Potentialities and pitfalls in the use of human brain material in molecular neuroanatomy

D.F. SWAAB and H.B.M. UYLINGS

1. Introduction

Transmitter systems in the brain have a very specific localization pattern. In addition, subsystems of the same transmitter may show differential changes in physiology and disease. Therefore, data obtained by means of assays in homogenized and extracted tissue only have a limited value, and techniques leaving the anatomical structures intact are preferable. Various molecular neuroanatomical techniques are now so far developed that they may successfully be applied on the human brain. However, many patient-related factors which are in animal experiments either non-existent as a problem or self-evident and rather automatically taken care of, may complicate the use of human material in such procedures. In this respect, some observations on the human brain concerning ante- and post-mortem factors, freezing procedures, fixation and storage time will be mentioned.

2. Factors to control for

2.1. Ante-mortem factors

Although a long time past the literature seems to have been in agreement that controls should be matched for sex (for review see Swaab and Hofman, 1984), this is often a neglected point in work on the human brain. Recent literature gives reason enough to take this point into consideration. We have demonstrated for example a sex difference in volume and cell number of a cell group in the hypothalamus, the sexually dimorphic nucleus of the preoptic area (SDN). This nucleus was 2.6 times as large in men as in women and contained 2.2 times as many cells in men. Since sex

Correspondence to: Prof. Dr. D.F. Swaab, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands.
differences have also been described with respect to the shape of the corpus callosum (De Lacoste-Utamsing and Holloway, 1982), and the cell number of Onuf's nucleus in the spinal cord in man (Forger and Breedlove, 1986) we may expect sex differences anywhere in the central nervous system, so that our controls should indeed be matched for sex. Recent allometric studies on the SDN make it clear that factors like body height and brain weight should also be taken into account. The SDN data illustrate also clearly that we should match controls for age. Already after the first neonatal years the SDN begins to decrease massively in volume and cell number. On the other hand, in immunocytochemically identified VP neurons in the suprachiasmatic nucleus (SCN) a decrease in number was found from the age of 80 onwards (Swaab et al., 1985). Apart from these four factors, additional information is needed on the subjects from which brain tissue was obtained. The corpus callosum was demonstrated to be larger in left-handed and ambidextrous people than in those with consistent right-hand preference (Witelson, 1985). In the author's opinion the greater bihemispheric representation of cognitive functions in lefthanders and mixed-handers may be associated with this difference. Such differences may be represented in many neuronal systems. Therefore, hand preference and laterality should be taken into consideration for measurements in the human brain. Recently, higher concentrations of thyroid-releasing hormone (TRH) were reported in the left side of the human hypothalamus i.e., in the ventromedial dorsal and paraventricular nuclei (Borson-Chazot et al., 1986). Earlier reports mentioned already lateralization of dopamine, CAT, noradrenaline and GABA in the human brain (Amaducci et al., 1981; Glick et al., 1982; Oke et al., 1978) Consequently, the side at which measurements are made should be mentioned.

Alcohol addiction is accompanied by smaller brain weight, at least in men (Skullerud, 1985), and may consequently affect measurements. Since the ventricle:brain ratio may increase not only in alcoholism, but also following long-term use of benzodiazepines (Lader and Petursson, 1983), a case history of medicine use may provide vital information. This also becomes evident from the observation of Spokes (1979), who found that in opiate-treated patients brain glutamate decarboxylase (GAD) levels were lower, while no difference was found in dopamine, noradrenaline or choline acetyltransferase (CAT).

The agonal state is also an important factor in human brain studies. Lower GAD levels were found throughout the brain in cases of death following protracted illness, as compared to sudden death (Spokes, 1979). Subjects who die after a long terminal illness have a lower pH in the brain, cerebrospinal fluid and blood. This general acidosis corresponds to high lactic acid concentrations (Hardy et al., 1985). This is not only an important point for biochemical studies on the human brain, but has its counterpart also in morphological parameters (Braak and Braak, 1985). Williams et al. (1978) reported that no satisfactory Golgi preparations are obtained when preterminal encephalopathy exceeds 12 h. Even the increase in brain weight during fixation and washing is higher in cases with sudden death than in cases with death resulting from protracted illness (Skullerud, 1985). This might be a factor of importance, e.g., in allometric studies. Brain pH should therefore be measured in human post-mortem material as an index of agonal state (Hardy et al., 1985) and
serve as a factor to match for. Human beings are most certainly not free from seasonal influences, as becomes apparent, e.g., from data on human reproduction. Live deliveries in Finland showed an annual cycle with a minimum of 10% below mean around the shortest day. Multiple pregnancies even showed a range varying between +20% and -20% (Vaughan et al., 1978). It is therefore hardly surprising that Carlsson et al. (1980) reported a seasonal variation that was essentially limited to hypothalamic 5-HT with a nadir during the winter months. We found recently indications for seasonal alterations in cell number in the paraventricular nucleus of the hypothalamus (PVN) and SCN (Hofman, Goudsmit and Swaab, unpubl. observ.). Also clock time of death was found to be a significant factor for the level of noradrenaline, serotonin and dopamine and their metabolites (Carlsson et al., 1980). Perry et al. (1977) found that cerebral GAD and CAT activities had circadian fluctuations. This could, however, not be confirmed by Spokes (1979). We recently found a clear-cut circadian pattern in the volume of the PVN, with higher values around midnight and lowest values at noon (Goudsmit, unpubl. observations). Time of death should therefore be regarded as a possible factor to match for.

