

The Netherlands brain bank — a clinico-pathological link in aging and dementia research

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Summary. The number of sophisticated neurobiological techniques which can be applied on human brain has rapidly increased and causes an increased demand for post-mortem human brain tissue for research purposes. Brain banks, which collect post-mortem tissue from patients who suffered from neurological and psychiatric disorders, have become an important link between clinicians, scientists and neuropathologists involved in aging and dementia research. Due to the large variability of the material, there are many drawbacks in the use of post-mortem brains. Therefore, collecting human brain tissue for research should include matching for several factors, both ante-mortem and post-mortem. Some of the most important ante-mortem factors include age, sex, agonal state, seasonal alterations, circadian variation and clock time of death. The post-mortem factors which should be matched for include the post-mortem delay, fixation and storage time and lateralization.

The material and data on aging and dementia, collected by the Netherlands Brain Bank in the past six years will serve in the present paper to illustrate the wide variety of potentialities and pitfalls in the use of post-mortem human brain tissue.

Brain Bank organizations for various neurological diseases form at present an important clinico-pathological link in aging and dementia research and the availability of post-mortem human brain tissue makes it possible to investigate those diseases for which no animal model is available. In order to provide research groups with post-mortem brain tissue from Alzheimer's disease (AD) patients and controls, a brain bank was established by the end of 1985 in The Netherlands Institute for Brain Research. This brain bank is based upon research projects submitted in advance, specifying a variety of requirements such as: total number of brains needed, kind of fixation, agonal state, post-mortem delay, exact anatomical boundaries of the brain region, kind of fixation and other treatment requirements of the tissue. This Brain Bank has got two unique features:

1. Human brain tissue is obtained by means of rapid autopsies with a very short post-mortem delay, ranging between 2–4 hour.
2. Fresh brain dissection procedure is used, which is a difficult regime to establish, requiring qualified staff at inconvenient times. This dissection procedure is necessary for the immediate use of fresh tissue and advantageous in increasing the range of morphological, neurochemical, immunocytochemical, metabolic and other procedures which can be applied to tissues fixed in a different way or rapidly frozen brain tissue free of freezing artifacts.

The brain tissue obtained at each autopsy is immediately dissected according to a protocol into 70 different structures, of which 14 are used for neuropathology while the rest is sent to various research groups.

The majority of the specimens are frozen in liquid N₂, stored at -80°C and shipped on dry ice. The diagnosis is confirmed clinically and neuropathologically. The neuropathological diagnosis is sent to the patient's physician as well as to the various research groups who make use of the tissue.

In the past six years, The Netherlands Brain Bank supplied tissue obtained from 620 autopsies of Alzheimer's disease and control patients to 116 different research projects in Europe, the USA and Canada. The Brain Bank also has at its disposal a growing collection of post-mortem cerebrospinal fluid (CSF) samples of AD patients and controls as well as of series of sections of fixed brain tissue. The collection includes series of brain areas of AD patients, clinically and neuropathologically documented and series of control brain areas from the fetal period up to the age of 93.

As appeared from applications of many research groups, similar brain bank facilities are needed for research on neurological and psychiatric diseases other than AD, such as Parkinson's disease, Multiple Sclerosis, Depression, Schizophrenia, Huntington's disease, Cerebro Vascular Accidents, Multiple Infarct Dementia, Binswanger disease, Creutzfeldt-Jacob disease and Korsakoff and in addition, plasma and CSF collections are required as well.

The various enzymes, transmitter systems and other active substances in the brain have their specific localization. Therefore, data obtained by biochemical assays in homogenates or tissue extracts have only a limited value and brain banks collecting human brain specimens for research purposes should strive to develop techniques which leave the morphology of the tissue intact (Swaab et al., 1986, 1987; Swaab and Uylings, 1988).

The various techniques which are applied on human brain have a common drawback; many patient-related factors introduce a huge variation and systematic errors, which have to be corrected and carefully matched for. There are several factors one should be aware of when collecting and handling human specimens and the data accumulated in The Netherlands Brain Bank will be used here to illustrate the need for matching samples for various factors.

