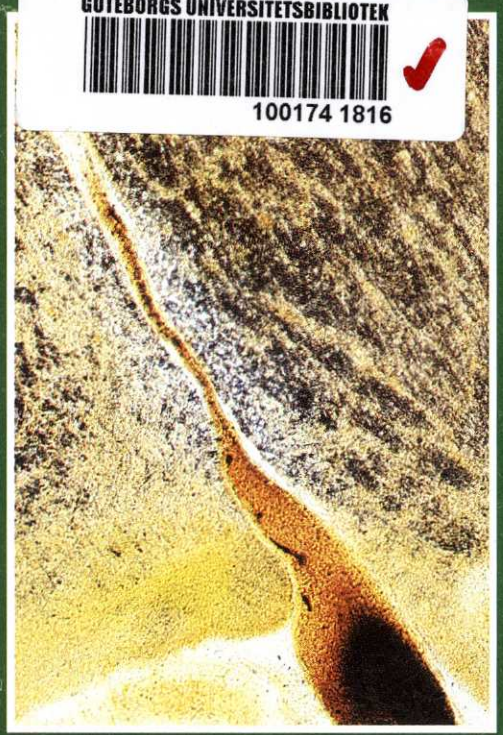
Capillary Electrophoresis

a Tool in Neuroscience and Immunology

Jonas Bergquist



Göteborg 1996

Doctoral thesis from the Institute of Clinical Neuroscience,
Department of Psychiatry and Neurochemistry, Göteborg
University, Mölndal Hospital, Sweden

Cover illustration: The light microscopy picture on the cover page shows a capillary blood vessel in medulla oblongata from human brain. This photograph was choosen to illustrate the connection between the central nervous system and the immune system. Further, the capillary could be seen as the capillary tube used for the electrophoretic technique described in this thesis. This is also a histological piece of history that I have found among the histological slides that I inherited from my grandfather Dr. Gerhard Widlund. The medulla oblongata came from the first (and last) convict that was executed with a guillotine at Långholmen, Stockholm, 08.07 in the morning, 23 november 1910.
Cover page colour: British racing green

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AKADEMISK AVHANDLING

som för avläggande av doktorsexamen i medicinsk vetenskap offentligen kommer att försvaras i Aulan, Mölndals sjukhus, fredagen den 31 maj 1996, kl 13.00

av

Jonas Bergquist

Avhandlingen baseras på följande delarbeten:

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Abstract

One of the key objectives of modern neuroscience is finding methods for detecting and analysing specific molecules that help us understand the mechanisms of signal transduction between cells both within the central nervous system (CNS), and between the CNS and peripheral systems.

This thesis describes the development and application of capillary electrophoresis (CE) to the neurochemical analysis of biological fluids and tissues, with examples in neurodegenerative disorders, neuropharmacology, and in neuroimmunology. CE was developed in the late 1980's, more than twenty years after the dawn of high performance liquid chromatography (HPLC). This technique offers a possibility for fast, efficient separation of a variety of molecules, ranging from metallic ions to macromolecules. The method permits sampling from microenvironments (i.e. single cells and subcellular compartments), provides excellent efficiency ($N>10^6$), high sensitivity (single molecules), all of which are of great importance to the areas of neurobiology and neurochemistry. The presented applications make use of three different detection methods; laser-induced flourescence, UV-, and electrochemical detection. These include separation and analysis of molecules from the nervous system using human lumbar cerebrospinal fluid (CSF), extracellular fluid obtained by microdialysis sampling in rat cerebrum, single human CSF lymphocytes, and of molecules in microextracts of peripheral immunocompetent cells of human and murine origin. Employing laser-induced fluorescence detection, analysis of amino acids in CSF has been performed in patients with various neurodegenerative disorders, and the results indicate a clinical application. Furthermore, in two neuropharmacological approaches, transient changes of amino acids after potassium or morphine stimulation in specific brain regions were recorded using microdialysis. By using UV-detection, analysis of the synaptic vesicle protein, synaptotagmin, in lumbar CSF has been performed. Finally, by using electrochemical detection, easily oxidised species like catecholamines were detected, and intracellular and intranuclear catecholamines have been for the first time detected in immunocompetent cells. These catecholamines were shown to have a regulatory function upon the cells by reducing proliferation and differentiation, and finally by induction of apoptosis - programmed cell death. These findings provide support for the existence of a mechanism connecting the CNS and the immune system, whereby the CNS may influence the immune system and vice versa. In summary, CE is shown to be a powerful tool in both neuroscience and immunology.

Key words: absorbance, amino acids, amperometric, apoptosis, capillary electrophoresis, detection, electrochemical, laser-induced fluorescence, lymphocytes, microdialysis, neurodegenerative disorders, neuroimmunology, neurotransmitters, synaptic vesicle proteins

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List of papers

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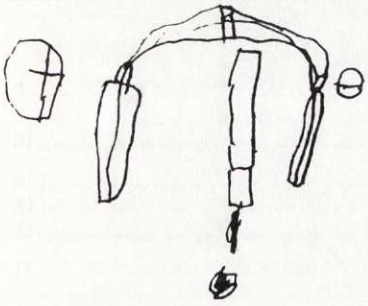

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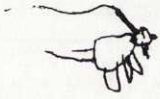

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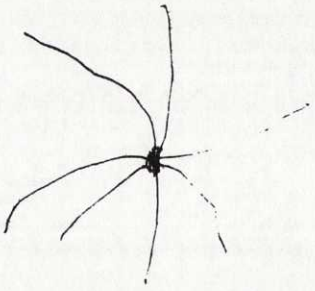

NERVCELL

Table of contents

Abbreviations

Ab	antibody
AD	Alzheimer's disease
Ag	antigen
amol	attomole (10^{-18} mole)
AU	arbitrary units
BBB	blood-brain barrier
BCIP	5-bromo-4-chloro-3-indolyl phosphate
B-END	β-endorphin
BSA	bovine serum albumin
CBQCA	3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CNS	central nervous system
COMT	catechol-*o*-methyltransferase
Con A	concanavaline A
CSF	cerebrospinal fluid
CZE	capillary zone electrophoresis
Da	dalton (unit of molecular mass)
DA	dopamine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DSM-III	diagnostic statistical manual, 3rd ed.
DTT	dithiothreitol
EAD	early onset Alzheimer disease
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraaceticacid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
ENK	enkephalin
EOF	electroosmotic flow
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
fmol	femtomole (10^{-15} mole)
GABA	γ-amino-n-butyric acid
HEPES	n-(2-hydroxyethyl)piperazine-n'-(2-ethanesulfonic acid)
HF	hydrofluoric acid
HPLC	high performance liquid chromatography
HV	high voltage
i.d.	inner diameter
IEF	isoelectric focusing
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
ITP	isotachophoresis
LAD	late onset Alzheimer disease
L-DOPA	3,4-dihydroxy-L-phenylalanine
LIF	laser-induced fluorescence
LPS	lipopolysaccharide
MAb	monoclonal antibody
MAO	monoamine oxidase
MEKC	micellar electrokinetic chromatography
MES	2-(*n*-morpholino)ethanesulfonic acid
MeTyr	α-methyl-*p*-tyrosine
MHC	major histocompatibility complex
MHPG	3-methoxy-4-hydroxyphenylglycol
μmol	micromole (10^{-6} mole)
MNC	mononuclear cells
NBT	nitro-blue tetrazolium
NDA	naphthalene-2,3-dicarboxaldehyde
NE	norepinephrine
nmol	nanomole (10^{-9} mole)
o.d.	outer diameter
OPA	*o*-phtaldialdehyde
PAG	periaqueductal grey matter
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PVDF	polyvinyl difluoride
pmol	picomole (10^{-12} mole)
PWM	pokeweed mitogen
RIA	radioimmunoassay
RNAse	ribonucleic acidase
RP	reversed phase
SDS	sodium dodecyl sulphate
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEM	standard error of the mean
TFA	trifluoroacetic acid
TNF-α	tumour necrosis factor-α
Tris	tris(hydroxymethyl)-aminomethane
UA	uric acid
UV	ultraviolet light
ymol	yoctomole (10^{-24} mole)
zmol	zeptomole (10^{-21} mole)

1. Introduction

Grundforskning är vad jag gör när jag inte vet vad jag gör
Wernher von Braun

The complexity of the mammalian brain and sensory organs is immense. In no other system is the signalling within and between cells so poorly understood. This signalling, or neurotransmission, is based on substances that, upon release from nerve terminals, act on receptor sites at postsynaptic membranes to produce either excitation or inhibition of the target cell. Often endogenously produced neuroactive substances, neurotransmitters like catecholamines, amino acids, peptides, and proteins, are present in picomolar to millimolar intracellular concentrations, resulting in extracellular levels at the femtomolar to micromolar range. In order to monitor the small amounts of biomolecules in small volumes, and to detect minor changes in these trace levels, sensitive and selective analytical methods need to be developed.

The application of different chromatographic techniques[1] began with the work of Runge in 1850[2]. He observed that certain coloured substances when spotted onto a filter paper spread out into concentric rings. Afterwards Rounge, Schönbein in 1861[3], and his student Goppelsröder[4], together developed the capillary analysis based upon the height to which the various components are sucked up by a filter paper. However, modern chromatography as we know it today was first presented by Tsvett in 1906[5], resulting in the development of paper-, thin-layer-, gas- and liquid chromatography. These techniques have contributed to structural and funtional data on many new biomolecules during the last century.

Due to the heterogeneity of cell types in the nervous system, it would be ideal to have methods that allow studies of these biomolecules at the single cell level. The analysis of neurotransmitters both at the single cell level, and in tissue samples comprised of various preparations of cells from the nervous system is an area of great importance, where capillary electrophoresis (CE) has emerged as a powerful tool. This has been the subject of recent reviews[6, 7]. Briefly, CE has been employed in the

analysis of neuroactive substances (i.e. proteins, neuropeptides, amino acids, catecholamines, thiols and polyamines) in combination with a large variety of sampling techniques. For instance, in microdialysis sampling (for references see Paper III and Gilman and Ewing, 1995[7]), sensitivity and selectivity, low sample volume, and fast analysis time of the CE method, has increased the temporal resolution of this sampling technique dramatically. Also, in combination with the push-pull cannula sampling technique, CE analysis of, for example, neuropeptides have been performed[8]. Furthermore, the direct analysis of human cerebrospinal fluid (CSF) has been accomplished as well as the analysis of neurotransmitters in tissue, single cells, and cell cultures. The detection of neuropeptides has been a most challenging goal since they exist in very low concentrations and are difficult to detect with most analytical methods[9-12].

With the developement of new CE separation and detection methods, their application to neuroscience and neuroimmune interactions has moved forward. The following is a more thorough description of the applications, where CE has been employed in this thesis.

CSF, secreted mainly by the choroid plexus, is the body fluid which is most likely to reflect disturbances in the central nervous system (CNS). The CSF is a serum filtrate, i.e. ~70% of the proteins it contains are plasma derived and ~30% of the proteins are related to the brain tissue. The CSF exists at equilibrium with the extracellular fluid surrounding the CNS cells, and is known to provide a constant chemical environment for the cells within the brain. Among the molecules found in CSF are a wide spectrum of neurotransmitters or neurotransmission derived molecules, e.g. amino acids, amino sugars, short peptide fragments, neuropeptides and larger proteins. Measurement of such molecules might potentially be useful as markers, to elucidate the CNS involvement in various diseases. Furthermore, these markers may assess the degree of damage in the CNS and enable an estimation of the prognosis. Finally, they might be used to evaluate the effects of pharmacological interventions.

As an example changes in amino acid levels in CSF (as described in Paper I) have been noticed in many CNS diseases, i.e. chronic schizophrenia[13], multiple sclerosis[14], Parkinson's disease[15], Alzheimer's disease (AD)[16], and other CNS related disorders[17-22]. These changes have been analysed employing a large variety of techniques, including reversed-phase chromatography, ion-exchange chromatography and isotachophoresis. There are, however, only a few reports on the analysis of human CSF by CE, using either of the UV-detection[23, 24] or laser-induced fluorescence detection (LIF)[25] technique.

Our study (Paper I) was the first to present any quantitative data from patients with different disorders. Just recently a study of free amino acids and primary amines in CSF from leukaemic children was reported[26], further indicating the usefulness of CE in clinical studies.

During an ongoing neurodegenerative process, a higher turnover rate within nerve cells and of synapses may be reflected as an increase, or decrease, in related molecules. The turnover rate of synapses might be monitored by measurements of synaptic vesicle proteins in CSF. The process of synaptic transmission involves many different proteins, e.g. rab3a, synaptophysin and synaptotagmin, all of which have important functions in vesicle trafficking, docking to, and fusion with the synaptic plasma membrane.

In Paper II the development of a method for analysing one of these synaptic proteins (i.e. synaptotagmin) in CSF was presented. In this process, CE was employed as a tool to study the purity and the characteristics of the protein. CSF markers might help to guide clinicians in their decisions regarding treatment, and help to indicate the disease prognosis. Furthermore the CSF markers might give valuable clues to solve the pathophysiological mechanism behind CNS diseases.

One of the most exacting methods used to monitor CNS changes is the microdialysis technique[27], which permits continuous monitoring of extracellular levels of biomolecules in vivo.

Microdialysis is a technique where a small probe tip with a semi-permeable membrane is placed inside the tissue of interest. On the inside of the probe a solution is pumped past the inner surface of the membrane. Analytes are osmotically transported across this membrane from high concentration to low. This technique offers the advantage that no volume of fluid is being removed from the tissue. The probe may also be used to deliver drugs or bioactive substances to a local area of the brain, with subsequent sampling by the same probe. Most studies using microdialysis sampling have been performed in rat cerebrum, however clinical applications in humans have been reported[28]. As this sampling technique involves a continuous flow of perfusate fluid across a membrane, a rather high dilution factor for sampled molecules occurs. Thus sensitive and selective analytical methods are required to be able to monitor small changes in trace levels.

In Papers III-IV the use of a sensitive CE method is described, employing laser-induced fluorescence detection and pre-column derivatization of the samples. In Paper III potassium induced release of neuroactive amino acids in the periaqueductal grey (PAG) matter is studied. This data is directly compared with two different HPLC methods which are commonly used for these kinds of analyses. In Paper IV the effect of morphine on the basal release of γ-amino-n-butyric acid (GABA) is monitored, and the activation of the μ-receptor on the GABA interneuron in the PAG has been studied. The high sensitivity of the CE-LIF method is one tremendous advantage which made it possible to follow these small changes at the nanomolar level.

As stated above, CE has become an effective analytical tool with many applications to bona fide neurobiological questions. Its application in immunology, however, has not been widespread. There is a wide variety of molecules to study in neuroimmunological interactions, with a multitude of characteristics. The most examined class of molecules are the cytokines, or neurokines, due to their regulatory functions in nervous tissue as well as in the immune system. It is becoming increasingly clear that the connections between the immune and nervous systems are involved in many functions of the mammalian life cycle[29]. Classical neurotransmitters

regulate the central and peripheral nervous systems, but these regulators may in fact be acting as immunotransmitters or neuroimmune transmitters. CE should be a very powerful technique for the separation and detection of neuroimmune transmitters.

The main analytical tool for immune molecule detection is enzyme linked immunosorbent assay (ELISA)[30]. This method provides good sensitivity with detection limits down to picogram levels. An alternative to this method is the ELISPOT assay[31], where products from a single cell can be detected. In addition some cell clones specifically responding to a specific interleukin by proliferation can be used to detect a given cytokine. The proliferative response can then be analysed by ^{3}H-thymidine incorporation. In many immunological methods the results are given as titers, not as absolute concentrations. As our knowledge of immunological interactions deepens, the need for quantitative methods increase.

In the few published applications of CE in immunology to date, immunological properties, e.g. antibody-antigen interactions, have been used as tools in CE, but CE has not been used as a tool in immunology. The possibility of resolving a large variety of molecules, i.e. small ions to large macromolecules, has been another advantage of this method. Earlier applications of CE were more concerned with pure protein chemistry than with immunology, especially concerning the analyses of recombinant proteins and hydrolysis of these proteins[32-36]. The purities of antigen binding $F(ab')_2$ regions, antibodies, as well as the thermal stability of antibodies have all been assessed using CE[37-41]. The technique has also been applied to the analysis of the nucleotide pools in lymphoma cells, in ucon-coated columns with on-column UV-detection[42]. Yet another important application of CE has been in immunoaffinity assays where the mobility shift due to the binding of an antibody to an antigen is used for detection and quantification. CE with laser-induced fluorescence detection, having low detection limits and good separation capacity, is a more than suitable system for this type of assay. However, this technique requires an antibody with a high specificity and that the labelling of the antigen results in a complex of antigen-antibody that is well separated from the non-labelled complex (Figure 1).

