dauphin and Hamel 1992). In a recent pharmacological study, the M1 and M3 receptor subtypes were characterized in human major cerebral arteries and pial vessels (Dauphin and Hamel 1992). Interestingly, heterogeneous muscarinic binding sites have recently been identified in bovine and human microvessels and capillaries (Garcia-Villaton 1989, Linville and Hamel 1992), namely, the M1 and M3 subtypes (Linville et al 1993). Although the

M1 amd M3 receptors have been shown in different species to mediate contraction and dilatation, respectively, of cerebral blood vessels (Dauphin and Hamel 1990, 1991) including small pial arterioles (Shimizu et al 1993), there is no information of subtype-specific functions in human cerebrovascular bed. Indeed, ACh only induces dilatation in isolated cerebral arteries (Tsukahara et al 1989) and the muscarinic receptors involved in this effect has not yet been characterized. It is also unknown if the contractile M1 subtype would be downregulated in the face of the vasodilatory M3 subtype, as recently reported in mouse (Shimizu et al 1993).

1.4. The cerebral blood vessels: Effects of aging and dementia.

1.4.a. Structural alterations:

Age and AD-related morphological changes in the blood vessel wall of the brain have been reported. In vertebral and basilar arteries in humans, wall thickness and the ratio of collagen to elastin increase with aging while vessel wall distensibility is reduced (Busky and Burton 1964, Nagasawa et al 1979). The increased stiffness in the intracranial arteries predisposes for the development of cerebral arterial aneurysm in the elderly (Lockstey 1966). Similar cerebrovascular structural alterations has been documented in aged animals. The ratio of collagen to elastin increases in the internal carotid artery between 2 and 24 months of age in rats (Cox 1977). Furthermore, pial arterioles obtained from aged rats showed atrophy, reduced distensibility and reduced relative proportion of the distensible elements, elastin and smooth muscle (Hajdu et al 1990). These findings suggest that the effects of aging on the distensibility of the cerebral arterioles may be similar to the effects of aging on the larger arteries (see above). Additional studies of the intraparenchymal vascular compartment also revealed the presence of age-related structural abnormalities. Ravens (1978), described three types of vascular changes in human

senile brain: 1) convoluted blood vessels involving small arteries, arterioles and precapillaries, 2) perivascular gliofibrilar proliferation of the entire vaacular tree, 3) adventitial proliferation of connective tissue fibers of small arteioles and capillaries. On the other hand, the presence of microvascular structural changes specifically encountered in the brains of AD patients have been well documented. Earlier investigators (Hassler 1965) described an abundant occurence of "glomerular loop formation", "vascular wickerworks" and "vascular bundles" in 16 brains from individuals with senile or presenile dementia. More recently, Higushi et al (1987) detected the presence of a degenerative state of the endothelial cells together with hypertrophy and irregularities of the basement membrane of small blood vessels and capillaries in the cortices of brains obtained from proved AD cases. Scheibel and colleagues (1987a) have also described a "denervating microangiopathy " in the brains of five patients with clinical and neuropsychological diagnosis of AD. Scheibel described this angiopathy as thickened capillary walls with irregular lumpy modulated contour which appeared to be due to infiltration of the vascular wall with rounded cell-like bodies. In each case there was no trace of the perivascular neural plexus which normally innervates the microvasculature of the brain parenchyma. He postulated that the loss of the plexus originating in the locus ceruleus and the basal forebrain may be related to the changes in the capillary wall structure and these in turn may lead to profound alterations in blood-brain-barrier functions. In another study, Scheibel (1987b) reported perforations of the vessel wall characterized by multiple openings that run through the complete thicknesss of the basement membrane but did not perforate the endothelial lining of the capillary lumen. Such perforations may provide a route for extravasation of serum constituents in the brain parenchyma reported by other investigators (Wisniwewski and Kozlowski 1982). In addition to these structural alterations, Bell and Ball (1981) measured the changes in diameters and densities of microvasculature in different age groups as well as in AD patients. They reported a diminished diameter and a lower

capillary density of the brain regions mostly affected by the degenerative neuronal lesions. This finding was replicated later on by Mann and his collaborators. (1986).

1.4.b. Biochemical changes:

The study of neurotransmitter biochemical and anatomical markers provides a useful way through which the nature and the density of the innervation in a specific organ can be evaluated. Until recently, there was no clear understanding of the age-related changes in the cerebrovascular innervation due to the scarcity of the litterature devoted to this subject. Neverthless, during the past few years , several investigators have reported various alterations in the cerebrovascular bed which are associated with the process of aging (Mione et al 1988, Hamel et al 1990). There is, however, virtually no report on the possible AD-related changes in large cerebral artery and pial vessel innervation and function despite the growing body of evidence suggesting alterations at this level in both aged animals as well as in man.

At the level of intraparenchymal microvessels, age-related alterations have also been reported. Radioligand binding assays aimed at detecting possible changes in receptor density related to the ageing process, nave focussed mainly on the adrenergic receptors, and more specifically the ß subtypes. Reports from two studies would support an age-related decrease in ß-adrenoceptors density in cortical microvessels (Koyabashi et al 1982, Mooradian and Scarpace 1991). In another study on cholinergic innervation of the microcirculation, a decline in ChAT activity has been observed in rat cortical capillaries after 18 months of age (Santos-Benito and Gonzalez 1985). In animal models used to reproduce some of the effects of degenerating neuronal systems encountered in AD inconsistent results have been obtained. Lesioning of the nucleus basalis magnocellularis in rats resulted in either a three fold increase (Santos-Benito et al 1985) or in no change (Galea et al 1991) in cortical microvessels ChAT activity. Following noradrenergic

denervation induced by destruction of the locus ceruleus, the density of α1-adrenergic binding sites in cortical microvessel was not altered , in contrast, microvascular ß2-adrenergic receptors were significantly increased following such lesion (Harik et al 1991). Interestingly, the same group of researchers measured the density of α and ß-adrenergic binding sites in microvessels isolated from the prefrontal cortex of AD patients and reported significant increases in both α2 and ß2 adrenoceptors. They attributed these changes to a possible noradrenergic deafferentiation of the microvessels (Kalaria and Harik 1989).

1.4.c. Functional cerebrovascular alterations in aging and dementia:

Age-dependent changes have been observed in the responsiveness of isolated cerebrovascular segments to stimulation by various pharmacological agents. Maximal contractile responses to noradrenaline of human basilar artery decreased with increasing age with no significant changes in the affinity of the adrenergic receptors (Hatake et al 1992). Diminished contractile responses to noradrenaline attributed to an age-related defect in α1 adrenoceptors were also observed in isolated monkey cerebral arteries (Toda 1991). Meanwhile, the vasodilatory mechanisms do not seem to be affected by senescence as evaluated in rat cerebral blood vessels (Hamel & MacKenzie 1991).

Additional evidence implicating a cerebrovascular component in the pathology of AD came from the measurement of regional cerebral blood flow (rCBF) changes in demented patients. Obrist et al (1970) who specifically investigated possible CBF abnormalities in patients with presenile and senile dementia of the Alzheimer type found low CBF values in both types of patients, when compared to controls, with a tendency for reduction in the frontotemporal region. The results from the majority of subsequent studies agreed on a reduction in CBF with a regional frontotemporal involvement in AD (Simmard et al 1971, Grubb et al 1977, Yamaguchi et 1980).

Some authors (Yamaguchi et al 1980) found a positive correlation between the CBF reduction and the duration and severity of dementia while others (Grubb et al 1977) failed to observe such correlation.

More recent studies with positron emission tomography (PET) offered a useful non-invasive in vivo technique that allowed for qualifying a variety of cerebral functions such as blood flow, metabolic rate of glucose or of oxygen. PET studies directed towards the evaluation of cerebral functions in normal ageing did not always yield consistent results. However, they have been recently summarized (Hoyer 1986) and, overall, they point to a relative stability in CBF from the third to the seventh decade of life. Thereafter, the parameters may decrease. On the other hand, PET proved to be an increasingly useful procedure in the study of Alzheimer's disease. CBF measurements with PET in AD patients (Grady et al 1990) confirmed the average decline observed by the previous techniques, but could not correlate those with the severity of the dementia. The decline mainly involved the temporal and parietal regions in mild degenerative dementia and the frontal and parietal regions in severe dementia. This pattern parallels that frequently reported for global (Ferris et al 1980,, Kuhl et al 1985, de Leon et al 1986) and focal (Foster et al 1984) reductions in glucose utilization most severely affecting the temporoparietal and occipital cortex even in the early stages of the disease. It remained to be determined if the changes in CBF are merely consecutive to the reduced metabolic activity or if they reflect primary dysregulations in the neurogenic control of CBF in the process of AD.

2. Experimental procedures

2.1. Biochemical markers for cerebrovascular innervation and receptors:

2.1.a. Human cerebrovascular tissues:

Pial vessels were removed from the cortical surface of human brains obtained from the Douglas hospital brain bank at different post-mortem intervals. The brains were from eight histopathologically proven cases of AD (27 ± 8 hours) and eight non-demented and/or neurologically normal elderly subjects (27 ± 6 hours) matched for age and, whenever possible, sex. Post-mortem diagnosis was performed by Dr. Yves Robitaille, neuropathologist at the Montreal Neurological Hospital, based on the presence of the following criteria: frontotemporal degeneration, high densities of both senile plaques and neurofibrillary tangles in addition to the presence of amyloid angiopathies. Two out of the eight AD brains were diagnosed as pre-senile AD and this corresponded with the pre-senile clinical deterioration of the patients cognitive functions. Clinical characteristics on the individuals used in the study are summarized in table 1.

2.1.b. Vascular tissue preparations:

The pial vessels were rapidly removed from the cortical surface of each human brain, they were frozen in isopentane (-45°C) and stored (-80°C) until assayed. A small part of the tissue was kept for ChAT activity determination. The remaining vessels were homogenized with a polytron (setting 3) in 10 volumes of 50 mM tris-HCl buffer (pH=7.4 at 4°C), centrifuged (3000 rpm for 10 minutes) and the resulting supernatant was recentrifuged (35,000 rpm for 90 minutes). The final pellet was resuspended in buffer to obtain a final protein concentration of 80μg/25μl, as determined according Lowry et al (1951). These membrane fractions were stored

Table 1: Clinical and post-mortem information on AD and control subjects included in the study. The age of death and the post-mortem delay are expressed as mean ± standard error of the mean, the range is also given within brackets. The number of individuals who died from different causes of death listed is demonstrated within parentheses.

	AD	Controls
Number of samples	8	8
Sex ratio (Male/Female)	2/6	4/4
Age at death (years)	75 ± 4 (66 - 93)	78 ± 4 (67 - 96)
post-mortem delay (hours)	27 ± 8 (11 - 72)	27 ± 6 (11 - 60)
Cause of death	Myocardial insufficiency (1) Cardiopulmonary arrest (2) Bronchopneumonia (2) Respiratory failure (1) Septic shock (1) pulmonary infection (1)	Pneumonia (2) Pulmonary embolism (1) Pulmonary edema (1) Respiratory failure (1) Cardiac tamponade (1) Myocardial insufficiency (1) Myocardial infarction (1)

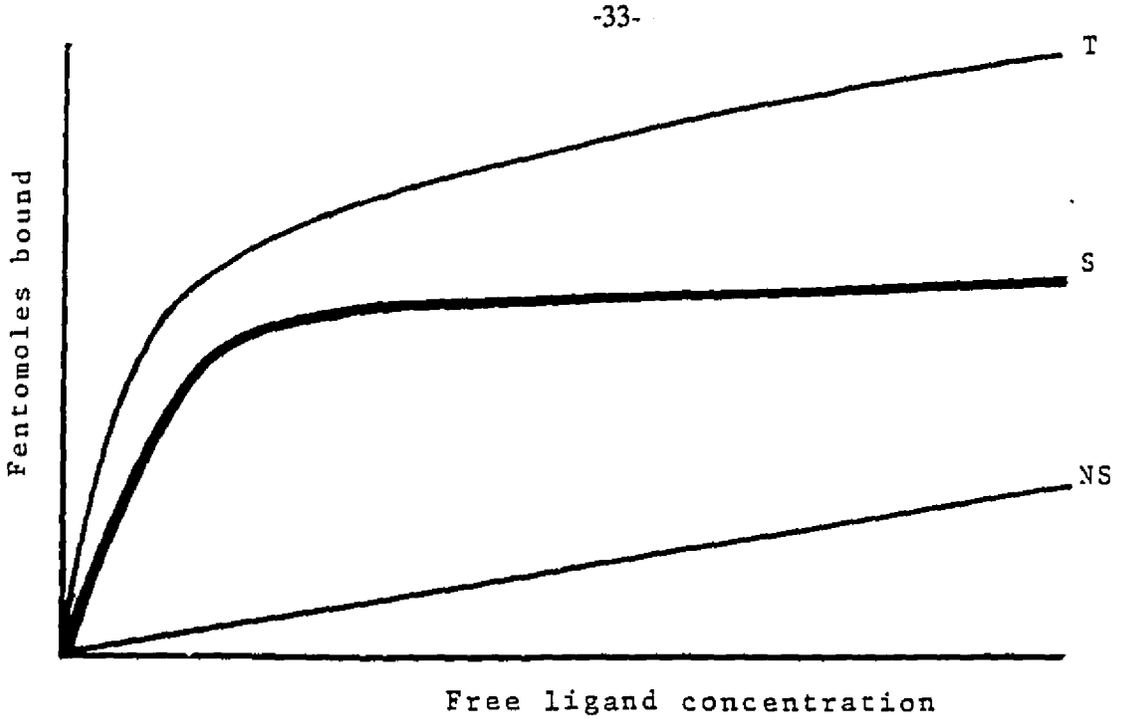
frozen (-80°C) until used for binding assays.

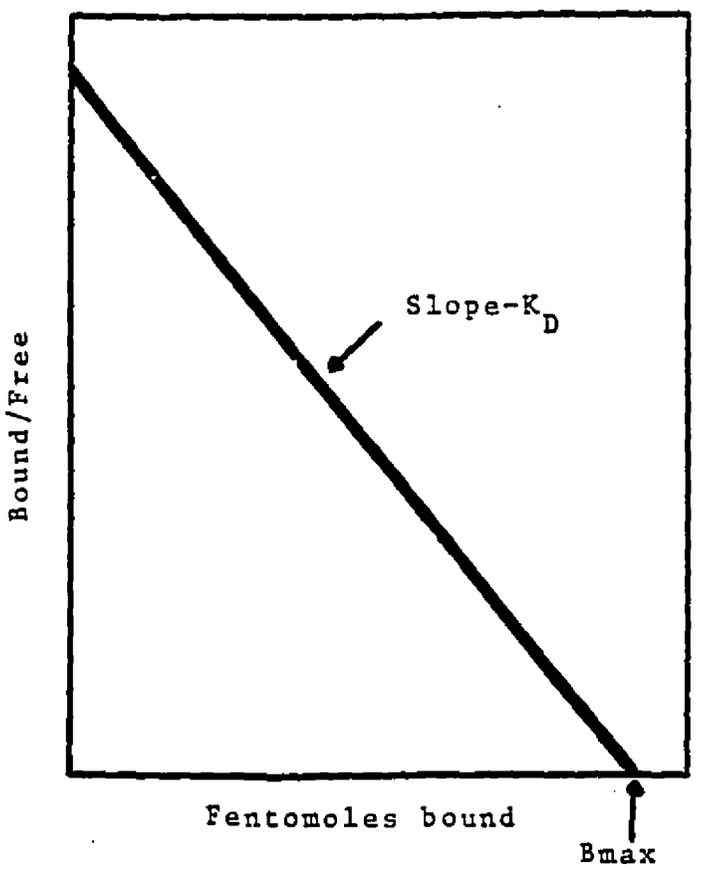
2.1.c. Principle of radioligand binding :

Radioligand Saturation binding studies depend upon gradual saturation of specific binding sites with a radiolabelled compound for which the receptor of interest has a high affinity. A fixed amount of receptor protein is incubated with increasing concentrations of radioligand in both the presence (non-specific binding) and the absence (total binding) of a concentration of an unlabelled drug that exhibits high affinity at the receptor. Proteins in tissue preparations have been found to bind to nitrocellulose filters and advantage can be taken from this phenomenon to measure binding of the radioligand receptors. The ligand-receptor complex is subjected to partial filtration through the nitrocellulose membrane, the amount of bound radioligand is then counted by liquid scintillation. In the absence of the unlabelled compound, the radioligand binds to both specific and non-specific sites whereas in its presence only the non-specific sites are available for labelling. The specific binding, which represents the sites of interest, is then calculated by substracting the non-specific from the total binding. The values obtained are used to draw a saturation binding curve where the values for total, specific and non-specific binding are plotted against the concentrations of the radioligand (fig. 7). Such analysis shows the saturability of the specific binding sites at high concentrations whereas the non-specific binding increases linearly with increasing concentrations. The calculated values for specific binding are used in the Scatchard linear transformation by plotting the ratio between the bound over the free fentomoles in the medium against the specific binding (fig. 8). A best fitting line is drawn between the points. The Scatchard analysis of the binding results is used for the caculation of two important binding parameters: 1) Maximal binding capacity (Bmax) representing the total amount of the specific binding sites in a given preparation. In a Scatchard representation the Bmax corresponds

Fig. 7: Typical saturation binding curve where the concentrations of the total (T), specific (S) and non-specific (NS) binding sites are plotted against the free ligand concentrations. Notice the saturability of the specific binding and the linear increase of the non-specific binding.

Fig. 8: Scatchard transformation obtained by plotting the ratio between bound over free fentomoles against the specific binding. The Bmax is the intercept of the line on the X-axis. The K_D is calculated from the slope of the line.


T
S
NS
Fentomoles bound
Free ligand concentration


Slope-K_D
Bound/Free
Fentomoles bound
Bmax

to the intercept of the line on the X-axis (Fig.8) and 2) The dissociation constant (K_D): which is an expression of binding sites affinity for the radioligand is calculated from the slope of the best fitting line (Fig.8).

In the present study, Bmax and K_D were caculated by computerized nonlinear curve fitting using the commercial software EBDA-LIGAND program of Munson and Rodbard (1980), modified by McPherson (1985). Raw data were initially processed by the EBDA program in order to give the best fitting line for the points entered and calculate the first estimates of the binding parameters. The file produced by the program EBDA is used by the program LIGAND for the final evaluation of the binding iotherms.

2.1.d. Total muscarinic binding sites in pial vessels:

Saturation experiments for total muscarinic binding sites were performed by incubating aliquots of the pial vessels homogenate (40μg) with increasing concentrations (0.01 - 10 nM) of the non-selective muscarinic antagonist [^{3}H]-N-methylscopolamine ([^{3}H]-NMS specific activity = 79.5 mCi/mmole). Incubation was performed (90 min.) in 50 mM tris -HCl buffer (pH 7.4) in a final incubation volume of 0.25 ml. Non-specific binding was determined in the presence of 1000 X excess of atropine. Termination of the binding was achieved by vacuum filtration with G/B filters presoaked overnight with 0.3% polyethylenimine followed by three 5 ml rinses with cold buffer. The filters were left to dry and transferred to plastic scintillation vials containing 10 ml of scintillation liquid (Ecolite). The radioactivity on the filters was determined by liquid scintillation counting (LKB spectrometer) with 45 - 50% efficiency.

2.1.e. Saturation binding assays under suppressive conditions:

Direct labelling of M1 sites in cerebrovascular preparations using [^{3}H]-pirenzepine(specific activity = 70.5 Ci/mmol) has also been attempted. An initial trial on bovine cortex with 600 μg protein / tube yielded a Bmax of 90 fmoles/mg protein and a $K_D$of 32 nM . Those values were similar to what have been previously reported for pirenzepine (10 nM). Four successive trials on human cerebrovascular tissue homogenates containing different protein concentrations (550 μg, 500 μg, 350 μg and 300 μg respectively) yielded controversial results. The total and non-specific binding both increased linearly and the values for non-specific binding exceeded those of the specific binding by many folds. In three out of four trials, the results obtained could not be analyzed either manually or using the LIGAND/EBDA program. Analysis of the binding results in a single preparation gave a K_D of 25 nM and a Bmax value (250 fmoles/mg protein) that exceeded the average values for total muscarinic binding sites labelled with [^{3}H]-NMS. Due to the unreliability of the results obtained with [^{3}H]-pirenzepine, we opted for binding assays under suppressive conditions as an attempt to discriminate more than one subtype of muscarinic binding sites in cerebrovascular tissues. Thus, a subset of muscarinic binding sites were labelled with [^{3}H]-NMS (0.01 - 10 nM) in the presence of 75 nM non-labelled pirenzepine. At this concentration, pirenzepine is expected to block all the M1 sites as well as parts of the M3 and M4 sites as determined by its affinity at the various muscarinic receptor subtypes (Buckley et al 1989) leaving a fraction of the M3 as well as a significant proportion of the M4 and M5 muscarinic receptor subtypes available for binding.

