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Glutamate, Microdialysis, and Cerebral Ischemia

Lost in Translation?

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Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. By Helene Benveniste, Jørgen Drejer, Arne Schousboe, Nils H. Diemer. *J Neurochem* 1984; 43:1369-74. Reprinted with permission of Blackwell Publishing.

Rats were implanted with 0.3-mm-diameter dialysis tubing through the hippocampus and subsequently perfused with Ringer's solution at a flow rate of 2 ml/min. Samples of the perfusate representing the extracellular fluid were collected over 5-min periods and subsequently analyzed for contents of the amino acids glutamate, aspartate, glutamine, taurine, alanine, and serine. Samples were collected before, during, and after a 10-min period of transient com-

plete cerebral ischemia. The extracellular contents of glutamate and aspartate were increased, respectively, eight- and threefold during the ischemic period; taurine concentration also was increased 2.6-fold. During the same period the extracellular content of glutamine was significantly decreased (to 68% of the control value), whereas the concentrations of alanine and serine did not change significantly during the ischemic period. The concentrations of γ -aminobutyric acid (GABA) were too low to be measured reliably. It is suggested that the large increase in the content of extracellular glutamate and aspartate in the hippocampus induced by the ischemia may be one of the causal factors in the damage to certain neurons observed after ischemia.

WHEN Dr. Warner approached me regarding writing a Classic Papers Revisited contribution for *Anesthesiology* on my glutamate, microdialysis, and cerebral ischemia work from 1984,¹ the first thought that ran through my mind was that I was getting really old! The second was that I should not be the one writing it; Nils-Henrik Diemer, M.D., D.Sc. (Professor, University of Copenhagen, Denmark), my mentor at the time and who generated the idea for the project, would be much more appropriate for the task. Then I thought some more about it. As with any other scientific project its execution is critically dependent on a team effort, and as a team player perhaps I did have a story to tell; ideally, the narrative should be constructed like the classic

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Dr. Helene Benveniste; photo taken in 2008 at her laboratory, Medical Department, Brookhaven National Laboratory.

Kurosawa movie *Rashomon*, where the story is told from four different viewpoints. Of course in reality you are only getting my version, which in the interest of accuracy and



Fig. 1. I kept the old box that held the amicon microdialysis tubes. All along I used the box for all the small accessories that I needed for the experiments. I have only a few (and worn!) gadgets left; a = custom made devices for securing (via the stereotaxic device) the microdialysis fiber and internal stylet; b = spinal needle stylet attached to one of the devices; c = small piece of sandpaper that I used to clean off the stylet after use (epoxy occasionally got stuck during the processing).

nostalgia may appear overly detailed and somewhat long winded.

In 1982, I was a third year medical student at the University of Copenhagen. I was keenly interested in doing a research project and fortunate enough to be introduced to Professor Nils-Henrik Diemer, a.k.a. Nils, at the Institute of Neuropathology in Copenhagen. (See Supplemental Digital Content 1, which shows Nils in 1991 while attending the defense of Dr. Benveniste's doctoral dissertation "Exitotoxin Hypothesis in Relation to Cerebral Ischemia" at the University of Copenhagen, Denmark, <http://links.lww.com/A679>.) I clearly remember my first encounter with Nils; he was standing with a small black box in his hands (fig. 1), which he explained contained a bundle of small dialysis fibers. He and his colleague Martin Balslev Jørgensen, M.D., D.Sc. (Professor, Department of Psychiatry, Rigshospitalet, Denmark), had been working on a hypothesis of selective neuronal loss after cerebral ischemia and the role of the neurotransmitter glutamate.² They had discovered that the pattern of selective neuronal loss after transient cerebral ischemia corresponded closely with areas of high affinity glutamate uptake and hypothesized that glutamate might play a decisive role in selective ischemia-induced neuronal damage.² My project was to follow up on this investigation with a study demonstrating the release and/or increase of the extracellular neurotransmitter glutamate in one of the 'ischemia-sensitive' brain regions such as the dorsal hippocampus of the rat. Nils also handed me two papers published by Urban Ungerstedt, M.D., D.Sc. (Professor, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden), and his colleagues, one which had used "intracerebral" dialysis and another describing "an implanted hollow perfused fiber"^{3,4} to measure amino acids and

dopamine *in vivo*. I remember opening the box and looking at the plastic tube, which contained hundreds of tiny hollow fibers with a molecular cut-off of 50,000 D. I thought about booking an eye exam.