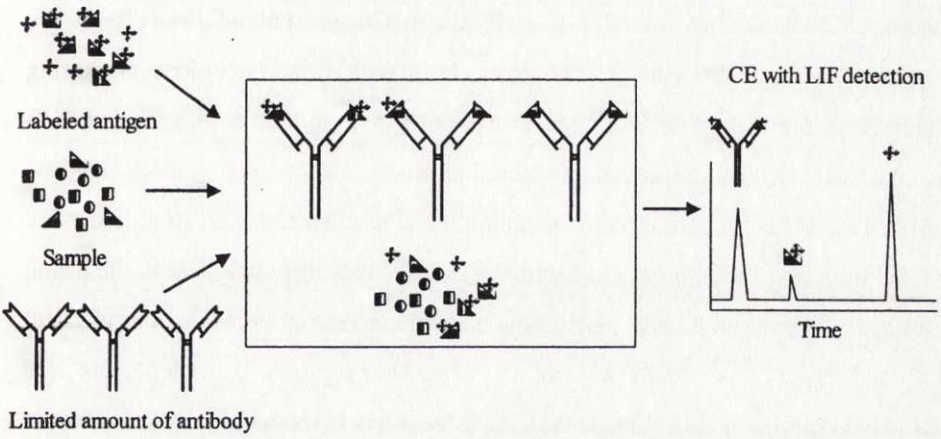

Figure 1. A schematic drawing of the competitive immunoassay procedure.

In this competitive immunoassay, the amount of free labelled antigen is directly proportional to the amount of antigen present in the sample, as seen in expression (1), where Ag=antigen, Ag*=labeled antigen, and Ab=antibody:

$$Ag + Ag^* + Ab \Leftrightarrow Ag\text{-}Ab + Ag^*\text{-}Ab \qquad (1)$$

This technique has been used to assay several different antigens (for background see[43-46]). By using affinity CE enzyme interactions[47], protein-protein interactions[48-52], antibody-antigen interactions[53-60], protein-carbohydrate interactions[61-63], and protein-drug interactions[64-68] have been determined. A modification of the affinity methods is the use of protein G immunoaffinity for preconcentration in CE[69]. To use CE as a tool in immunology, we reported the analysis of single lymphocytes from human CSF by CE with amperometric detection (Paper V). This analysis led to the discovery of endogenous catecholamines in cells derived from the immune system. Thus extracts of ex-vivo and cloned lymphocytes were also examined for their catecholamine content. The possible effects of catecholamines on lymphocytes were studied by analysing their

proliferative response and differentiation (Papers V-VI). Both the proliferation and the differentiation of the immunocompetent cells were significantly suppressed by exposure to catecholamine. In order to clarify the mechanism behind these effects, the cells were examined for signs of apoptosis, both with flow cytometry, measuring genomic DNA fragmentation, and by measuring of apoptotic marker proteins (i.e. Bcl-2/Bax and Fas/FasL) using western blot techniques.

Apoptosis is the process whereby developmental or environmental stimuli activate a genetic program to accomplish a specific series of events that culminate in the death, and efficient disposal of a cell. Necrosis is usually considered to result from physical injury, and is not genetically controlled, whereas apoptosis is a deliberate and genetically controlled cellular response. Also, necrosis is recognised by cytoplasmic organelle destruction and loss of plasma membrane integrity, whereas apoptosis is associated with cytoplasmic boiling, chromatin condensation, and nuclear DNA fragmentation[70]. However an event that produces necrosis may trigger apoptosis in surrounding tissue as a result of the accumulatation of cellular debris. Likewise apoptosis may indirectly produce necrosis under some conditions. As seen in Paper VI, an apoptotic mechanism involves a rather complicated series of actions, with up- and down-regulation of different proteins (such as Bcl-2/Bax and Fas/FasL). The Bcl-2 protein is known to protect against apoptosis[71, 72], so a decrease of this protein with an increase of Bax, an apoptosis induced protein[73] that forms heteromers with Bcl-2, strongly suggests an ongoing apoptotic process. The same is valid for the expression of Fas. The Fas/FasL interaction is believed to be one of the mechanisms involved in suppression of the immune response and in peripheral tolerance[74, 75]. Antigen presenting cells express on their surface an autoreactive antigen as a complex with the major histocompatibility complex (MHC). The antigen-MHC complex interacts with the T cell receptor on autoreactive T cells. This activates the cells and induces the expression of Fas and FasL. These cells then undergo apoptosis through the interactions between Fas and FasL. Apoptosis of T cells can take place when Fas and FasL are located on different cells, on the same cell, or by release of soluble FasL.

Both the flow cytometric method and the analysis of apoptotic marker proteins provided evidence of a specific induction of apoptosis in PBMC by catecholamines (Paper VI). In a parallel study, the presence and effects of catecholamines in cells of murine origin, were examined (Paper VII). This clearly demonstrates that the mechanism is general within mammals, and may become an important factor to consider in neuroimmunology.

In this thesis, consisting of seven separate papers, three different detection modes for CE have been used. These include two different utilisations of CE in neuroscience with special applications for the detection of early markers for progressive neurodegenerative disorders. In addition, two different applications of CE in neuropharmacology, where neuroactive amino acids have been monitored combining CE and microdialysis in rat cerebrum, are presented. Finally, three different applications of CE in neuroimmunology are reported.

The main goal with these studies has been to further develop available CE methods, and by further employing this versatile technique, attempt to clarify some of the questions that have evolved in neuroscience today. The primary issue has not been to push for analytical bench-top markings, but rather to apply the technique to relevant biological and clinical inquiries, believing that the quality and applicability of an analytical method can not solely be judged from separations of pure standards.

Hjärnan är ett underbart organ. Den börjar arbeta i samma stund som du vaknar på morgonen och slutar inte förrän du kommer till jobbet

Robert Frost

2. Principles of capillary electrophoresis

A large number of review articles[6, 7, 76-99] and books[100-106] have been published, describing various aspects of CE. Hence, only a short background and description of the CE method is given. There are several different modes of CE, i.e. capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), isotachophoresis (ITP), and isoelectric focusing (IEF). In the present thesis only two of these modes have been used, CZE and MEKC.

Free zone, or CZE, can be used for many different types of molecules. Separations are based on variations in mass-to-charge ratios of the analytes. Optimising a CZE separation involves selection of a buffer pH which maximises the differences in mass-to-charge ratios, selecting a polarity appropriate for the sample charge, and applying a field strength which provides the best compromise between analysis time and zone sharpness.

MEKC is a separation technique, which in contrast to CZE, permits resolution of neutral as well as charged molecules. This is possible due to a combined effect of electrophoretic migration and micellar partitioning. Micelles are spherical aggregates of surfactant molecules, with an hydrophilic outer surface and hydrophobic interior. Sample components have different degrees of interaction with the micelles, which in turn serve as a pseudostationary phase within the capillary. Sodium dodecyl sulfate (SDS) is the most commonly used surfactant, and forms an anionic micelle which has an electrophoretic mobility opposite to the bulk flow in the capillary. The following principles are applicable for most CE modes; however, some exception do exist.

Historical perspective of electrophoresis and its development

In Sweden there has been a long tradition of electrophoresis from the early part of this century. Electrophoresis has been defined as the differential movement of ions by attraction or repulsion in an electric field. Arne Tiselius' development of the moving

boundary electrophoresis technique[107], based on the first electrophoretic experiments performed by the Russian physicist Reuss in 1809[108], and the application of the method for examination of molecules in biological fluids was a major breakthrough for biochemistry. Placing protein mixtures between buffer solutions in a tube and applying an electric field, he found that sample components migrated in a direction and at a rate determined by their charge and mobility. This innovation rendered Tiselius the Noble Prize in 1948. In combination with the ultracentrifugation technique[109] developed by Tiselius' mentor The Svedberg (Noble Prize in 1926), this technique allowed for complex biological solutions to be both qualitatively and quantitatively analysed. However, the moving boundary technique had its limitations and the major drawback was the large inner diameter (i.d) of the glass tubes used.

Already as a preliminary note in 1958[110] and later in his thesis in 1967[111], the student of Tiselius, Stellan Hjertén, described a free zone electrophoresis method in rotating quartz capillaries (along their longitudinal axis), offering several methods for fast, efficient separation of ionic species and separation of macromolecules important in the area of analytical biotechnology. The lack of sensitive UV-detectors postponed the use of narrow-bore capillaries for some time; however, this together with the invention of fused silica capillaries in 1978 was the groundstone for the capillary electrophoretical methods we know today. During this period in Tiselius' and later Hjertén's laboratory, many other important bioanalytical techniques were designed: including adsorption chromatography, size exclusion gel chromatography, hydrophobic-interaction chromatography and polyacrylamide gel electrophoresis, which are still used today.

In 1968, Everaerts described an apparatus for displacement electrophoresis in his thesis[112], still in rather large i.d. (0.6 mm) tubes with the disadvantage of convection, and thermal zone deformation. In 1974 Virtanen reported on potentiometric detection of electrophoretically separated solutes in 200 to 500 μm i.d. Pyrex tubes[113]. This work dealt with zone electrophoresis, and pointed out many of the unique advantages of using small diameter tubes. In 1979 Mikkers et al. refined this technique to 200 μm

i.d. teflon tubes, and obtained separations with plate heights of less than 10 μm[114]. Jorgenson and Lukacs[115] advanced the area using even narrower capillaries (75 μm) which resulted in plate heights of just a few micrometers. Hjertén is still very active in research and has, over the last thirty years contributed with many new theoretical and practical approaches to CE, including coating the capillary walls with polymers in order to reduce the absorption of large molecules[116, 117], isoelectric focusing techniques[118], and improved sample handling[119].

Although CE was not offered as a commercially available instrument until the end of the 1980's, there is already an impressive number of applications of CE in a large number of areas, including biotechnology, pharmacology, neurobiology, etc. The number of CE papers is increasing exponentially, with approximately 50 new applications published per month. This has produced a total number in excess of 3000 published papers by the spring of 1996.

The theory of capillary electrophoresis

As CE technology has evolved, it has become apparent that an immense versatility in separation selectivity can be achieved by simple changes in the composition of the electrophoresis buffer. A CE instrument is a simple apparatus, both commercially available but also well suited for "home building" (Papers V-VII, Figure 2). In brief, the ends of a buffer filled fused silica capillary are placed in buffer reservoirs. Sample is loaded onto the capillary by replacing one of the reservoirs (i.e. normally the anodic one) with a reservoir containing the sample and applying an external pressure (Papers I-IV), an electric field (Papers V-VII), a vacuum at the outlet end, or by just causing a siphoning effect by elevating the sample reservoir. A high-voltage power supply is connected across the capillary creating an electric field that will cause the separation. Towards the cathodic end of the capillary, a detector is placed.

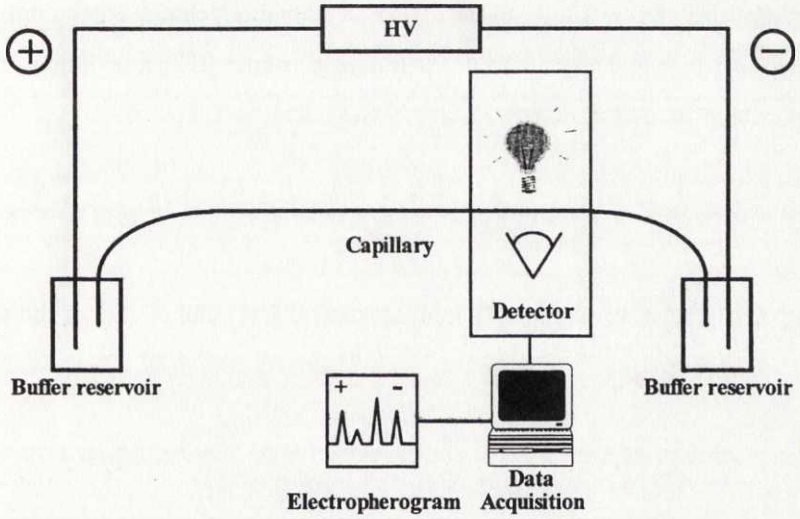

Figure 2. Schematical drawing of a principle CE instrumentation with a buffer-filled fused silica capillary positioned in-between two buffer reservoirs, across which a high potential is connected in order to create an electric field inside the capillary and cause a separation. Towards the cathodic end of the capillary a detector is arranged and often a computer is employed for the data aquisition.

Electrophoresis

Electrophoretic separation is based on differences in ion velocity in an applied electric field. This velocity can be calculated by equation (2), where v_e=ion velocity (cm/s), μ_e=electrophoretic mobility (cm^2/Vs), and E=applied electric field (V/cm):

$$v_e = \mu_e E \qquad (2)$$

The electrophoretic mobility (μ_e) can be calculated by use of the Stokes equation, where the electrical force acting upon an ion is balanced by the frictional drag it experiences passing through a medium. During electrophoresis a steady state is attained, where the forces are equal but opposite. The electric force can be calculated by equation (3), where F_E=electric force (N), and q=ion charge (C):

$$F_E = q E \qquad (3)$$

The frictional force for a spherical ion in a hydrodynamic continuum can be calculated by equation (4), where F_F=frictional force (N), η=solution viscosity (Ns/cm^2), and r=ion radius (cm):

$$F_F = -6 \pi \eta r v_e \quad (4)$$

At a steady state, the combination of equation (2), (3) and (4), gives the equation (5):

$$\mu_e = q / 6 \pi \eta r \quad (5)$$

From this equation it is evident that small, highly charged species have high mobilities whereas large, minimally charged species have lower ones. However this equation does not take into account the electric forces between analyte and solvent molecules, and does not apply to ions similar in size to the solvent molecules. Furthermore, the ionic shape, the attached solvent shell, the ζ-potential (the zeta-potential, i.e. the potential at the slip plane between the ion and the solution; the higher the surface charge, the higher the ζ-potential), the ionic strength, the pH (i.e. solute pK_a), and the temperature are all significant factors. Therefore, the physical constant of μ_e found in standard tables, determined at the point of full solute charge and extrapolated to infinite dilution often differs from the empirically found μ_e. The apparent electrophoretic mobility (μ_a in cm^2/Vs) can be calculated by equation (6), where L_{det}=length of the capillary to the detector (cm), L_{tot}=total length of the capillary (cm), V=applied voltage (V), and t_m=migration time (s):

$$\mu_a = L_{det} L_{tot} / t_m V \quad (6)$$

In order to calculate the actual mobility for a given substance in an electrophoretic separation the electroosmotic flow must be accounted for.

Electroosmosis

The phenomenon of electroosmotic flow (EOF) is a consequence of the silica surface charge inside the capillary. The ionizable acidic silanol groups on the capillary wall will dissociate and the surface will be negatively charged at pH values above 2. Positive ions in the buffer will act as counter ions and will be attracted to the negative surface. Some ions are adsorbed onto the surface, but no complete charge neutralisation will occur. The excess of hydrated positive ions in the vicinity will form a diffuse electrical double layer (Figure 3). The potential difference close to the wall is the ζ-potential. The ζ-potential (V) can be calculated by the equation (7), where μ_{eo}=electroosmotic mobility (cm^2/Vs), and ε=dielectric constant (As/Vm):

$$\zeta = 4 \pi \eta \mu_{eo} / \varepsilon \qquad (7)$$

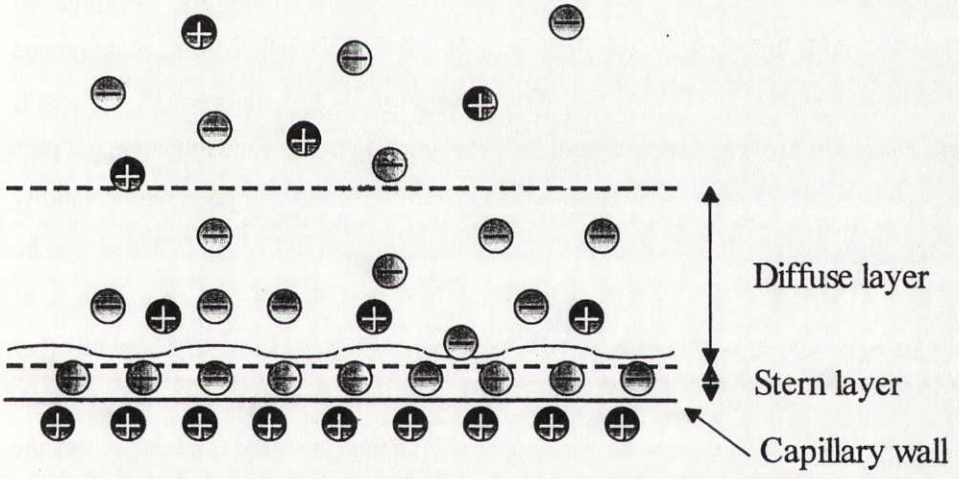

Figure 3. Schematic drawing of the double layer at the capillary wall.

The EOF is the bulk flow of liquid in the capillary, and results from the effect that the applied electric field has on the cations in this diffuse mobile part of the double layer, carrying water with them. Since the pH determines the charge of the surface, and the charge of the surface determines the ζ-potential, the EOF increases with increasing pH. The ζ-potential is related to the charge per unit surface area, the number of valence electrons, and the square root concentration of the electrolyte. Since this is an inverse relationship, increasing the concentration of the electrolyte, resulting in a compression of the double layer, will decrease the EOF.

There are many features to consider with the EOF in the capillary, among those, the flat flow profile (Figure 4). Since the driving force of the flow is uniform already close to the wall (at a distance approximately two times the thickness of the double layer), the pressure drop over the capillary is almost zero. Therefore, little or no dispersion of solute zones are found, in contrast to systems with parabolic flow.