2.1.f. ß-adrenergic binding sites:

Total ß-adrenergic binding sites have been traditionally labelled with the radioligand [^{3}H]-Dihydroalprenolol ([^{3}H]-DHA). Previous studies, however, reported a higher affinity of

[^{3}H]-DHA for ß2 adrenergic binding sites (Neve et al 1986) or the possibility that other sites (5HT-1B) (Tsuchihashi et al 1990) are also labelled. On the other hand, binding characteristics of the novel radioligand 4-(3-Tertiarybutylamine-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride) ([^{3}H]-CGP 12177) to the central ß-adrenergic binding sites revealed a higher specifity for both ß-adrenergic subtypes and a lower non-specific binding as compared to [^{3}H]-DHA (de Paermentier et al 1989). This was confirmed by a recent study re-evaluating the selectivity of both radioligands (Riva & Creese 1989). We thus elected to use this ligand for labelling total ß-adrenergic binding sites in cerebral blood vessels.

Pial vessels were processed as described above. Aliquots of the final membrane preparations (60μg) were incubated in 50 mM tris-HCl (pH=7.6) with various concentrations (0.02 - 2 nM) of [^{3}H]-CGP 12177 for 120 min at 25°C in a final incubation volume of 0.25 ml. Termination of the binding assays was as described for [^{3}H-NMSbinding. Non-specific binding was determined in the presence of 2000 X excess of isoproterenol.

2.1.g. α1-adrenergic binding sites:

Prazosin is a post-junctional α-adrenoreceptor (α1) antagonist in peripheral vascular tissues. However, unlike phentolamine and phenoxybenzamine, prazosin has little affinity for pre-junctional α-adrenoreceptors in these tissues (Cambridge et al 1977). [^{3}H]-Prazosin has been widely used for the characterization of α1 - adrenergic receptors. Its advantage over other radioligands (such as [^{3}H]-Clonidine and [^{125}I]-HEAT) include a higher selectivity, a shorter incubation period (30 min) and a lower non-specific binding (Greengrass & Bremner 1979).

Aliquots of pial vessels preparations (80 μg) were incubated (30 min) in 50 mM tris-HCl (pH=7.4 at 25°C) in the presence of increasing concentrations of [^{3}H]-Prazosin (0.02 - 2 nM).

Non-specific binding was determined in the presence of 2000 X excess of phentolamione.

2.1.h. Analysis of the binding results:

The counts obtained for total and non-specific binding at each of the radioligand concentrations were averaged from triplicate determinations. The total amount of binding sites was determined and expressed as fentomoles/mg protein and the three values (total, specific and non-specific) were used to draw a saturation curve. Bmax and K_D values were calculated by the EBDA/LIGAND program as already mentioned in section 2.1.c.

2.1.i. ChAT activity determination:

Choline acetyltransferase (ChAT) is the enyme involved in the biosynthesis of acetylcholine (ACh) in nerve cell bodies and terminals. This enzyme catalizes the acetylation of choline to acetylcholine using acetyl-CoA as a source of acetyl group (fig.6). ChAT activity is generally accepted as a reliable and specific marker for cholinergic systems. In this study, the cerebrovascular ChAT activity was measured as an index of cerebrovascular cholinergic innervation (Hamel et al 1987) and was determined by the radiochemical method of Fonum (1975).

In order to insure adequate estimation of ChAT activity in the vascular bed examined, small segments of pial vessels were obtained from vessels at different locations on the cortical mantle and these were pooled and assayed collectively. The cerebrovascular tissues were cut into small pieces and then homogenized by sonication (30 seconds) in approximately an equal volume (100 - 150 μl) of Triton X-100/EDTA (0.5% / 10 mM) at 4°C. This crude homogenate was centrifuged (60 sec at 12,000 x g) and the resulting supernatant used undiluted for the assay. A 10μl sample of homogenates was incubated for 25 min at 37°C with a 10μl of 50 mM sodium phosphate

buffer containing 0.2 mM S-acetyl coenzyme A (NEN, Dupont Canada, specific activity = 51.5 mCi/mmole), choline 8 mM and eserine 0.1 mM at a final specific activity of 12 - 15 mCi/mmole. Blanks were prepared by replacing the tissue with 10μl TritonX-100/EDTA. The reaction is terminated by adding 150μl Ethyl Butyl Cetone/Tetra Phenyl Borate (EBC/TPB) to the contents of the vials. After vigorous agitation followed by centrifugation, 100μl of the organic (top) phase (containing the labelled acetylcholine) were transfered to scintillation vials containing 50 μl of water. They are then filled with 10 ml of a liquid scintillation solution (Ecolite) for radioactivity determined by liquid scintillation spectrometry. The protein concentration in the tissue homogenates was determined by the method of Lowry et al (1951) and the ChAT activity expressed as nmole of ACh formed/mg of protein/hour.

2.2. Sudomotor activity:

2.2.a. Subjects:

Sweat responses were studied in 30 AD patients (75.7 $\pm$ 1.5y, 12 males / 18 females), 30 elderly (79.4 $\pm$ 1.3 y / 12 males,18 females) and 30 young (30.2 $\pm$ 1.3 y / 13 males, 17 females) controls. The AD patients included in the study were chosen from inpatients of the geriatric floor of the Montreal General Hospital and from patients followed by Dr. Serge Gauthier in the Alzheimer's disease clinic belonging to McGill center for studies in ageing. All the AD patients were diagnosed as probable AD of the late onset form. None of the patients had any clinical manifestations of accompanying central or peripheral neurological disorders that may interfere with the interpretation of the sweat response obtained. A written consent form was signed by the patients or their caretakers before undergoing the experiment.

The clinical data of the 30 patients included in the study is represented in table 2.

The elderly controls were chosen from inpatients of the geriatrics unit of the Montreal General Hospital as well as relatives and spouses of the AD patients. The criteria for elderly control inclusion were the following: age > 70 years, no medical history of dementia or related neurological or psychological disorders, no medical history or clinical manifestations of autonomic dysfunction and no peripheral neuropathies. The exclusion criteria included the following: chronic diseases with autonomic neuropathies (e.g. diabetes mellitus, Parkinson's disease, and chronic alcoholism), multi-infarct dementia, oncological disorders, stroke, hyper- or hypothyroidism, peripheral vascular disorders, patients on ß-blockers and patients on medications with anticholinergic effects. The young controls were medical students, residents, members of the geriatric medicine team of the MGH as well as patient's relatives.

None of the 90 subjects were taking any medications with anticholinergic effects for at least 3 days before being tested. The age (in mean ± SEM) and sex ratio of the controls are represented in table 3.

2.2.b. Iontophoresis of acetylcholine:

Iontophoresis is the process of introducing ionized substances into tissues by mean of an electric current. The method of iontophoresis used is a modification of the quantitative sudomotor axon reflex test (QSART) described by Low et al (1983). For the purpose of this study, all the tests were undertaken at room temperature (≈ 22°C). While humidity was not specifically controlled for, all subjects were tested in similar non-humidified hospital area with seemingly little difference in humidity. The controls were in a sitting position whereas the AD patients were in a sitting or a supine position. A three chambered round plastic device (Fig. 9) connected to a constant power generator was tightly applied by mean of velcro straps to the volar surface of

Table 2: Clinical data on 30 AD patients included in this study.

Table 3: Average (± SEM) age and sex ratio of 30 elderly and 30 young controls included in this study.

Number	Sex ratio (M/F)	Age (Mean ± SEM)	MMSE score (Mean ± SEM)
30	12/18	75.7 ± 1.5	16 ± 1.5

Data	Elderly controls	Young controls
Number	30	30
Age (Mean ± SEM)	79.4 ± 1.3	30.2 ± 1.3
Sex ratio (M/F)	13/17	13/17

one of the subjects forearm at a distance approximately midway between the skin crease of the wrist and the cubital fossa (Fig. 10). The outer chamber of the device was filled with a freshly prepared 10% solution of acetylcholine disolved in normal saline (1 g acetylcholine, Sigma, disolved in 10 ml normal saline) by mean of a plastic syringe. An electrical stimulus of 3 mA obtained from the constant power generator was applied for 5 minutes after which the electric power was discontinued, the device was removed and the remainder of the solution removed using gauze. A low viscosity polyvinylsilicone impression material (Kerr company, Canada) prepared by homogeneously mixing an equal volume of base and catalyst was spread by mean of a metallic spatula to cover a skin area with a diameter at least twice that of the plastic device. The purpose of this wide area of coverage is to detect sweat drops produced by the population of sweat glands directly stimulated by the iontophoresed acetylcholine as well as much of the axonal reflex sweating produced outside the borders of the device. The material was left for 5 minutes during which the impression of each sweat droplet as it emerges from its sweat duct is retained as a minute hole in the rubberizing mold. The dry material is peeled off the skin; an impression of the device appears and the number of holes formed by the sweat droplets were counted under a dissecting microscope and were tabulated into four differnt zones:

Zone 1: Axon reflex zone. This zone corresponds to the inner chamber of the device where no ACh was injected.

Zone 2: Buffer zone between zone 1 and the iontophoretic zone. Corresponds to the narrow chamber separating the innermost and the outermost chambers of the device.

Zone 3: Iontophoretic zone. Represents the area of the skin directly stimulated by the iontophoresed ACh.

Zone 4: The area whereby a reflection of axonal reflex sweating in zone 1 and zone 2 beyond the limits of the device can be detected.

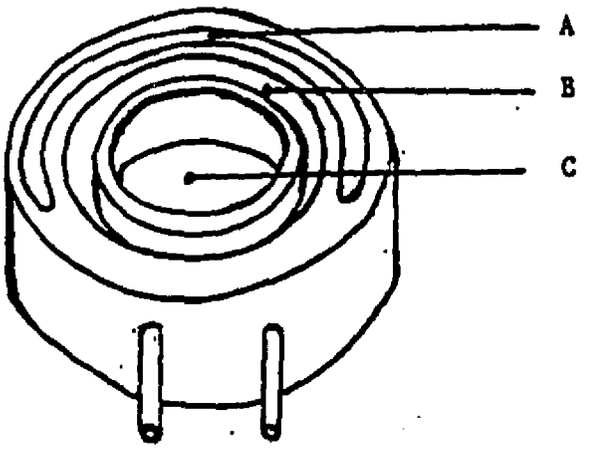
Fig. 9: Diagramatic representation of the plastic device used in this study demonstrating its different chambers:

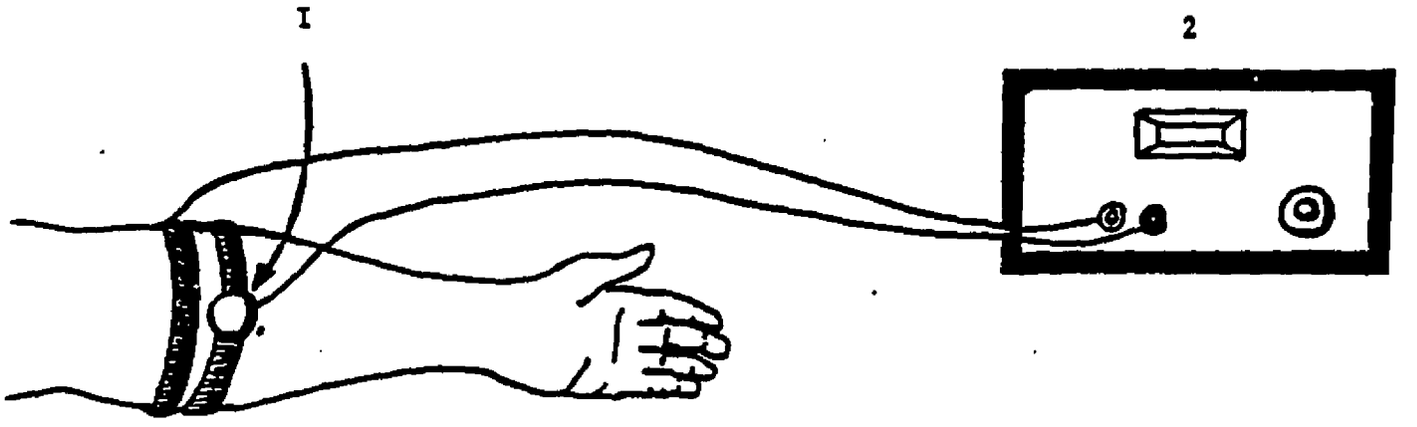
A- Iontophoresis chamber.

B- Intermediate chamber.

C- Innermost chamber.

Figure 10: Setting for the iontophoresis of acetylcholine showing 1) the plastic device applied to the subject's forearm and 2) the constant power generator.


A
B
C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2

2.3. Statistical analysis of the results:

2.3.a. Binding assays and ChAT activity determination:

Comparison between the average Bmax and K_D values obtained from the binding assays on 8 control and 8 AD pial vessels preparations was determined by the unpaired Student's t-test included in the software statistical package EPISTAT. The level of significance was established at $p \leq 0.05$. Comparison of the results of the ChAT activity obtained from the 7 pairs of pial vessels was also determined using the same test of statistical significance.

2.3.b. Sudomotor activity:

One of our interests in quantifying sudomotor activity in AD and control subjects is to evaluate the different components of sweat responses (direct and axonal responses). Our second interest is to try to verify the authenticity of the previously reported significant difference between sweat responses in AD and elderly subjects. Consequently, we classified the average responses obtained into 5 different classes:

1- Standard Quantitative Sudomotor Axon Reflex sweating Test (QSART) response: The sum of the responses in zone 1, 2 and 3. The response represents the total number of sweat glands activated either directly or through axon reflex stimulation within the boundaries of the device.

2- Pure axon reflex sweating: values obtained in zone 1. Represents sweat produced through pure activation of axon reflex.

3- Direct response: values obtained in zone 3. Represents the number of sweat glands directly activated by the iontophoresed ACh.

4- Total reflex sweating: the sum of the responses in zone 1, 2, and 4. Represents the sweat responses obtained in all areas where no ACh was iontophoresed.

5- Global sweat response: The sum of the responses in zone 1, zone 2, zone 3 and zone 4. Indicates all the responses within and beyond the limits of the device.

Calculated averages from different zones were tested for statistical significance by one-way analysis of variance implicated in the statistical software package EPISTAT.

3.Results:

3.1.Biochemical changes in post-mortem cerebrovascular tissue obtained from AD patients:

3.1.a Total muscarinic binding sites:

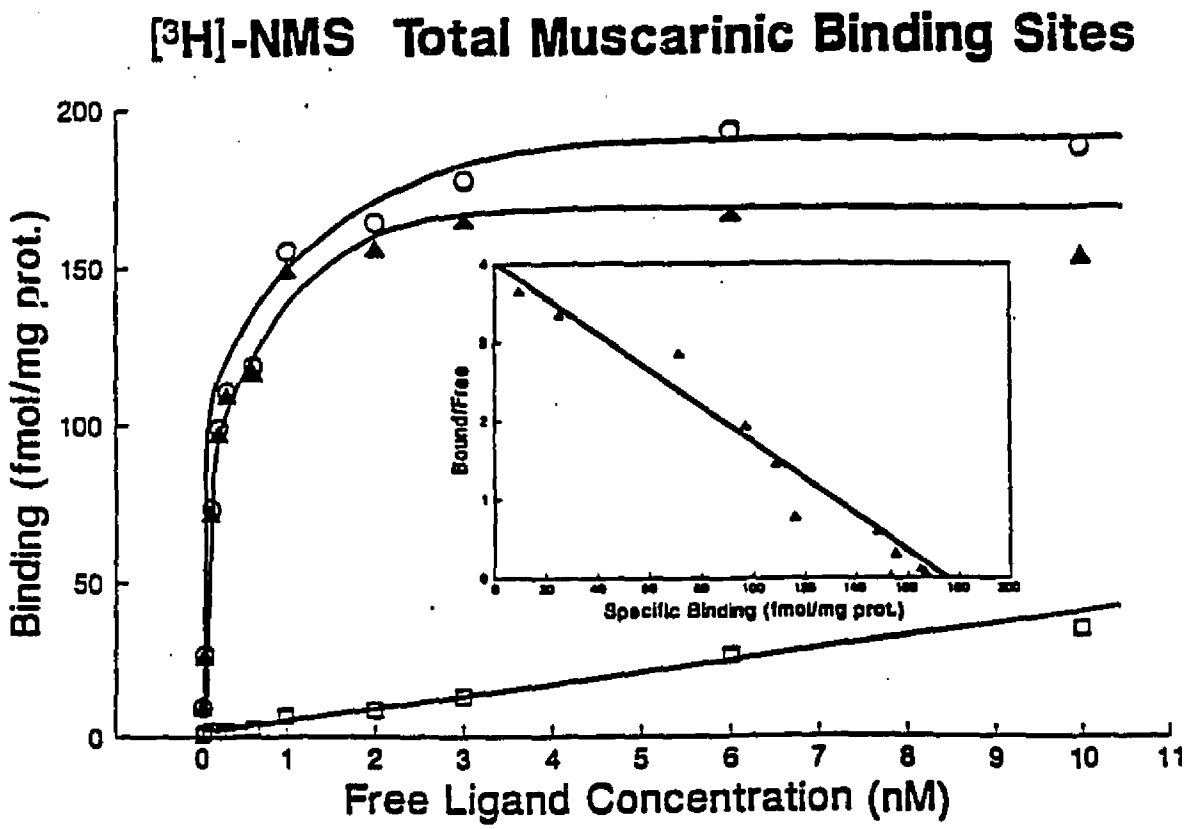
Specific binding of [^{3}H]-NMS was found to be saturable and of high affinity in both AD and control pial vessel preparations whereas the non-specific binding increased linearly as a function of [^{3}H]-NMS concentration (figure 11). The specific binding represented approximately 90% of the total binding at concentrations of the radioligand close to the K_D value (see below). The specific binding of [^{3}H]-NMS was best defined by the interaction of the radioligand with a single population of binding sites as indicated by the linear Scatchard plot representation (figure 11) and the Hill coefficient close to 1 for both the AD (0.94 $\pm$ 0.02) and the control (0.91 $\pm$ 0.05) preparations.

When the binding isotherms of [^{3}H]-NMS to the cerebrovascular preparations were ranked as a function of the post-mortem delay, no difference in Bmax nor K_D values could be evidenced (table 4).

Binding capacity (Bmax) of [^{3}H]-NMS in pial vessels obtained from controls varied from 33.8 to 166.8 fmoles/mg protein with an overall mean of 88.7 $\pm$ 7 fmoles/mg protein. In AD, the average Bmax for [^{3}H]-NMS binding to cerebrovascular tissues was 50.2 $\pm$ 7 fmoles/mg protein with a variation ranging from 30.3 to 66.3 fmoles/mg protein. The [^{3}H]-NMS binding capacity in pial vessels preparations from eight pathologically confirmed cases of AD was significantly reduced (43%, $p < 0.05$) as compared to their matched controls (figure 12). In contrast, the K_D value for [^{3}H]-NMS at cerebrovascular muscarinic receptors were similar in pial vessels obtained from control and AD brains (143 $\pm$ 43 pM and 195 $\pm$ 49 pM respectively).

Fig. 11: Typical saturation curve of [^{3}H]-NMSbinding to human pial vessel membranes as shown here from a control preparation. Abscissa: concentration of [^{3}H]-NMS (nM); Ordinate: radioactivity bound expressed as fmol/mg protein. Insert shows the scatchard plot analysis for the binding of [^{3}H]-NMS in which the amount of radioligand is expressed as a function of a given free concentration of [^{3}H]-NMS.

Fig. 12: Histogram representing Bmax for [^{3}H]-NMS binding in eight pairs of pial vessels obtained from AD patients and matching non-demented controls showing a significant ($p < 0.05$) 43% reduction in the total muscarinic binding capacity obtained from AD patients compared to controls.


[3H]-NMS Total Muscarinic Binding Sites
Binding (fmol/mg prot.)
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
11
Free Ligand Concentration (nM)
Bound/Free
Specific Binding (fmol/mg prot.)