2.2. Post-mortem delay

Another factor of considerable importance in the analysis of human brain tissue is post-mortem delay. The time between death and fixation or freezing of the brain tissue has received attention mainly from a neurochemical standpoint. There are, of course, compounds that can never be measured fast enough after death to obtain the actual in vivo levels in human autopsy material; P-creatinn, for instance, reaches a low and basal level already 30 seconds after death (Swaab and Boer, 1972). Other compounds, such as GABA and glycine, show changes within several hours, while aspartate, glutamate, taurine levels and enzymes such as GAD and CAT remain quite stable in this time-interval (Perry et al., 1981; Spokes, 1979). This does not necessarily mean, of course, that such compounds remain in the same cellular compartment for hours. Dopamine and noradrenaline were quite stable during routine post-mortem handling (Spokes, 1979). However, a substantial loss of catecholamines may occur already during the first hours after death (Sloviter and Connor, 1977).

Post-mortem interval is not only important for neurochemistry, but also has considerable bearing on some morphological parameters. This may be illustrated by data obtained with Golgi techniques, which have become increasingly important in neurosciences. The generally accepted idea that with aging dendrites would degenerate, as proposed by Scheibel et al. (1975), had to be revised when quantitative techniques were applied. Coleman and Flood (1986) even found that dendrites grow also in the brain of old human subjects during normal aging, and hypothesized that the formation of new branches might serve as a compensation for cell loss in neighbouring cells. In Alzheimer’s disease the size of the dendritic tree is reduced in the dentate gyrus (Coleman and Flood, 1986; De Ruiter and Uylings, 1987; Flood et al., 1987). An important point in the current discrepancies, e.g., on the absolute size of the dendritic tree in the human brain, is the possible influence of post-mortem
Fig. 1. Number of spines per 50 μm segment as a function of the delay between death and fixation in 28 guinea pigs on the apical and basal dendrite of pyramids in layer V and on the oblique branches of the apical dendrite (from De Ruiter (1983) with permission).

Fig. 2. Total length of the basal dendrites as a function of the delay between death and fixation in 28 guinea pigs (from De Ruiter (1983) with permission).
Fig. 3. Golgi-Cox neocortical tissue of immersion fixed adult rats after a post-mortem delay of 6 h. No deleterious effect is visible.

Fig. 4. After a post-mortem delay of 32 h cell bodies with degenerating dendrites or without any dendrite at all may be observed in this Golgi-Cox neocortical rat preparation. Such deleterious effects are found following a post-mortem delay of 24 h or longer.
delay and the difference in sensitivity between the silver rapid-Golgi technique and the mercury Golgi-Cox technique. Using guinea pig brains, and applying the rapid-Golgi technique after formaldehyde fixation, De Ruiter (1983) varied this delay between 5 min to 24 h. He observed a considerable drop in density of the spines (Fig. 1), the structures that are most susceptible to fixation delay, on the apical main shaft of pyramidal layer V cells, starting from a delay of 1.5 h onwards. This is, of course, a period that in general is not feasible when human autopsy material is studied, so that unreliable or very variable results are to be expected in spine counting. Already after 4 h did the spine counts and total length of the impregnated basal dendrite arborization in Golgi-rapid stained sections show a reduction (Fig. 2). Williams et al. (1978) concluded that human material remained relatively free of degenerative changes when fixation is delayed for no longer than 6 h. However, the Golgi-Cox method seems to be less sensitive to post-mortem delay than the Golgi-rapid method (Buell, 1982). Using the former procedure, Uylings and Evers observed qualitatively completely impregnated dendritic trees in rat brains (Figs. 3, and 4), even after a post-mortem delay of 24 h. The post-mortem delay might also be prolonged to 24–48 h by the use of an alternative silver Golgi procedure developed by Von Kopsch in 1896. He left rat heads on his table at room temperature for 24 h, followed by his modification of Golgi staining and reported in 1896: "... färben sich die Pyramidenzellen in derselben Schönheit wie bei ganz frischen Materialien". Using the Golgi-Kopsch staining Uylings and Evers observed after a post-mortem delay of 32 h that the few impregnated neurons in rat cerebral cortex did not show the degenerative features of dendrites seen after 6 h post-mortem delay in Golgi-rapid stained tissue (De Ruiter, 1983; Williams, 1978). However, the staining results of immersed fixed tissue appear to be much more capricious than the other Golgi mentioned above.