Antemortem factors

Age

Age related changes are present in normal aging and in Alzheimer's disease (AD), e.g. the decreased number of vasopressin-immunoreactive neurons in the supra chiasmatic nucleus (SCN) above 80 years of age in both sexes (Swaab et al., 1985). A decrease in volume and cell number was observed in this nucleus in senescence (80–100 years) and was even more pronounced in Alzheimer's disease. Another brain region which shows clear age related changes is the Sexually dimorphic nucleus, SDN (Swaab and Hofman, 1984; Hofman and Swaab, 1989). Between the ages of 10 and 93 years, this nucleus decreases greatly in volume and in cell number (Swaab and Fliers, 1985).

Age must be also taken into consideration when studying monoamines, their metabolites and enzyme activities in postmortem investigation of the human brain (Adolfsson et al., 1979). Monoamines were reported to be decreased in brains from patients suffering from AD (Hardy et al., 1985) and in vascular dementia (Wallin et al., 1991).

Sex

There are a lot of data supporting sex differences in the human brain. Morphometric analysis revealed that the shape of the SCN is sexually dimorphic (Swaab et al., 1985) and there is a striking sexual dimorphism in the size, shape and cellular morphology of the SDN-POA. The volume of this nucleus was larger in men than in women and contained a significantly larger number of cells (Swaab and Hofman, 1984; Swaab and Fliers, 1985; Hofman and Swaab, 1989).

Brain weight

Recent allometric studies made it clear that brain weight should be taken into account (Swaab and Hofman, 1984) in studying aging and dementia. We know that although in the average, males have larger brains than their female counterparts, one should also take into account the differences in age, body weight and length (Dekaban and Sadowsky, 1978; Haug, 1984). Brain weight and ventricular volume are considered to be objective measures of brain atrophy. Brain weight is significantly influenced by various fixatives and the duration of fixation and is known to increase during fixation in formalin and this increase is directly correlated to the fresh brain weight (Skullerud, 1985).

Agonal state

The agonal effects associated with death of the patient may influence the concentrations of certain chemical substances in the brain. Lower levels of pH 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, CSF and blood, and this acidosis corresponds to a high lactic acid concentrations (Perry et al., 1982; Hardy et al., 1985). Various enzymatic activities were also found to be related to pH and lactate in postmortem brain in Alzheimer's disease and Down's syndrome as well as other dementias (Yates et al., 1990). Strong positive correlations were obtained between the concentration of tryptophan, a putative agonal status marker of postmortem brain tissue and the concentration of γ -amino butyric acid (GABA) in all brain areas (Korpi et al., 1987).

To prevent the use of unsuitable tissue for research, brain pH should always be measured at autopsy as an index of agonal state.

Seasonal variation

Seasonal alterations have been found in cell numbers of the paraventricular and suprachiasmatic nuclei (Hofman, Goudsmit and Swaab, unpublished). The level of hypothalamic 5-HT was also found to change according to the time of the year with a minimum during the months December-January and a maximum during October-November (Carlsson et al., 1980a).

Circadian variation

This has been found to be a significant factor for the levels of monoamines and neuropeptides in dementia. Circadian changes were also observed when measuring noradrenaline (NA), 5-HT and dopamine (DA) and their metabolites (Carlsson et al., 1980b). There is evidence of diminished concentrations of 5-HT and DA in various brain regions in Alzheimer's disease (Hardy et al., 1985), suggesting reduced activity of both systems. In a recent study, the concentrations of monoamines and various peptides in Alzheimer's disease and vascular dementia were measured (Wallin et al., 1991). They suggest that these changes may be important for the changes in circadian symptoms in dementia. 5-HT has been claimed to play an important role as a sleep-inducing neurotransmitter (Koella, 1974) and the high nocturnal activity of 5-HT favours its active role in sleep.

Lateralization

Fixing one hemisphere and freezing the other as currently done by many brain banks does not enable to recognize left-right differences.

Several functions are asymmetrically represented in the left or right hemisphere; lateralization of norepinephrine has been demonstrated in the human brain (Oke et al., 1978) and there is evidence for a left prominence in the distribution of Thyroid releasing hormone (TRH) (Borson-Chazot et al., 1986) with higher concentrations in the left side. Consequently it is preferential to sample bilaterally and if not possible, mention on which hemisphere the measurements have been performed.