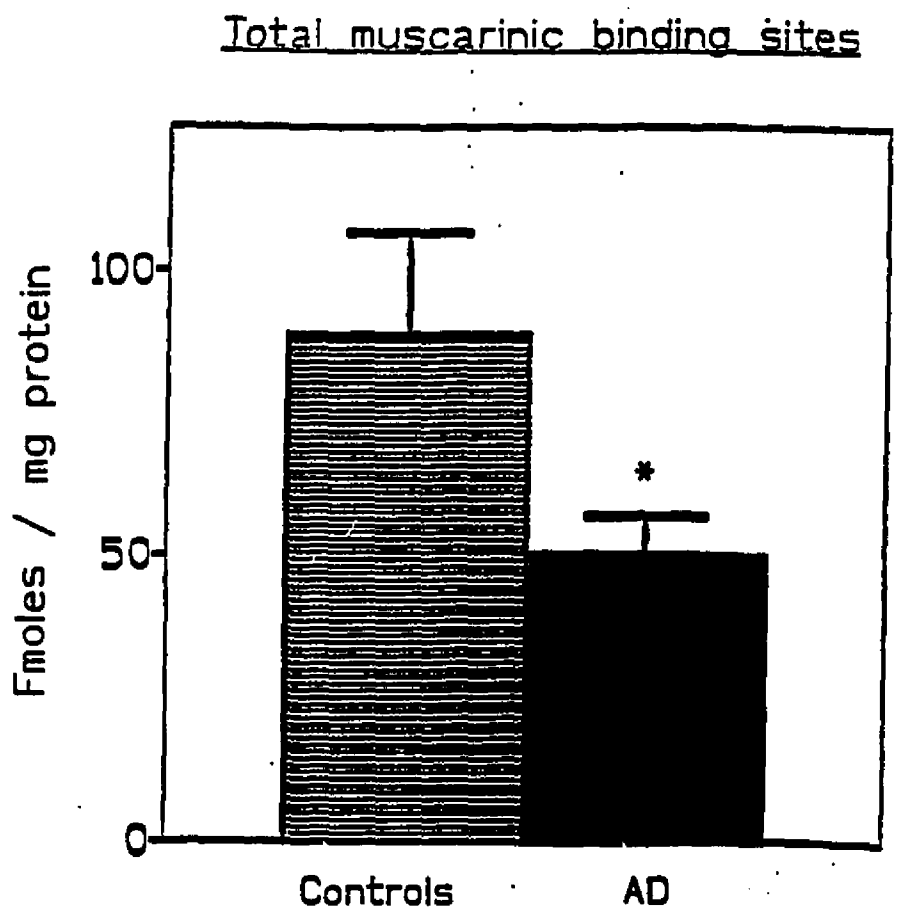

Total muscarinic binding sites
Fmoles / mg protein
100
50
0
*
Controls
AD

Table 4: Parameters of [^{3}H]-NMS binding in 8 pairs of pial vessels obtained from AD and control subjects. As can be seen, there was no apparent effect of the increased post-mortem delay on binding capacity or affinity.

TOTAL MUSCARINIC ([^{3}H]-NMS) BINDING SITES IN PIAL VESSELS.

Pair number	Post-mortem delay (hours) Control / AD		Bmax (fmoles/mg protein)		K_D (pM)	
			Control	AD	Control	AD
1	11	11	122.7	45.6	226.0	110.2
2	13	11	33.8	30.3	416.0	194.6
3	14	14	64.2	32.0	102.0	181.1
4	24	21	166.8	58.2	140.0	260.0
5	27	27	57.9	79.5	132.0	48.8
6	27	28	114.4	66.3	356.0	361.0
7	39	34	44.8	36.2	112.0	23.7
8	60	72	100.0	54.0	77.2	84.2
Mean ± SEM	27±6	27±8	88.7 ± 18	50.3 ±7 *	147.0 ± 49	195 ± 49

* = $p \leq 0.05$ by Student t-test.

3.1.b. Binding of [^{3}H]-NMS under suppressive conditions:

Binding of [^{3}H]-NMS was repeated on the same control and AD pial membrane preparations but this time in the presence of 75 nM pirenzepine, in order to block the M1 (as well as part of M3 and M4) muscarinic receptor subtypes (see experimental procedures). Under these suppressive conditions, the binding capacity of [^{3}H]-NMS, analyzed by Scatchard transformation, corresponded to 50% (controls) and 48% (AD) of that determined for total muscarinic binding (figure 13) labelled with [^{3}H]-NMS alone.

In the presence of 75 nM pirenzepine, the specific [^{3}H]-NMS binding to pial vessel membranes was found to be decreased (from 26% to 80%) in seven out of eight AD preparations as compared to their matched controls (table 5). Only one AD preparation exhibited a modest (20%) increase over the matched control (table 5). Incidently, this AD preparation also showed no change, as compared to its control, in the total muscarinic binding capacity labelled with [^{3}H]-NMS alone (see pair 5 in table 4). However, the overall binding capacity was significantly decreased (46%, $p \leq 0.05$) in the AD pial vessels (Bmax = 24.2 $\pm$ 2 fmoles/mg protein) as compared to those from matched controls (Bmax = 44.8 fmoles/mg protein) (figure 13). There was no significant difference in the binding site affinity (K_D)between control (1.33 $\pm$ 0.7 nM) and AD (1.41 $\pm$ 0.5 nM) (table 5) even though the K_D for [^{3}H]-NMS in the presence of 75 nM pirenzepine was significantly increased ($p < 0.05$) as compared to the K_D value obtained when the binding was performed with [^{3}H]-NMS alone (see section 3.1.a. above).

Fig. 13: Histogram illustrating the maximal binding capacity (Bmax) for [^{3}H]-NMS in the presence of 75 nM pirenzepine in both the control (n = 8) and AD (n = 8) preparations. A significant ($p < 0.05$) reduction is observed in AD compared to control tis sues.

Binding of [^{3}H]-NMS binding under suppressive conditions.

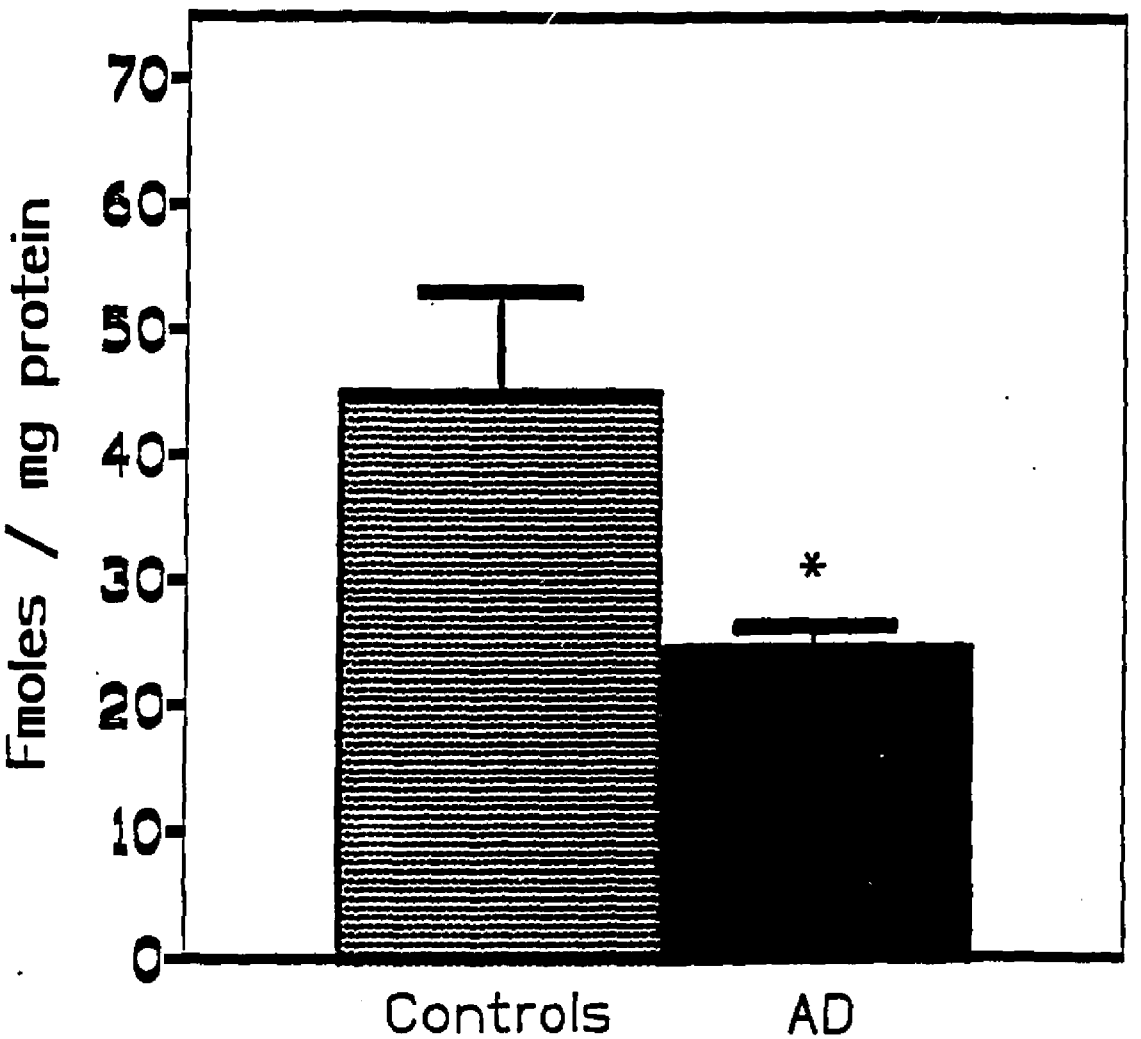


Table 5: Parameters of [^{3}H]-NMS binding in the presence of 75 nM pirenzepine in AD and control pial vessels.

BINDING OF [^{3}H]-NMS UNDER SUPPRESSIVE CONDITIONS IN PIAL VESSELS OBTAINED POST-MORTEM FROM 8 AD PATIENT AND 8 AGE-MATCHED ELDERLY CONTROLS.

PAIR NUMBER	CONTROL		AD	
	Bmax (fmoles/mg protein)	K_D (nM)	Bmax (fmoles/mg protein)	K_D (nM)
1	50.1	1.38	36.9	0.76
2	17.4	0.33	12.5	0.20
3	54.4	0.29	18.8	0.35
4	83.8	6.05	16.6	0.15
5	25.3	1.11	30.1	3.57
6	60.6	0.61	31.7	2.53
7	36.2	0.44	26.7	1.07
8	30.6	0.12	20.2	2.70
MEAN ± SEM	44.8 ± 8.3	1.33 ± 0.7	24.2 ± 2.0 *	1.41 ± 0.5

* $p \leq 0.05$ by Student t-test.

3.1.c. Cerebrovascular ChAT activity:

ChAT activity was measured in seven out of the eight pairs used in the saturation binding assays. In addition, in one pair, the control was obtained from vessels other than those used in the binding experiments since there was no tissues left. This newly introduced control (from pair number 6 in table 6), however, was of the same sex and had a similar post-mortem delay as the missing control. The reported results are the average of three to four independent determinations from pial vessels taken on different locations over the cerebral cortex.

ChAT activity in human pial vessels was variable and ranged from 0.88 to 5.37 nmol/mg protein/hour in the AD and from 0.55 to 5.22 nmol/mg protein/hour in the controls (table 6). In four AD, cerebrovascular ChAT activity was decreased (20% to 84%) as compared to that of their matched controls. In the remaining three AD cerebrovascular preparations, ChAT activity was slightly increased as compared to controls. Thus, the overall ChAT activity was found to be slightly diminished (25%) in AD pial vessels in comparison to controls (2.1 $\pm$ 0.6 and 2.8 $\pm$ 0.6 nmol/mg protein/hour, respectively) but, due to the high variability, this difference did not reach statistical significance (figure 14).

Fig. 14: Histogram representing the average values of ChAT activity in 7 AD and 7 control pial vessels. A non-significant ($p = 0.1$, 25%) reduction in ChAT acitivty in AD pial vessels was noted.

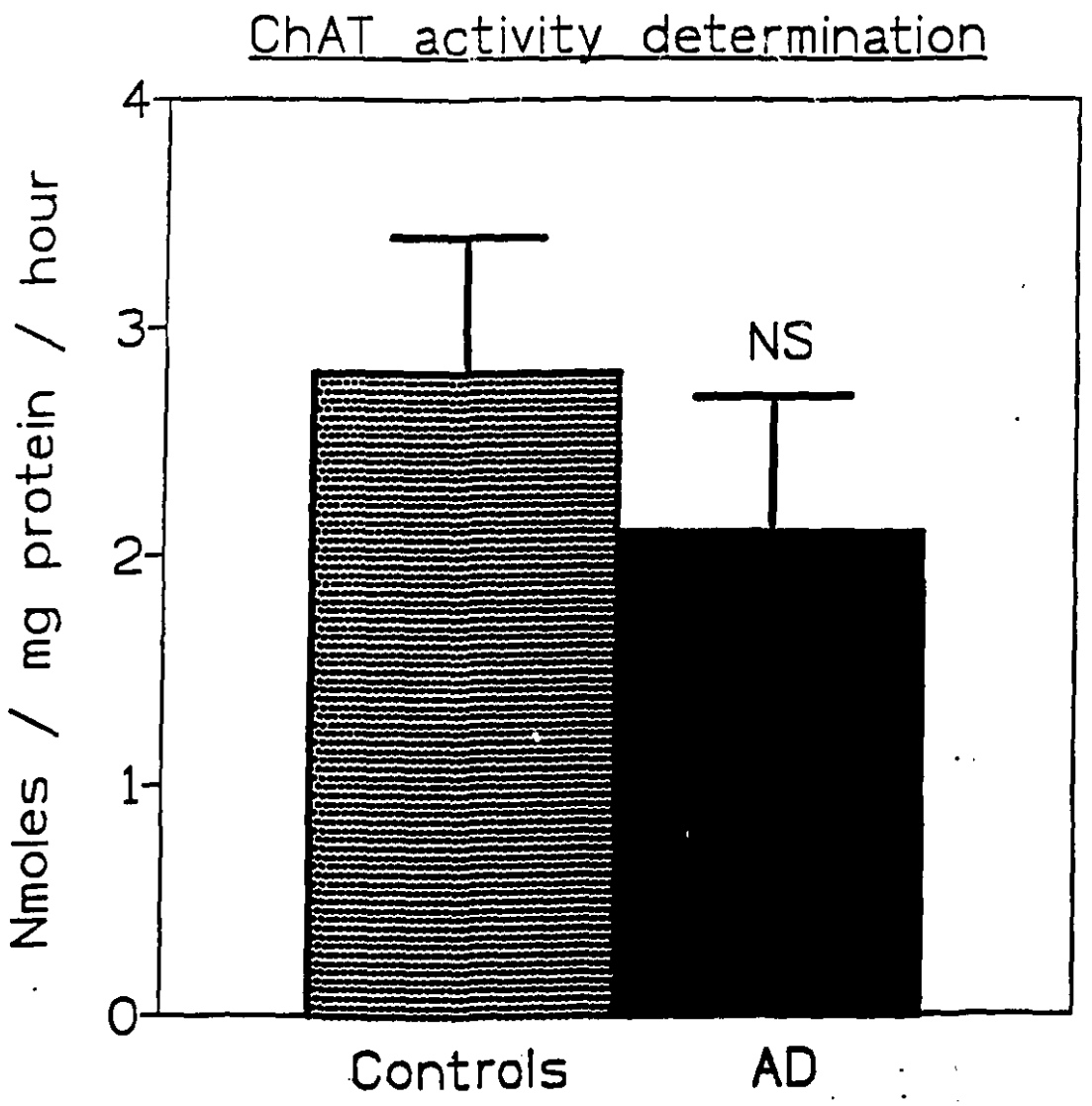

ChAT activity determination
Nmoles / mg protein / hour
4
3
2
1
0
NS
Controls
AD

Table 6: Individual and average (± SEM) ChAT activity values determined in 7 AD and 7 control pial vessels.

CHAT ACTIVITY (NMOL/ MG PROTEIN/ HOUR) IN PIAL VESSELS.

Pair number	CONTROL	AD
1	5.2	2.0
2	4.0	5.4
3	1.5	1.2
4	2.9	1.0
5	0.6	1.7
6	1.8	2.5
7	3.3	0.6
MEAN ± SEM	2.8 ± 0.6	2.1 ± 0.6

Difference in ChAT activity is not statistically significant.

3.1.d. [^{3}H]-CGP 12177 binding to ß-adrenergic binding sites:

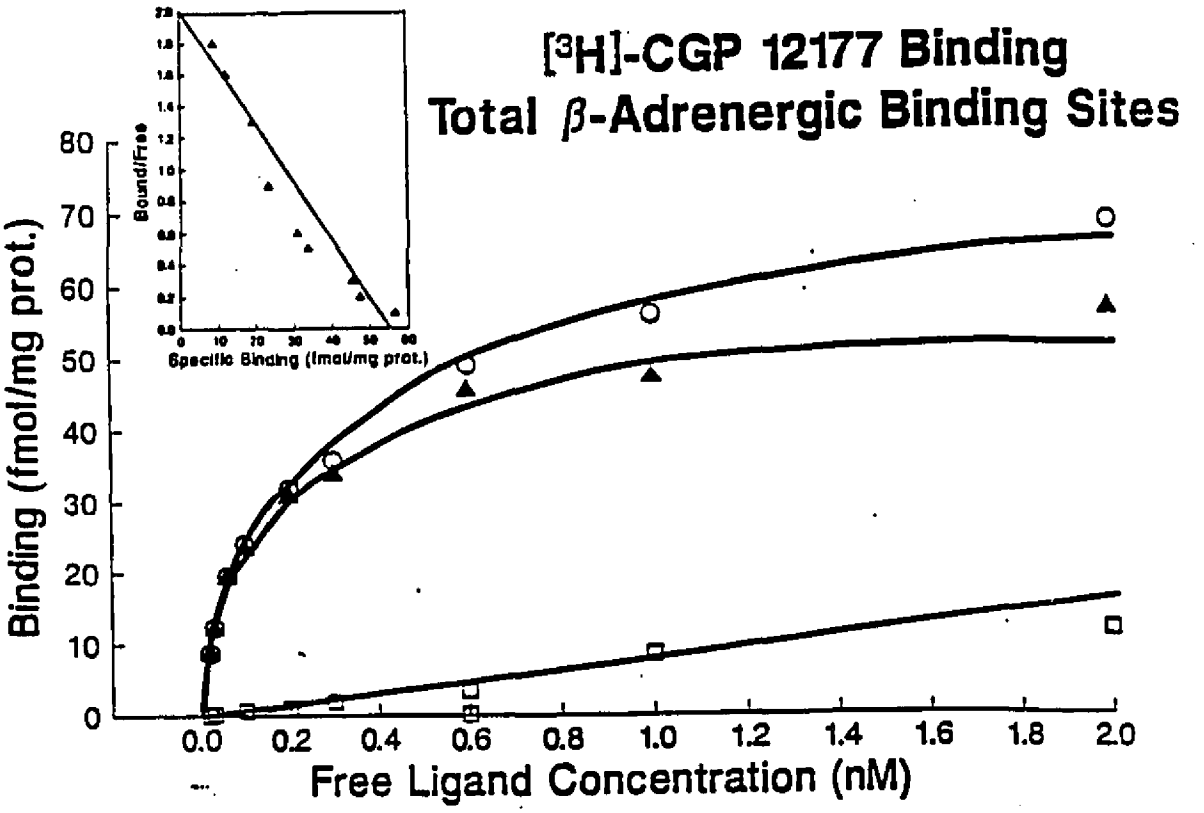
A first series of experiments was aimed at establishing the best conditions for [^{3}H]-CGP 12177 in pial vessels. Different protein concentrations were tested. The binding was found to increase linearly with protein concentrations up to 250 μg protein per tube. Since we were limited by the availability of AD and control pial vessels as well as the amount of protein obtained from a single cerebrovascular preparation, we used 60 μg of protein in each assay. At this concentration, an appreciable and reproducible binding was obtained with [^{3}H]-CGP 12177. Using a concentration of the radioligand close to the reported K_D (0.2 nM), equilibrium was reached in 120 minutes and the non-specific binding (determined in the presence of 200 μM isoproterenol) represented only 7% of the total binding. Under these conditions, saturation studies showed that specific [^{3}H]-CGP 12177 binding was saturable in both the AD and control preparations whereas non-specific binding increased lineraly with increasing radioligand concentrations (figure 15).