Also for immunocytochemistry in the human brain one generally strives at short post-mortem intervals (e.g., Bouras et al., 1986). For immunocytochemistry on amines, a rapid perfusion fixation is indeed essential. However, immunocytochemical procedures for peptides are generally much less sensitive to post-mortem delay than Golgi procedures (e.g., Emson et al., 1984). We could still obtain excellent staining of vasopressin neurons when the tissue was fixed up to 48 h after death (Fliers et al., 1985; Swaab et al., 1985). A similar stability upto post-mortem times of more than 60 h was found for extrahypothalamic vasopressin fibres (Fliers et al., 1986; Swaab, 1982). Although for some antigens perfusion fixation of the human brain may be preferable (Beach et al., 1987), it is in fact amazing how much one can accomplish on routine autopsy material that has been fixed by immersion. For the immunocytochemical demonstration of some compounds a longer post-mortem interval might even be advantageous. ACTH cells in the pars distalis of the rat pituitary stained increasingly better during a period of post-mortem storage at 4°C for 48 h (Visser and Swaab, 1979). A similar phenomenon was observed for vasopressin producing neurons in extrahypothalamic areas in the rat brain, that stained better following incubation of fresh tissue slices in saline for 6 h. Indications for new sites of vasopressin production have been found this way recently in the rat brain (Ravid et al., in prep.). One might question, therefore, whether it is always
wise to strive a priori, to a post-mortem interval that is as short as possible. On the other hand, one may question the physiological importance of compounds that only become visible after long post-mortem intervals. Immunocytochemistry of peptides or enzymes might therefore sometimes be preferable to those for monoamines or Golgi-rapid procedures on human tissue where post-mortem intervals are never really short. It would be an important development indeed for brain-banking if more neurobiological techniques would be adapted for human tissue to relatively long post-mortem intervals.

3. Freezing procedures, fixation and storage time

Generally tissue for biochemically work is rapidly frozen and slowly thawed. However, Hardy et al. (1983) showed that just the opposite procedure should be used to obtain synaptosomes from human brain tissue with relatively good retention of morphology and metabolic performance. One should freeze slowly by adding 0.32 M sucrose to whole pieces of brain tissue and put them in an insulated container in a \(-70^\circ C\) deep freezer, and rapidly thaw in a 37\(^\circ C\) waterbath in order to obtain good synaptosomes. Protection from freeze-thaw injury of human brain prisms has also been obtained by dimethyl sulfoxide (Haan and Bowen, 1981).

Changes in fixation and storage procedure may certainly affect the potentialities of staining procedures considerably. The use of ethylene glycol-based cryoprotectant has recently been reported to be effective for keeping the immunoreactivity intact during long-term storage of brain tissue either in block form or as freely floating sections (Watson et al., 1986). On the other hand, our experience is that storage in glutaraldehyde-paraformaldehyde fixative also preserves immunoreactivity of vasopressin, oxytocin and a-MSH for more than a year (Ravid, unpubl. results). We could even still show some vasopressin immunoreactivity in a human brain that had been stored in our museum for some 50 years, although the staining was anything but optimal (Swaab, 1982). Conventional formaldehyde fixation for one month to one year enabled excellent vasopressin and oxytocin staining in neurons in the supraoptic nucleus (SON) and suprachiasmatic nucleus (SCN) of the human hypothalamus (Fliers et al., 1985; Swaab et al, 1985). This allowed measurements to be done of the neurosecretory activity of immunocytochemically identified neurons by measurements of nucleolar size (Hoogendijk et al., 1985). However, this procedure was not suitable for studying the extrahypothalamic fibres of these peptidergic neurons. In the rat, extensive networks of extrahypothalamic vasopressin and oxytocin fibres have been described (e.g., De Vries et al., 1985). Glutaraldehyde-paraformaldehyde perfusion fixation gives an excellent vasopressin fibre staining. Since immersion in this fixative did not fully penetrate the human brain, fresh tissue blocks of 1 cm\(^3\) of human brain tissue were fixed by immersion in 2.5% glutaraldehyde and 1% paraformaldehyde for one week. Subsequently, the blocks were frozen and stored in sealed plastic at \(-80^\circ C\). Cryostat sections (40 \(\mu\)m) were cut, mounted on uncoated glass slides and stored in series at \(-20^\circ C\) in standard slide boxes. For orientation every 20th section was stained with thionin.
The other sections were incubated free-floating for immunocytochemistry. This procedure increased the immunocytochemical sensitivity considerably, and extrahypothalamic vasopressin fibres could in fact be found in the human brain. In the septum and other limbic structures only few fibres were found in the human brain, in contrast to the dense innervation found in the rat. However, the locus coeruleus contained denser networks than were observed in the rat (Fliers et al., 1986). The extrahypothalamic vasopressin innervation in the human brain is, consequently, certainly not identical to that in the rat, as was implicitly supposed in some books on human chemoarchitecture (Nieuwenhuys, 1985) and in some studies on the effects of vasopressin and its analogues in man.