Also, the EOF permits simultaneous analysis of cations, anions and neutral species in a single run. At high and neutral pHs, when the silanol groups are mostly deprotonated, the EOF is stronger than the electrophoretic mobility, sweeping all species towards the negative cathode (Figure 4). At low pH, when most silanol groups become protonated, the EOF is still directed against the cathode even if it is small; however, most zwitterions and cations will also migrate in the same direction and pass the detector. The EOF is sometimes hard to control, resulting in poor reproducibility of the separations. In order to control and change the EOF several factors can be varied. As already mentioned, the electric field strength, the pH, the ionic strength of the solvent, and the temperature (changes viscosity 2-3% per °C), are all very important for the EOF. Also, organic modifiers like dimethyl sulfoxide (DMSO, Papers I, III-IV) when added to the buffer, will change both the ζ-potential and the viscosity, and normally lead to a decreased EOF. Finally the EOF can be controlled by adding dynamic coatings (e.g. surfactants and hydrophilic polymers) to the buffer, or by covalently coating the capillary wall.

There are a number of other factors effecting the efficiency of a capillary electrophoretic separation, (i.e. sample adsorption, electrodispersion, laminar flow due to unlevelled buffer reservoirs or Joule heating, the injection plug length, and longitudinal diffusion), which in most cases can be controlled and calculated for under ideal conditions. However empirical experiments are often both simpler and faster.

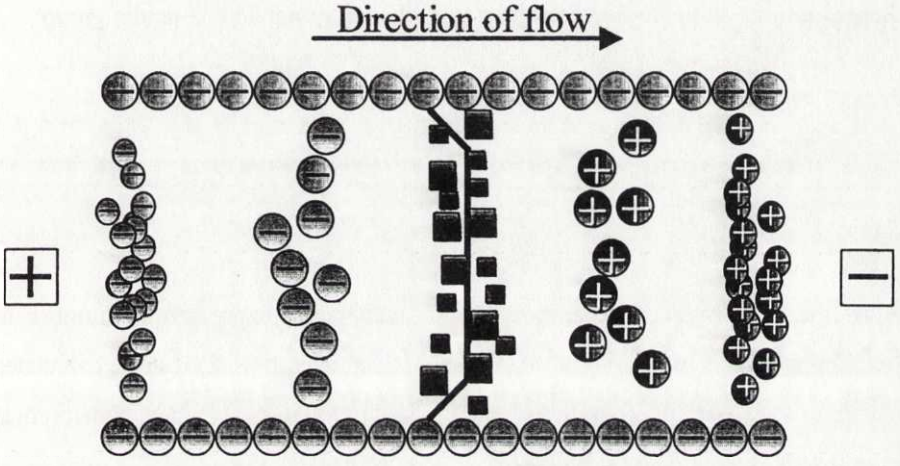

Figure 4. Differential analyte migration zones in a capillary, where small cations migrate fastest, small anions migrate slowest, and non separated neutrals are all carried at the velocity of the flat flow profile electroosmotic flow.

Analytical parameters in capillary electrophoresis

By using an electroosmotic flow marker (a neutral species), the EOF velocity can be empirically determined and a true or effective electrophoretic mobility for a given substance can be calculated by the equation (8), where μ_e=effective electrophoretic mobility (cm^2/Vs), t_{eo}=migration time for the electroosmotic marker (s), and where μ_e will be positive for a cation and negative for a anion:

$$\mu_e = (1 / t_m - 1 / t_{eo}) L_{det} L_{tot} / V \qquad (8)$$

The relative retention time (t_m/t_{eo}) for an analyte can also be calculated from equation (9)[120], where k= constant, M=molecular weight (g/mole) and Z=net-charge (C):

$$t_m/t_{eo} = k\, M^{2/3} / Z \qquad (9)$$

The net-charge for an analyte, can be calculated from equation (10)[121] by summing the charged groups on the molecule, where u_+= alkaline group and u_-=acidic group:

$$Z = u_+ + u_- \qquad (10)$$
$$u_+ = 1 / (1 + 10^{(pH - pKa)})$$
$$u_- = -(10^{(pH - pKa)} / (1 + 10^{(pH - pKa)}))$$

Also, the efficiency of separation for a given peak, expressed in number of theoretical plates (sometimes over 10^6 of them are achieved by CE), can be calculated directly from the electropherogram by equation (11), where N=number of theoretical plates, and w_b=baseline peak width (s) for a Gaussian peak:

$$N = 16\,(t_m/w_b)^2 \qquad (11)$$

Depending on the peak shape and signal to noise ratio, it is sometimes better to use the peak width at half the peak height ($w_{1/2}$), as shown in equation (12):

$$N = 5.54\,(t_m/w_{1/2})^2 \qquad (12)$$

or at the peak width at 10% of the peak height ($w_{10\%}$), as shown in equation (13):

$$N = 18.5\,(t_m/w_{10\%})^2 \qquad (13)$$

However, none of the above provided equations take into account the skewed peak in the determination of N.

Finally, the resolution between two adjacent peaks (R_s), can be calculated by equation (14), where t_{m1}, t_{m2}, w_{b1} and w_{b2}, are the migration time (s) and baseline peak width (s) for each peak respectively:

$$R_s = 2\,(t_{m2}-t_{m1}) / (w_{b1}+w_{b2}) \qquad (14)$$

Depending on the injection technique applied, the amount of injected material can be calculated or measured. When using pressure injection as in Papers I-IV, the minimum injection volume is approximately 1 nL. This is a rather large volume compared to the volumes injected by electrokinetic injection (Papers V-VII), where injection volumes down to 270 fL have been reported[122]. However, it should be noted that the pressure injection technique, in contrast to electrokinetic injection, delivers a more representative sample without bias according to the charge of the injected species. The volume injected with pressure injection can be calculated using equation (15), where Vol_i=injected volume (L), ΔP=pressure difference across the capillary (mbar), $d_{i.d.}$=inside diameter of the capillary (m), and t_i=injection time (s):

$$Vol_i = \Delta P\, d_{i.d.}^{4}\, \pi\, t_i / 128\, \eta\, L_{tot} \qquad (15)$$

The electrokinetic injection techniques involve both electrophoresis and electroosmosis, and the injected amount can be calculated by equation (16), where Q_i=quantity injected (mole), A=cross sectional area of the capillary (m^2), and C=concentration of the analyte (mole/L):

$$Q_i = (\mu_e + \mu_{eo})\, V\, A\, C\, t_i / L_{tot} \qquad (16)$$

Under normal electrophoresis conditions, cations will move faster than the EOF and anions slower. Thus, the apparent injection volume for cations and anions differs from the volume removed from the system. Thus, the apparent injection volume for each component may be calculated by equation (17), where Vol_a=apparent injection volume (L):

$$Vol_a = (\mu_e + \mu_{eo}) V A t_i / L_{tot} \quad (17)$$

Detection in capillary electrophoresis

In this thesis three different detection modes have been utilised (i.e. laser-induced fluorescence in Papers I and III-IV, absorption detection in Paper II, and electrochemical or amperometric detection in Papers V-VII). These will be shortly described below.

Laser-induced fluorescence detection

Optical detection techniques for CE have recently been reviewed by Pentoney and Sweedler[123]. The laser-induced fluorescence technique (LIF) has proven to be the most sensitive detection scheme for CE with reports of single molecule detection[124-127]. Despite the exceptional detection limits available, this technique is only beginning to be applied to neurochemically interesting materials. The laser provides a fine source of excitation due to its high intensity of monochromatic light and low beam divergence. In contrast with UV-detection, LIF directly obtains the signal against a dark background and is thus many orders of magnitude as sensitive. However, unless the natural fluorescence of a compound can be exploited[128], it is necessary to derivatize the analyte with a fluorescent label or "tag".

The explanation of fluorescence is easily found in physical chemistry textbooks[129]. Briefly, in an often used set-up, a fibre-optic cable transmits laser light from the laser to the detector and illuminates a section of the capillary. Thus, the initial absorbance

takes the molecule to an excited electron state by the transfer of an electron from its singlet ground electron state (S_0) to the higher singlet excited electron state (S_1). The fluorescence is the emission of photons due to the S_1-S_0 transition, when the molecule returns to its singlet ground electron state again. The emitted light (at a longer wavelenght compared with excitation light) is then collected by an ellipsoidal mirror and focused back onto a photomultiplier tube. In the excited state the molecule is subjected to collisions with the surrounding molecules (e.g. of the solvent), and as it gives up energy it steps down the ladder of vibrational levels. If the surrounding molecules can not accept the larger energy difference needed to lower the molecule to the ground electron state, it may undergo spontaneous emission, emitting the remaining excess energy as radiation.

The majority of CE-LIF applications involve pre-capillary labelling with reagents similar to those used with fluorescence detection in HPLC, although on-column derivatization for CE has been developed[130, 131]. Normally, the choice of agent is dependent on the nature of analyte and the laser available. Schwartz et al. recently gave an overview of available lasers for CE-LIF detection[132]. The derivatizing agent used in Papers I and III-IV, 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde (CBQCA)[133], forms highly fluorescent isoindole products, analogous to the products of *o*-phtaldialdehyde (OPA) and naphthalene-2,3-dicarboxaldehyde (NDA)[134], when reacting with a suitable nucleophile or compounds containing primary amines.

There are several important advantages realised by the use of CBQCA, including detection limits in the nanomolar range, excellent linearity, compatibility with MEKC, fast reaction time, and good product stability. Background fluorescence is minimal because CBQCA itself does not fluoresce. Hence, no purification of CBQCA derivatives is required prior to analysis. Unfortunately, most peptides and proteins present multiple sites, which can be tagged resulting in many labelled species. Interesting new developments include the employment of fluorescently labelled immunochemicals, described earlier in the introduction, and post-column derivatization[135, 136].

Absorption Detection

Detection by UV or visible absorption is the most common, commercially available technique used with CE, primarily due to its nearly universal detection nature. Typically, a small portion of the polyimide coating on the capillary is removed by heat or acid leaching to form an on-column detection window (see Figure 2). Detection is most often carried out by absorbance measurements from below 200 nm up through the visible spectrum, and many array and scanning detection systems are available. Since the optical window is directly in the capillary there is no zone broadening as a result of dead-volume or component mixing. However, due to the short path length resulting from the narrow bore of the capillary, it is a relatively insensitive technique, when compared to most other techniques, and is therefore usually not useful for the low-levels of neurochemicals typically extracted in neuroscience experiments. In fact, due to the curvature of the capillary, the actual pathlength in the capillary is less than the i.d., since only a fraction of the light passes directly through the centre. Since UV-detection is universal (i.e., suitable for many types of analytes), it is suitable to use for purity analysis and characterisation as in Paper II.

Electrochemical or Amperometric Detection

CE with electrochemical detection has been widely used to answer neurologically interesting questions, and was recently reviewed by Ewing et al.[98] This detection scheme has allowed the use of extremely small capillaries (down to 2 μm i.d.). In short, electrochemical detection involves electron transfer from an analyte, at a surface of an electrode. If the potential at the electrode surface is greater (more positive for oxidations, more negative for reductions) than that required for the electrolysis of the analyte, an oxidation of the analyte takes place and released electron(s) reach the electrode surface. This current is subsequently converted to a voltage which, through an interface is recorded, e.g. by a computer, and converted to an electropherogram. The resulting current is directly proportional to the concentration of solute reaching the

electrode surface, as can be seen from equation (18), where Q=number of charges (C), n=number of moles of electrons lost or gained in the transfer process per mole of material, N=moles of material (mol), and F=Faraday's constant (96485 C/mol of electrons):

$$Q = n\,N\,F \qquad (18)$$

Differentiation of equation (18) with respect to time yields the current, which is the measure of the rate at which material is converted. Equation (19) therefore relates a measurable quantity, the current (i), to the fundamental redox process occurring in the cell:

$$dQ/dt = i = nF\,dN/dt \qquad (19)$$

Fortunately, many neurochemicals of interest are easily oxidised in their natural state. This gives electrochemical detection two distinct advantages; first, no derivatization is needed and, second, as a consequence of the first, ultrasensitive detection (comparable to that offered by laser-induced fluorescence detection) is available for direct in vivo measurements. Amperometric detection has been accomplished both off-column (Paper V) and on-column (Papers VI-VII) in this thesis. The experimental set-up for off-column detection was earlier described in great detail by Wallingford and Ewing[137-142]. This apparatus employs a piece of porous glass tubing over a small crack in the capillary to allow electrical isolation of the separation potential field from the potential applied to the amperometric electrode.

The experimental set-up for on-column or end-column detection has been described in detail by Sloss and Ewing[143]. Briefly, the apparatus consisted of a capillary placed between two buffer reservoirs with high voltage applied at the injection end, and the detection reservoir containing the electrochemical detector was held at ground potential (see Figure 5 in Methods). Detection of the easily oxidised

analytes was performed in the amperometric mode with a two-electrode configuration. A carbon-fibre microelectrode was inserted into an etched funnel structure in the end of the capillary and held at the amperometric detection potential versus a reference electrode. Due to the large resistance in small i.d. capillaries ($\sim 10^{12}$ Ω), no decoupler is needed to shield the small electric current produced in the column upon application of the separation potential.

3. Aims of the study

Det är med idéer som med småbarn. Man tycker bäst om sina egna

Moa Martinson

- to explore CE with laser-induced fluorescence detection, as a new analytical tool in medical neuroscience for quantitative analysis of neuroactive amino acids in CSF (Paper I), and to monitor local transient changes of amino acid levels in microdialysis samples from rat cerebrum (Papers III-IV).

- to apply CE with UV detection as part of the analysis of synaptic vesicle proteins in CSF (Paper II).

- to develop CE with electrochemical detection as a tool in neuroimmunology, for the analysis of catecholamine neurotransmitters down to the single cell level (Papers V-VII).

- to apply these techniques to previously unanswered neuroimmunological questions, and to study the production of catecholamines in immunocompetent cells (Papers V-VII).

- to investigate how catecholamines can influence the reactivity of immunocompetent cells by regulation of proliferation, differentiation and induction of apoptosis (Papers V-VII).

4. Materials

Jag använder inte bara den hjärna jag har, utan också alla dem som jag kan låna
Woodrow Wilson

Patients (Papers I-II)

The studies performed with patient material was approved by the Ethics Committee, Göteborg University.

The pooled control

The control pool (pool CCSF, Paper I) consisted of 80 human CSF samples. The samples were sent to the laboratory for protein analysis, but had normal values of albumin and albumin ratio, and no signs of intrathecal production of immunoglobulin G or M. The samples were from individuals older than 15 years of age.

The Alzheimer's type I patient pool

The Alzheimer's pool (Pool ADCSF, Paper I) consisted of five samples (from 2 men and 3 women; 60 to 68 years of age; mean ± standard error of the mean (SEM) age, 62.8 ± 1.6, duration of dementia; mean ± SEM duration in years, 5.8 ± 1.5). All patients underwent thorough clinical investigation. The diagnosis of Alzheimer's disease was made in accordance with the NINCDS-ADRDA criteria[144]. All the AD patients belonged to the subgroup AD type I[145].

The AD-matched healthy control pool

The Alzheimer's age matched control pool (Pool AMCCSF, Paper I) consisted of five samples (from 4 men and 1 woman; 66 to 70 years of age; mean ± SEM age, 67.4 ± 0.7). These individuals had no symptoms or signs of psychiatric or neurologic disease, normal values of albumin and albumin ratio, and no signs of intrathecal production of immunoglobulin G or M

CSF sample from a 4 year old girl with undiagnosed muscular pain

Patient A (Paper I), admitted to the Department of Paediatrics for neuro-muscular investigation, was included in this study. She was screened and showed no oligoclonal bands in CSF, and no changes in monoamine metabolites. No pathological values considering albumin, IgG or IgM, β-endorphin, and neuropeptide Y, were found in her CSF.

CSF sample from a 3 year old girl with a diagnosis of progressive epilepsy

Patient B (Paper I), was admitted to the Neurochemical laboratory for protein and monoamine metabolite analysis, but no pathological values was found in her CSF.

CSF sample from a 7 year old girl with suspected autism and mental retardation

Patient C (Paper I), was included in this study. She was admitted to the Department of Paediatrics for investigation and diagnosis. Routine analysis of proteins and monoamine metabolites in CSF showed no pathological values.

CSF sample from a 9 year old boy subjected to allergic investigation

Patient D (Paper I), was included in this study. He suffered from undiagnosed headache and a swollen forehead. The CSF was admitted to the Neurochemical laboratory for routine analysis of proteins and monoamine metabolites in CSF. No pathological values were found for IgG, IgM, albumin, β-endorphin, neuropeptide Y and somatostatin. However, he had high values for eosinophile cells and IgE in his blood and was diagnosed to have a parasitic infection.

CSF samples used for synaptotagmin analysis (Paper II)

In Paper II, pooled CSF from 4 patients with early onset (before 65 years of age) AD, 2 men and 2 women, aged 61-67 years, was analysed for synaptotagmin. The diagnosis was made in accordance with the NINCDS-ADRDA criteria[144]. Also,

pooled CSF from 4 healthy individuals, 2 men and 2 women, aged 59-66 years, was analysed for comparison. This group was described previously in detail[146].