[^{3}H]-CGP 12177 binding performed in control and AD vessels was best represented by a linear Scatchard plot (figure 15) and a Hill coefficient of 0.92 $\pm$ 0.03 for the controls and 0.94 $\pm$0.02 for AD, both indicating the interaction of the radioligand with a homogeneous population of binding sites with high affinity for [^{3}H]-CGP 12177. The binding capacity exhibited some variability in AD (31.8 to 81.6 fmoles/mg protein) and control (38.8 to 77.9 fmoles/mg protein) preparations. Small decreases (11 - 34%) in Bmax values were obsereved in five AD preparations, one showed no change while the remaining two exhibited considerable increase in the binding sites density (196% and 181% respectively) (table 7). Thus the overall binding capacities in AD and control cerebrovascular membranes were comparable (57.9 $\pm$ 6.8 and 58.6 $\pm$ 5.4 fmoles/mg protein respectively) (figure 16). Similarly, the K_D for [^{3}H]-CGP 12177 binding was similar in cerebrovacular AD preparations as compared to matched controls (0.45 $\pm$ 0.1 and 0.49 $\pm$ 0.17 nM respectively) (table 7).

Similar to the previous observations with total muscarinic binding sites, the post-mortem delay appeared to have no effect on the maximal binding capacity of total ß-adrenergic binding sites (table 7).

Fig. 15: Representative saturation curve of [^{3}H]-CGP 12177 binding to human pial vessels. The insert represents the Scatchard plot transformation for specific binding and shows interaction of the radioligand with a homogeneous population of binding sites.

Fig. 16: Histogram representing Bmax for [^{3}H]-CGP 12177 binding. Similar values were obtained from both AD (n = 8) and control (n = 8) vessels.


[3H]-CGP 12177 Binding
Total β-Adrenergic Binding Sites
Binding (fmol/mg prot.)
Free Ligand Concentration (nM)
Bound/Free
Specific Binding (fmol/mg prot.)

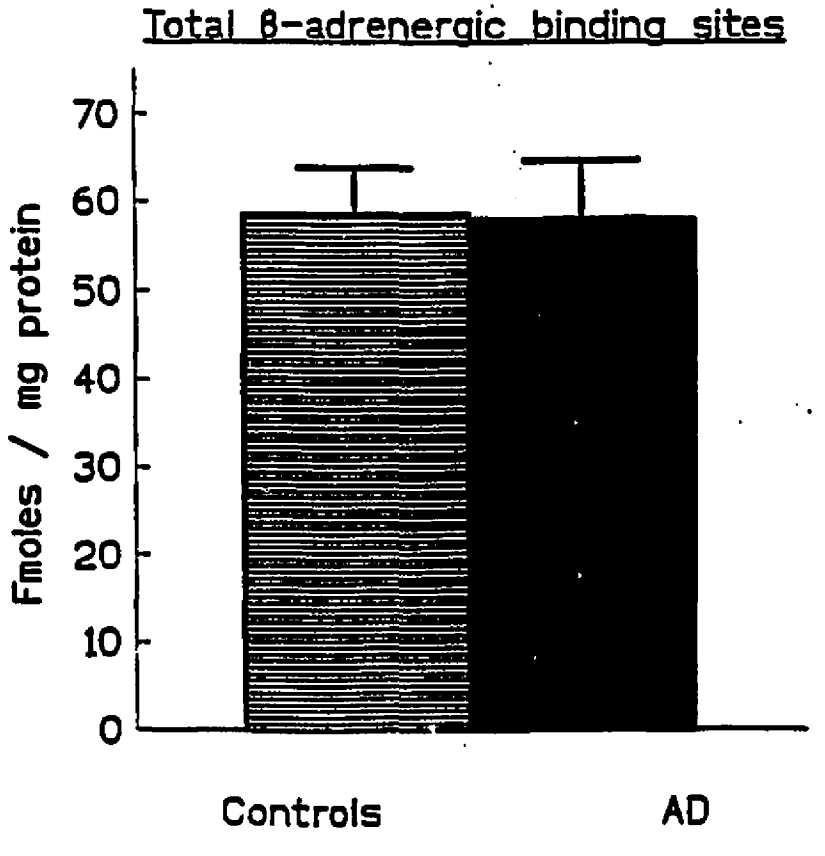

Total β-adrenergic binding sites
Fmoles / mg protein
Controls
AD

Table 7: Binding capacity (Bmax) and affinity (K_D) of [^{3}H]-CGP 12177 in pial vessel membranes obtained from control and AD subjects. Total capacity was reduced in five out of eight AD preparations, considerably increased in two and showed no change in the remaining preparations. The K_D values were comparable in the AD and control group.

TOTAL ß-ADRENERGIC ([^{3}H]-CGP 12177) BINDING SITES IN PIAL VESSELS.

PAIR NUMBER	Bmax (fmoles /mg protein)		K_D (nM)	
	Control	AD	Control	AD
1	51.4	41.4	0.15	0.12
2	45.0	81.6	0.33	0.18
3	47.8	31.8	0.94	0.12
4	77.9	75.9	0.88	0.36
5	38.8	75.9	0.38	0.93
6	62.9	48.6	0.11	0.90
7	64.6	57.2	1.00	0.91
8	75.0	52.8	0.14	0.10
MEAN ± SEM	58.6 ± 5.4	57.9 ± 6.8	0.49 ± 0.17	0.45 ± 0.17

3.1.e. [^{3}H]-Prazosin binding to α1-adrenergic binding sites:

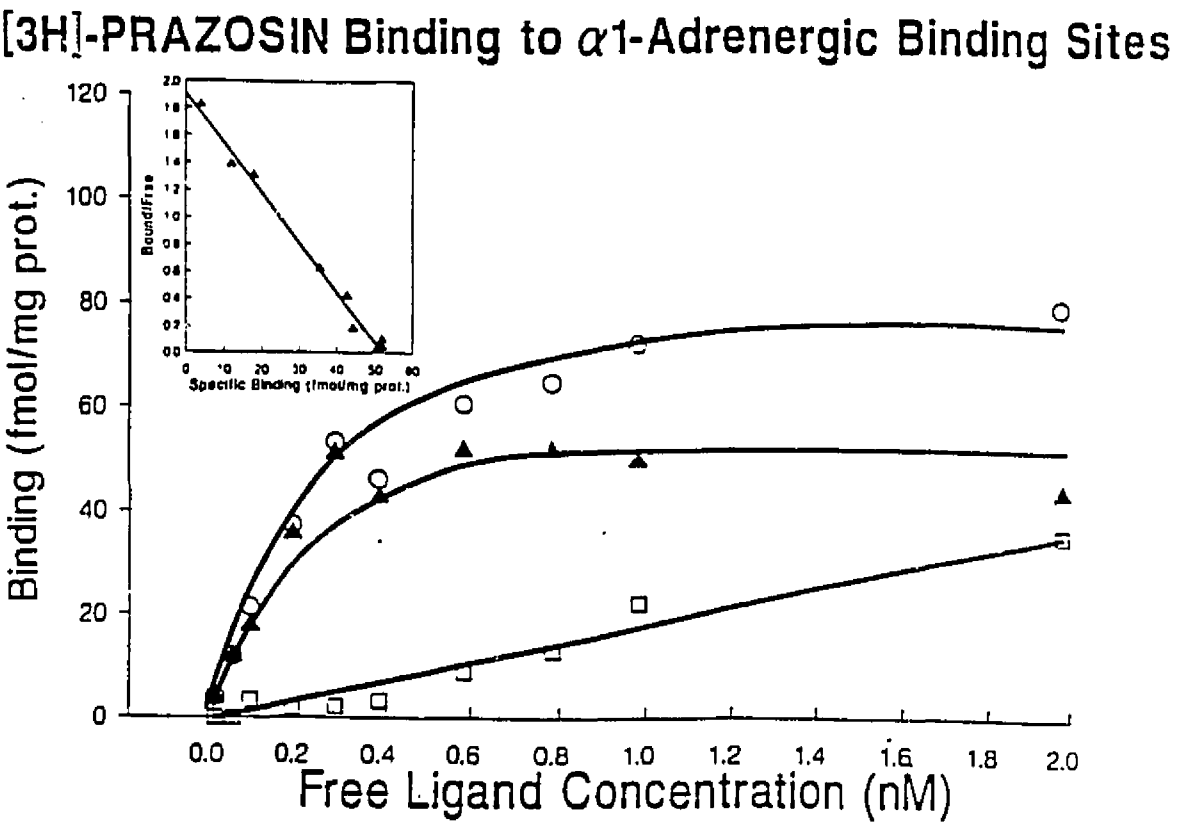
As for [^{3}H]-CGP 12177 binding, initial experiments with [^{3}H]-Prazosin were directed at establishing the best experimental conditions in pial vessels . Two different protein concentrations (80 μg and 160 μg) were incubated with a constant concentration of the radioligand (0.2 nM) for 30 minutes, 45 minutes and 60 minutes, respectively, at a temperatue of 25°C. The fraction of the non-specific binding determined in the presence of 10 μM phentolamine tended to increase with longer incubation periods. At 80 μg protein in the assay for an incubation period of 30 minutes, we obtained the least non-specific binding (14% of the total binding). Due to the limited amount of protein in our preparations, we opted for the use of 80 μg protein in the assays. Under these conditions, specific binding was saturable specific in both the AD and control preparations whereas non-specific binding increased linearly with increasing concentrations of the radioligand (fig. 17). Specific saturation isotherms of [^{3}H]-Prazosin binding performed in control and AD membranes were best represented by linear Scatchard plot (fig.17) and a Hill coefficient value of 0.87 $\pm$ 0.1 for AD and 0.9 $\pm$ 0.07 for the controls indicating the interaction of the radioligand with a homogeneous population of binding sites.

Binding isotherms with [^{3}H]-Prazosin demonstrated a small variability of the binding capacity in both the AD (13.3 - 29.2 fmoles/ mg protein) and control (23.1 - 44.3 fmoles/ mg protein) preparations. The Bmax value was diminished in six AD preparations as compared to their matched controls whereas one preparation showed no change (table 8). One last AD preparation repeatedly (on three different saturation experiments) gave counts that could not be analyzed suggesting no detectable α1-adrenergic binding sites. In the final analysis, we have discarded this AD preparation and its corresponding control. The overall binding capacities in the seven pairs considered for analysis showed a significant diminution ($p < 0.05$, 35%) in AD as compared to control cerebrovascular membranes (32.6 for the controls and 21.3 fmoles/mg protein for the AD

preparations) (figure 18). However, the average K_D for [^{3}H]-Prazosin was not significantly different between the AD and control preparations (0.02 $\pm$ 0.03 nM and 0.08 $\pm$ 0.02 nM respectively) (table 8).

Fig. 17: Representative saturation curve of [^{3}H]-prazosin binding to human pial vessels. The insert represents the Scatchard plot transformation for specific binding and shows interaction of the radioligand with a homogeneous population of binding sites.

Fig. 18: Histogram representing Bmax for [^{3}H]-Prazosin binding. There was a significant ($p < 0.05$) 35% rduction between AD and control vessels.


[3H]-PRAZOSIN Binding to α1-Adrenergic Binding Sites
Binding (fmol/mg prot.)
Free Ligand Concentration (nM)
Bound/Free
Specific Binding (fmol/mg prot.)

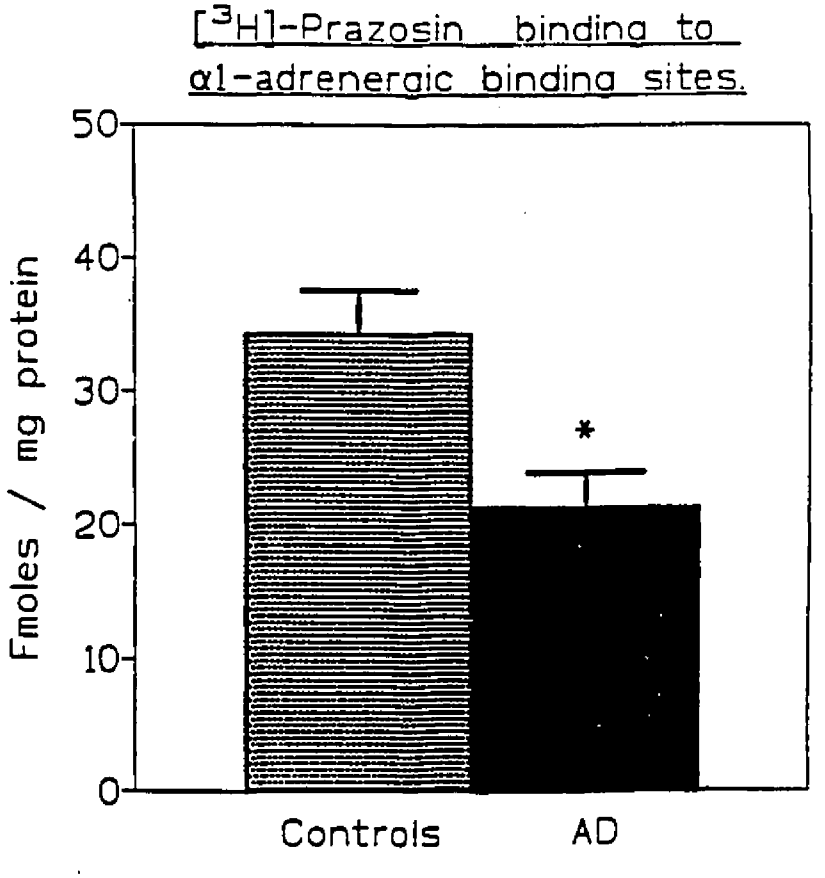

[3H]-Prazosin binding to
α1-adrenergic binding sites.
Fmoles / mg protein
Controls
AD
*

Table 8: Binding capacity (Bmax) and affinity of [^{3}H]-prazosin in pial vessel membranes from 7 AD and 7 control subjects. Bmax was reduced in 6 out of 7 AD preparations as compared to control vessels whereas the average affinity for [^{3}H]-prazosin showed no significant change between both groups.

α1-ADRENERGIC ([^{3}H]-PRAZOSIN) BINDING IN PIAL VESSELS

PAIR NUMBER	Bmax (fmoles/mg protein)		K_D (nM)	
	Control	AD	Control	AD
1	39.9	29.2	0.10	0.05
2	31.9	16.1	0.25	0.04
3	25.9	22.1	0.09	0.10
4	44.3	27.0	0.07	0.11
5	26.6	27.0	0.07	0.09
6	28.2	13.3	0.08	0.15
7	31.1	14.7	0.21	0.04
Mean ± SEM	32.6 ± 2.9	21.3 ± 2.7 *	0.12 ± 0.03	0.08 ± 0.02

* = $p \leq 0.05$ by Student t-test.

3.2. Sudomotor responses in AD patients, elderly and young controls:

3.2.a. AD patients:

The average (± SEM) value of response in zone 1, zone 2, zone 3 and zone 4 as counted in 30 AD patients as well as male and female AD patients are represented in table 9. The average (± SEM) value for standard QSART response, pure axon reflex sweating, direct response and total reflex sweating are shown in table 10, table 11, figure 19 and figure 20.

As expected, due to differences in physiological constitution (age, sex, physical condition...etc), there was a great variability of the results obtained indicated by the large value of SEM calculated.

The mean standard QSART response, pure axon reflex sweating as well as total reflex sweating were similar in male (554.7 ± 90.7, 46.1 ± 9.5 and 405.3 ± 76.8 respectively) and female (549.2 ± 129.5, 48.2 ± 9.5 and 395.4 ± 55.9 respectively) AD patients, whereas the average direct response was slightly higher in male (275.2 ± 54.9) compared to female (252.8 ± 65.4) AD patients. The difference was not found to be statistically significant. There was, however, a considerable 130% increase in the global response obtained from male compared to female AD patients (figure 19) but this difference was also not statistically significant.

Pure axon reflex sweating and direct response represented 12% and 66% of the average standard QSART response (table 10) obtained from 30 AD patients. Similar percentage for pure axon reflex sweating and direct response were also obtained in male (12% and 66% respectively) and female (12% and 64% respectively) AD patients.

Table 9: Average (± SEM) sweat responses obtained from 30 AD patients and counted in zone 1, zone 2, zone 3 and zone 4.

AD patients

	Total number (n = 30)	Male AD patients (n = 12)	Female AD patients (n = 18)
Zone 1	47.4 ± 6.8	46.8 ± 9.5	48.2 ± 9.5
Zone 2	90.2 ± 10.4	83.9 ± 17.4	94.4 ± 13.2
Zone 3	261.8 ± 29.7	275.2 ± 54.9	252.8 ± 65.4
Zone 3	413.7 ± 54.5	424.5 ± 69.9	386.6 ± 69.7

Table 10: Average (± SEM) standard QSART, pure axon reflex sweating and direct response obtained from 30 probable AD patients composed of 12 male and 18 female AD patients. The % of pure axon reflex sweating and direct response of the standard QSART response are also shown.

Fif. 19: Histogram representing pure axon reflex sweating (P.A.R.S.) and direct response (D.R.) obtained from 30 AD patients. Similar responses as well as standard QSART response (S.Q.R.) obtained from male (M) and female (F) AD patients are also represented.

AD patients

Response	Total number (n = 30)		Male AD patients (n = 12)		Female AD patients (n =18)	
	Average response (± SEM)	% of standard QSART	Average response (± SEM)	% of standard QSART	Average response (± SEM)	% of global response
Standard QSART	399.4 ± 43.7	100	405.3 ± 76.8	100	395.4 ± 55.9	100
Pure axon reflex	47.4 ± 6.8	12	46.8 ± 9.5	12	48.2 ± 9.5	12
Direct	261.8 ± 29.7	66	275.2 ± 54.9	66	252.8 ± 65.4	64

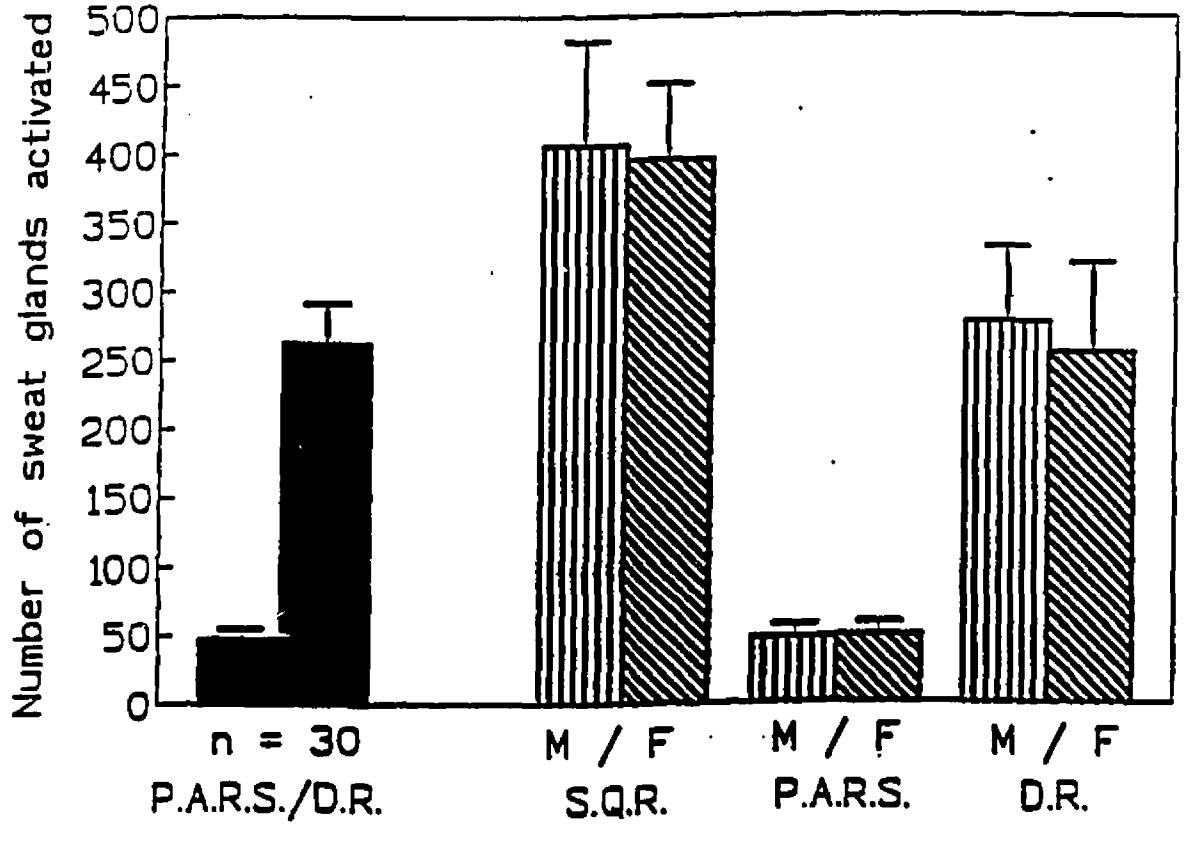
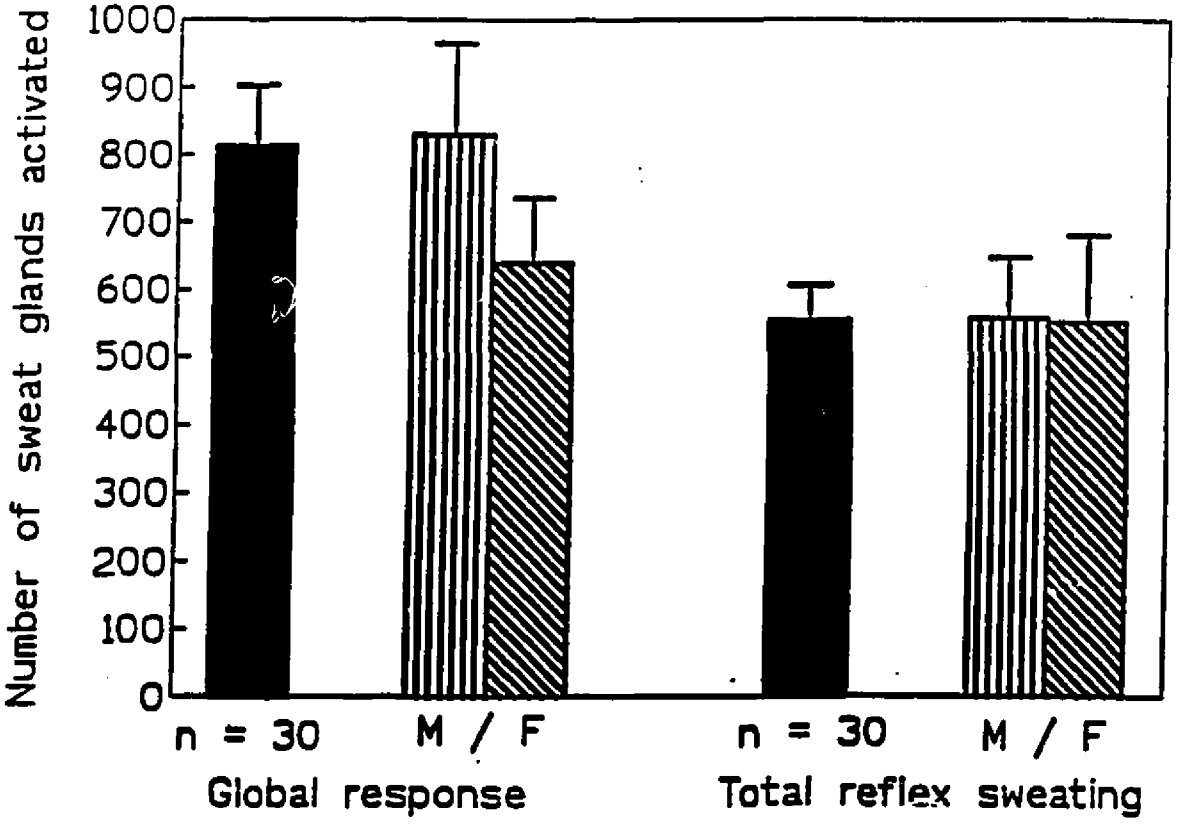


Table 11: Average (± SEM) global response and total reflex sweating obtained from 30 AD patients as well as male and female AD patients.