4. Selection of parameters and procedures

4.1. Morphological parameters

Not only the factors mentioned above have been taken into account (e.g., by analyzing their effect on the measurements), but also the value of the parameters used in the study of the human brain. The question which parameter to choose is hard to answer in any general way, but in our experience there is at least one morphological parameter on which one should certainly not base one's conclusions, namely cell density. In spite of the fact that so far neuropathologists have used this parameter almost exclusively to assess, e.g., cell loss in aging and Alzheimer's disease, this seems to be a highly insensitive measure. We found no changes with aging or in Alzheimer's disease in vasopressin cell density and total cell density in the SCN of the human brain. Morphometry showed, however, that the SCN volume decreased strongly in senescence in Alzheimer's disease, and the same was observed for vasopressin cell numbers and total cell numbers in the SCN. This may explain the loss of circadian rhythms in both conditions (Swaab et al., 1985). Consequently, in case of cell loss the remaining cells may group together, resulting in the same density but in a smaller SCN volume. By judging cell density in a few sections through a structure obviously essential information can be overlooked.

Fixation and staining may affect the size of brain structures differentially at different ages (Haug, 1986; Uylings et al., 1986). For comparison of brain region volumes of subjects of different ages, two kinds of size-changes have to be corrected for, viz. (a) histological shrinkage (see for procedures of estimation Uylings et al., 1986) and (b) the "secular acceleration". This means that in general the brain weight of the older generation was primarily smaller than that of the younger and taller generation. The increase in brain weight between generations is estimated by Haug (1986) as 40 g/100 years. When age-matched groups of control and diseased subjects are compared for differences in brain size or brain region size the implicit assumption is that histological shrinkage of both groups is similar.

When thickness or diameter differences are determined in cortical areas or brain regions, different planes of cutting might interfere with comparisons (e.g., Uylings et al., 1984).
An interesting but also frightening additional point concerning the neurons stained by the mercury Golgi-Cox procedure is that impregnation may in some cases takes place preferentially in healthy cells. This would imply that in human pathology the major changes may be overlooked when measurements are performed only on Golgi-Cox-impregnated material and no silver-Golgi techniques are used. This statement is based upon histological observations of a brain biopsy taken from a 2-year-old mentally retarded child with microcephaly and dyskinesia, a heredodegenerative disease of the neonatal period. Bodian staining in the cerebral cortex revealed several corkscrew-shaped apical dendrites. Such clearly pathological shapes, could, however, not be retrieved from Golgi-Cox-impregnated sections (H.B.M. Uylings and P.G. Barth, unpubl. observ.). Since pathological changes have often been observed in Golgi-rapid stained tissue (e.g., Braak and Braak, 1985), it may be preferable to stain pathological tissue with both a silver-Golgi and the Golgi-Cox procedure. Golgi-Cox provides in general consistent results and reveals a larger percentage of impregnated nerve cells, but their ability to impregnate the very fine details of spines is disputed.