Postmortem factors

Postmortem delay

The time between death and fixation or freezing of the tissue is important not only from a neurochemical viewpoint but also for several morphological parameters. Some substances in the brain are very unstable but there are many important neurochemical substances which are very stable, and neuropeptides appear to share this property. Correlating the post-mortem delay to various chemical variables revealed that there was a significant negative correlation between this time variable and noradrenaline (NA) and normetanephrine (NM) levels. On the other hand, a positive correlation was found for the levels of the amino acids tryptophane and tyrosine (Gottfries et al., 1980). Immunocytochemical (ICC) procedures are less sensible to the postmortem delay; an excellent staining of vasopressin neurons in the SON and PVN is obtained on tissue which was fixed 48 hours after death. A similar stability up to postmortem time of more than 60 hrs was found for extrahypothalamic vasopressin fibers (Swaab, 1982; Fliers et al., 1986). ICC staining of formaline fixed postmortem tissue using the monoclonal antibody Alz-50, results in excellent staining of the three main hallmarks of AD, namely: senile plaques, neurofibrillary tangles and dystrophic neurites (Fig. 1).

Quantitative autoradiography has been used to study the localization and regional distribution of transmitters, enzymes and receptor binding sites in postmortem human brain in both normal and diseased tissue. This approach appears to be particularly valuable as most transmitters and binding sites appear to be quite stable in postmortem tissue (Hardy and Dodd, 1983). Binding studies of imipramine and desmethylimipramine (DMI) in postmortem human brain have revealed the highest density in the hypothalamus (Langer et al., 1981; Gross-Isseroff and Biegon, 1988; Gross-Isseroff et al., 1988; Cortes et al., 1988). The postmortem delay and sex did not affect imipramine or desmethylimipramine binding.

It is possible to use postmortem tissue to examine the antemortem regulation of a human neuroendocrine gene. Neuropeptides are known to play a major role in neural transmission in the brain and changes in these compounds occur in various neurodegenerative disorders. To be able to interpret the dynamics of neuropeptides, it is also important to investigate neuropeptide messenger RNA (mRNA) in human brain in addition to the