Fig. 20: Histogram representing global response and total reflex sweating in 30 AD patients as well as male (M) and female (F) AD patients.

AD patients

Response	Total number (n = 30)	Male AD patients (n = 12)	Female AD patients (n = 18)
Global response	813.1 ± 90.7	829.8 ± 135.1	639.4 ± 97.5
Total reflex sweating	551.4 ± 54.5	554.7 ± 90.5	549.2 ± 129.5



3.2.b. Elderly controls:

The average (± SEM) value of response in zone 1, zone 2, zone 3 and zone 4 as counted in 30 elderly controls as well as male and female elderly controls are represented in table 12. Average (± SEM) standard QSART response, axon reflex sweating and direct response obtained from 30 non-demented elderly controls are represented in table 13, table 14, figure 21 and figure 22.

As in the AD patients, a wide variability of the results were also obtained from the elderly population due to constitutional variabilities such as exercise, basal metabolic rate and gender.

The average standard QSART response and direct responses were both higher in male (539.5 ± 68.0 and 342.2 ± 42.4 respectively) compared to female (464.8 ± 69.0 and 281.7 ± 46.3 respectively) elderly controls. The reduced response in females was not statistically significant.

In contrast, both pure axon reflex sweating and total reflex sweating were slightly lower in male (72.8 ± 12.0 and 746.1 ± 107.5 respectively) compared to female (82.6 ± 42.6 and 778.9 ± 116.9 respectively) elderly controls. These differences were also not statistically significant.

Meanwhile, there was a non-significant 124% increase in the average global response obtained from male compared to female elderly controls (1088.3 ± 147.1 for males and 877.6 ± 136.5 for females). The extent of the diminution in the female response, though not statistically significant, was similar to that obtained in female AD patients. The percentage occupied by pure axon reflex sweating and direct response of the standard QSART response were close to the values obtained in AD patients and are represented in table 13.

Table 12: Average (± SEM) sweat responses obtained from 30 elderly controls included in the study as counted in zone 1, zone 2, zone 3 and zone 4.

Elderly controls

	Total number (n = 30)	Male AD patients (n = 12)	Female AD patients (n = 18)
Zone 1	78.7 ± 8.9	72.8 ± 12.0	82.6 ± 12.6
Zone 2	109.4 ± 11.1	122.8 ± 17.1	100.4 ± 14.6
Zone 3	305.0 ± 32.3	342.2 ± 42.4	281.7 ± 46.3
Zone 4	581.5 ± 65.8	425.5 ± 69.9	595.6 ± 94.5

Table 13: Average (± SEM) standard QSART, pure axon reflex sweating and direct response obtained from 30 elderly controls as well as male and female elderly controls. The % of pure axon reflex sweating and direct response of the standard QSART response are also shown.

Fif. 21: Histogram representing pure axon reflex sweating (P.A.R.S.) and direct response (D.R.) obtained from 30 elderly controls. Similar responses as well as standard QSART response (S.Q.R.) obtained from male (M) and female (F) AD patients are also represented.

Elderly controls

Response	Total number (n = 30)		Male elderly controls (n = 12)		Female elderly controls (n = 18)	
	Average response (± SEM)	% of standard QSART	Average response (± SEM)	% of standard QSART	Average response (± SEM)	% of standard QSART
Standard QSART	504.7 ± 48.6	100	539.5 ± 68.0	100	464.8 ± 69.0	100
Pure axon reflex	78.7 ± 8.9	16	72.8 ± 12.0	14	82.6 ± 12.6	18
Direct	305.0 ± 32.3	60	342.2 ± 42.4	63	281.7 ± 46.3	61

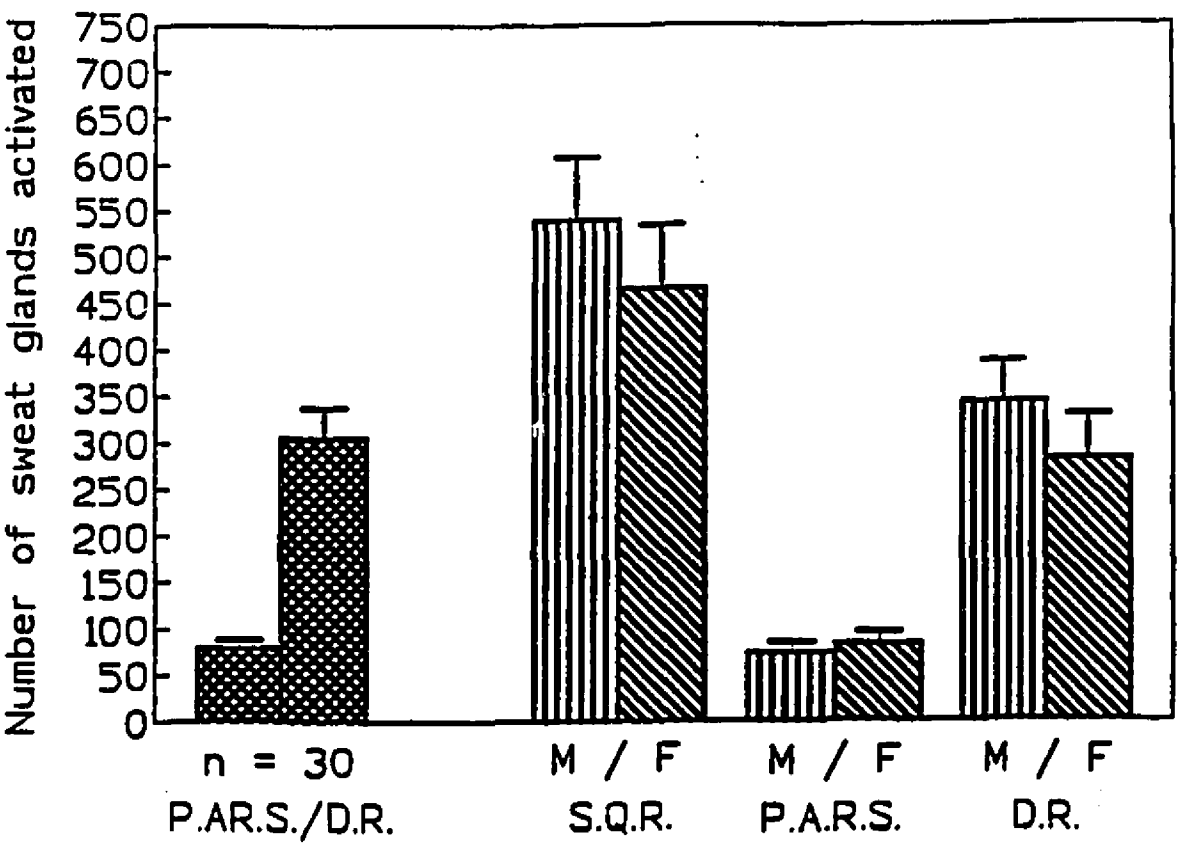
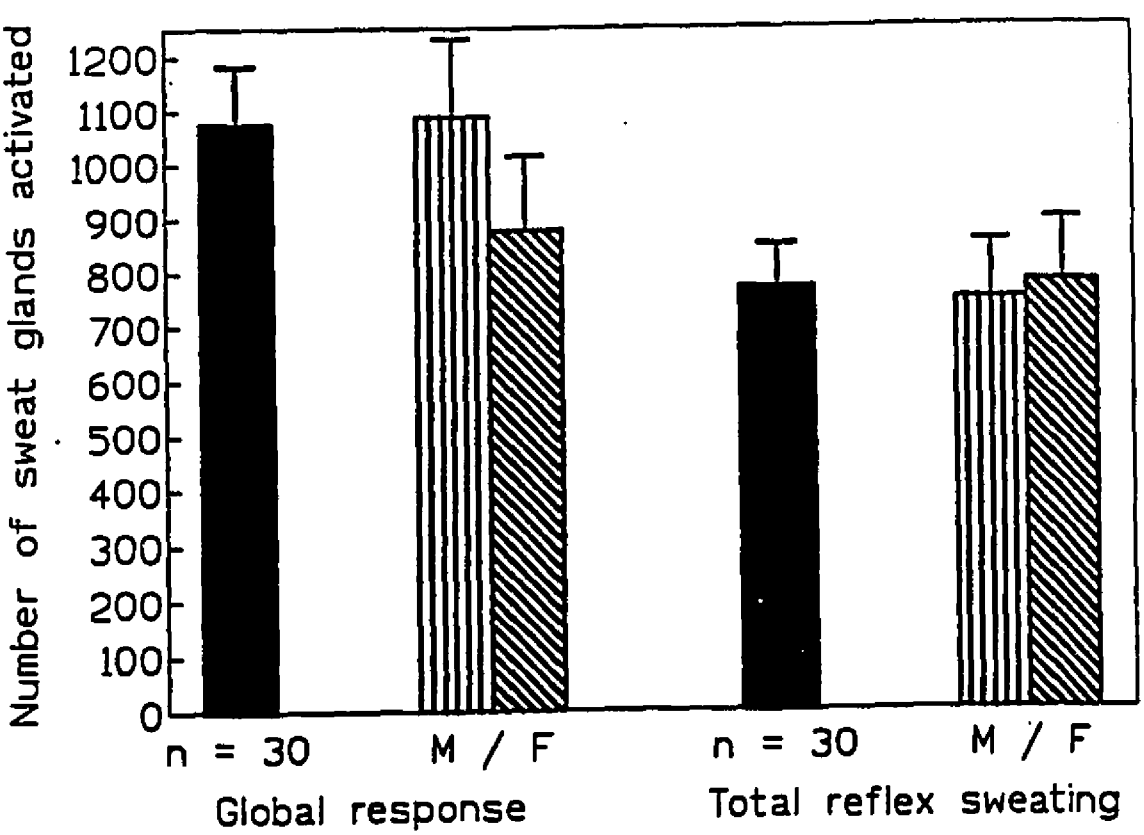


Table 14: Average (± SEM) global response and total reflex sweating obtained from 30 elderly controls as well as male and female elderly controls.

Fig. 22: Histogram representing global response and total reflex sweating in 30 elderly controls as well as male (M) and female (F) elderly controls.

Elderly controls

Response	Total number (n = 30)	Male AD patients (n = 12)	Female AD patients (n = 18)
Global response	1074.6 ± 108.6	1088.3 ± 142.1	877.6 ± 136.5
Total reflex sweating	769.6 ± 81.0	746.1 ± 107.5	778.9 ± 116.9



3.2.c. Young controls:

The average (±SEM) value of response in zone 1, zone 2, zone 3 and zone 4 as counted in 30 young controls as well as male and female young controls are shown in table 15. Mean (± SEM) values for standard QSART response, axon reflex sweating, direct response and total reflex sweating counted in 30 young controls are representd in table 16, table 17, figure 23 and figure 24.

Similar to AD patients and elderly controls, responses obtained from young controls demonstrated a high variability because of gender, exercise, dietary intake and basal metabolic rate.

In general, the average standard QSART response as well as direct response were slightly higher in male (585.3 ± 55.4 and 369.3 ± 36.6 respectively) compared to female (527.2 ± 79.7 and 322.4 ± 50.9 respectively) young controls. The differences however were not statistically significant. Whereas similar values for pure axon reflex sweating and total reflex sweating were obtained from male (90.3 ± 16.0 and 864.2 ± 110.7 respectively) and female (86.0 ± 16.9 and 41.4 ± 103.6 respectively) young controls.

On the other hand, a non-significant 129% increase was found when global responses in male young controls were compared to global responses of female young controls. The diminution is similar to the one calculated in female AD patients and female elderly controls. Average (±SEM) values for global responses are represented in table 17.

The calculated percentages of pure axon reflex sweating and direct response from the standard QSART response (table 16) were comparable to those obtained from AD patients and elderly controls.

Table 15: Average (± SEM) sweat responses obtained from 30 young controls as counted in zone 1, zone 2, zone 3 and zone 4.

	Total number (n = 30)	Male young controls (n = 13)	Female young controls (n =17)
Zone 1	85.0 ± 11.9	90.3 ± 16.0	86.0 ± 16.9
Zone 2	120.4 ± 12.5	125.7 ± 15.6	118.8 ± 18.7
Zone 3	336.5 ± 33.1	369.3 ± 36.6	322.4 ± 50.9
Zone 4	614.0 ± 61.3	648.2 ± 88.7	636.5 ± 84.2

Table 16: Average (± SEM) standard QSART, pure axon reflex sweating and direct response obtained from 30 young controls as well as male and female young controls. The % of pure axon reflex sweating and direct response of the standard QSART response are also shown.

Fig. 23: Histogram representing pure axon reflex sweating (P.A.R.S.) and direct response (D.R.) obtained from 30 young controls. Similar responses as well as standard QSART response (S.Q.R.) obtained from male (M) nd female (F) young controls are also represented.

Young controls

Response	Total number (n = 30)		Male young controls (n = 13)		Female young controls (n = 17)	
	Average response (± SEM)	% of standard QSART	Average response (± SEM)	% of standard QSART	Average response (± SEM)	% of standard QSART
Standard QSART	497.7 ± 49.0	100	585.3 ± 55.4	100	527.2 ± 79.7	100
Pure axon reflex	85.0 ± 11.9	15	90.3 ± 16.0	15	86.0 ± 16.9	16
Direct	336.5 ± 33.1	61	369.3 ± 36.6	63	322.4 ± 50.9	61

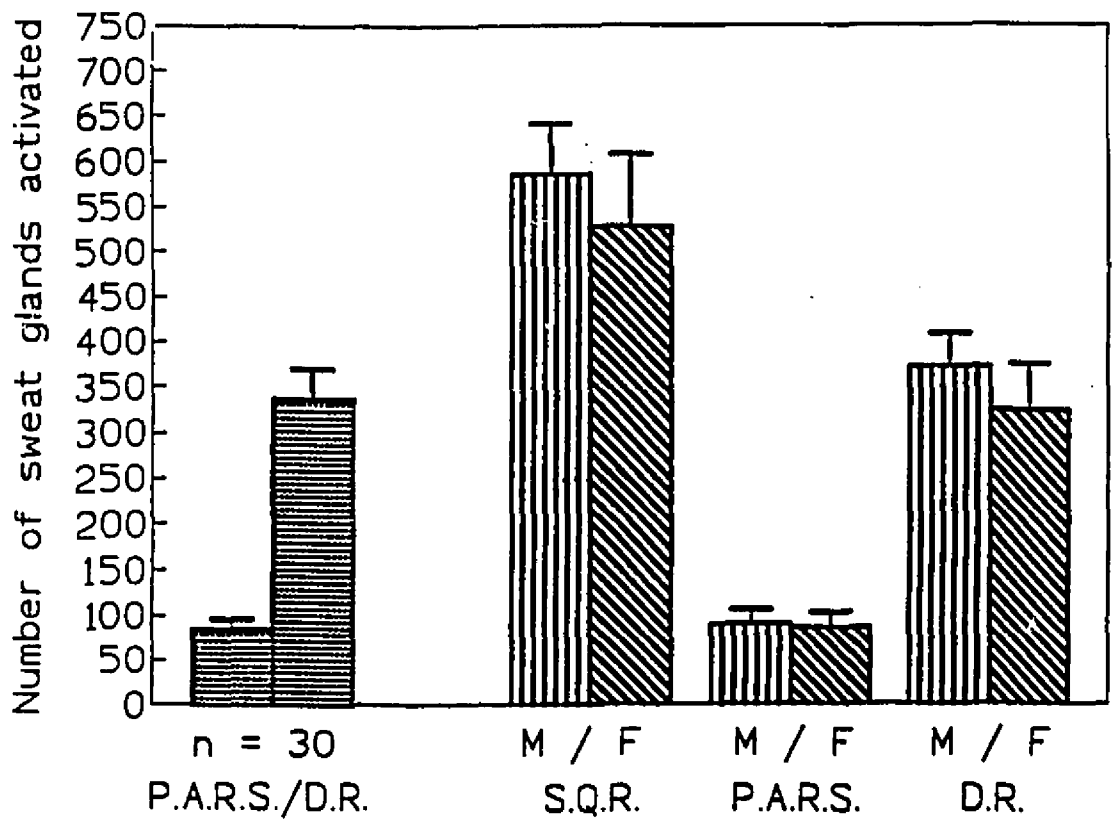
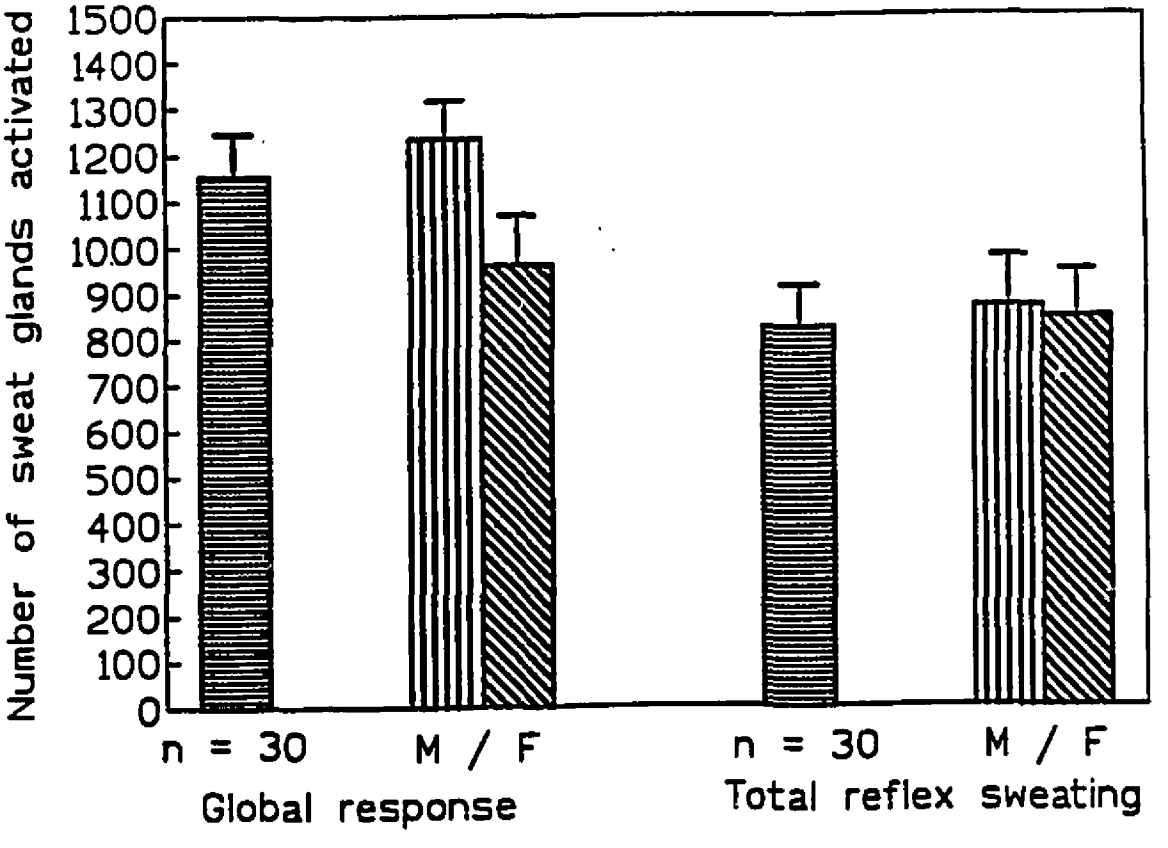


Table 17: Average (± SEM) global response and total reflex sweating obtained from 30 young controls as well as male and female young controls.