I read the Dr. Ungerstedt papers several (make that hundreds of) times. They briefly described implanting a dialysis tube into the rabbit/rat brain and also gave the composition of the dialysis perfusion fluid, but very little detail was given as to how to actually do it surgically. They had used a horizontal dialysis tube approach where the implantation requires two bilateral craniotomies placed at the level of the dorsal hippocampus. I decided that I needed some bioengineering help and immediately contacted my father (Daniel Benveniste, M.D., anesthesiologist, [1917–1983]) who at that time worked as an anesthesiologist at a regional hospital in Denmark. (See Supplemental Digital Content 2, which shows Dr. D. Benveniste in 1978 while working in the Department of Anesthesiology, Nykøbing Falster Hospital, Denmark, <http://links.lww.com/A680>.)

He was keenly interested in bioengineering and had designed multiple instruments, including humidifiers, ventilators, a cardiac resuscitation device, and a pediatric continuous positive airway pressure valve.^{5–7} While growing up I spent hours with my father in his various workshops at home and in the hospital surrounded by tubes, valves, fitting devices, needles, cylinders, and gadgets of all sizes. In parenthesis, many of my father's inventions required testing on humans and by all means why not keep the experiments in the family? Luckily, the modern day Institutional review board had yet to be invented.

The internal diameter of one single hollow diaflo fiber from the Amicon unit was 200 μm , very fragile due to its large pore size, and clearly needed to be supported. I spent several days digging through boxes of gadgets and finally settled on a series of spinal needle stylets. The 150 μm stylet was sufficiently thin, straight, and sturdy to act as an enforcing internal wire for the fragile dialysis fiber so that it would remain in position when implanted into the brain. The second step required sealing parts of the dialysis tube to assure that it only exchanged metabolites from the hippocampus and not, for example, from the adjacent cortex. I used epoxy to cover the nonexchangeable parts, leaving an 8-mm long middle section uncovered. The third step required some kind of holder, which could be attached to the stereotaxic frame, which could fix the dialysis fiber in the horizontal position at the level of the correct stereotaxic coordinates and after implantation would allow the internal stylet to be removed from the dialysis membrane now positioned in the hippocampus. My father actually designed this simple device using a 16-gauge needle combined with a tiny coil cylinder with a small screw device (one version actually using a catheter 'fixer' from an epidural kit) that could be tightened and loosened easily. I returned happily to

the Institute of Neuropathology in Copenhagen with all my new devices ready for the first experiment.

My colleagues in the cerebral ischemia laboratory had taught me how to anesthetize, tracheotomize, and mechanically ventilate a rat. I managed to do all these procedures, position the rat in the stereotaxic frame, and also drill two craniotomies bilaterally in the temporal bone so that the dialysis fiber would be correctly positioned in the CA1 region of the hippocampus. The idea was to implant the dialysis fiber, let it perfuse with artificial cerebrospinal fluid for 1 or 2 h at a perfusion flow of 2 $\mu\text{l}/\text{min}$ and then do the experiment. Nils was present for this first experiment to show me how to induce transient global cerebral ischemia which was a first for me. I clearly remember being very nervous at the time of implanting the fiber and the dental cement, which was used to secure the dialysis fiber bilaterally was difficult to work with; it would be misleading to say that things looked neat. I also discovered that it was not so easy to remove the stylet from the dialysis fiber without applying quite a bit of force. I had decided to use a temporal resolution of 5 min intervals, collect 2–4 baseline samples, two during the 10 min cerebral ischemia episode and then another 2–4 samples postischemia. The transient global ischemia model previously designed by Siemkowitz⁸ was effective but not as elegant as the later 2- or 4-vessel occlusion models.⁹ It used a cuff/plastic tube positioned around the neck and occluded while arterial blood was withdrawn until mean arterial blood pressure reached 50 mmHg. After the experiment I collected and froze all the dialysates (collected in small sealed tubes).

The analysis of the dialysate samples was done using high-performance liquid chromatography, and this crucial part of the project was done by Jørgen Drejer, Ph.D. (Director of Cell Biology, NeuroSearch A/S, Denmark), and Arne Schousboe, Ph.D. (Professor, Department of Pharmacology and Pharmacotherapy, University of Copenhagen, Denmark), at the Pharmacology Institute in Copenhagen. Our team met some weeks later to discuss the data and Nils was very excited, because the results clearly demonstrated a significant increase of extracellular glutamate during ischemia. I was obviously also very happy with the results, but truth be told I am sure I did not understand the full impact of this discovery at the time. We also added a series of experiments, where we tested the integrity of the blood brain barrier (measured as a transfer constant for α -amino-isobutrate) after the implantation procedure. The α -amino-isobutrate experiments showed that the blood brain barrier was intact in the area of interest, *i.e.*, the dorsal hippocampus indicating that the metabolites extracted *via* the dialysate process originated from the brain and not from the vascular compartment.