Brain tissue samples used for synaptotagmin analysis (Paper II)

Synaptotagmin was analysed in brain tissue specimens from AD patients, aged matched controls, and patients with schizophrenia (Paper II). All AD patients had a histopathological score[147] of five or above. AD was subdivided based on the age at onset into early-onset AD (EAD, age at onset before 65 years of age), in total 8 patients, mean age 71 ± 5.9 years, and late-onset AD (LAD, with age at onset after 65 years of age), in total 11 patients, mean age 83 ± 7.6 years. The severity of dementia was estimated using the intellectual subscale in a geriatric rating scale[148].

The control group consisted 9 patients, mean age 71 ± 14 years, who had died from cardiac or malignant disease. Their medical records revealed no history of dementia, psychiatric or neurological diseases. All control patients had a histopathological score[147] of four or lower.

The diagnosis of schizophrenia was made in accordance with the DSM-III-R criteria[149]. This group consisted of 7 patients, mean age 80 ± 11 years, who had schizophrenia for several years, but no symptoms or signs of dementia, cerebrovascular disease, or other neurological disorders. A histopathological examination was not performed in this group.

Animals (Papers III-IV, VII)

The studies performed with animal material were approved by the Local Animal Ethics Committee of northern Stockholm and the Ethics Committee, Göteborg University.

Rats

In Papers III and IV, male Sprague Dawley rats (ALAB, Södertälje or B&K Universal, Sollentuna) were housed at 12 h light and dark cycle, with food and water ad libitum. After the surgery the animals were allowed to recover for two days in single cages before the experiment started.

Mice

In Paper VII, female DBA/1 mice, 12-19 weeks old were bred in the animal facility of the Department of Clinical Immunology in Göteborg, but originally purchased from Harlan Olac farm (Bicester, UK). The mice were housed 10 in each cage and were fed standard laboratory chow and water ad libitum under standard conditions of temperature and light.

Cerebrospinal fluid sampling (Papers I-II)

In Papers I and II, the first 12 mL of the CSF (3 mL for children under 16 years of age), was collected in plastic tubes and gently mixed to avoid gradient effects. Lumbar punctures were performed in the L3-4 or L4-5 interspace, in the morning with the patient in a recumbent position.

All CSF samples with more than 500 erythrocytes per µL were excluded. A blood sample was taken at the same time. CSF and serum samples were stored in 1 mL portions at -20°C or -80°C prior to analysis. Quantitative determination of albumin in CSF and serum was performed by rocket immunoelectrophoresis[150]. The albumin ratio (CSF albumin in mg/L divided by serum albumin in g/L) was used as the measure of

the blood-brain barrier function[151], to be able to exclude samples from patients with blood-brain barrier dysfunction, with an increased influence of molecules from serum in their CSF.

Human brain tissue sampling (Paper II)

At autopsy the brains were weighed, and infarcts, lacunas, or other macroscopical pathological changes other than brain atrophy were not noted on circumspect gross examination. The left hippocampal formation and frontal cortex (Brodmann area 9) was dissected out, homogenised in liquid nitrogen, and stored at -80°C prior to biochemical analyses.

Microdialysis samples from rat cerebrum (Papers III-IV)

Microdialysis was used together with CE-LIF in Papers III-IV. A stereotaxic instrument (David Kopf, Tujunga, CA, USA) was used for implantation of a guide cannula (CMA/12 Guide/CMA Microdialysis, Stockholm, Sweden) under halothane anaesthesia. A hole was drilled in the skull (2.5 mm (Paper III) or 1.5 mm (Paper IV) lateral to the midline and 7.3 mm caudal to bregma) and the guide cannula with a stylet in place was inserted with a trajectory, angled 22° (Paper III) or 14° (Paper IV) from the midsagittal plane to a depth of 4.6 mm (Paper III) or 4.0 mm (Paper IV) ventrally from the dura. The tip of the guide cannula was placed in immediate vicinity of the ventrolateral PAG. By this approach, the microdialysis probe (CMA/12, membrane diameter 0.5 mm, membrane length 2 mm, molecular cut off 20000 D) could be placed with the dialysis membrane in the ventrolateral PAG without interfering with the aqueduct. Dental cement and two anchoring screws in the scull fixed the guide cannula. The stylet was left in the guide cannula until the insertion of the microdialysis probe one hour before the start of the dialysis experiment.

The perfusion fluid contained 148 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$ and 0.85 mM $MgCl_2$. Potassium induced release was obtained by switching to a perfusion

medium containing 100 mM KCl for 15 min. For the study of calcium independent basal and potassium induced release, calcium was removed and 12 mM $MgCl_2$ was added to the perfusion medium. When studying the effect of morphine and naloxone , the rats were perfused with a modified Ringer's solution containing 100 μM morphine alone or in combination with 100 μM naloxone. All dialysis fluids maintained the same osmolarity.

The experiments were conducted after two days of postoperative recovery. After the insertion of the microdialysis probe and a washout period of one hour, allowing for the stabilisation of the system, sampling was started. Dialysis samples were collected every 15 min at a perfusion rate of 7 μL/min and immediately stored at -20°C. The rats were kept in a device for microdialysis in freely moving rats (CMA/120, CMA Microdialysis, Stockholm, Sweden) throughout the experiment.

The rats were sacrificed by an overdose of pentobarbital, the brains were dissected out, immediately frozen and stored at -20°C. The brain was later cut in frontal sections in a cryo-microtome and examined under magnification for verification of the position of the probe.

Human immunocompetent cells (Papers V-VI)

Determination of cell concentration (Papers V-VII)

Cell concentration was determined with three different methods; (i) with a FACSort flow cytometer (Becton and Dickinson, San Jose´, CA), (ii) with a cellcounter Cysmex F300 (Toa Medical Electronic Comp., Kobe, Japan), (iii) or by using a Bürker chamber and a light microscope[152].

Isolation of human cerebrospinal fluid lymphocytes (Paper V)

CSF were prepared by centrifugation of 12 mL of CSF at 100×g for 20 min at 4°C. The upper 10 mL of CSF was drawn off and used for biochemical determinations. The lower 2 mL was used for cell counting and analysis by CE.

Isolation of human CD4$^+$ T- and B-Cell Extracts (Paper V)

Mononuclear cells were eluted from a prostate cancer specimen after enzymatic digestion of the tissue as previously described[153, 154]. T-cell clones were established by the limiting dilution technique[155] in the presence of irradiated, autologous mononuclear feeder cells. Interleukin-2 (IL-2, Amersham, Bucksh, UK) and anti-CD3 antibodies (Ortho Diagnostics, Raritan, New Jersey, USA) were used to promote growth of the clones. Immunophenotyping was performed by incubation with fluorescein isothiocyanate (FITC)-labelled anti-CD3 (Leu-4, Becton Dickinson, Mountain View, CA, USA) or with FITC-labelled anti-CD4 (Leu-3a) together with phycoerythrin-labelled anti-CD8 (Leu-2a). A Becton Dickinson FACSort flow cytometer was used for analysis. Cells were incubated for 1 h with α-methyl-*p*-tyrosine (MeTyr) or dopamine at 37°C with 95% relative humidity and 5% CO_2. B cell clones were grown autonomously. Extraction of catecholamines from cloned T- and B-lymphocyte populations were performed as later will be described in detail.

Isolation of human peripheral blood mononuclear cells (Papers V-VI)

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy blood donors by centrifugation on a Ficoll-Hypaque (Sigma, St. Louis, MO, Paper V) or a Lymphoprep (Nycomed Pharma AS, Norway, Paper VI) density gradient, washed twice in PBS and resuspended in culture medium. The culture medium used throughout the human studies was Iscove's medium (GIBCO, Paisley, UK) supplemented with 10% AB, Rh+ human serum, 2 mM L-glutamine, 50 μg/mL gentamycin and 50 μM mercaptoethanol.

Murine immunocompetent cells (Paper VII)

Isolation of murine hybridoma cells and leukocytes (Paper VII)

The B cell hybridomas were raised and subcloned at the Department of Clinical Immunology, the clone 6B9E4 was found to be specific for staphylococcal collagen

adhesin whereas the specificity of 4A12 is unknown. The T cell hybridomas were obtained from Dr. Rickard Holmdahl, Lund, hybridoma HCQ.6 is Vβ 8.1+, HCQ.11 is Vβ 8.2+ and HCQ.9 consists of several clones.

To obtain macrophages 1% of sucrose in PBS was injected intraperitoneally, after 15 min the mouse was sacrificed, the abdomen massaged and the intraperitoneal fluid aspirated. The cells were centrifuged at 515×g for 5 min. The pelleted cells were resuspended for 10 min in Tris-buffered 0.83% ammonium chloride to lyse erythrocytes and then washed twice in PBS. Differential counts were performed on May-Grünewald-Giemsa-stained smears and displayed that more than 90% of the cells had the morphology of macrophages.

To obtain mononuclear cells, spleens of mice were teased and passed through a nylon sieve. The cells were suspended in PBS and centrifuged. Erythrocytes were lysed and the cells washed in PBS twice.

Mononuclear cells of either origin mentioned above were washed once in culture medium and counted before the extraction procedure. Extraction of catecholamines from mononuclear cells was performed as later will be described in detail.

The culture medium used throughout the murine study was Iscove´s medium (GIBCO) supplemented with 10% FCS (Biological Ind., Beit Haemek, Israel), 2 mM L-glutamine, 50 μg/mL gentamycin and 50 μM mercaptoethanol (complete medium).

5. Methods

Intellektet har ett vaket öga för metoder och verktyg men är blint för mål och värden

Albert Einstein

Capillary electrophoresis with laser-induced fluorescence (Papers I, III-IV)

A P/ACE 2100 instrument (Beckman Instruments; Fullerton, CA, USA) equipped with a P/ACE-LIF detector was used to obtain all CE separations in Papers I, III-IV. The LIF detector used an Ar ion laser for excitation at 488 nm (4 mW), and a 560 ± 40 nm band-pass filter provided with the LIF detector was used to reject scattered laser light. Fused silica capillaries (75 μm i.d., 360 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ), and all separations were performed in 57 cm lengths (50 cm to detector) of untreated capillary.

The buffer consisted of 50 mM borate, and 30 mM SDS was added as a pseudostationary phase. The pH was adjusted to 9.0 by addition of sodium hydroxide. The pH was measured, using a Beckman pH-meter (Φ32), equipped with a combination electrode and calibrated using a two-point calibration curve (pH 7.0-10.0). The organic modifier dimethyl sulfoxide (DMSO) was added to this solution (20% DMSO v/v) to form the final separation buffer.

The instrument was programmed to rinse the capillary with 0.1 M NaOH at high pressure for 2.0 min, followed by separation buffer at high pressure for 3.0 min prior to each injection in order to obtain consistent retention times in-between runs. Samples were injected automatically by pressure injection for 1.0 s (5.9 nL volume) in Papers I and III, or for 5.0 s (30 nL volume) in Paper IV. A separation potential of 20 kV was used, and the capillary was held at 25°C. The electrophoretic current was typically 33 μA.

The samples were kept frozen at -20°C until derivatized for CE-LIF analysis. One μL of internal standard, (somatostatin 3-6, 198 μM in water in Papers I and III,

histamine, 5 μM in Paper IV), 5 μL of CBQCA (3 mg/mL in MeOH) and 2 μL KCN (50 mM in 50 mM borate buffer, pH 9.0) were added to 10 μL of microdialysis sample in 100 μL polycarbonate vials provided for use with the CE instrument (Beckman). The vial housings were filled with water and capped with the provided rubber injection septum in order to reduce solvent evaporation during the reaction time. Solutions were left to react for 2 h (Papers I and III) or 1 h (Paper IV) at room temperature before direct injection from the reaction vial, without prior purification of the reaction products. Standard solutions were made in water and derivatized under conditions identical to those used for CSF or microdialysis samples. In order to assure uniform reactivity, additional standards were derivatized in sample fluid with known amino acid concentrations.

Capillary electrophoresis with UV-absorbance detection (Paper II)

Capillary electrophoretic analysis of the anti-synaptotagmin immunoreactive fraction from the μRP-HPLC was performed on a P/ACE 2100 instrument (Beckman Instruments) equipped with P/ACE-UV detection at 200 nm, and a fused silica capillary (75μm i.d., 360 μm o.d., length 57 cm, and length to detector 50 cm) (Polymicro Technologies). The separation buffer (50 mM borate, pH 9.0) contained 50 mM SDS in order to provide a micellar pseudo stationary phase and to increase protein solubility. A separation potential of 10 kV was used, the electrophoretic current was 30 μA, and the capillary was held at 25°C. The sample was injected automatically by pressure injection for 10.0 s (vol. 59 nL).

Capillary electrophoresis with electrochemical detection (Papers V-VII)

The system used for CE with electrochemical detection in small bore capillaries was similar to that described earlier by Ewing[156]. In Paper V, a decoupler was used to allow electrical isolation of the separation potential field from the detector, but later in

Papers VI-VII, the system was equipped with the modified optimised end-column detection[143] (Figure 5). Fused silica capillaries with 5-10 μm i.d. and 65-80 cm length were obtained from Polymicro Technologies. Electrokinetic injection was used for all samples, 5 s at 30 kV. The separation potential was 30 kV and the amperometric detection potential was 0.8 V versus a sodium-saturated calomel reference electrode. The electrophoresis buffer was 25 mM 2-(N-morpholino) ethanesulfonic acid (MES; Sigma) adjusted to pH 5.65 with NaOH. Calibration standards of 3,4-dihydroxy-L-phenylalanine (L-DOPA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), 3-methoxy-4-hydroxyphenylglycol (MHPG), and uric acid (UA) all from Sigma, were prepared as 10 mM stock solutions in 0.1 M $HClO_4$ and diluted to the desired concentration in operating buffer.

Microinjectors for capillary electrophoresis (Papers V-VII)

A significant advance in injection of ultra-small sample volumes involved the construction of a microinjector from the CE capillary itself. This is accomplished by removing a small portion of the polyimide coating near the tip of a 5-10 μm i.d. capillary and etching to an o.d. of 8-10 μm (Figure 5). Microinjectors are prepared by removing 5 mm of the polyimide polymer coating with a flame, from one end of the capillary to expose the fused silica. The capillary is then connected to a helium tank and purged with 50 psi during the etching process, which only allows some of the silica etching hydrofluoric acid (HF) to diffuse into the capillary tip. To etch the microinjector and the detector end of the capillary hydrogen fluoride is used, obtained as a 40% aqueous solution from Aldrich Chemicals (Steinheim, Germany). By altering the applied He pressure, the concentration of HF, and etching time it is possible to achieve the microinjector tip that best serves the experiment. After etching, the tip is placed into a concentrated solution of calcium carbonate to neutralize the acid and then washed with water.

The smaller tip permits viewing under a microscope as the capillary tip is manipulated to a specific cell. Injection of the cell is accomplished by electroosmotic suction which is followed by cell lysing and subsequent separation of the released components. A recent improvement of this technique involves etching both the outside and a small portion of the inside of the capillary tip to provide a conical external form and a funnel-like internal structure to capture the cell. This etching procedure is also used when constructing the optimised form of end-column electrochemical detection[143] (Figure 5), used in Papers VI-VII.

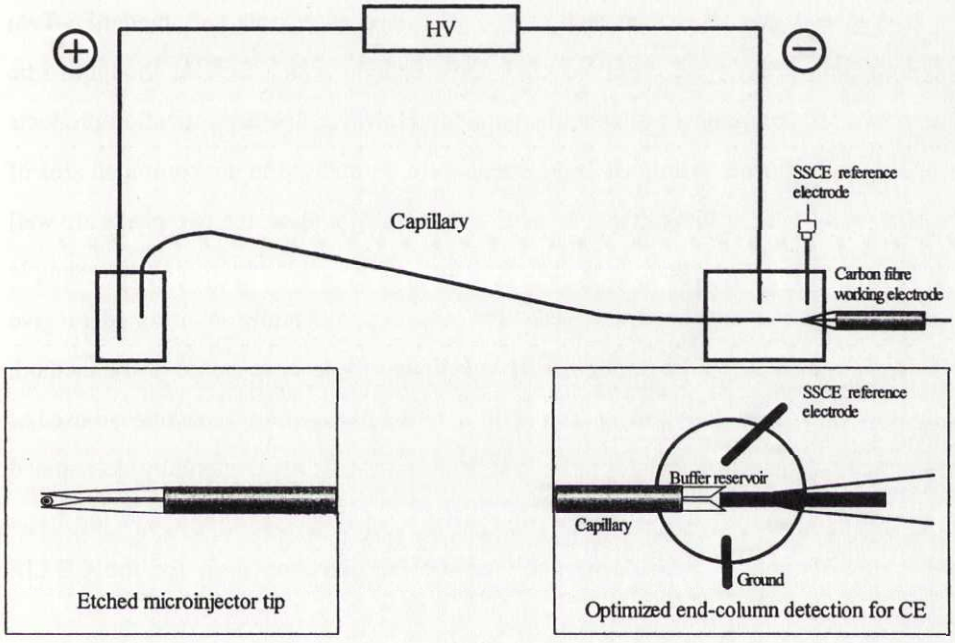

Figure 5. Schematic drawing of the capillary electrophoretic system used for electrochemical detection of catecholamines. At the injection end of the capillary, a microinjector is etched, in order to allow analysis of single cells. At the detection end of the capillary an electrochemical optimised end-column detector is used.