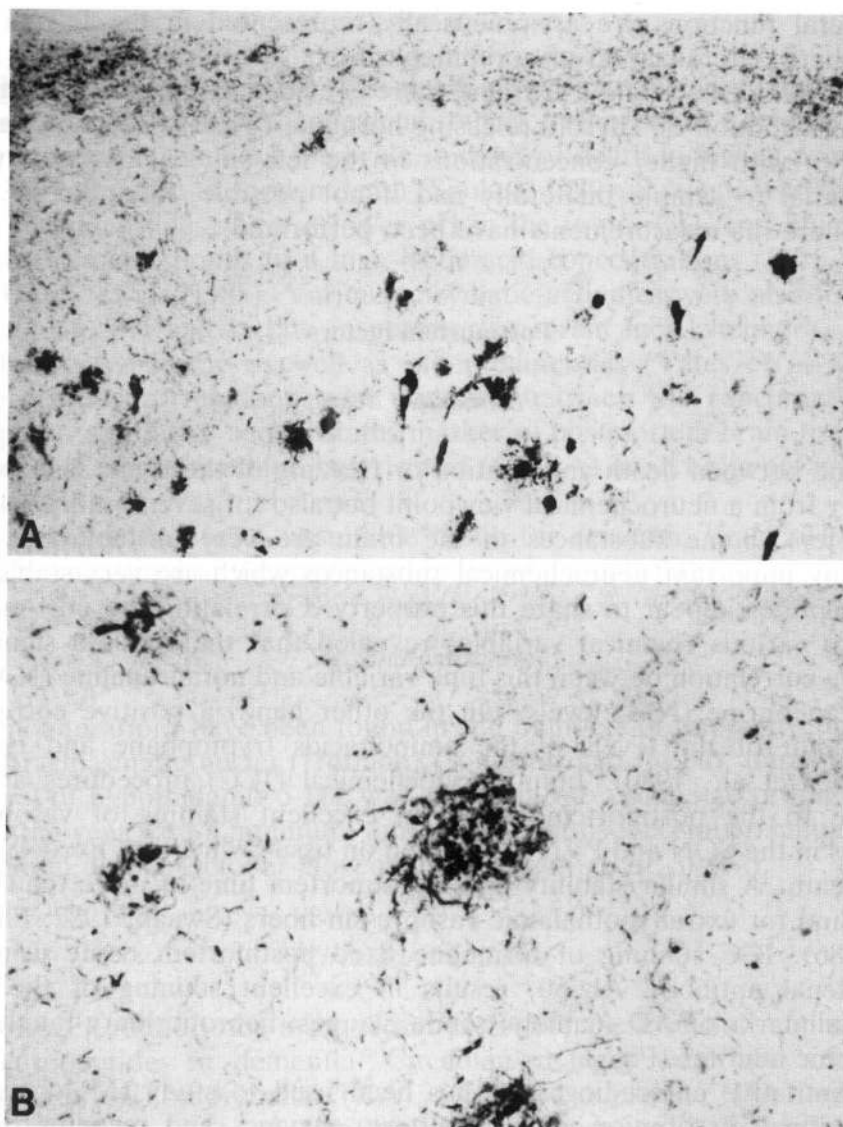


Fig. 1. Immunocytochemical staining with Alz-50 in frontal cortex of a 88-year old woman affected by Alzheimer's disease. **A** Neurofibrillary tangles — note the heavily stained neurons and the densely stained dystrophic neurites all through the section. **B** A plaque — note the enormous amount of dystrophic neurites surrounding the plaque. (Photograph by G. van der Meulen)

neuropeptides themselves. Modern molecular biological techniques allow the study and quantification of those mRNA's by using various hybridization techniques. RNA stability has been investigated in several studies, with some discrepancy between results. An extensive postmortem stability of total RNA has been reported in rat and human brain up to 48 hrs and 36 hrs respectively. Both the yield and the integrity of RNA stayed unchanged during the postmortem period (Johnson et al., 1986; Kobayashi et al., 1990).

Vasopressin (preproressophysin) m-RNA has been detected in neurons of the SON, PVN, SCN by quantified in situ hybridization (Rivkees et al., 1989). This technique has been also applied for the study of other neuropeptide gene expression in postmortem tissue. No significant correlations are found between the density of the hybridization signal and parameters such as postmortem delay, age and sex (Mengod et al., 1990). The post-mortem stability of arginine-vasopressin (AVP) messenger RNA (mRNA) in the rat brain was studied and revealed that AVP mRNA was degraded postmortem more rapidly than rRNA (Noguchi et al., 1991). These results suggest that autopsied human brain should be used for AVP mRNA study within a short postmortem time.

Alterations in neurotransmitter and drug receptors in various neurodegenerative disorders can be studied on autopsy material. The effect of postmortem delay and prolonged storage of the tissue prior to performing the binding assays may limit the interpretation of the disease related changes in receptor populations. The understanding of receptor changes associated with the disease have implications for the development of therapeutic strategies by using drugs that modify receptor function. In order to be able to claim that changes in receptors found in human brain result from the disease process being studied, it is important to exclude other factors which may affect ligand binding. Autolysis of tissue due to the postmortem delay and the freezing process of the tissue may both contribute to such changes, alter the receptor and affect the binding of a ligand.

The post-mortem delay is an essential variable due to the degradation of various tissue constituents. It would be therefore an important development for brain banking if more neurobiological techniques would be adapted for human tissue with a relatively long postmortem delay.

Freezing procedures, fixation and storage time

Changes in those factors may affect many of the parameters used to assess changes in the brain and the potentialities of staining procedures considerably. On the other hand, some tissue components are not very sensitive to these factors.

Human brain tissue used for biochemical studies is usually rapidly frozen and slowly thawed. However, to isolate synaptosomes which are morphologically well preserved and have retained their metabolic performance one should use the opposite procedure as snap-freezing generally yields metabolically and functionally inactive preparations (Hardy et al., 1983).

It is noteworthy that a large number of metabolic and functional processes as well as binding capacity of various receptors are retained surprisingly well in frozen tissue. That way it becomes possible to study regional variations, distribution and comparative activities of various transmitters or drugs in normal and diseased brain and correlate them to the anatomical changes.