4.2. Functional parameters

Classical morphological markers for neuronal activity, i.e., cell size and nucleolar size, have been used in the human supraoptic and paraventricular nuclei, in order to monitor changes in peptide production in vasopressin and oxytocin neurons with age (Fliers et al., 1985; Hoogendijk et al., 1985). These two measures for cellular activity revealed similar results, i.e., an enlargement in AVP cells in senescence. In addition, these two measures showed an excellent correlation (e.g., for the AVP neurons in the PVN $n = 0.82; \ p < 0.0001$). Both parameters appeared to be a good measure for neurosecretory activity. Molecular biological techniques bear great promises for more specific measures and new insights when applied on human brain tissue in the coming years. Human cerebral cortex RNA, obtained following post-mortem intervals of 7–36 h, demonstrated an extensive stability (Johnson et al., 1986). A regional distribution in the human brain of messenger RNA encoding glutamic acid decarboxylase has been described (Wood et al., 1986) and a rapid microprocedure for isolating RNA from human brain has been reported (llaria et al., 1985).

Also receptors in the brain are found to be amazingly stable. Somatostatin binding sites in the rat brain were not altered up to 24 h after decapitation when the brain was left in situ while stored at 4°C (Reubi et al., 1986). A 4-h post-mortem interval did not change nicotine binding in the rat cerebral cortex (Benwell and Belfour, 1985). Human cerebral cortex muscarinic receptors were stable up to 51 h post-mortem when stored at 4°C (Undén et al., 1983). Cooling of the brain to 4°C may prevent to a large extent the decrease of receptor binding during a post-mortem interval of 96 h (Benwell and Belfour, 1985; Whitehouse et al., 1984). It goes without saying that the determination of these stable functional measures in the human brain in relation to various psychopathologies bears great promises for the coming decennia.
5. Internal and disease-related controls

When differences are found in certain areas in the brain of, e.g., Alzheimer patients as compared to matched controls that did not have a neurological or psychiatric disorder, the question arises whether this difference is indeed restricted to the measured area, or forms part of a generalized change, and whether the change observed is specific for Alzheimer’s disease or is also present in other psychopathological conditions.

As an internal control for the first question one may study the same parameter in other brain areas of the same subjects. As discussed before, a strong decrease in cell number was observed in the clock of the brain (the SCN), in senescence, and an even stronger cell loss was found in this area in Alzheimer’s disease. This alteration was supposed to be the morphological basis for sleep disturbances and other circadian changes in senescence and Alzheimer’s disease (Swaab et al., 1985). This pattern of cell loss seemed to be region-specific, since in the same sections the SDN showed a much earlier decrease in cell number, viz., at least already from the age of 40 onwards (Swaab and Fliers, 1985). On the other hand, the cell number in the neurosecretory nuclei, the PVN and SON, appeared to be remarkably stable. Neither of these showed any significant alterations with age or in Alzheimer's disease (Goudsmit et al., 1988). The changes observed in the SCN in senescence and Alzheimer’s disease thus appeared to be quite specific for the region. The question whether any other diseases should serve as controls in order to investigate the specificity of the changes for the disease studied is more difficult to answer. The premise that each disease entity should be reflected by specific alterations in different parts of the brain or different transmitter systems is certainly too simple. Parkinson’s disease has often been mentioned as a useful disease control for Alzheimer’s disease. Many investigations from the last decade have shown, however, that these two diseases have many alterations in the brain in common. There is a marked loss of cortical cholinergic innervation, measured as CAT, not only in Alzheimer’s disease, but also in cognitively impaired Parkinson patients. In addition, some CAT loss also occurs during aging in non-demented patients (Candy et al., 1986). The loss of CAT is therefore not restricted to Alzheimer’s disease. Although there are differences between the CAT loss in Alzheimer’s and Parkinson’s disease, they are subtle. The problem is that one does not know a priori what to expect from the parameter of interest in other psychopathologies. So as long as not a great deal more basic information is available on other neurologic or psychiatric diseases, they can hardly serve as a “good control”. The same holds good for other types of dementia that are often used as controls.

The relationship between course of the disease and the changes in the brain may also reveal valuable information. Brain biopsy data of Alzheimer patients have been compared with changes in their brains after death (Francis et al., 1985). This procedure may of course provide information on the natural course of the disease. However, since the information obtained from such biopsies does not have any direct bearing on the treatment of the Alzheimer patients, this procedure is, in our opinion, ethically not justifiable in this condition.
Although, as will be clear from the present paper, the possible pitfalls are numerous when human brain tissue is studied with molecular neuroanatomical techniques, the potentialities of such studies in neurology and psychiatry are great. Since the techniques are currently available, such studies will undoubtedly take place in rapidly increasing numbers during the next decennia. A well-organized Brain Bank for the collection of human brain tissue of clinically well-described patients that, in addition, takes into consideration ante- and post-mortem factors, selects carefully the conditions for the freezing-fixation and storage procedures, and takes care that the material is systematically diagnosed by neuropathologists, will be a conditio sine qua non for good research in this field.

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References