Capillary electrophoretic data collection and analysis (Papers I-VII)

Data collection at a rate of 5 Hz, processing and analysis were performed using the System Gold software package (Beckman). Peaks were identified by both their electrophoretic mobility, and by spiking samples with standard solutions of the analysed molecules. The analytes were subsequently quantitated using linear calibration plots based on peak area versus concentration. Each calibration consisted of at least 6 different concentrations, spanning the range of concentrations found in the samples. Detection limits were estimated at two times the peak to peak noise by extrapolation from plots of peak height versus concentration.

In Papers I and III, somatostatin (3-6) was used as an internal standard. Two corresponding peaks were observed after derivatization with CBQCA, thought to be due to two primary amine groups in the peptide. However, the derivatization products are going to be further examined. Peak areas were normalised to the combined area of the observed peaks. Although this internal standard is not ideal, the two peaks are well separated from any major peaks in the CSF and microdialysis sample electropherograms, and the total peak area was experimentally determined to give consistent values over a large number of injections. In order to improve the method, and allow for shorter derivatizing time (from 2 h to 1 h), histamine was instead used as an internal standard in Paper IV. The histamine peak was experimentally determined to be well separated from other known substances in the separation, and the basal levels of histamine in brain is normally below the detection limit for the CE-LIF assay[157].

In Paper II where UV detection was used, no standard was applied since the synaptic vesicle protein synaptotagmin is not commercially obtainable.

In Papers V-VII where electrochemical detection was used, catecholamines and their metabolites, were run as standards in-between each sample. Also, since electrokinetic injection was used, the injected amounts of analyte were corrected for injection discrimination of cations and anions. In-between series of runs, the capillary

was flushed with 0.1 M NaOH to refresh the inner capillary surface and to maintain reproducible separation conditions.

Affinity chromatography (Paper II)

The affinity chromatographic removal of serum proteins was performed in three steps: In the first step, a Blue Sepharose column (Pharmacia, Uppsala, Sweden) was used for selective removal of albumin from CSF[158]. The column was equilibrated with 0.05 M Tris-HCl, 0.1 M KCl, pH 7.0, and 2.0 mL of CSF was loaded onto the column. The albumin-free CSF fraction was collected until the absorbance at 280 nm (A_{280}) was less than 0.05 arbitrary units (AU).

In the second step, a column with staphylococcal Protein G covalently linked to Sepharose 4B (Pharmacia) was used for selective removal of IgG from CSF[159]. The column was equilibrated with 0.05 M Tris-HCl, 0.1 M KCl, pH 7.0, and the albumin-free CSF fraction from step one was loaded onto the column. The albumin/IgG-free CSF fraction was collected until the A_{280} was less than 0.05 AU.

In the third step, other major serum proteins were removed by loading the albumin/Ig-free CSF fraction onto a divinylsulfone-activated agarose column (Mini-leak Low, Kem-En-Tec A/S, Copenhagen, Denmark) coupled with rabbit antiserum against human serum, cross-reacting with more than 50 serum proteins (catalogue #A206, Dako, Glostrup, Denmark). The column was equilibrated with 0.10 M Tris-HCl, 0.5 M NaCl, pH 8.6, and the albumin/IgG-free CSF fraction from step two was loaded onto the column. The albumin/IgG/serum protein-free CSF fraction was collected after elution with the equilibrium buffer. Finally, the albumin/IgG/serum protein-free CSF fraction was vacuum dried by evaporation in a Savant Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY, USA).

Micro-reversed phase-high performance liquid chromatography (Paper II)

For separation of the CSF proteins, untreated CSF (0.5 mL), or the albumin/IgG/serum protein-free CSF fraction (dissolved in 500 µL of 0.14% trifluoroacetic acid (TFA) in ultrapure water), corresponding to 2.0 mL of untreated CSF, was injected into a µRP-HPLC system (SMARTSystem™, Pharmacia), equipped with a µRPC C_2/C_{18} column (dim. 2.1×100 mm, gel volume 0.35 mL, particle size 3 µm). The eluents consisted of 0.14% TFA in ultrapure water (eluent A) and 0.12% TFA in 60% acetonitrile (eluent B). Elution was achieved with isocratic elution (46% B) in 10 min, two linear gradients of 46-83% B in 30 min and 83-100% B in 5 min respectively, and finally with isocratic elution (100% B) in 5 min with a flow rate of 200 µL/min. The eluent after the void volume was collected in 40 fractions of 150 µL, which thereafter were vacuum dried.

Electrospray ionisation mass spectrometry (Paper II)

The fractions from the µRP-HPLC containing the major peaks were analysed by electrospray ionisation mass spectrometry (VG BIO-Q, Fisons, Loughborough, UK.) to determine the molecular weights of β_2-microglobulin, prealbumin, cystatin C and asialotransferrin.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Papers II, VI)

Samples subjected for SDS-PAGE were dissolved in sample buffer (0.08 M Tris-HCl, pH 6.8, containing 0.17 M SDS, 6.5 mM dithiothreitol (DTT), 0.5 M urea, and 0.1% bromphenol blue), ultrasonicated for 15 min, and boiled for 5 min. The proteins were separated on a 12% polyacrylamide gel (Ready Gels, BioRad, Richmond, CA).

The electrophoresis was run for 30 min at 200 V constant voltage in a Mini-PROTEAN^{II} Cell (BioRad, Richmond, CA), using the buffer systems of Laemmli[160].

Western blotting of brain specific proteins (Paper II)

For Western blotting of the albumin/IgG/serum protein-free CSF fractions separated by μRP-HPLC, the proteins in the SDS-PAGE gel were transferred from the gel onto a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), by the semidry technique using the Multiphor II NovaBlot Unit (Pharmacia) at 0.8 mA/cm^2 for 30 min. After blotting, the PVDF membrane was blocked with 5% non-fatty milk powder in phosphate buffered saline (PBS, 58 mM Na_2HPO_4, 17 mM $Na_2HPO_4 \times H_2O$, 68 mM NaCl, pH 7.4), containing 0.05% Tween 20. After that, the PVDF membrane was incubated with specific antiserum against human β_2-microglobulin[161], cystatin C[162], prealbumin[163, 164], and transferrin[165], all from Dako (Glostrup, Denmark). After incubation with the secondary alkaline phosphatase conjugated goat anti-rabbit IgG (Jackson, West Grove, PA), the colour reaction was developed with 0.015% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.030% nitro-blue tetrazolium (NBT), both from Sigma, in 0.1 M carbonate buffer containing 1.0 mM $MgCl_2$[166].

Enhanced chemiluminescence (ECL) immunoblotting (Paper II)

For ECL immunoblotting of the synaptic vesicle specific proteins in the albumin/IgG/serum protein-free CSF fractions separated by μRP-HPLC, the proteins in the SDS-PAGE gel were transferred from the gel onto a Hybond nitro-cellulose membrane, blocked with 5% milk powder in 0.05% PBS-Tween, and then incubated with the specific MAbs against synaptophysin (Cl. 7.2)[167], diluted 1:500, synaptotagmin I (Cl. 41.1)[168-170] diluted 1:500, or rab3a protein (Cl. 42.2)[171] diluted 1:500, all in PBS-Tween. The membrane was incubated for 1 h with alkaline phosphatase conjugated goat anti-mouse IgG+M (H+L, Jackson) diluted 1:4000.

Detection was performed using ECL (Amersham), according to the manufacturers instructions.

Quantitative Western blotting of synaptotagmin in brain tissue (Paper II)

Samples of homogenised brain tissue (≈ 100 mg wet weight) were delipidised in chloroform/methanol/water (4/8/3, v/v)[172], and centrifuged at 2000×g for 10 min. The pellet was resuspended in chloroform/methanol/water, centrifuged, and the supernatant containing the lipids was discarded. After that, the pellet was dried under a stream of nitrogen, dissolved in SDS sample buffer, and subjected to SDS-PAGE and Western blotting to a PVDF membrane, as described above, using the anti-synaptotagmin MAb. The total protein concentration was determined in each sample using the bicinchoninic acid method[173], and the volume of each sample was adjusted to get a total protein concentration of 5 μg/mL. A volume of 20 μL (corresponding to 100 ng of total protein) was loaded onto the gel.

Synaptotagmin was quantified by scanning of the PVDF membrane on a Camag Scanner II (Mattenz, Switzerland) at 620 nm. In order to make a standard curve, hippocampal or frontal cortex brain homogenate from one control case was prepared as described above, and three dilutions (2.5, 5.0, and 10.0 μg/mL total protein) of the standards were run on each gel. The synaptotagmin level in the 5.0 μg/mL hippocampal and frontal cortex standards (corresponding to 100 ng of total protein) was set as 1 AU, and after that, all values were corrected by dividing with the mean band intensity in controls in hippocampus and frontal cortex respectively.

High performance liquid chromatography for detection of amino acids (Paper III)

The system employed for the analysis of GABA consisted of a high-pressure pump (Knauer 64) connected to a CMA/200 refrigerated microsampler (Carnegie Medicin, Stockholm, Sweden) working at 4°C, an on line CMA 260 degasser, and a Spectra Physics 4290 integrator[174]. The derivatization reagent used for the electrochemical detection of GABA was prepared by dissolving 27 mg of *o*-phthaldialdehyde in 10 mL of 0.1 M carbonate buffer, pH 9.6, containing 50% methanol and 45 μL of t-butylthiol. A robotics program was set to add 0.5 μL of the reagent to 20 μL of standard or dialysis sample. The reagent was mixed with the sample three times and, after 120 s, 20 μL were injected onto the column. The 100×4 mm column, packed with Nucleosil-3 (particle size 3 μm) C_{18} (Knauer, Berlin, Germany), was equipped with a 5×4 mm guard column packed with the same material but of 5 μm particle size. The mobile phase was 0.15 M sodium acetate buffer, 1 mM EDTA (pH 5.4), and 50% acetonitrile at a flow rate of 0.8 mL/min. An electrochemical detector, BAS LC4C (Bioanalytical Systems, West Lafayette, IN, USA) set at 80 nA was used, with the glassy carbon working electrodes set at + 0.75 V. Analysis time was 8-10 min per sample. External standards or blanks (water) were injected following every fourth sample to confirm the reliability and selectivity of the derivatization. GABA was detected 3 min after injection. In addition, a standard curve ranging from 1 nM to 1000 nM was constructed and observed to be linear. The detection limit for GABA was approximately 0.5 nM at an injection volume of 10 μL.

The assay of glutamate started with a pre-column derivatization with *o*-pthaldialdehyde/mercaptoethanol reagents followed by fluorometric detection[175]. Briefly, 10 μL of reagent (0.4 M borate, 0.04 M *o*-pthaldialdehyde, 0.4 M mercaptoethanol, pH 10.4) was added to 10 μL of the perfusate and following 60 s reaction time, 15 μL of the derivatized aliquot was injected into the column. The system consisted of a cartridge column (i.d. 4 mm; length 60 mm) containing 5 μm Nucleosil-100 C_{18} packing material (Knauer), a Spectra Physics 8800 ternary precision

pump with a two way valve (Spectra Physics, San Jose, CA, USA), an on line CMA 260 degasser and a Spectra Physics 4220 integrator. The system used a Hitachi F1000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) with the excitation wavelength set at 370 nm and emission cut-off at 450 nm for detection. The mobile phase was 0.1 M sodium acetate with 8% (v/v) methanol and 1.5% (v/v) of tetrahydrofuran adjusted to pH 6.95. The flow rate was 1 mL/min. A multilinear gradient (100% mobile phase to 100% methanol in 6 min, returning to 100% mobile phase after 2 min) was used to clean the column after glutamate elution. An integrator (SP 4220, Spectra-Physics, San Jose, CA, USA) was used to determine concentrations of glutamate. The integrator was calibrated with a 1.875 μM solution of 32 amino acids, and the limit of detection for glutamate was 100 nM at a sample volume of 10 μL.

High performance liquid chromatography for detection of morphine (Paper IV)

An automated ion-pair HPLC-system with electrochemical detection was used for morphine quantification[176]. Ten μL of the dialysis sample was diluted (1:10) with perfusion medium and further diluted (1:10) with mobile phase prior to injection into the chromatographic system.

Immunological cell assays (Papers V-VII)

Proliferative responses of lymphocytes (Papers V-VII)

Proliferation and differentiation of human peripheral blood mononuclear cells (PBMC) were induced by mitogen stimulation. PBMC were incubated with 25 μg/mL of Con A or 10 μg/mL of Pokeweed mitogen (PWM) (GIBCO) for T- and B-cell stimulation, respectively. In proliferation assays PBMC were incubated at a concentration of 1×10^6 mononuclear cells/mL in 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) in 0.1 mL culture medium at 37°C in 5 % CO_2 and

95 % humidity. To the PBMC cultures were added 0-500 μM of L-DOPA, DA or NE, all compounds obtained from Sigma. The cells were either preincubated with the respective compounds at 37°C for 2 h, then washed, recounted and resuspended in culture medium, before stimulation with mitogens, or cultured together with the respective compounds throughout the mitogen stimulation. The cells were cultured for 72 h. During the final 18 h of culture 1 μCi ^{3}H-thymidine (Radiochemical Centre, Amersham) was included in each well. The cultures were harvested on glass fibre filters, processed and counted in a β-counter. The cultures were set up in triplicate and results expressed as percentage of control mean counts/min.

Mouse spleen mononuclear cells were incubated at a concentration of 1×10^{6} mononuclear cells/mL in 96-well flat-bottomed microtitre plates (Nunc) in 0.1 mL complete medium at 37°C in 5% CO_2 and 95% humidity. Tyrosine, L-DOPA, DA and NE in concentrations ranging from 0-500 μM and Con A (Miles Yeda, Rehovot, Israel) or LPS (Sigma) were included in the medium. The cells were cultured for 48-72 h which was previously found to be the optimal culture time[177]. During the final 8-18 h of culture, 1 μCi ^{3}H-labelled thymidine (Radiochemical Centre) was included in each well. The cultures were harvested, processed and counted as above. Preliminary experiments showed that for murine cells, the optimal proliferative responses to Con A and LPS were obtained using 2.5 μg/mL and 10 μg/mL of the stimulants, respectively.

Differentiation of immunocompetent cells (Papers V-VII)

In differentiation assays human peripheral blood mononuclear cells and murine splenocytes were stimulated with mitogens in 1 mL culture medium. Supernatant from stimulated cell cultures, were harvested, and analysed for IL-4 and IFN-γ (Papers V-VI) or IL-2, IL-6 and IFN-γ (Paper VII) production. The cytokine levels in supernatants were determined by bioassays (using indicator cells for IL-2 and IL-6) or by enzyme-linked immunosorbent assays (ELISA, see below). The ELISPOT technique[31] was used to determine the numbers of immunoglobulin secreting cells of a given isotype (see below).

IL-2 assay (Paper VII)

Supernatants from murine splenocytes cultured for 24 h with Con A were analysed for the content of IL-2 using the CTLL-2 cell line[178]. The particular CTLL-2 line used was chosen because it does not proliferate in response to recombinant IL-4 and thus provides a selective assay for IL-2[179]. Indicator cells were cultured in supernatants for 24 h and ^{3}H-labelled thymidine was included during the last 4 h of culture. IL-2 activity was determined in triplicate cultures and expressed as mean counts/min of ^{3}H uptake by CTLL-2 cells. In each experiment a standard IL-2 curve was generated using mouse recombinant IL-2 (Genzyme, Cambridge, MA) and the IL-2 content of the supernatant was calculated from the linear part of the standard curve.

IL-4 assay (Paper VI)

To determine IL-4 levels, 96-well plates (MAXISORP, Nunc) were coated overnight at 4°C with 50 µL (2 µg/mL, diluted in 0.1 M carbonate buffer pH 9.6) of monoclonal anti-human IL-4 antibody (MABTECH, Stockholm, Sweden). The coated plates were washed with 0.05 M Tris-HCl (pH 7.4) + 0.05% Tween 20 and saturated with 100 µL 1% BSA in 0.05 M Tris-HCl for 1 h at room temperature. After washing, the plates were incubated for 2 h at 37°C with different samples (50 µL/well), followed by further washes. Biotinylated anti-human IL-4 (MABTECH, 50 µL, 2 µg/mL in 0.05 M Tris-HCl + 0.015 M NaCl) was added and incubated overnight at 4°C. The plates were washed again and re-incubated with 50 µL (4 µg/mL) of StreptAvidin Alkaline Phosphatase (Sigma) for 1 h at room temperature, followed by thorough washes. The enzyme substrate (p-nitrophenyl-phosphate (Sigma), 1 mg/mL in 100 µL diethanolamine buffer) was then added and optical density at 405 nm was determined in a Titertek Multiscan photometer (Flow Laboratories). The concentration of the IL-4 was calculated using standard curve based on known quantities of recombinant human IL-4 (Genzyme). The samples were assayed in triplicate and results expressed as percentage of control IL-4 production.