Fig. 24: Histogram representing global response and total reflex sweating in 30 young controls as well as male (M) and female (F) young controls.

Young controls

	Total numbver (n = 30)	Male young controls (n = 13)	Female young controls (n =17)
Global response	1153.1 ± 94.2	1233.5 ± 82.6	959.1 ± 109.5
Total reflex sweating	818.7 ± 94.2	864.2 ± 110.7	841.4 ± 103.6



3.2.d. AD versus control (Young and elderly) responses:

Mean pure axon reflex sweating in 30 AD patients showed a significant ($p < 0.05$) 44% reduction compared to young controls and a significant ($p < 0.05$) 40% reduction compared to elderly controls (fig 25). When the results were analyzed according to sex, pure axon reflex sweating was significantly ($p < 0.05$) reduced by 48% in male AD patients compared to male young controls (figure 27) while in female AD patients, a 42% significant reduction ($p < 0.05$) was found (figure 28).

Total reflex sweating was also signicantly reduced ($p < 0.05$) in AD patients (33% compared to young controls and $p < 0.05$, 29% compared to elderly controls) (figure 26). There was also a significant ($p < 0.05$) reduction in total reflex sweating obtained from male AD patients compared to male young and male elderly controls (30% and 26% respectively) (figure 29) as well as from female AD patients compared to female young and female elderly controls (35% and 30% respectively) (figure 30).

Direct responses were also reduced in AD patients (22% compared to young controls and 14% compared to elderly controls) (figure 25) but the differnces were not statistically significant. Similarly, the reduced direct response obtained from male AD patients (25% compared to male young controls and 20% compared to male elderly controls) (figure 27) and in female AD patients (22% compared to female young controls and 10% compared to female elderly controls) (figure 28) were also not statistically significant.

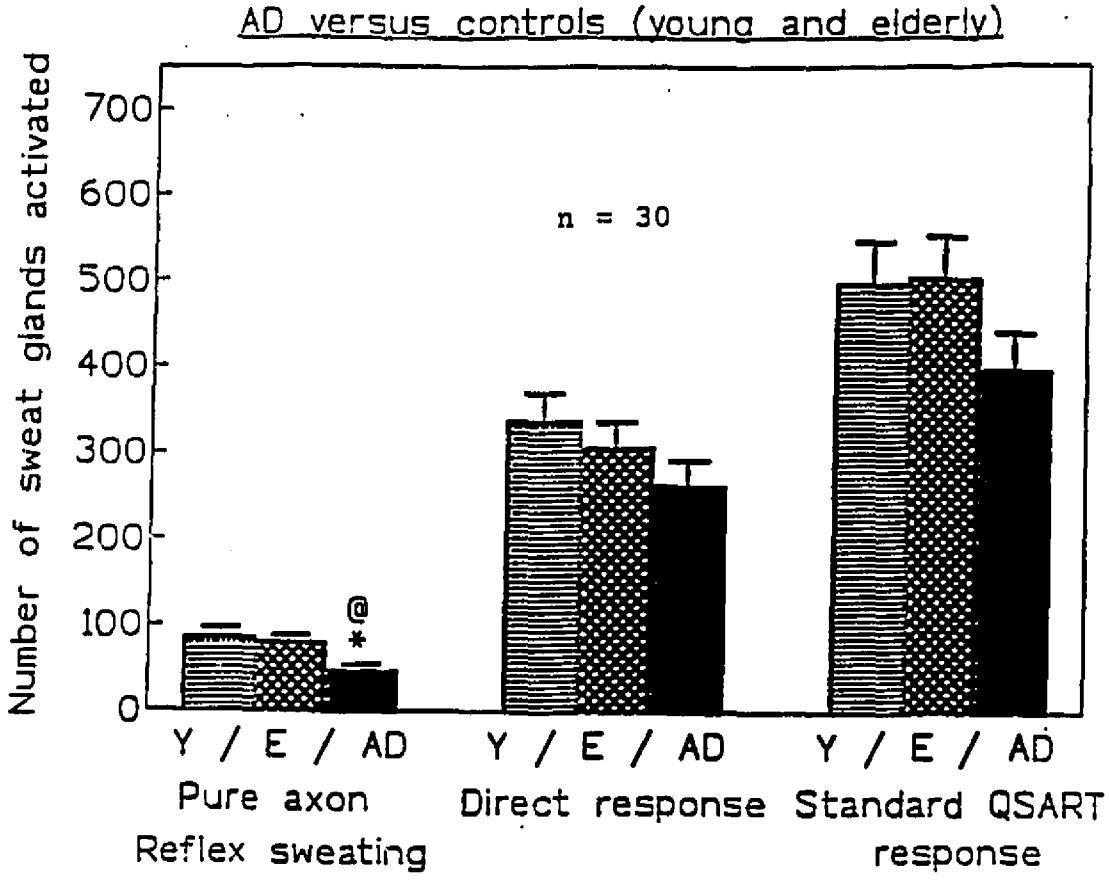
Meanwhile, average standard QSART responses obtained from 30 AD patients showed a non- significant 20% reduction compared to the same response obtained from young and elderly controls (figure 25).

Global responses were also reduced in AD aptients. The diminution was statistically significant

controls (24% non-significant reduction) (figure 26). Male and female global responses showed a significant diminution between AD and young controls ($p < 0.05$, 34% between male AD patients and male young controls & $p < 0.05$, 33% between female AD patients and female young controls) (figure 29 and figure 30). While a similar trend was found between male and female AD patients compared with male and female elderly controls (24% and 27% respectively) however this was not statistically significant.

Fig. 25: Histogram representing pure axon reflex sweating, direct response and standard QSART response obtained from 30 probable AD patients, as well as 30 elderly (E) and 30 young (Y) controls included in the study. Pure axon reflex sweating obtained from 30 AD patients was significantly ($p < 0.05$) reduced by 44% as compared to young controls and by 40% as compared to elderly controls.

Fig. 26: Histogram representing global response as well as total reflex sweating in 30 AD patients, 30 elderly controls (E) and 30 young controls (Y). Total reflex sweating obtained from AD patients also showed a significant ($p < 0.05$) 33% reduction as compared to young controls and 29% as compared to elderly controls.


AD versus controls (young and elderly)
Number of sweat glands activated
700
600
500
400
300
200
100
0
n = 30
@
*
Y / E / AD
Y / E / AD
Y / E / AD
Pure axon
Reflex sweating
Direct response
Standard QSART
response

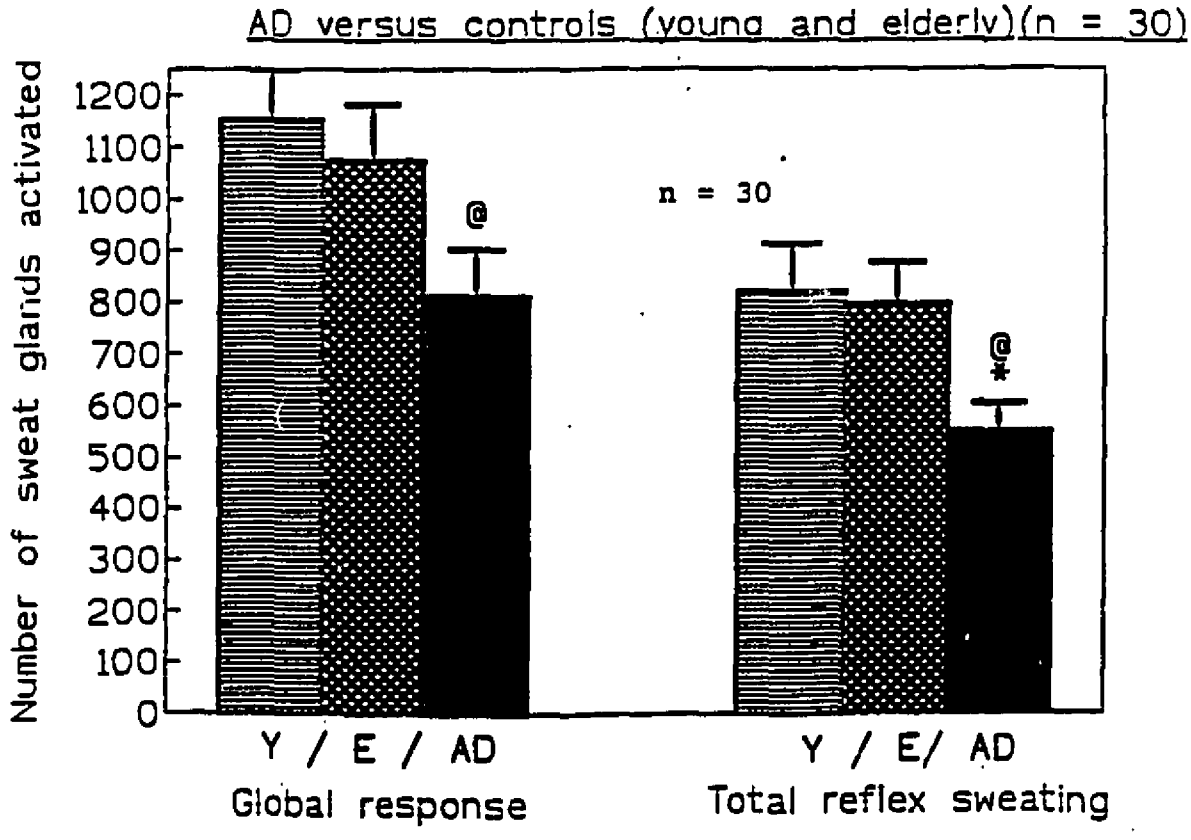
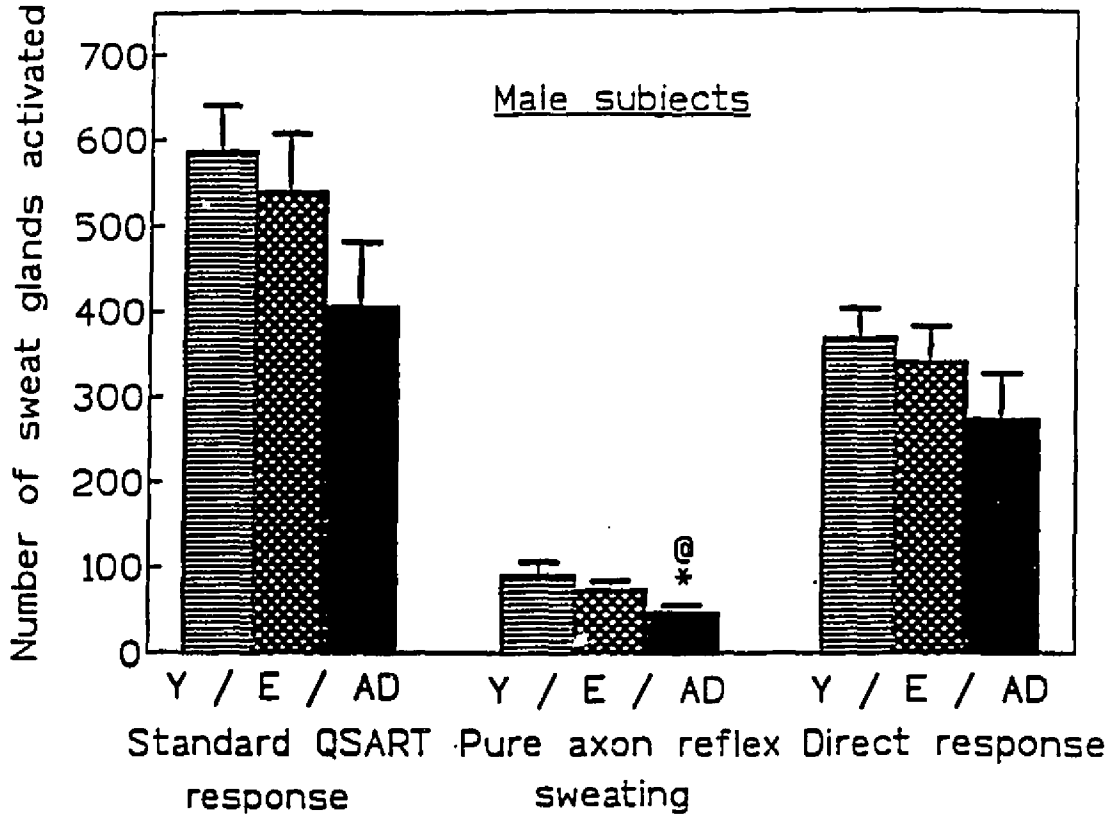

AD versus controls (young and elderly)(n = 30)
Number of sweat glands activated
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
n = 30
@
@
*
Y / E / AD
Y / E/ AD
Global response
Total reflex sweating

Fig. 27: Histogram representing standard QSART response, pure axon reflex sweating and direct response in male AD patients (AD), male elderly controls (E) and male young controls (Y). only pure axon reflex sweating showed a significant ($p < 0.05$) reduction between male AD patients and male controls (young and elderly).

Fig. 28: Histogram representing standard QSART response, pure axon reflex sweating and direct response in female AD patients, female elderly controls and female young controls. Similar to male subjects, only pure axon reflex sweating showed a significant diminution between female AD patients and female controis (young and elderly).

AD versus controls (young and elderly)



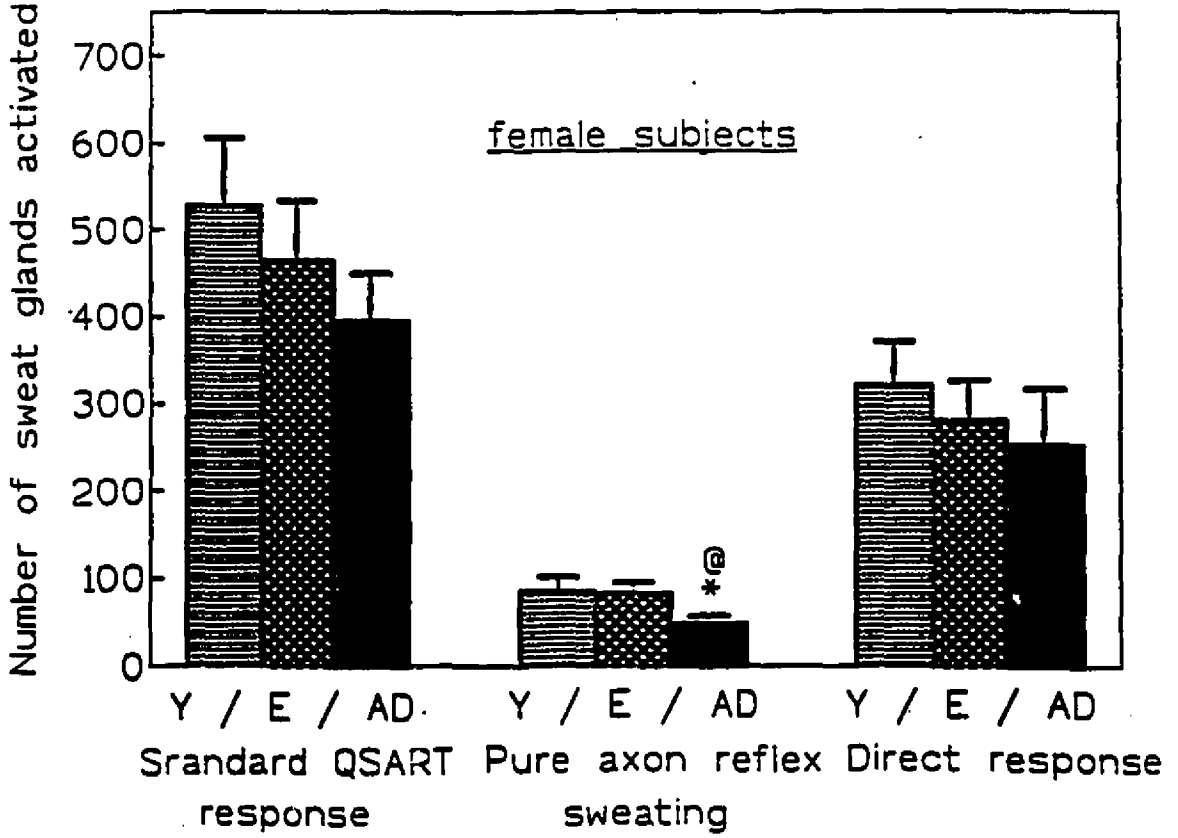
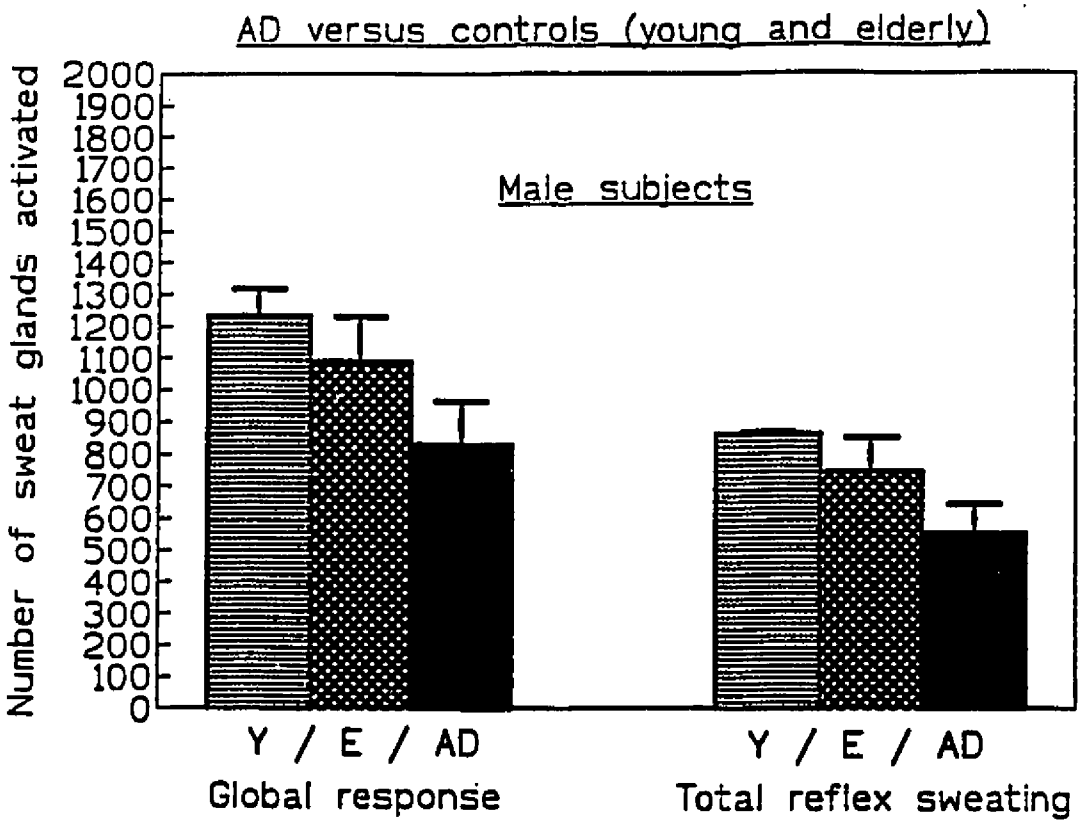
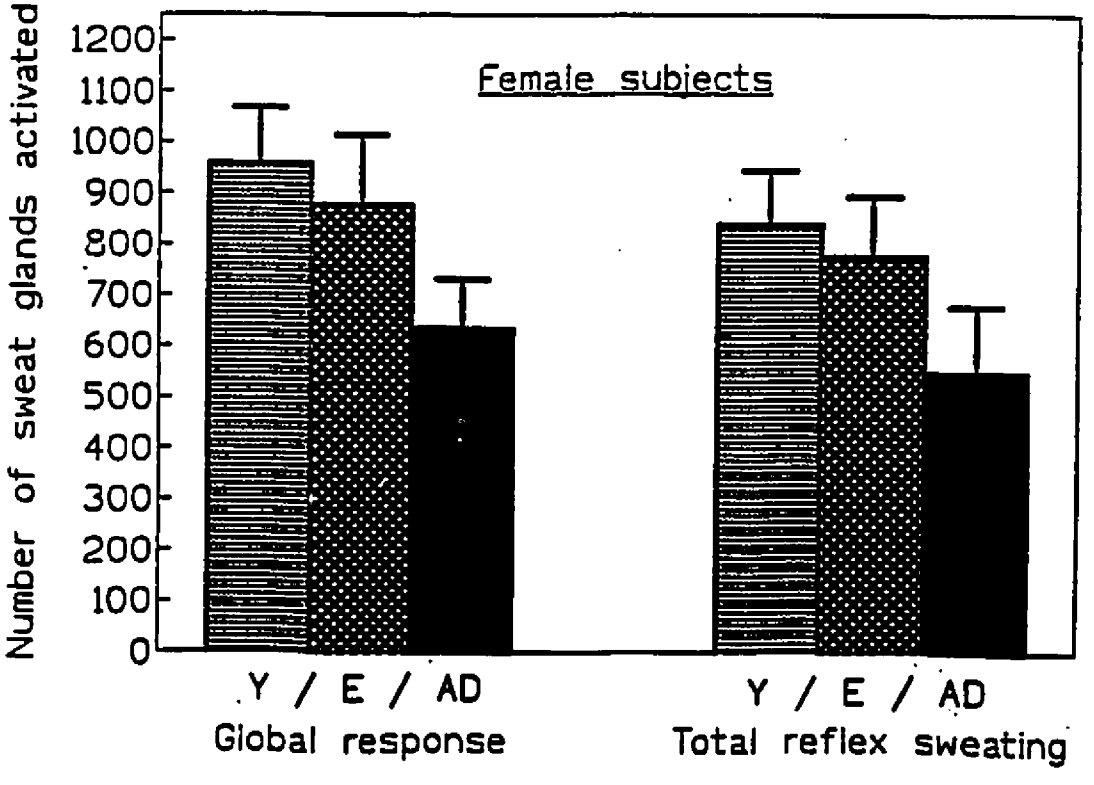