A first draft manuscript was written, and in parallel we also submitted an abstract, which was accepted as a poster presentation at a cerebral ischemia meeting in Toulouse, France. This was my first participation in a

scientific meeting, and I wanted to be well prepared, so I essentially memorized the manuscript and brought it along to the meeting. I attended talks and discussions of then (and now) famous and world renowned scientists in the field, including Siesjo B, Plum F, Hossman AK, Pulsinelli W, Klatzo I, and Ito U, among others, and thrived in the stardust. As I recall, not too many scientists at the meeting looked at my poster despite my enthusiasm. The few that did come by looked mighty skeptical, and someone even suggested the unspeakable word “artifact.” To round off my brilliant debut, the manuscript disappeared into thin air right out of my bag; needless to say that Nils took a very professorial dim view, and the rush to publish became acute.

The paper, entitled “Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis,” was accepted a few months later for publication in the *Journal of Neurochemistry*. Of note, the title carries the term “intracerebral microdialysis,” which I believe is the first use of this term we came up with and which has been adapted and implemented in the brain microdialysis literature ever since and became an established keyword. (See Supplemental Digital Content 3, which shows Dr. Helene Benveniste in 1991 defending her doctoral dissertation entitled “Excitotoxins in Relation to Cerebral Ischemia,” <http://links.lww.com/A681>.)

Since its publication in 1984, the paper has been cited 2,146 times (Science Citation Index), which makes it a classic by definition. However, what has been the impact of the results? Our discovery instigated an essential series of studies first reproducing our early results of extracellular glutamate increases in a wide variety of animal models closely mimicking the clinical condition of stroke.^{10–13} Later extracellular glutamate increases were also confirmed in the live human brain under ischemic conditions.^{14,15} Various “excitotoxic” hypotheses in relation to cerebral ischemia were formulated^{16–22} and expanded rapidly as an increasing number of potentially deleterious molecules in the cascade leading to cell death were characterized. The early “basic” concept stated that neurons exposed to excessive glutamate (and other potentially toxic neurotransmitters) during ischemia died from a complex, deleterious “amplified” signal of mixed molecular origin, of which intracellular calcium accumulation played a key role. Today all of these studies would have been deemed truly “translational,” since they inspired the development of a wide range of glutamate and calcium antagonists for subsequent clinical trials.

I have not personally been scientifically active in the field of glutamate, cerebral ischemia, and stroke therapy for more than 15 yr but have followed the literature out of curiosity and necessity. In the early 90’s and onwards as the clinical trials progressed, it became clear that most of the N-methyl-D-aspartate (NMDA) antagonists produced malig-

nant neuropsychological effects in humans even when administered at suboptimal dose ranges (see the recent and excellent review by Ginsberg, 2008²³). This could have been predicted (and probably was at least for the noncompetitive NMDA antagonists considering the known psychotic effects of ketamine and other phencyclidine agents. Another disappointing fact was that, although most glutamate and calcium antagonists worked in animal models of stroke, they have not changed outcome in humans and, in fact, in some instances they have increased mortality.²³ In the review by Ginsberg, the list of “culprits” with regard to the lack of drug efficacy in stroke therapy trials include the window of opportunity for treatment, the lack of solid preclinical evidence of neuro-protective efficacy and possible drug delivery problems²³; these issues are obviously extremely important for the proper evaluation of the trials. Nevertheless, there is as yet no effective protective strategy for preventing a possible deleterious effect of glutamate in human stroke. This is perhaps akin to having identified a bacteria or viral organism deemed responsible for a deadly infection without having the appropriate antibiotic or antiviral therapy available. Alternatively and retrospectively, given the complex pathophysiological chain reactions during and after brain ischemia, some might argue that promoting glutamate to the standout role in human stroke was somewhat hasty. Personally I have no stake in such a debate, but I am reminded of two basic things here with respect to general scientific theory and the practice of medicine; first, even the most enticing correlation does not prove causation; and second, *primum non nocere*.

In closing, I would like to draw your attention to two papers, which I find intriguing and thought provoking. One is directly related to the topic at hand; the other is not; at least not directly. The first paper relates to experimental head injury, glutamate release and the demonstration of a long-lasting NMDA receptor inactivation postinjury.²⁴ The results seem ironic in the context of my presentation, but demonstrated that stimulation of NMDA receptors by the glutamate agonist NMDA 24 and 48 h post injury reduced neurologic deficits and restored cognitive performance; these beneficial effects were blocked by the coadministration of the glutamate antagonist dizocilpine or (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine maleate.²⁴ Despite possible ‘pathophysiological’ objections to comparing head injury with stroke I find the results quite thought provoking. The other paper is a recent report describing the “awakening” of a closed head injury patient in the vegetative state following repetitive deep brain stimulation²⁵; the result is nothing less than astounding and suggests a rethinking of the much debated therapeutic window for the recovery of function in stroke victims; moreover the result sparks

the imagination with respect to brain plasticity and the creation of novel neural pathways and in fact the whole concept of the physiologic basis of consciousness.

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