IL-6 assay (Paper VII)

Cell line B13.29, which is dependent on interleukin-6 (IL-6) for growth, has been previously described[180]. For IL-6 determinations, the more sensitive subclone B9 was used[181, 182]. B9 cells were harvested from tissue culture flasks, seeded into microtitre plates (Nunc) at a concentration of 5000 cells per well, and cultured in complete Iscove's medium with supernatants from splenocytes cultured for 24 h with Con A or for 48 h with LPS. After 68 h of culture ^{3}H-labelled thymidine was added and the cells were harvested 4 h later. The samples were tested in twofold dilutions and compared with an recombinant mouse IL-6 standard (Genzyme)[183]. B9 cells were previously shown not to react with several recombinant cytokines, including IL-1α, IL-1β, IL-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor, TNF-α and IFN-γ. There was only weak reactivity with IL-4[182].

IFN-γ assay (Papers V-VII)

When determining IFN-γ production in human peripheral blood mononuclear cells, 96-well plates (MAXISORP, Nunc) were coated overnight at 4°C with 50 µL (5 µg/mL, diluted in 0.1 M carbonate buffer pH 9.6) of monoclonal anti-human IFN-γ antibody (Kabi Diagnostica AB, Mölndal, Sweden). The coated plates were washed with 0.05 M Tris-HCl (pH 7.4) + 0.05% Tween 20, and saturated with 100 µL 1% BSA in 0.05 M Tris-HCl (pH 7.4) for 1 h at 37°C. After washing, the plates were incubated for 2 h at 37°C with different samples diluted in PBS (50 µL/well), followed by further washes. Biotinylated anti-human IFN-γ (Kabi Diagnostica AB, 50 µL, 1.2 µg/mL) was added and incubated overnight at 4°C. The plates were washed again and re-incubated with 50 µL (4 µg/mL) of ExtraAvidin Alkaline Phosphatase (Sigma) in 0.05 M Tris-HCl (pH 7.4) + 0.015 M NaCl for 1 h at room temperature, followed by thorough washes. The enzyme substrate (*p*-nitrophenyl-phosphate (Sigma), 1 mg/mL in 100 µL diethanolamine buffer) was then added and optical density at 405 nm was determined in a Titertek Multiscan photometer (Flow Laboratories). The concentration of the IFN-γ was calculated using standard curve based on known quantities of

recombinant human IFN-γ (Genzyme). The samples were assayed in triplicate and results expressed as percentage of control IFN-γ production.

When determining IFN-γ production in murine cells, polystyrene flat-bottomed plates were coated overnight at 4°C with 2 μg/mL of purified anti-mouse IFN-γ (PharMingen, San Diego, CA). The coated plates were washed and saturated with 1% BSA for 1 h at 37°C. After washing, the plates were incubated for 2 h at 37°C with different dilutions of samples. Biotinylated anti-mouse IFN-γ (PharMingen) (2 μg/mL) was added and incubated overnight at 4°C. The plates were washed again and re-incubated with 4 μg/mL of ExtrAvidin Alkaline Phosphatase (Sigma). The enzyme substrate was then added and optical density was determined in a Titertek Multiscan photometer (Flow Laboratories, McLean, VA). The concentration of the IFN-γ was calculated using standard curve based on known quantities of recombinant mouse IFN-γ (Genzyme).

Immunoglobulin assay (Papers V-VII)

Number of immunoglobulin producing cells, was determined by the enzyme-linked immunospot assay (ELISPOT). Briefly, wells in the lids of 24 well culture cluster plates (Costar, Cambridge, MA) were coated overnight at 4°C with affinity-purified $F(ab')_2$ fragments of goat anti-human or goat anti-mouse IgG and IgM (Cappel, Cochraneville, PA) at a concentration of 5 μg/mL. The plates were then washed with tap water and blocked with 5% FCS in PBS for 1 h. Mononuclear cells from the mitogen stimulation assay were washed and viability was assessed by trypan blue. One hundred μL of mononuclear cells resuspended in Iscove´s complete medium was added into each coated well and incubated for 3.5 hours at 37°C. After another wash, affinity-purified biotinylated $F(ab')_2$ fragments of goat anti-human or goat anti-mouse IgG or IgM antibodies (Jackson), diluted 1:750 in PBS containing 0.05% Tween 20 were applied followed by incubation with 0.5 μg/mL of ExtrAvidin-alkaline phosphatase (Sigma) in PBS-Tween. After addition of the phosphatase substrate solution (BCIP; Sigma) spots were enumerated under low magnification.

Each spot represents one immunoglobulin producing cell (spot forming cell). All the assays were done in duplicates and at cell concentrations of 1×10^4, 1×10^5, 1×10^6 cells/mL for the IgG spots and 1×10^3, 1×10^4 and 1×10^5 cells/mL for the IgM spots.

Incubation of human peripheral blood mononuclear cells, murine hybridoma cells and splenocytes with the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (Papers V, VII)

Catecholamine production by PBMC, HCQ.6 hybridoma cells and splenocytes was blocked by the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine (MeTyr). Cells were incubated for 1 h with 10 μM of MeTyr, L-DOPA and DA respectively in complete medium at 37°C. The cells were washed, recounted and the catecholamines were extracted as described below. In proliferation assays spleen cells were incubated with 10 μM MeTyr for 1 h, washed and stimulated with Con A or LPS, or incubated with 10 μM MeTyr over night before Con A and LPS was added.

Incubation of human peripheral blood mononuclear cells with dopamine uptake and storage blockers (Paper VI)

The effects of nomifensine maleate (Hoecht-Roussel Pharmaceuticals, Somerville, NJ) and tetrabenazine (Fluka AG, Switzerland) were examined by incubating isolated PBMC (1×10^6 cells per sample) with 10 μM DA or 10 μM DA in combination with 10 μM nomifensine or 10 μM tetrabenazine, respectively for 1 h at 37°C. The cells were then washed twice in PBS and extracted as described below.

Extraction of catecholamines from human and murine mononuclear cells (Papers V-VII)

The cells were washed twice in PBS and extracted by addition of 10 μL of 0.1 M perchloric acid with 1 mM sodium EDTA and 1 mM sodium sulphite to the cell pellet in an Eppendorf tube, to end up at a total volume of 15 μL. This was ultrasonicated for

1 min on ice using a MSE Soniprep 150 probe, and the extract was centrifuged for 30 min (35000×g) at 4°C. All samples were prepared and analysed in triplicate.

Induction and detection of apoptosis in human mononuclear cells and murine splenocytes (Papers VI-VII)

Human PBMC, IL-2 dependent human lymphoid cell clone (CTLL-2)[178] and murine splenocytes, 1×10^6 mononuclear cells/well, were incubated in 96-well flat-bottomed microtitre plates with 0-500 μM of L-DOPA, DA or NE in a total volume of 0.1 mL at 37°C. The cells were either treated for 24-72 h followed by 2 washes in PBS or pre-treated for 4 h in 37°C, washed twice, incubated for 24-72 h and rewashed. Cells were stained with propidium iodide in a hypotonic staining solution[184] with 0.1 mg/mL of RNAse added and propidium iodide fluorescence of individual nuclei was measured on a FACSort flow cytometer with a Lysys II software program (Becton and Dickinson, San Jose´, CA). All samples were prepared and analysed in triplicate and data expressed as percent apoptotic (i.e. hypodiploid) nuclei.

The analysis of Bcl-2/Bax and Fas/FasL expression in control and catecholamine induced apoptotic human PBMCs was performed by western blotting (see below), using antibodies purchased from Santa Cruz Biotechnology (CA).

Western blot for analysis of apoptotic markers (Paper VI)

For Western blotting of the apoptotic regulatory proteins (Bcl-2, Bax, Fas and FasL), the proteins separated in a SDS-PAGE gel were transferred from the gel onto a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), by the semidry technique using the Multiphor II NovaBlot Unit (Pharmacia) at 0.8 mA/cm^2 for 30 min. After blotting, the PVDF membrane was blocked with 5% non-fatty milk powder in PBS, containing 0.05% Tween 20 for 1 h. After that, the PVDF membrane was incubated with specific antiserum against the different apoptotic regulatory proteins Bcl-2 (mouse monoclonal IgG from Santa Cruz Biotechnology, CA), Bax,

Fas, FasL (all rabbit polyclonal IgG from Santa Cruz Biotechnology, CA), in dilution 1:1000 over night. After incubation with the secondary alkaline phosphatase conjugated goat anti-mouse IgG (1:2000, Jackson) for 2 h or the biotinylated goat anti-rabbit IgG (1:4000, Jackson) for 1 h, followed by alkaline phosphatase conjugated ExtrAvidin (1:4000, Sigma) for 1 h, the colour reaction was developed with 0.015% BCIP (Sigma) and 0.030% NBT (Sigma) in 0.1 M carbonate buffer containing 1.0 mM $MgCl_2$[166].

Statistics (Papers I-VII)

In Papers I, V-VII the level of significance of the differences between groups was calculated using a paired Students two-tailed *t*-test. In Paper II the level of significance of the differences between groups was calculated using the Mann-Whitney U-test, and the Spearman correlation coefficient was used for correlations. In Papers III and IV, the Friedman nonparametric ANOVA[185] was used, followed by either pairwise comparisons, Dunnetts test, or unpaired t-test.[186]

Values are presented as mean ± standard error of the mean (mean ± SEM) unless otherwise indicated.

6. Summary of the results and their implications

Egentligen vet man något först när man nästan inget vet. Tvivlet växer med kunskaperna

Johann Wolfgang von Goethe

CE-LIF detection of amino acids in human cerebrospinal fluid (Paper I)

A rapid and simple procedure for analysis of amino acids in human CSF was developed. Capillary electropherograms containing more than 50 peaks were obtained in less than 40 min from 10 μL samples of unconcentrated human CSF. Many of these peaks, including arginine, glutamine, threonine, valine, GABA, serine, alanine, glycine, glutamic, and aspartic acid, were identified and quantitated. Capillary electropherograms obtained from patients with Alzheimer's disease, and from children with various neurological disorders, were compared to healthy controls. The CSF from patients and healthy controls revealed significant differences. For example, GABA levels were decreased in CSF samples from Alzheimer patients, while glutamic and aspartic acid levels were decreased in CSF samples from children compared to the CSF samples from elderly patients.

These results indicate that CE with laser-induced fluorescence detection has a significant potential as a tool in the evaluation of disorders related to the CNS.

Isolation, identification and analysis of synaptotagmin in human cerebrospinal fluid (Paper II)

With the use of affinity chromatography, reversed-phase chromatography, and chemiluminescence immunoblotting of human CSF, the synaptic vesicle protein, synaptotagmin I, was isolated, identified, and quantitated. By using the chromatographic procedures mentioned above, enrichment of CSF proteins was accomplished, an approach that may be applied to many brain-specific proteins. CE with UV-detection, with its high resolving power and large peak capacity, was used to

further chromatographically characterize the isolated anti-synaptotagmin reactive protein fraction. Only one peak was found in the electropherogram, which showed the homogeneity of the isolated fraction. A lower concentration of synaptotagmin in CSF samples from patients with early-onset Alzheimer's (EAD) was demonstrated. Results showing a decrease of this protein in the hippocampus and frontal cortex from EAD patients support that pathological events in the brain involving this protein, are likely to be reflected in the CSF.

CE-LIF detection of amino acids in microdialysis samples of rat periaqueductal grey (PAG) matter (Papers III-IV)

In the electropherograms obtained from the CE-LIF system, over 30 shoulders and peaks were detected in 20 min. Among these peaks, 8 amino acids were identified and quantified (arginine, glutamine, valine, GABA, alanine, glycine, glutamate, and aspartate). GABA and glutamate were also analysed with HPLC in order to be able to compare with the CE results. A good correlation between the two methods was found. The results of the present study indicate that CE can be used for the detection of amino acids in microdialysis samples of the rat brain. Among the amino acids analysed, the potassium induced release of GABA was found to be calcium dependent, while significant decreases in glutamine and valine levels after the potassium stimulation were found, the latter being under calcium free conditions.

Considering the low detection limit and the low volume needed for detection of GABA by the CE assay, this method together with microdialysis, seems to be especially useful for the detection of fast and transient changes of extracellular transmitter levels.

The capillary electrophoretic method developed in Paper III was, with some minor modifications (see Methods), used in Paper IV to study the effect of morphine on the GABA release in the PAG of freely moving rats. The basal GABA level was significantly decreased after perfusion of the dialysis probe with morphine for 30 min. The effect of morphine was reversed by co-perfusion with naloxone. The present

results thus provide direct experimental evidence for an opioid induced inhibition of a tonic GABA release in the PAG, which may in turn lead to a disinhibition of descending pain inhibitory pathways.

CE analysis of single human lymphocytes from cerebrospinal fluid (Paper V)

The analysis by CE of single lymphocytes, isolated from human CSF, has led to the discovery that these cells contain catecholamines. The electropherogram in Figure 6 shows the analysis of one single human lymphocyte with a volume of ~180 fL. The cell was injected into an etched 10 μm i.d. capillary by electroosmotic flow. After lysing the cell, a potential of 25 kV was applied to separate and mobilise the cytoplasmic components towards the electrochemical detector. The cationic peak at 10.8 min (peak 1) was tentatively identified as DA, based on electrophoretic mobility, and an average DA content of 2.3 ± 1.7 attomoles (mean±SEM, n=3) was found for CSF lymphocytes. Peak 1 might also have contained other electroactive amines with similar electrophoretic mobilities such as NE, epinephrine, or serotonin. However, the electrophoretic mobility of peak 4 corresponds to dihydroxyphenylacetic acid (DOPAC), a metabolite of DA, which strongly suggested that peak 1 contained DA.

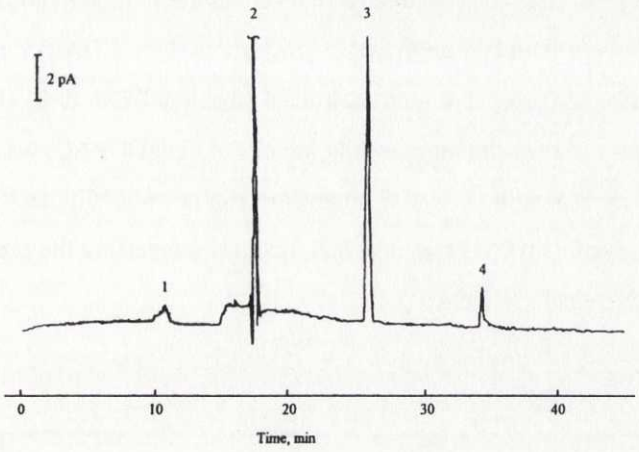

Figure 6. Electropherogram of a single human CSF lymphocyte. Conditions were as follows: separation capillary 10 μm i.d., 80 cm length with decoupler[156]; buffer, 25 mM MES (pH 5.65); injection, 60 s at 1 kV to draw the lymphocyte into the capillary tip followed by a 15 s injection of digitonin to permeabilize the cell; separation potential, 25 kV; amperometric detection potential, 0.8 V vs a sodium-saturated calomel reference electrode. Electrophoretic mobilities of the major peaks correspond to the calculated electrophoretic mobilities of dopamine (peak 1), a neutral species (peak 2), uric acid (peak 3), and DOPAC (peak 4). (Reproduced with permission from Proc. Natl. Acad. Sci. USA).

CE of single cloned human lymphocytes (Paper V)

Single cloned $CD4^+$ T lymphocytes and B lymphocytes have been examined by CE and found to contain catecholamines. Cloned $CD4^+$ T lymphocytes were found to hold catecholamines at a level of 31 ± 29 attomoles (mean ± SEM, n=3), while a single B lymphocyte was found to contain 310 zeptomoles of catecholamines.

CE analysis of catecholamines in immunocompetent cells (Papers V-VII)

The levels of catecholamines have also been quantitated in extracts from cloned human $CD4^+$ T lymphocyte populations, and found to be 1.8 ± 1.0 attomoles per cell (mean ± SEM, n=13), within the range of catecholamine levels found in single $CD4^+$ T cells (Paper V). The average catecholamine level in extracts of cloned B cells was 2.0

± 1.5 attomoles per cell (mean ± SEM, n=3). Using a modified analytical set-up, the DA and the NE peak could be separated based on electrophoretic mobility, and freshly isolated PBMC were found to hold 1.61 ± 0.72 attomoles of DA per cell, while the basal NE levels were 0.96 ± 0.86 attomoles/cell (mean ± SEM, n=3) (Paper VI). In these separations, not only the main metabolite of DA (i.e. DOPAC) was detected, but also a peak corresponding to the main metabolite of NE, i.e. 3-methoxy-4-hydroxyphenylglycol (MHPG) was detected, strongly suggesting the presence of both DA and NE in these cells (Figure 7.)