Fig. 29: Histogram representing global response and total reflex sweating obtained from male AD patients (AD), male elderly (E) and male young (Y) controls.

Fig. 30: Histogram representing global response and total reflex sweating obtained from female AD patients, female elderly (E) and female young (Y) controls.


AD versus controls (young and elderly)
Male subjects
Number of sweat glands activated
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Y / E / AD
Global response
Y / E / AD
Total reflex sweating


Female subjects
Number of sweat glands activated
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Y / E / AD
Global response
Y / E / AD
Total reflex sweating

4. Discussion

4.1. Muscarinic cholinergic receptors and ChAT activity in cerebral blood vessels:

Biochemical evaluation of the total muscarinic receptors in pial vessels of brains obtained from confirmed cases of AD revealed a significant reduction (43%, $p < 0.05$) in total muscarinic binding sites density. When the assays were repeated in the presence of 75 nM pirenzepine, there was a similar significant reduction (46%, $p < 0.05$) in the density of the labelled subpopulation of muscarinic binding sites. The same pial vessel preparation from AD showed a modest non-significant 25% reduction in ChAT activity as compared to vessels obtained from elderly, non-demented controls.

Although ChAT activity has been found in several non-neuronal tissues such as the placenta and corneal epithelium, it has been shown to be a specific biochemical marker for the cholinergic innervation of major cerebral arteries and small pial vessels (Hardebo et al 1978, Florence and Bevan 1979, Hamel et al 1987). The recent development in some laboratories of antibodies raised against ChAT has made the immunocytochemical demonstration of cerebrovascular cholinergic nerve fibers possible in several species (Saito et al 1985, Suzuki et al 1990). As well, a population of ChAT neurons projecting to cerebral arteries was identified in the sphenopalatine ganglia with additional contribution from the otic ganglia as well as the miniganglia of the internal carotid artery (Suzuki et al 1990). All together, these tract tracing and lesion studies have all attested the parasympathetic nature of the cholinergic innervation of extra-cerebral blood vessels and demonstrated its partial colocalization with perivascular nerves containing VIP.

In the present study, pial vessels obtained from confirmed cases of AD showed a non-significant 25% reduction in ChAT activity when compared to vessels obtained from non-demented elderly subjects. The statistical non-significance of the results cannot be attributed to

the small sample size since the same number of subjects was sufficient to statistically detect differences in binding sites density (see below). It is, however, evident that there was a great variability in cerebrovascular ChAT activity measured in both the AD and control groups. This variability may be attributed to the irregular distribution of cholinergic nerve terminals in the walls of pial vessels as has been previously documented (Saito et al 1985). Consequently, cerebrovascular ChAT activity values would vary according to the segment isolated for the evaluation with high values in segments rich in cholinergic nerves and low ones where these terminals are scarse or deficient. We have tried to overcome this difficulty by including vascular segments from different parts of the pial circulation in the assay. However, it appears that this procedure was not completely successful in eliminating the variability in cholinergic innervation and thus ChAT activity. The average value of ChAT activity obtained from 7 pial vessels belonging to the AD group showed a trend towards a diminution as compared to the aged vessels. This trend would agree with the reduction in ChAT activity detected in brain regions showing the most dramatic loss of cholinergic innervation in AD (Araujo et al 1988). Although the results from the present study do not unequivocally indicate a deficit in cholinergic parasympathetic innervation, they could nonetheless suggest such a possibility. Interestingly, similar conclusions have been reached by other investigators who determined ChAT activity in peripheral tissues in AD as reviewed before (section 4.3, see also below). All together, those findings could be compatible with a degeneration of cholinergic neurons located outside the CNS.

If the limited reduction in ChAT activity does not provide a definitive answer to possible alterations in peripheral cholinergic innervation of pial vessels, substantial support for a dysfunction at this level was obtained by the marked reduction in cerebrovascular muscarinic receptors. Total as well as a subpopulation of non-M1 muscarinic receptors subtypes were significantly and selectively reduced in pial vessels from AD patients. Previous studies from our

group had demonstrated the heterogeneity of muscarinic receptors in human pial vessels (Dauphin and Hamel 1992). Our results agree with these previous studies and confirmed that approximately 40 - 50% of the muscarinic binding sites could be blocked by 75 nM pirenzepine (Dauphin and Hamel 1992) suggesting a high proportion of M1 receptors (and, to a lesser extent of M3 sites) since this latter subpopulation would be partly blocked by such a high concentration of pirenzepine in human extra-cerebral blood vessels However, a small disparity was observed between our findings and those of Dauphin and Hamel in the binding under suppressive conditions. They observed no major changes in binding sites affinity while we found close to a ten fold decrease in the affinity (K_D) of [^{3}H]-NMS in the presence of pirenzepine. We presently have no explanation for this apparent discrepancy, and more detailed studies only could help clarify the mechanisms underlying the changes that we have observed in ligand affinity in the presence of a high concentration of pirenzepine.

The muscarinic binding sites in AD and control cerebral vessels were determined in vessels included within the pia-arachnoid membrane. Although we assume that most of the binding sites are associated with vascular elements (smooth muscle and/or endothelial cells), we cannot exclude the presence of a subpopulation of sites on other cellular components of the arachnoid membrane, as recently reported (Lasbennes et al 1992). However, it would appear that only a minute fraction of muscarinic binding sites can be attributed to such origin and, as such, it is unlikely that the considerable decrease in pial vessels muscarinic binding sites could be explained solely on the basis of the alterations in non-vascular component. Similarly, although muscarinic binding sites have been associated with brain astrocytes (Hosli & Hosli 1993), these are not known to be associated with the major cerebral and small pial vessels and should be disregarded as possible cellular elements endowed with muscarinic binding sites measured in the pial membrane fractions. Thus, we believe that it is justifiable to assume that smooth muscle and/or endothelial muscarinic

receptors, including the M1 subtype, are selectively affected in AD. However, it is also possible that some of these sites correspond to pre-synaptic receptors on cerebrovascular nerve terminals.

The decrease, most likely to be primarily in post-synaptic muscarinic binding sites, is reminiscent of that observed in rat parotid gland following unilateral post-ganglionic parasympathetic denervation (Talamo et al 1979). Similarly, reduced sudorific responses were observed following post-ganglionic lesions to sweat glands (Coon and Rothman 1941). It is therefore possible that pial vessels display a comparable response to denervation, namely an absence of receptor upregulation, following parasympathetic cholinergic denervation. This situation would contrast with that observed in the CNS following a loss of cholinergic input (Quirion et al 1989). The fact that AD is accompanied by structural changes in the blood vessel wall (see section 1.4.c.) distinct from those observed in normal aging, including the accumulation of ß-amyloid in pial vessels (Yamaguchi et al 1992, Kawai et al 1993) may, however, suggest an abnormal composition of the vessel wall in AD. Such alterations could result in inappropriate receptor integration within the membrane. It is therefore possible that the apparent and significant decrease in cerebrovascular muscarinic receptors observed here merely reflects a loss in the amount of binding sites due to degeneration of smooth muscle cells in vessels containing ß-amyloid, as recently reported (Yamaguchi et al 1992, Kawai et al 1993). In fact, amyloid deposition in vessels of non-neuronal tissues is far less than that of cerebral vessels and the reasons for such specificity for brain vascular tissues remain unexplained (Yamaguchi et al 1992). Alternatively, thickening of the vessel wall (Higushi et al 1987) and ß-amyloid deposits in leptomeningeal vessels (Yamaguchi et al 1992, Roher et al 1993) may contribute to artificially increase vascular protein content and thus a preserved amount of binding sites would appear decreased when expressed per mg protein as we did in the present study.

It is interesting to note that M1 and M3 receptor subtypes have been identified in cerebral blood vessels from human and other species (Dauphin and Hamel 1990, Dauphin et al 1991, Shimizu et al 1993) and that they have also been shown to stimulate the release of amyloid precusor protein (Nitsch et al 1992). Such abnormal behaviour of these muscarinic receptor subtypes in AD could contribute to the thickening of the vessel wall and apparent reduction in receptor population without any deficit in cerebrovascular parasympathetic innervation.

4.2. Adrenoceptors in cerebral blood vessels from AD patients:

Another important neurogenic system involved in the control of vasomotricity of brain major cerebral arteries is the sympathetic system. As described in details in the introduction, the superior cervical ganglion provides most of this cerebrovascular innervation. Having observed pronounced changes in the cerebrovascular receptor population related to the parasympathetic cholinergic system (see previous section), it appeared important to investigate if similar dysfunctions could be associated with cerebrovascular adrenoceptors.

The α and ß- adrenoceptors have been detected in cerebral arteries in various species (see introduction section 1.3.c.). Interspecies differences exist in the subtype of α receptors which dominates in cerebral blood vessels. There is a general agreement for a majority of α2-receptors in cat, dog and piglet (Medgett and Langer 1983, Toda 1983, Busji and Leffler 1987) whereas the α1 subtype seems to predominate in vessels of rabbit and non-human primate (Toda 1983). In the human cerebral blood vessels, a general concensus has not been reached. In fact, while many investigators agree on the predominance of α1-adrenoceptors (Toda 1983, Skarby and Anderson 1984), others have also suggested the existence of a population of α2 subtypes (Ferrari-Di Leao and Potter 1985, Usui et al 1985). In our own experiments, labelling of α2 receptors with the specific ligand [^{3}H]-RX781094 did not yield significant specific binding which was

saturable in human cerebrovascular membranes. Thus, it appears that our results would further indicate that α2-adrenoceptors represent a minor population, if any, of the cerebrovascular α-adrenoceptors in human cerebral blood vessels. The possibility that α2 receptors are pre-synaptic receptors located on noradrenergic perivascular nerve terminals and not directly related to vasomotor functions, as reported in cortical microvessels (Kalaria and Harik 1989) could agree with a minor population of α2-adrenoceptors in human cerebral blood vessels that would not be detected readily by binding studies.

In contrast, specific α1 adrenoceptors were easily evidenced in human cerebral blood vessels by radioligand binding studies and they exhibited a 35% decrease in density in AD patients. This alteration appears specific since ß-adrenoceptors measured in the same membrane preparations (see below) were of similar density in both elderly controls and AD. The α1-receptor subtype has been shown to mediate the noradrenaline-induced contraction in human cerebral arteries (Skarby and Anderson 1984). Although we have not performed any functional studies in AD vessels, the important loss in cerebrovascular α1-binding sites noted here could possibly lead to abnormal vasomotor responses. Interestingly, reduced α1-mediated cutaneous responses (erythema and blanching) has been observed in skin vessels of AD patients (Hörnqvist et al 1987) suggesting a reduced peripheral adrenergic reactivity. Alternatively, an apparent decrease in receptor density could partly be the consequence of a greater protein content in the thickened vascular wall as discussed in detail above. Although we cannot exclude this possibility, it appears unlikely since the ß-adrenoceptors were unaltered in the same cerebrovascular membrane preparations from AD patients.

Cerebrovascular ß-adrenoceptors are composed of ß1 and ß2 subtypes and different down and upregulation in these two subtypes may result in no detectable changes in the level of the total ß receptors. However, previous studies together with our findings strongly suggest

that AD is accompanied by degeneration of smooth muscle cells in the leptomeningeal blood vessels which result in the observed and significant changes in specific population of vascular α1 and muscarinic receptors.

Although it is not clear why ß receptors would not be affected by this process, one explanation might reside in the localization of ß receptors in the vessel wall. Indeed, ß adrenergic receptors appear to be located in the endothelial and, to a smaller extent, the smooth muscle cells of the cerebral blood vessels (Nakau et al 1986). In fact, ß1 and ß2 adenergic receptors have been identified in cerebrovascular endothelial cells (Bacic et al 1992). If, as suggested, the damaged vascular wall and ß-amyloid deposit takes place outside the abluminal vascular basement membrane (Yamaguchi et al 1992), the endothelial receptors would be spared to the expense of smooth muscle receptors. Although this might represent one possible explanation, other factors are surely involved in order to have no detectable changes in human cerebrovascular ß-adrenoceptors. Indeed, other studies on the changes in ß-mediated responses with aging have demonstrated that, while total salivary protein secretion (a ß-mediated function) decreased with age, there was no change in the density of the ß-receptors in the parotid or the submandibular glands (in rats) (Rajakumar et al 1992). This was attributed to a deficit in ß-adrenergic transduction mechanisms. Interestingly, ß-adrenergic stimulated increase in cAMP was reduced by approximately 80% in fibroblasts from AD patients compared to age-matched controls (Huang and Gibson 1993).

4.3. Comparison with other changes detected peripherally in AD:

In view of the detected cholinergic and adrenergic changes in pial vessels, it would be interesting to see how these findings compare with other peripheral alterations reported in AD. As mentioned before (section 1.1.d.), blood elements obtained from clinically diagnosed AD

patients, have been repeatedly used as a mean for screening peripheral alterations in AD. Binding studies on isolated lymphocytes obtained from AD patients have demonstrated a significant diminution in both muscarinic (Rabey et al 1986) and nicotinic (Adem et al 1986) sites compared to lymphocytes isolated from elderly controls. The reduction in muscarinic binding sites density in lymphocytes of AD patients agrees with the diminished total muscarinic binding sites in pial vessels. In the present study, we have not attempted to evaluate nicotinic binding sites in human pial vessels since preliminary studies in our laboratory indicated either a very low density or an absence of these receptors in human pial vessels. However, functional studies on the sweat responses in AD patients (see later) suggest a diminution in the nicotinic-mediated axon reflex which would argue in favor of a decline in nicotine-mediated mechanisms in AD.

ChAT activity has not been evaluated before in peripheral tissues of AD, but it has been reported that sympathetic neurons cultured in a medium conditioned by exposure to skin fibroblasts obtained from AD patients exhibit a significant reduction in ChAT activity (Kessler 1987). This observation emphasized the peripheral AD-related alterations in the metabolism of ACh. Such statement is further substantiated by the finding of a lower influx of choline in both red blood cells (Miller et al 1985) and fibroblasts (Makrash 1989) isolated from AD patients.

On the other hand, study of the peripheral adrenergic alterations in AD showed a trend for an increase in $\alpha 2$ binding as well as MAO activity in platelets (Adolfson et al 1980, Adunsky et al 1989). Similar alterations were obtained following sympathetic denervation of cerebral blood vessels. Indeed, superior cervical ganglionectomy resulted in a supersensitivity to NE in rat cerebral arteries. This supersensitivity reaction gradually disappeared with time and returned to control levels (Edvinson et al 1975, Lobato et al 1980). Meanwhile, the effects of a degenerating sympathetic innervation has been investigated in patients with multiple system atrophy (MSA) (Davies et al 1982). There was a 10 fold increase in the $\alpha 1$-mediated pressor effect of NE

infusion on peripheral blood vessels. In contrast, Hornqvist et al (1987) observed that peripheral vascular reactivity evaluated through cutaneous responses to α1 and ß agonists is reduced in AD patients compared to age-matched controls, a response mediated by these adrenoceptors located in superficial dermal blood vessels (Hornqvist et al 1984). The reduction in α1-adrenergic binding sites detected in pial vessels of AD patients and reported in the present study would therefore agree with the results of Hornqvist and colleagues. Assuming that a progressive degeneration of the neurogenic control of cerebral arteries occurs in AD, this tissue appears to lose its ability to upregulate its receptor population.

4.4. Changes in AD as compared to those reported in normal aging:

Whether the observed changes in pial vessels are the reflection of a sympathetic and/or parasympathetic denervation or of structural alterations within the wall of pial vessels from AD patients, they are obviously different from the age-related changes observed in cerebral and extracerebral vessels.

There is an indication from biochemical studies that aging is associated with an increase in cerebrovascular ChAT activity in rats (Hamel et al 1990). However, no age-related changes in the response of rat basilar artery to ACh have been observed in that same study. On the other hand, peripheral blood vessels (isolated mesenteric artery of the beagle, Shimizu and Toda 1986) (human coronary artery, Egashira et al 1993) demonstrated an age-related decrease in their distensibility to ACh.

Meanwhile, there is considerable evidence in man for a diminished responsiveness in peripheral ß-mediated autonomic functions with advancing age. For example, Conway (1970) provided evidence that older people have smaller vasodilatory responses to propranolol both at rest and during exercise. This also seems to be the case in extracranial blood vessels in human

as well as in animals. For instance, vascular relaxation induced by isoproterenol (ß-agonist) in human superficial hand veins was found to be inversely related to age (Hörnqvist et al 1987). Similar age-related reduction in the responses induced by NE or isoproterenol were observed in the beagle coronary artery (Shimizu and Toda 1986, Toda and Miyazaki 1987).

Interestingly, in the CNS, ß-adrenoceptors density has also been found to diminish with age both in the cortex as well as in the brain microvessels (Koyabashi et al 1982, Mooradian and Scarpace 1991). The changes suggest alterations of the ß-adrenergic component both centrally and peripherally with age.

On the other hand, α-mediated contractility of extracranial blood vessels does not seem to show similar age-related alterations. Isolated peripheral human arteries and veins, like those in animals (Duckles et al 1985) showed no change in α-adrenergic vasocontractile response with age (Scott and Reid 1982). Thus, aging appears to be associated with a decrement in the ß-adrenergic component whereas the α-adrenergic component does not show similar alterations.