Fixation in formalin causes increase in brain weight and the subsequent washing in water introduces a systematic error in brain weight, e.g. larger brains gain more weight than small brains, brains from younger individuals do not gain more in weight than older ones when the difference in fresh brain weight between the two groups was taken into account.

Similarly, the increase in brain weight is not sexually dimorphic when the fresh brain weight is taken into account (Skullerud, 1985). On the other hand, a strikingly interesting difference was observed by the same author in increased brain weight after fixation in patients dying after a protracted illness than in those who had a sudden death.

The fixation procedure used and storage of the fixed tissue prior to the application of ICC procedures may affect the staining. Vasopressin immunoreactivity was present in material which has been fixed and stored for more than 50 years (Swaab, 1982). Conventional formaldehyde fixation for one month results in excellent vasopressin and oxytocin staining of the SON and SCN neurons (Fliers et al., 1985; Swaab et al., 1985). However, this procedure was not suitable for studying the extrahypothalamic fibers of these peptidergic neurons which had been observed in rat brain. Our experience on rat material is that storage in glutaraldehyde-paraformaldehyde fixative preserves immunoreactivity of vasopressin, oxytocin and alpha-MSH for more than a year (Ravid, unpublished results). Since immersion in this fixative does not fully penetrate the human brain, fresh tissue blocks can be fixed by immersion in 2.5% glutaraldehyde and 1% paraformaldehyde for one week. Subsequently the blocks are frozen and stored in sealed plastic at -80°C and cryostat sections are used for ICC. This procedure gave an increased sensitivity of the ICC procedure and resulted in staining of VP fibers in the human brain (Fliers et al., 1986).

All the observed changes in postmortem brain mentioned in this paper have clear consequences for brain banking procedures. One should use a constant protocol of fixation and subsequent washing and embedding procedures in order to minimize the systematic errors and variables. Our group had used in the past 10 years a constant procedure; brains obtained at autopsy are fixed for 1 month in 10% buffered formalin at room temperature before further washing and embedding in paraffin blocks (Fliers et al., 1985).

Conclusions

The study of the various processes occurring in the human brain can be undoubtedly best carried out on autopsy material. The availability of this material, whether fresh, frozen or fixed, makes it possible to develop methodologies for studying the neuroanatomical, neurochemical and functional aspects of the human brain. It has also become possible in recent years to correlate functional changes with neurochemical changes and with neuroanatomical abnormalities in disease states.

Some brain functions are damaged irreversibly within minutes after death and some brain components are known to disintegrate within seconds. This led to the wide spread idea that autopsy material obtained after death would not be suitable for research purposes and would not supply the necessary answers on the various relevant questions regarding processes occurring in normal or diseased brain. However, reading the literature and data published in recent years in which autopsy material has been routinely used, it becomes more and more evident that this is a misconception. It also became evident that when using the proper fixation procedures, sufficient structural integrity is retained in the tissue to allow morphological and morphometrical studies (Swaab and Uylings, 1988). Electron microscopic examination of synaptosomal preparations from postmortem human brain showed them to be only slightly less pure than preparations from fresh tissue although there was some degree of damage (Hardy et al., 1982).

Agonal state affects the stability of brain compounds and causes brain hypoxia. This creates again a tremendous difficulty for the study of human neurological and psychiatric diseases as one of the frequent causes of death is bronchopneumonia which leads to brain hypoxia and results in pronounced lactic acidosis. The Netherlands Brain Bank has succeeded to partly circumvent some of the serious problems encountered in providing human tissue for research by performing rapid autopsies with an average postmortem delay of 2–4 hours. We also test the pH of the tissue and discard all unsuitable specimens.

The analysis of postmortem human brain data is extremely difficult. The interpretation of the various results must be done with great care to exclude false correlations due to the heterogeneity of the material with respect to the various factors mentioned above in detail. It is evident that there are numerous possible pitfalls to be encountered when human brain tissue is studied with the conventional neuroanatomical techniques. Matching for the various ante and postmortem factors is an essential step towards obtaining meaningful results.

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