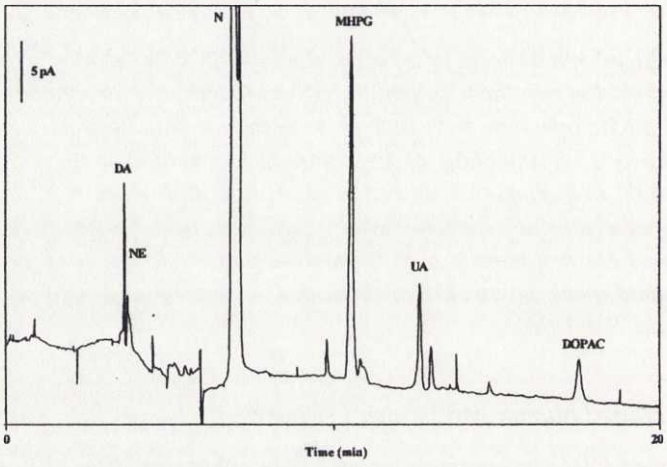

Figure 7. Separation and detection of easily oxidized species in microextracts of isolated human peripheral blood mononuclear cells by CE. Conditions: separation capillary, 10 μm i.d., 65 cm length with optimized end-column detection[143]; buffer, 25 mM MES (pH 5.65); injection was 5 s at 30 kV; separation potential, 30 kV; amperometric detection potential, 0.8 V vs a sodium saturated calomel reference electrode. The electrophoretic mobilities of the major peaks shown correspond to the calculated electrophoretic mobilities of dopamine (DA), norepinephrine (NE), a neutral species (N), 3-methoxy-4-hydroxyphenylglycol (MHPG), uric acid (UA), 3,4-dihydroxyphenylacetic acid (DOPAC). (Reproduced from Paper VI).

Furthermore, the catecholamine levels in mouse spleen cells, in peritoneal macrophages, and in T- and B-cell hybridomas were determined with CE (Paper VII).

The spleen cells contained 66 ± 57 attomoles of DA and 23 ± 12 attomoles of NE per cell, while the peritoneal macrophages contained 20 ± 18 attomoles of DA and no detectable amounts of NE (mean ± SEM, n=3). The variation among different hybridoma cell clones was found to be notable, ranging from 1.9 ± 0.7 attomoles of DA/cell down to below the detection limit (approximately 100 zmol). Also the NE levels varied from 280 ± 80 zeptomoles/cell down to undetectable levels. In order to examine the uptake and biosynthesis of catecholamines in lymphocytes, both human and murine T cell clones were incubated with either DA, or the DA synthesis inhibitor α-methyl-*p*-tyrosine (MeTyr, Papers V, VII). After incubation with DA or L-DOPA (the precursor to DA), a large increase in the level of DA per cell was observed. However, incubation with the synthesis inhibitor decreased the level of DA. Further, the uptake of DA into PBMC was significantly decreased ($p<0.01$) by co-incubating the cells with nomifensine[187], a DA transporter antagonist (Paper VI). The corresponding incubation with DA in the presence of, tetrabenazine[188], a catecholamine storage blocker, led to similar results, with a significant decrease of intracellular catecholamines ($p<0.05$). None of these substances affected the basal levels of catecholamines in PBMCs.

Analysis of catecholamine induced apoptosis in immunocompetent cells (Papers VI-VII)

The possible ability of catecholamines to suppress lymphocyte proliferation and differentiation by triggering of apoptosis, was assayed by two different methods. First, the DNA damage in cells after incubation with L-DOPA, DA and NE was assessed by measuring of an intercalating fluorescent agent in a flow cytometer. Apoptosis was dose-dependently induced, both in human PBMC and in murine spleen cells. Second, a dose-dependent decrease of the apoptosis inhibitor protein Bcl-2, and dose-dependent increase of the apoptosis inducing proteins Bax, Fas and FasL were determined by western blotting.

7. General discussion

Methodological aspects

CE is becoming accepted as a new separation technique, that is and will be of great importance in bioanalytical chemistry. There are many characteristics of CE, some of them are listed in Table 1.

Table 1. Characteristics of CE

- Capillary electrophoresis is performed in narrow-bore (2 to 200 μm i.d.) fused silica capillaries
- High voltages (5 to 40 kV) and high electric fields (100 to 500 V/cm) are applied across the capillary
- High efficiency ($N>10^5$ to 10^6) and short analysis time
- High peak capacity (can separate 10 times more substances, with 10 times higher selectivity compared with HPLC)
- Numerous modes to vary selectivity and wide application range
- Numerous modes of detection, performed on-column
- Small sample volume required (10^{-13} to 10^{-9} L injected)
- Consumes limited quantities of reagents
- Operates in aqueous media

Many parameters in CE can be altered however, and will consequently influence on the efficiency, resolution, and selectivity of the method. Some of these aspects have been mentioned in the introduction (see Principles of CE). Briefly, there is a number of practical things to consider when constructing a separation method for CE. Some of these are listed in Table 2.

Table 2. Variables in CE

- Capillary length
- Capillary diameter and wall coatings
- Detection method to use
- Applied field strength
- Temperature control
- Ionic strength of the separation buffer and in the sample
- The pH of the separation buffer
- Additives and organic modifiers in the separation buffer
- Injection mode

Since many factors are involved during the electrophoretic separation, reproducibility and quantification may in some cases be problematic. Difficulty in maintaining a constant temperature inside the capillary, viscosity changes, pH variations, ion depletion in the buffer reservoirs, binding of sample molecules to the capillary wall etc., may all affect the electroosmotic flow and in turn make identification and quantification elaborate. Furthermore, since one of the major advantages with CE is the possibility to handle small sample volumes and analysis of extremely low amounts (down to single molecules), one needs to consider problems associated with evaporation and contamination. Also, working with complex biological systems may introduce problems due to sample instabilities.

In order to minimise these analytical problems it is important to control a number of parameters, some of them listed in Table 3.

Table 3. Parameters to consider to achieve good results in CE

- Attend to effective temperature control, especially important in capillaries with >25 μm i.d., since internal heating could dramatically effect the separation
- Use low heat producing buffers
- Regenerate buffers frequently (in-between every 10 runs, depending on reservoir size)
- Regenerate the capillary wall with base or acid in-between runs
- Compensate for mobility changes by using internal standards (also important in derivatization procedures to control the degree of reaction equilibrium established)
- Ensure a correct identification by spiking samples with known amounts of standards or by blocking with known synthesis inhibitors
- Seal and store samples cold in order to reduce evaporation
- Eliminate the risk of contamination, by running blank runs of buffers, medium, etc.
- Consider the sampling procedure and sample treatment

Finally, the interpretation of data obtained from the electropherogram, is of great importance. Here, the use of a chromatography software is of help, by keeping track of current, voltage, capillary dimensions and other experimental parameters. The software can provide actual peak migration time, corrected peak migration time, peak height, and the peak area. It can calculate the electrophoretic mobility for a given peak and also calculate concentrations based on peak height or peak area.

By controlling all of the above parameters, CE fulfils all the requirements for a valid and reliable analytical tool.

Discussion of results

In our first paper the major objective was to show the capability of the CE-LIF technique for the direct analysis of amino acids in a biological fluid as CSF, without prior purification. Clearly, it emphasises the advantages of the separation and sampling capabilities of CE, and the sensitivity of the LIF detection. The separation conditions and derivatizing procedures were optimized in order to determine GABA levels. GABA is a very challenging neurotransmitter to assay due to its low concentration and instability. The procedure used for the GABA determination gave a rather large variation in detection limits for the other amino acids. Plausible explanations are that there are varied amounts of individual amino acids available, and that they have differing steric conformations. These differences in properties, will result in heterogeneous derivatizing reaction times for individual amino acids. It is also possible that amino acids interact to different degrees with other molecules in the sample, e.g. proteins. The recovery data presented in Papers I and III, to some extent suggest this phenomenon.

Derivatization times between 5 min and 7 h were tested. Two hours was chosen because the GABA peak was maximal and stable following this derivatization time. However, sufficient analysis of the other amino acids was also permitted following this procedure.

It is important to consider the problems of instability in the amino acid levels of CSF. Especially GABA, glutamine, glutamic acid, and aspartic acid levels are easily influenced by sample handling. Therefore, in the literature, the reported levels of amino acids in CSF vary widely[189-191]. In order to be able to perform valid bioanalytical investigations of clinical samples, it is crucial that a strict sampling procedure is followed. All the samples need to be obtained in exactly the same, controlled way, avoiding unintentional degradation, stored at -80°C, and analysed at the same time, etc.

Another problem which might influence the results is the risk of comigrating substances (less common in CE than in most other separation techniques, due to the

high peak capacity and selectivity obtained with CE) which have to be carefully thought about, and, if possible, excluded by changing the ionic strength or the pH in the separation system, or by running a second analytical method (e.g. HPLC) separately, in parallel, and comparing the data (see Paper III).

Maybe the low sample consumption in CE (with only 5.9 nL injection volume), does not fully realize its potential in this application, since CSF samples often are of rather large volume (12 mL from adults and ~3 mL from children under the age of 16). However, the consumption of expensive derivatizing agents is kept low in CE. As a comparison, a conventional HPLC method for GABA analysis in CSF consumes approximately 2.5 μL of sample and 25 μL of reagents (OPA, 10 mg/mL)[192].

The results from the analysis of CSF from Alzheimer's patients and children with neurological disorders must be seen as preliminary. In order to be able to interpret anything definite about the differences between patients and controls individual samples in larger numbers need to be investigated. Preferably consecutive samples, from the same patient, are drawn over a period of time in order to evaluate the individual fluctuations. Notable, is the recent study of CSF from leukaemic children by Nouadje et al. (1995)[26], where basically the same method for separation and detection of primary amines and amino acids as shown in this thesis, was used. The only difference was the derivatizing agent (FITC instead of CBQCA), and the lack of an internal standard to control the reaction equilibrium and the injected amount of material in their system. There was good agreement of their results with ours, except for aspartic acid. Nouadje et al.[26] found 10 times higher levels of aspartic acid. The reason for this discrepancy is not obvious. However, their congruent data strengthens our findings and based on this and a number of other recent reports of clinical applications of CE[193, 194], it is our belief that CE might become a powerful tool in clinical laboratories in the near future.

In the second paper we showed that proteins specific for CNS may be analyzed in CSF after a specially designed purification scheme, including affinity- and reversed-phase chromatography. Trace levels of the synaptic vesicle specific protein, synaptotagmin, can be quantitated with chemiluminescence immunoblotting, and a decrease of this protein has been found in CSF from patients with early onset Alzheimer disease patients. This data is preliminary, in view of the small number of patients and the fact that their CSF was pooled. However a decreased concentration is also present in hippocampus and frontal cortex of these patients, further supporting the findings in the CSF.

Because of the high abundance of plasma proteins in CSF (~70% of the protein content in CSF is derived from plasma), purification procedures like the one described here, are of great importance, enabling the analysis of the trace level proteins derived from the CNS. In conjunction with the HPLC purification step, CE with UV-detection has been used for further characterisation and purity analysis of these proteins. The unique and complementary selectivity differences of CE and HPLC make them an ideal combination for purity cross confirmation.

As seen in the electropherogram in Paper II, one single peak was detected in the HPLC fraction, supporting the presence of only one protein in that specific fraction. In order to increase the detection, the sample has been dissolved in low ionic strength solvent in order to cause sample stacking inside the capillary, and a rather long injection time (10 s, 59 nL) has been used. There is little reason to believe that there could be problems with comigration in this separation, with very high efficiency (>290000 calculated number of theoretical plates) for the resulting peak.

It is not known whether the entire synaptotagmin molecule or only a N-truncated form of the protein is present in CSF. Since there is no synaptotagmin standard available we could not confirm comigration with the CE peak. The separation efficiency of the SDS-PAGE can only give an approximate molecular weight, and the isolated amount was not sufficient for mass spectrometric determination.

Synaptotagmin is normally anchored in the membrane[167] and therefore ultracentrifugation was performed to investigate whether the isolated protein was membrane bound or soluble. Since the protein stayed in solution it might be free or possibly complexed with another protein, not large enough to form precipitates.

Alzheimer's disease is characterized by a number of pathological findings. A loss of synapses is one of them[195]. The amount of synaptotagmin, being important in the docking of vesicles to the synaptic plasma membrane, is likely to be decreased as a result. It is important to find tracers that monitor synaptic function and pathology in vivo, in order to facilitate the early diagnosis and treatment of patients with neurodegenerative disorders.

In the two following papers the microdialysis technique in combination with CE-LIF was used to monitor changes of amino acids in the periaqueductal grey matter of freely moving rats. The microdialysis technique has been used in many experimental systems[27]. There are many factors to consider in these types of animal experiments, including probe size, type of dialysis membrane, insertion and positioning; perfusion buffer and rate; and animal maintenance. However this is not further discussed here. The analysis of microdialysis samples is often difficult due to the low amounts of most substances. The diffusion rates of molecules across the dialysis membrane is determined by the difference in their concentrations. Thus, low perfusion rate increases the concentration but decreases the absolute available amount obtained per time unit, and vice versa. It is also difficult to assess the true extracellular concentrations. Furthermore a sampling technique has not been available for the measurement of fast changes , since only the sum of the effects will be seen in the microdialysis sample due to a low rate constant. In most applications HPLC[196] or radioimmunoassays (RIA)[197, 198] are used for the analysis of neurotransmitters. However there are now several reports of the use of CE[199-208].

CE may permit a more sensitive analysis, wich may in turn permit a higher temporal resolution, usually one of the major problems with microdialysis when

monitoring transmitter release. Even on-line monitoring of amino acids has been published[205, 209].

In the first of these two papers CE-LIF was used in parallel with two different HPLC methods, one with electrochemical detection[174] and one with fluorescence detection[175], for the analysis of GABA and glutamic acid. The effect of potassium stimulation (100 mM in the perfusion fluid) on released amounts of amino acids was studied, with or without calcium present. The two methods gave similar results, especially concerning GABA release patterns. Perfusion with an elevated concentration of potassium resulted in an increased release of GABA but failed to increase glutamate levels. This may be due to the potent uptake mechanism for glutamate in the PAG brain region[210]. The time in storage of samples, the differences in derivatizing procedures, etc. also need to be considered as possible factors, that may contribute to the discrepancy between the absolute values obtained with the two methods. Yet the CE analysis of several amino acids is a good complement to conventional HPLC techniques.

In the second of the two microdialysis studies, the sensitivity of the CE-LIF method is used when the basal release of GABA is analysed. In this paper, the microdialysis probe is used both for sampling and for local administration of morphine and naloxone in the periaqueductal grey matter, a brain region strongly involved in nociceptive functions[211-213]. It was shown that morphine decreases the basal release of GABA suggesting an opioid inhibition of GABA, and resulting in a disinhibition of descending antinociceptive pathways (see Figure 8). This effect of morphine was blocked by the opioid receptor antagonist naloxone[214], further indicating an involvement of opioid receptors.

Due to the interference of both morphine and naloxone with the electrochemical detection in the HPLC method, HPLC could not be used in parallel with the CE method. The derivatizing agent CBQCA in the CE-LIF assay does not react with either substance, since they lack primary amines.

Some changes in the derivatizing procedure were made. Firstly the internal standard was changed from a somatostatin fragment to histamine, a molecule normally present at very low concentrations in brain tissue[157]. Standard histamine has a migration time well separated from the other major components. Histamine gives a single peak, instead of the two given by the somatostatin fragment, which facilitates data evaluation. Secondly, the derivatization time was decreased to 1 h from 2 h, and the separations were stopped after the GABA peak eluted, in order to shorten the intrasample time (from 40 to 20 min). Thirdly, by increasing the injected amount five times (from 6 to 30 nL), this only affected the detection limit to a minor extent (from 0.3 to 0.4 nM). Also the error in injected amount decreased from 6% to less than 2%.

Comparing the GABA levels obtained with the CE-LIF method in the two papers demonstrated some, although not dramatic, differences in the absolute levels. Nevertheless, they could be explained by the differences in probe positioning in the two experiments. As seen in the schematic drawings the probes in the second paper are much more centered as the result of a new insertion angle. Also there may be other differences occurring due to sample storage. Further analyses need to be performed in order to establish if the release of other amino acid transmitters is affected by the morphine treatment; for instance glutamate may be a good candidate. Thus, these two papers in combination show the great potential of CE-LIF in the analysis of microdialysis samples as a complement, or as an alternative to HPLC.