4.5. Possible correlates with brain perfusion in AD patients:

It is surprising that in view of the major contribution of extracerebral blood vessels (major basal and small pial vesels) in brain cortical perfusion and the reported landmark changes in cortical cerebral blood flow in AD patients (see below), there has been so few reports on possible changes, other than structural, in the cerebral vasculature in this disease. The present study provides direct evidence for neurotransmitter receptor alterations in cerebral blood vessels of well characterized cases of AD. In the elderly, there has been evidence for an increased incidence of deteriorating vasomotor responses which may impair, in whole or in part, adaptative responses to orthostatic stress as well as extreme environmental conditions (Lipsitz et al 1991, Collins et al 1980). These findings have argued in favor of a reduced sympathetic tone of peripheral vessels

in advanced age. However, evidence for increased sympathetic nerve activity with age (Christensen 1982) have been provided which would suggest that the deficit is not a consequence of a degenerating process but is rather due to changes in the post-synaptic vascular tissues. Our results would also indicate that selective changes in cerebral blood vessels, not found in normal aging (see section 4.4), are present in AD. We have not performed any functional studies on AD post-mortem blood vessels so it is impossible to attest that the changes observed here would result in abnormal vasomotor receptor-mediated functions. It is, however, most probable that such changes in muscarinic and adrenergic receptors in AD vessels would affect in one way or another cerebrovascular responses.

Muscarinic receptors are heterogeneous in human cerebral blood vessels and subtypes related to both vasoconstriction (M1) and dilatation (M3) have been identified (Dauphin and Hamel 1992). Interestingly, the functional response elicited by acetylcholine administered to isolated human cerebral arteries in vitro corresponds uniquely to a vasodilatation. As described earlier, α1-adrenoceptors in human cerebral arteries elicited vasoconstriction (Skärby and Anderson 1984). These two receptor-mediated vasomotor functions could thus be altered in AD vessels due to a reduced number of cerebrovascular receptors. In fact, our results tend to suggest, at least for the cerebrovascular cholinergic system, very minor deficits in cerebrovascular nerve density and/or ability to synthesize ACh, an observation which would favor a relatively preserved innervation in face of a diminished receptor population. If true, such interpretation would imply that the blood vessel itself is altered in AD. These conclusions would be in line with previous structural studies on cerebral blood vessels of AD patients (see section 1.4.a.) and, more importantly, would imply selective vascular deficits in AD that are not encountered in normal aging.

Measurements of CBF, although not in full agreement at all level, have indicated a reduction in brain perfusion generally restricted to or more pronounced in the frontoparietal cortex (Simmard et al 1971, Grubb et al 1977, Grady et al 1990). Although it has been suggested that the CBF decreases would become apparent quite late in the process of the disase (Rogers et al 1986) it appears that deficits in brain metabolism and/or perfusion could be unmasked relatively early in AD patients when subjected to selective neuropsychological tasks (Duara et al 1987, Miller et al 1987) or pharmacological manipulations (Gustafson et al 1987). Such statement would imply that disturbances in vasomotor functions regulating CBF would insure proper brain perfusion under basal conditions but could not adapt when submitted to activation or stressful stimuli. Based on such observations, it is justifiable to suggest that a loss of autonomic responses in cerebral blood vessels may result in cortical vascular deficits in AD that would not be apparent under resting neuronal conditions. However, it is clear that the alterations in cortical microvessels (Scheibel et al 1987) and receptor densities (Kalaria & Harik 1989) reported in AD would also contribute to any dysfunction in the regulation of local brain circulation . The deficits in AD major cerebral arteries and pial vessels identified in the present study, together with those reported earlier in the brain microcirculation could thus be responsible, albeit by acting at two different levels of the cerebrovascular bed (extra- versus intra-cerebral, respectively), for the abnormal CBF responses in AD (Grady et al 1990). It is not excluded, however, that at a later stage in the disease, combined alterations of the cerebrovascular innervation, neurotransmitter receptors and wall components would provide the basis for the basal fronto-parietal reduction in brain perfusion noted in AD.

It is possible that the role of the autonomic nervous system becomes more apparent when the integrity of the brain tissue and its circulation are jeopardized. Cerebrovascular parasympathetic nerves have indeed been shown recently to exert a protective role in focal cerebral ischemia

characterized cases of AD. In the elderly, there has been evidence for an increased incidence of deteriorating vasomotor responses which may impair, in whole or in part, adaptative responses to orthostatic stress as well as extreme environmental conditions (Lipsitz et al 1991, Collins et al 1980). These findings have argued in favor of a reduced sympathetic tone of peripheral vessels in advanced age. However, evidence for increased sympathetic nerve activity with age (Christensen 1982) have been provided which would suggest that the deficit is not a consequence of a degenerating process but is rather due to changes in the post-synaptic vascular tissues. Our results would also indicate that selective changes in cerebral blood vessels, not found in normal aging (see section 4.4), are present in AD. We have not performed any functional studies on AD post-mortem blood vessels so it is impossible to attest that the changes observed here would result in abnormal vasomotor receptor-mediated functions. It is, however, most probable that such changes in muscarinic and adrenergic receptors in AD vessels would affect in one way or another cerebrovascular responses.

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The iontophoresed acetylcholine will induce sweat production through two cholinergic mechanisms: a) direct binding to muscarinic receptors on sweat glands, b) activation of axonal reflex sweating. The stimulus for axonal reflex travels along post-ganglionic cholinergic fibers retrogradely from the point of stimulation then centrifugally back to sweat glands (figure 2). Sweat produced through axonal reflex stimulation are thought to be mediated by nicotinic receptors (see section 1.2.b). Adrenergic mechanisms are not known to be involved in axonal reflex sweating. Consequently, axonal reflex mechanisms are purely cholinergic in nature. Any defect in sweat production through axonal reflex stimulation is therefore indicative of a defective cholinergic mechanism.

The significant diminution in pure axon reflex sweating obtained from 30 AD patients therefore points to a defective peripheral cholinergic mechanism. This may add to other peripheral cholinergic alterations in AD (see section 1.1.d.) and give further support to the hypothesized generalized nature of the pathological process in AD.

More specifically, the diminished axon reflex sweating point to a defective post-ganglionic nicotinic function in AD. Although nicotinic receptors have not been identified biochemically or through molecular biology techniques in sweat glands, pharmacological studies are strongly suggestive of their involvement in mediating axonal reflex sweating (see section 1.2.b). Since the final response observed is the result of nicotinic receptor stimulation, the diminished sudorific responses obtained through axonal reflex stimulation is strongly pointing to a significantly diminished nicotinic receptor density in sweat glands of AD patients compared to young as well as elderly controls. This coincides with the reduced nicotinic binding sites in lymphocytes of AD patients (Adem et al 1986). The precise function of nicotinic receptors on lymphocytes and the physiological significance of axon reflex sweating are not known. However, the previous observations indicate the specificity of AD to involve peripheral nicotinic mechanisms in different

non-neuronal tissues. Interestingly, nicotinic receptor density has also been found to be significantly reduced in the cortex of AD patients (Quirion et al 1988, Rinne et al 1989). This has been attributed to the dramatic loss of cholinergic neurons which consists one of the major pathological features of AD.

Whether, as in the CNS, the reduced nicotinic receptors on sweat gland detected in this study is the result of a post-ganglionic cholinergic denervation inflicted by AD is controversial. The human eccrine sweat gland has often been signaled as a possible exception to Canon's rule of hypersensitivity following post-ganglionic denervation (Coon and Rothman 1941). This has also been recently observed in rats (Grant et al 1991). Neverthless, the responses to denervation observed in the previous studies have been tested following direct stimulation of sweat glands with cholinomimetic agents hence mediated by muscarinic rather than nicotinic receptors. It is most probable, however, that sweat glands would show a uniform reaction to denervation whether at the muscarinic or the nicotinic receptors level. In this respect, the reduction in nicotinic sudomotor function observed in the present study could provide a good reflection of an underlying AD-associated post-ganglionic cholinergic denervation involving axonal ramifications carrying efferent impulses to sweat gands. In contrast, other studies reported a marked hypersensitivity of sweat glands following denervation (Reas and Trendelenburg 1967). Similarly, denervation of other peripheral autonomic targets (rat parotid gland) resulted in a supersensitivity to cholinergic stimulation (Talamo et al 1979). Surprisingly, in the later report there was a slight reduction in receptor concentration indicating a possible uncoupling of the functional receptors from the denervation process. Increased sensitivity of the nicotinic receptors has not been noticed in this study. Since the final response is reproduced at the level of the effector organ, hence a reflection of the state of receptors involved, nicotinic receptors seem to be funtionally related to post-ganglionic sweat gland innervation. This contrasts with the non-significantly reduced direct

response (see later) more in favor of sparing of the muscarinic receptors. Sudorific responses produced through axonal reflex stimulation are therefore a more accurate index of the state of post-ganglionic sweat gland innervation.

There is also the possibility that the reduced response detected may indicate a disruption in the cellular responses to receptor activation in AD. The signal transduction pathway regulating secretion in the sweat glands has not yet been fully elucidated. Thus far, sweat secretion in rats has been shown to be calcium dependent and consist of high potassium and chloride effluent (Sato and Sato 1978). Studies performed on other exocrine gland cells suggest that secretion is stimulated via the phosphoinositol pathway. For example, the generation of inositol triphosphate in lacrimal glands promotes the release of intracellular Ca2+ which activates potassium chloride ion channels (Trautman and Marty 1984). In addition, the coupling of muscarinic receptors to secretion has been extensively investigated in the parotid gland - which bears a close structural resemblance to eccrine sweat glands- where it is also thought to be regulated through PI pathway (Haddas et al 1979). The uncoupling of the receptor signaling system can occur at three levels a) at the level of receptor recognition site, b) at the level of receptor-effector complex and c) at the level of intracellular actions of second messenger produced as a result of receptor stimulation. Although this area of research is relatively unexplored, evidence of disturbances in AD at all three levels is appearing in the literature (for a review, see Fowler et al 1992). Our study explored functional and not biochemical changes in nicotinic functions with AD hence we cannot relate our findings to signal transduction mechanisms. However, in one study (Margiotta et al 1987), cAMP was found to increase cellular responses in chicken ciliary ganglion neurons by recruiting a larger number of "functional" nicotinic receptors. Whether sweat glands react to cAMP in a similar fashion is not known. Neverthless, one of the functions of post-ganglionic innervation is to enhance cAMP activation of nicotinic receptors on sweat glands. A denervating

lesion would disrupt this cAMP mediated mechanism and consequently diminish the responsiveness of sweat glands following axonal reflex stimulation.

Total reflex sweating obtained from AD patients was also significantly reduced ($p < 0.05$) compared to elderly (28% reduction) and young (33% reduction) controls. Total reflex sweating includes sweat responses counted in zone 1, zone 2, and zone 4 where no acetylcholine was iontophoresed. Zone 4 is a modification of the standard QSART evaluation procedure. The reasons for its inclusion are: a) reflection of axon reflex sweating outside the limits of the device, b) estimation of the extent of axonal arborization carrying efferent signals to the sweat galnds.

In the initial QSART response, zone 2 was included as a buffer zone designed to buffer any inevitable overflow of acetylcholine towards zone 1 where pure axon reflex is detected. Therefore, although no acetylcholine was injected in this zone, there is a possibility that at least part of the response counted in zone 2 might be the result of direct activation of sweat glands by the exceding acetylcholine. Because of the doubtfull nature of the response in zone 2, we did not consider it separately. Similarly, responses counted in zone 4 may also include sweat glands activated by acetylcholine present outside the limits of the device. Since there is no buffer zone between zone 3 and zone 4, responses in zone 4 were not evaluated separately. Instead we included all sweat responses obtained in areas where no acetylcholine was iontophoresed under the notion "Total Reflex Sweating" which is most probably the mechanism by which sweat was reproduced in these zones. The spilling effect may explain why the percentage of total reflex sweating reduction in AD patients is lower than for the pure axon reflex sweating. However the statistical significance of the results indicate that this effect (if present) constitute a constant percentage of the calculated response that has no influence on the interpretation of the results.

On the other hand, although we counted reflex sweating in an area 3 times larger than the

surface area of the device itself, we were not able to include all axonal reflex responses. Sweat droplets were still seen outside the limits of zone 4. It is therefore obvious that the area covered by axonal arborization cannot be all included by our counting procedure.

4.7. Direct responses to iontophoresed ACh in AD patients and controls:

Direct responses produced by direct action of the iontophoresed ACh on sweat glands showed a trend (albeit not significant) for diminution in AD patients compared to young and elderly controls.

Direct responses are mediated by the action of acetylcholine on sweat gland muscarinic receptors (see section 1.2.c). A recent study (Grant et al 1991) revealed that, in rats, sweat gland receptors belong to the M2 pharmacological subtype whereas in situ hybridization indicated that rat sweat glands express the m3 molecular receptor subtype. The non-significant diminution in direct responses obtained indicates that muscarinic mediated sweat responses, although weakened, are not specifically affected by AD. Our experimental procedure involved the direct activation of sweat glands by acetylcholine molecules introduced through the skin pores therefore no nerve stimulation is involved. The responses obtained in zone 3 are consequently a quantitative expression of the receptor-function mechanisms involved in sweat production by the iontophoresed acetylcholine. A difference in direct sweat responses counted in the iontophoresis zone would , therefore, be attributed either to changes in the receptor density on sweat glands, altered functional responses following receptor activation or a defect at the receptor-function coupling level.

Most investigators agree on the decrement in sweat production with age (see section 1.2.f). This has been attributed to a decrement in sweat gland output rather than the recruitment of a lower density of sweat glands (Silver et al 1964, Foster et al 1976, Inoue et al 1991). Atrophic

changes in sweat glands are known to occur with age (Sato 1977). Similar structural alteration of sweat gland is also expected to be found in AD, a disease of the elderly population. In this study, only senile AD patients were tested. Consequently, any additional effect of AD would be superimposed on already present senile changes. Whether further atrophy of sweat glands, hence further decrement in sweat gland output, occur in AD cannot be concluded. The present study was aimed at quantifying rather than qualifying sweat responses in AD and control subjects. It is possible that the increased (though non-signficant) diminution in the counted direct responses obtained from AD patients compared to elderly controls is an indication of further changes in the effector organ (sweat gland) tested in AD. However, exagerated atrophy of sweat glands in AD would be expected to reduce the muscarinic receptor density as a result of tissue loss. The non-significant reduction in the counts obtained indicates sparing of the sweat gland muscarininc receptors in AD therefore argue against extensive AD-related structural alterations of sweat glands. Also, as far as the results indicate, there is probably no significant alterations of the intracellular mechanisms mediating functional responses following receptor activation in sweat glands. A more accurate statement about the receptor-function coupling can be made following sweat gland output measurement.

Unlike nicotinic function mediating axonal reflex sweating, there is a discrepency between the non-significant diminution in muscarinic receptors on sweat glands and the reported significant diminution in muscarinic cholinergic binding sites detected in lymphocytes (Rabey et al 1988) as well as in cerebrovascular tissue (binding results). This discrepancy can be explained on the basis of the differential alteration of muscarinic receptors in AD. Muscarinic receptors in sweat glands belong to the M3 subtype (Grant et al 1991). The significant diminution in quinuclidinyl benzilate (QNB) binding in lymphocytes of AD patients (Rabey et al 1988) does not specify whether there is a heterogeneous population of muscarinic receptors or whether this reduction

involves one specific subtype. Also results from pial vessels showed a significant diminution in in the binding sites unbloked by 75 nM pirenzepine. Whether this involves the M3 receptors as well entails further consideration. On the other hand, the state of the M3 subtype in the CNS of AD patients has not been studied. Therefore the apparent contradiction in the results obtained can be decieving.

Whether the M3 receptors in peripheral tissues are altered in AD still need to be investigated. However, the non-significant diminution in muscarinic receptors on sweat glands give further support to our conclusion on the accuracy of nicotinic over muscarinic receptors to detect a cholinergic denervation either centrally or peripherally indicating an intimate functional relationship between post-ganglionic cholinergic denervation and the state of the nicotinic receptors. Cholinergic denervation produced little change in the distribution or pharmacological properties of muscarinic receptors expressed by rat sweat glands however the glands no longer responded to administration of cholinergic agents (Grant et al 1991). A similar dicrepancy between the presence of muscarinic binding sites and cholinergic sensitivity has also been previously described (Siegel & Fishbach 1984). These observations suggest that sympathetic cholinergic innervation does not regulate the functional responsiveness of sweat glands at the level of receptor expression. Histochemical studies have revealed the presence of a diverse number of regulatory peptides colocalized in the same fibers with acetylcholine (see section 1.2.e). It is possible that physiological sweat production is a function of one of the peptides present in the post-ganglionic cholinergic fibers whereas acetylcholine mediates axonal reflex sweating by its action on nicotinic receptors.

5. Conclusion:

On a general basis, the changes in cerebrovascular receptor density as well as sudorific responses obtained in the present study add to the previously reported alterations in peripheral neurotransmitter-related functions in AD. The results of our study are strongly pointing to a deficit in autonomic function in AD different from that accompanying normal aging. The changes may represent an extra-cerebral expression of the central degenerative pathology involving both adrenergic and cholinergic systems. It is, however, obvious that some of the changes in the cerebrovascular bed from AD patients are not an exact replication of the central biochemical alterations but rather suggest profound alterations in the post-synaptic target namely the blood vessel wall itself. For instance, total muscarinic receptors seem to be preserved in the brain of AD patients (Quirion et al 1988) but they were significantly reduced in pial vessels (this study) as well as in lymphocytes obtained from AD patients (Adunsky 1989). Interestingly, sudorific responses mediated by muscarinic receptors were not significantly reduced in AD. These are located on the surface of the acinar cells and seem to be spared by the peripheral pathological process occuring in AD. On the other hand, the reduction in nicotinic binding sites in the CNS of AD patients seem to be accompanied by a parallel diminution in the nicotine-mediated axonal reflex sweating (this study) and a reduction in the amount of nicotine binding sites in lymphocytes of AD patients (Adem et al 1986). The similarities and differences between central and peripheral neurotransmitter receptor-related changes could possibly be attributed to the differences in the location of the receptor subtype along the synaptic unit.

The consistant reduction in nicotinic receptors in AD both in the CNS and in the autonomic pathways involving axonal reflex control of sudorific responses, could suggest that these receptors are reliable indicators of a degenerative cholinergic innervation. We did not evaluate the state of nicotinic receptors in pial vessels of AD patients. This could have been an interesting avenue

since cerebrovascular nicotinic receptors have been suggested to be pre-synaptically located on perivascular nerve fibers (Edvinsson et al 1981). However, radioligand binding studies performed in the laboratory on pial vessels obtained from both human and bovine brains have failed so far to detect any significant amount of [^{3}H]-nicotine labelled binding sites (Linville and Hamel 1994). These observations could in fact be compatible with a minor population of pre-synaptic receptors that would not be readily detectable by radioligand binding experiments. Although there is a clear need to clarify this issue in further details, it appears that cerebrovascular nicotinic binding sites cannot be used as an index of cerebrovacular innervation. Our results on ChAT activity in pial vessels would, however, tend to indicate a minor degeneration of cerebrovascular cholinergic nerves. Indeed, what was the most striking finding in human pial vessels was the generalized drop in post-synaptically located muscarinic binding sites. Together with other reports on degeneration of the vessel wall in AD (Yamaguchi et al 1992), initiated by various processes including ß-amyloid deposition (Yamaguchi et al 1992, Kawai et al 1993), the most logical explanation for these findings would be that cellular elements (endothelial and smooth muscle cells) primarly endowed with muscarinic receptors in the cerebral blood vessels are severely damaged in AD. The selective deterioration of the blood vessel structure could explain the unaltered post-synaptic muscarinic-mediated sweat response in AD. In contrast, the significant diminution in responses mediated by nicotinic mechanisms is strongly suggestive of a peripheral post-ganglionic degeneration similar to what have been reported in the CNS in AD (Whitehouse 1982) and is most probably indicative of a similar underlying process occuring both centrally and peripherally.

The significant reduction in the density of α1-adrenergic cerebrovascular receptors in AD, receptors known to mediate vasoconstriction, further support an alteration at the level of the blood vessel wall itself. Although we did not evaluate the functional aspects of these receptors,

their decrease is in keeping with other studies performed in peripheral blood vessels in AD. (Hörnqvist et al 1987). Although it is not clear to us at the present time why cerebrovascular ß-adrenergic receptors would not be modified in AD, the fact that we only assessed total ß-adrenergic receptors might have hampered detection of changes in a given subtype. Alternatively, their known endothelial location in cerebral blood vessels could suggest that these cells are not as severely affected as the smooth muscle layer in AD.

It can be finallly concluded that the peripheral AD-related changes can also be detected in structures receiving a well documented peripheral innervation. The biochemical changes shown in pial vessels are in keeping with a degenerative pathology involving the vessel wall whereas the diminution in sudorific responses point to a peripheral denervating process involving post-ganglionic sweat gland innervation. The peripheral alterations detected in AD may not be of a first degree importance in explaining the etiology of the disease however, they may constitute the basis on which a diagnostic procedure can be developped.

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