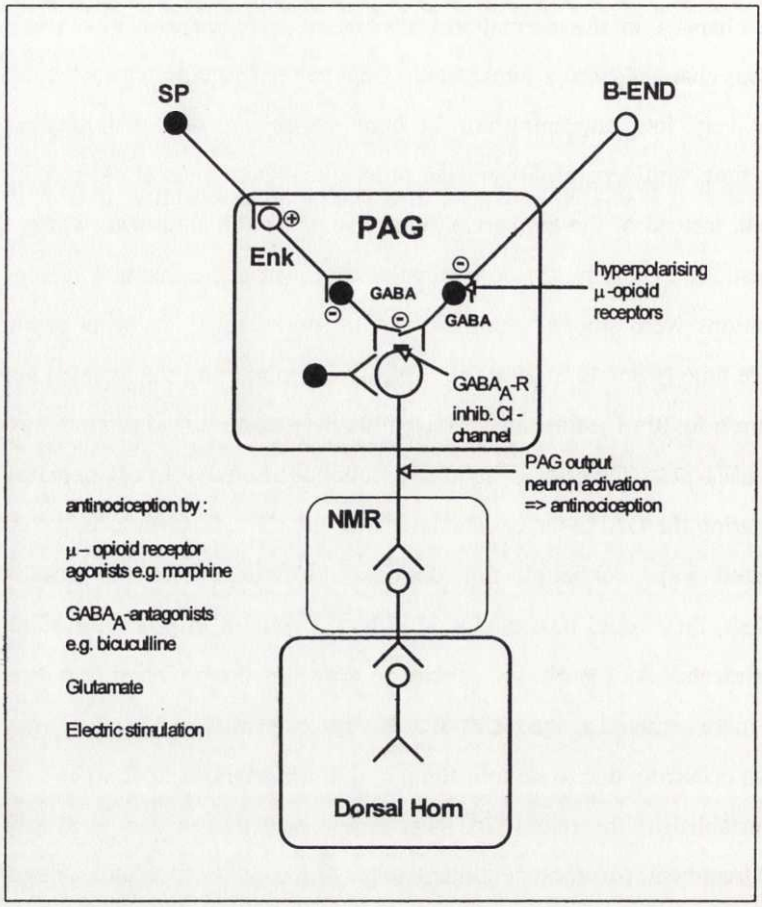

Figure 8. Schematic drawing of the periaqueductal grey matter (PAG) region. From this region, descending pain inhibitory pathways projects down to the nucleus raphe magnus (NRM) in the rostroventral medulla, which in turn contain neurons which project to the dorsal horn of the spinal cord. These output neurons are tonically inhibited by GABA interneurons (via $GABA_A$-receptors), which in turn may be regulated by either substance P (SP) via an enkephalin (ENK) neuron, or by β-endorphin (B-END). In the PAG region, antinociception can be induced in a number of different ways. As shown in Paper IV local administration of morphine decreases the extracellular level of GABA, suggesting a hyperpolarising effect on the GABA interneurons, inhibiting the basal GABA release.

The application of CE with electrochemical detection for detection of easily oxidized species is presented in Papers V-VII. These applications show that the use of

CE with electrochemical detection might be a tool which opens up new avenues to explore the interactions between the nervous and the immune systems.

The finding of catecholamines in lymphocytes derived from the immune system has produced many questions. The dramatic effects that catecholamines have on proliferation, and at differentiation, indicate an important regulatory role of these substances. Since we have shown that these cells produce catecholamines, both autocrine and paracrine routes may exist. The possibility that catecholamines might induce apoptosis in lymphocytes has also been measured, and a dose-dependent effect on DNA damage and apoptosis marker proteins was found.

The mechanism or mechanisms by which exogenous catecholamines affect different cells is still somewhat unclear. Since it is known that lymphocytes have both α- and β-receptors[215-219] one possible course could be a receptor mediated effect. In preliminary studies (unpublished results) different receptor blockers have been tested. However the results were unclear. The receptor antagonists appear to exert an effect of their own and no clear inhibitory effect on the catecholamine response could be demonstrated.

The regulatory effects induced by catecholamines are also induced by L-DOPA (Papers VI-VII, Slominski and Goodman-Snitkoff, 1992[220]), where the effects are probably not receptor mediated. In addition to this, we found an uptake of exogenous DA by lymphocytes, possibly indicating the presence of a DA transporter[221, 222]. This uptake can be blocked by the specific DA transport inhibitor, nomifensine[187], further supporting the transporter theory (Paper VI). Once taken up into the cell cytoplasm, catecholamines are degraded by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) if they are not protected through storage in vesicles or other compartments at low pH. This active storage mechanism can be blocked by incubation with tetrabenazine, depleting the vesicles and reducing the intracellular levels of catecholamines (Paper VI).

In preliminary experiments (unpublished results) the levels of catecholamines in isolated and extracted human lymphocyte cell nuclei were determined with CE and

electrochemical detection. The amount of catecholamines was found to be just a few zeptomoles per nucleus. No catecholamine metabolites were detected in the nuclear extract. The experimental data suggest that ~1-2% of the total amount of catecholamine may be situated inside the nuclear membrane. This finding of catecholamines inside the nuclear membrane in PBMC is remarkable. The risk of contamination from cytosolic compartments is low due to the rigorous isolation and purification procedure. The lack of detectable catecholamine metabolites in the extracts further reduces the possibility of contamination. Since both MAO and COMT, the two major catabolic enzymes for catecholamines (at least COMT has been found in PBMC[223]), are located at the outer membrane of the mitochondria or in the cytosolic compartment, the catecholamines inside the nuclear membrane are protected. A nuclear membrane transporter for catecholamines is also known to exist[224] which further strengthens this finding of intranuclear catecholamines. The role of the catecholamines inside the nuclear membrane is still to be resolved, but may involve interaction with steroid receptors or interference with transcription.

Other preliminary data (unpublished results) show that the levels of endogenous catecholamines are dependent on stimulation of human lymphocytes. The superantigen SEA initially increased the level of DA and then caused a decrease after 72 h of incubation. The two mitogens, Con A and aCD3, had opposite effects on catecholamine levels. Con A stimulation of lymphocytes increased the DA levels, while aCD3 decreased both the DA and the NE levels. When repeating the same experiments with mice spleen cells in Paper VII, no significant changes in the levels of endogenous catecholamines were found. The mice cells were stimulated with Con A, LPS, SEA, SEB and toxic shock syndrome toxin-1. At this stage, no obvious explanation for these results has been found. However, this data further emphasises the sensitivity and complexity of neuroimmunological interactions. Immunocompetent cells may change their neurotransmitter content due to a variety of exogenous influences. All the above mentioned findings are summarised in Figure 9.

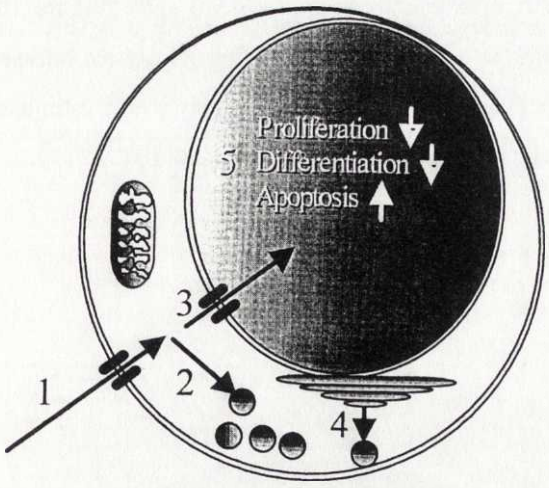

Figure 9. A schematic drawing of a lymphocyte, illustrating the neuroimmunological interactions and effects of catecholamines studied in Papers V-VII. (1) An uptake mechanism (Papers V-VII) that can be blocked with nomifensine (Paper VI). (2) A storage mechanism that can be blocked with tetrabenazine (Paper VI). (3) An uptake and presence of intranuclear catecholamines (unpublished results). (4) An endogenous production of catecholamines in lymphocytes (Papers V-VII) that can be blocked by α-methyl-p-tyrosine (Papers V, VII). (5) Finally, an inhibitory effect of exogenous catecholamines was found on proliferation and differentiation, in combination with an induction of apoptosis (Papers V-VII).

The regulatory effect of catecholamines on immunocompetent cells is a well studied phenomenon, both by us (Papers V-VII) and by others[220, 225-228]. The systemic concentrations of these catecholamines are rather low (~1 nM)[229], but may reach significant levels locally, i.e. in the immediate vicinity of the synapse or close to a sympathetic varicosity. In order to evaluate what concentrations of catecholamines could occur locally at a synapse, a rough calculation was performed. Typical synaptic vesicles have diameters in the range from 20 to 100 nm[230-232], (50 nm would give a volume of 65 zeptolitre) and contain 0.05-0.5 M of catecholamines[233]. This would result in ~2000-20000 molecules per 50 nm vesicle. If one vesicle fuses with the presynaptic membrane and releases its contents into the synapse (with an approximate volume of 10 attolitre, calculated as a cylinder with an height of 50 nm and a diameter

of 500 nm), this would result in a concentration of 0.3-3 mM (Figure 10). This concentration could be even higher (i.e. 30 mM) if 100 vesicles were released during the same time period. The concentrations presented are very rough estimates, and in addition, re-uptake and degradation mechanisms have been ignored[234].

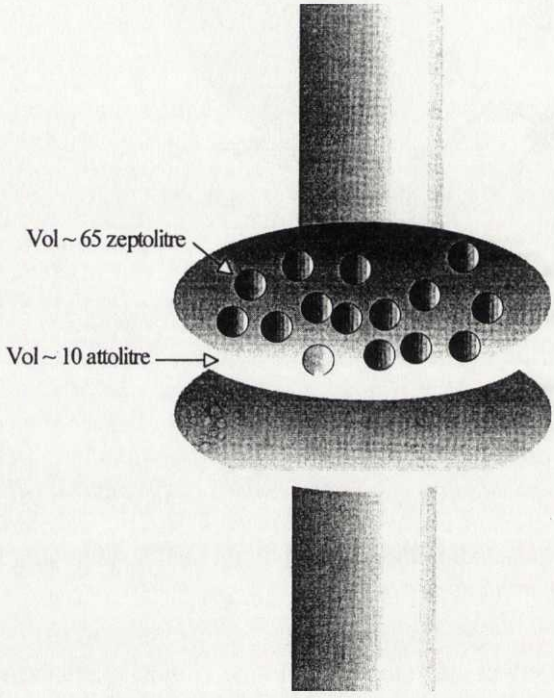

Figure 10. A schematic drawing of a synapse, where vesicles stored in the presynaptic terminal are filled with neurotransmitters, to be released into the synapse when fusion with the presynaptic membrane take place, and bind to receptors on the postsynaptic membrane, in order to forward the signal between nerve cells.

However, this data points out the possibility that lymphocytes could encounter high concentrations of catecholamines in a local environment near synapses in the brain or close to sympathetic varicosities. In this thesis, immunocompetent cells have been shown to produce and actively store catecholamines (Papers V-VII). Furthermore, an active uptake and storage of catecholamines in these cells has also been established (Paper VI). Both exogenous and possibly endogenous

catecholamines may suppress proliferation and differentiation of immunocompetent cells via the induction of apoptosis (Papers VI-VII). Together, this data suggests new mechanisms by which the central and peripheral nervous systems may downregulate, or drain, the immune system locally.

The small volume capabilities of CE and the sensitivity of electrochemical detection, make this an ideal tool to study the neuroimmunological network from the single cell aspect. Sample handling for lymphocytes with femtoliter volume is relatively straightforward and detection limits for catecholamines in the order of 10^{-19} to 10^{-20} moles can be achieved. Key areas of future work need to include laser-induced fluorescence, mass spectrometry, and perhaps immunoassay based detection schemes of determining molecular neuroimmune interactions.

8. Conclusions

Vi vet att den mänskliga hjärnan finns där för förhindra att öronen trasslar in sig i varandra

Peter de Vries

- CE with laser-induced fluorescence detection has been shown to be a new tool in medical neuroscience for the sensitive analysis of neuroactive amino acids in the CSF. Levels of individual amino acids can be determined, indicating the potential of CE for the analysis of CSF as a method both, for the study of CNS disorders and, for the examination of the pathophysiological mechanisms underlying these disorders (Paper I).

- Proteins specific to the CNS may be analysed in the CSF according to a specially designed purification scheme. Trace levels of the synaptic vesicle specific protein, synaptotagmin, can be quantified, and a decreased content of this protein has been shown in the CSF of patients with early onset Alzheimer's disease. This phenomenon is also seen in the hippocampus and frontal cortex of these patients, further supporting the CSF findings. CE with UV detection is a universal method for studying the purity and characteristics of analytes after other separation and purification methods. The unique complementary selectivity differences of CE and HPLC make them an ideal combination for cross confirmation of purity (Paper II).

- CE with laser-induced fluorescence detection, has become a new analytical tool in neuropharmacology, for the analysis of neuroactive amino acids in microdialysis samples, as shown by the analysis of local fast and transient changes in animal cerebrum (Paper III).

- CE with laser-induced fluorescence detection permits the detection of the basal release levels of GABA in the periaqueductal grey matter. Local administration of morphine in this region led to a significant decrease of the GABA level, providing direct experimental evidence for an opioid induced inhibition of a tonic GABA release, which may in turn lead to a disinhibition of the descending pain inhibitory pathways (Paper IV)

- CE with electrochemical detection permits analysis of neurotransmitters down to the single cell level. The technique has been shown to be instrumental in the discovery of a new neuroimmunological communication pathway, where catecholamines are endogenously produced in cells derived from the immune system of both human and murine origin (Papers V-VII).

- Catecholamines can act as autocrine and paracrine regulators of human and murine lymphocyte activity (Papers V-VII).

- An uptake mechanism for exogenous catecholamines in lymphocytes can be blocked by an uptake inhibitor (Paper VI)

- An intracellular storage mechanism for catecholamines in lymphocytes can be blocked by a storage blocker (Paper VI)

- Catecholamines may induce apoptosis in lymphocytes, involving changes in levels of apoptotic marker proteins like Bcl-2/Bax and Fas/FasL, indicating a possible regulatory mechanism for the nervous system to suppress the immune system locally (Papers VI-VII).

9. Summary in lay language (Svensk sammanfattning)

Den medicinska forskningen har gjort sådana enorma framsteg att det praktiskt taget inte finns några friska människor längre

Aldous Huxley

En viktig uppgift inom modern neurovetenskap, är att finna metoder för att kunna detektera och kvantifiera specifika molekyler, som reflekterar cellkommunikationen. Denna signalering pågår ständigt inom cellen, mellan celler inom nervsystemet, mellan nervsystemet och andra system och organ i kroppen.

Frågeställning:

- Kan kapillärelektroforetiska tekniker tillämpas för att studera förändringar i nivåer av olika neurotransmittorer och proteiner, i enskilda celler och kroppsvätskor?
- Kan dessa förändringar vara tidiga markörer för ett progredierande sjukdomsförlopp i CNS, som i förlängningen kan komma att leda till psykisk störning eller demens?
- Kan kopplingen mellan immun- och nervsystemet vara en viktig faktor i en rubbad biologisk jämvikt som uppträder vid ovan nämnda störningar?
- Kan en katekolamin inducerad apoptos vara en del av orsaken till att det centrala nervsystemet är immunologiskt privilegierat?

Metodik:

Vi har konstruerat och utvecklat ett eget kapillärelektrofores (CE) instrument, som parallellt med ett kommersiellt instrument, har använts för neurokemiska analyser av substanser i biologiska system. Denna relativt nya analytiska teknik utvecklades i slutet av 1980-talet, mer än 20 år efter genombrottet för högtrycks vätskekromatografi (HPLC). Kapillär elektrofores tekniken erbjuder flertalet olika system för snabba och effektiva separationer av alla möjliga molekyler, ifrån metalljoner till stora makromolekyler. Metoden tillåter provtagning från mycket små volymer (enstaka celler och subcellulära fraktioner), ger utsökt upplösning med upp till en miljon teoretiska bottnar, samt har mycket god känslighet (ner till enstaka molekyler). Alla

dessa egenskaper är av stor vikt vid studier av komplexa samband inom neurobiologin och neurokemin. Förutom CE har en rad biokemiska analys metoder använts (ELISA, ELISPOT, HPLC, flödescytometri, western blot).

Våra försök har utförts dels direkt på cerebrospinal vätska (CSF), på enskild cell nivå vid analys av lymfocyter, dels på extraherade perifera cellpopulationer av humant och murint ursprung, samt på mikrodialysat från hjärnregioner hos råtta.

Resultat och slutsatser:

- Genom att använda CE med laser-inducerad fluorescens detektion har vi analyserat ett flertal neurobiologiskt aktiva amino syror i mycket små provvolymer (6 nL injicerad volym). Denna metodik har visat sig vara användbar vid undersökningar av amino syra nivåer i CSF vid t.ex. neurodegenerativa sjukdomar.

- Genom att använda UV detektion, har kapillärelektrofores tekniken kunnat bidraga till fyndet av ett hjärnspecifikt protein i human CSF, synaptotagmin. Detta synapsvesikel protein kan komma att utgöra en tidig markör för neuronal skada och synapsförlust. Sänkta nivåer av proteinet konstaterades i CSF och hjärnvävnad från Alzheimer's patienter.

- I två neurofarkamologiska studier, fann vi att CE lämpar sig väl som metod för analys av snabba förändringar av frisatt mängd amino syror i specifika hjärnregioner. Detta ledde till fyndet av en verkningsmekanism för morfins smärtinhibitoriska funktion i den periaqueductala grå substansen.

- Genom att använda elektrokemisk detektion och CE, är vi först med att ha studerat katekolaminers produktion och lagring i immunkompetenta celler. Vidare har vi funnit belägg för att katekolaminer påverkar proliferation och differentiering hos dessa celler. Detta kan förklaras av en induktion av apoptos, som involverar Bcl-2/Bax och Fas/FasL. Detta innebär en ny väg för nervsystemet att påverka immunsystemet och vice versa. Resultaten antyder även i förlängningen nya patofysiologiska mekanismer vid neurodegenerativa sjukdomar.

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Maybe this part of the thesis has been the most difficult one to write. Since I had already enjoyed so many fun moments both in science and in reality, I have so many to thank for their great care, support and friendship they have shown to me. This gratefulness can not really be expressed in words, without getting a bit pathetic. However.....

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.....

(please fill in your name if I accidentally forgot you, terribly sorry for that, old sport!)

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Om man skriver av en bok är det plagiat, om man skriver av två är det forskning, om man skriver av flera är det forskning på ett högt plan

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