The origin of BSE according to the alternative „ammonia-magnesium theory“

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SUMMARY

Cellular prion protein and intracellular calcium

There are similarities between brain pathological changes caused by excitatory amino acids (EAAs) glutamate and aspartate toxicity and by transmisible spongiform encephalopathy (TSE) infection. Neurons mediating the neuroendocrine functions of the hypothalamus as well as Purkinje cells of the cerebellum are targets for damage by TSEs and EAAs. Both EAAs and TSE agents cause astrocytosis…

Early of prion diseases, neurons develop intracytoplasmic vacuoles. As the disease progresses, vacuolization becomes more pronounced and advanced cases show neuron loss, gliosis (astrocytosis), and brain atrophy. So, as a result prions multiply, are not broken down by proteases and accumulate in brain tissue, where damage results by one of two mechanisms: accumulation of the abnormal form of the protein itself causes the damage („vacuolization"); the loss of function of normal protein results in cell death („astrocytosis").

Cellular prion protein (PrPC) is associated with regulation of intracellular free calcium levels through an interaction with voltage-sensitive calcium channels, the PrPC is a protein involved in the neuronal metabolism of calcium. Toxic effects displayed by PrPSc (scrapie prion protein) and its peptide fragment (106- 126) can be blocked by antagonists of *N*-methyl-D -aspartate (NMDA) receptor channels. So, antagonists of the NMDA receptor – channel complex abolish the PrPSc – induced neuronal injury. The fragment 106-126 of PrPSc directly stimulates the proliferation of astrocytes via an increase in intracellular Ca2+ through the L-type voltage-sensitive calcium channels.

High protein intake and hyperammonemia

A high rate and extent of degradation of crude protein causing high concentrations of ammonia – N in rumen results in hyperammonemia, because of diminished capacity of liver to synthetise urea in ornithine cycle. Activity of the cycle is regulated by the rate of synthesis of N-acetylglutamate, the enzyme activator of carbamoyl- phosphate synthetase (CPS), which initiates incorporation of ammonia into the urea cycle.

Of prime importance in the control of CPS activity in ornithine cycle; is the mitochondrial concentration of N-acetylglutamate, a compound that is indispensable for enzyme activity. In addition to the absolute concentration of mitochondrial N-acetylglutamate, the concentration of liver mitochondrial free Mg^{2+} may be relevant, since binding N-acetylglutamate to CPS is dependent on this action.

The function of activity changes in CPS, via the well-documented alterations in the intramitochondrial concentration on N-acetylglutamate, is to buffer the intrahepatic ammonia concentration rather than to affect urea production per se. At constant concentration of ammonia the rate of urea production is entirely controlled by the activity of CPS. In other words; N-acetylglutamate synthetase (NAS) is a mitochondrial matrix enzyme which catalyses the synthesis of N-acetylglutamate, which activates CPS, which initiates the first step of urea synthesis from ammonia. Regulation of CPS activity depends upon the levels of N-acetylglutamate. In cases of homozygous deficiency of CPS, the ability to fix waste nitrogen is completely absent, which results in increasing levels of free ammonia with the attendant effects on the CNS.

Ammonia is a main factor in the pathogenesis of hepatic encephalopathy, the CNS is most sensitive to the toxic effects of ammonia. Many metabolic derangements occur as a consequence of high ammonia levels, including alteration of the metabolism of important compounds, such as pyruvate, lactate, glycogen, and glucose. High ammonia levels also induce changes in *N*-methyl D-aspartate (NMDA) and gamma-aminobutyric acid (GABA) receptors and causes downregulation in astroglial glutamate transporter molecules. Acute ammonia toxicity is mediated by activation of NMDA receptors. In this process of neuronal death is known that the rise of intracellular Ca2+ is an essential step.

Ammonia toxicity is involved in alterations of glutamatergic synaptic regulation which is implicated in the pathophysiology of hepatic encephalopathy (HE) in acute liver failure (ALF). A rapid increase in ammonia- acute exposure to ammonia; results in an increase in pH_i (intracellular alkanization) in all cell types, including astrocytes. This results in cytosolic alkalinization (pH action) and leads to calcium-dependent glutamate release from astrocytes. A deregulation of glutamate release from astrocytes by ammonia could contribute to glutamate dysfunction consistently observed in acute hepatic encephalopathy. Intracellular alkalinization is accompanied with an increase in $(Ca^{2+})_i$ in neurons. The role of ammonia in the glutamatergic dysfunction demonstrated in HE is supported with a positive correlation between extracellular brain concentrations of glutamate and arterial ammonia concentrations in ALF. So, the effects of ammonia may be responsible for the reduced astrocytic uptake of neuronally-released glutamate and high extracellular glutamate levels consistently seen in experimental models of HE

Astrocytes in the brain form an intimately associated network with neurons. They respond to neuronal activity and synaptically released glutamate by raising intracellular calcium concentration Ca2+. Ability of most neurotransmitters to increase astrocytic Ca^{2+} levels is firmly established. Astrocytes regulate neuronal calcium levels through the calcium-dependent release of glutamate. Astrocytic glutamate release pathway is engaged at physiological levels of internal calcium. Astrocytic glutamate release can be triggered by any ligand that stimulates an increase in Ca2+.

During ammonia intoxication, NMDA receptors are excessively stimulated, resulting in a larger influx of Ca^{2+} than usual into neurons. This would elicit a cascade of reactions and eventually lead to neuronal cell death. It has been shown that NH_4^+ induced depolarization in cultured rat cortical astrocytes . This ammonia-induced depolarization could also take place in neuronal membranes and result in removal of Mg^{2+} that normally blocks the NMDA receptor channel, leading to excessive activation of the NMDA receptor.

The MK-801, an antagonist of NMDA receptors prevents ammonia-induced changes in superoxide dismutase, glutathione peroxidase and catalase. Ammonia intoxication also induces a depletion of glutathione and an increase in lipid peroxidation. Both effects, as well as ammonia-induced increase in superoxide formation are prevented by MK-801. These results indicate that ammonia-induced oxidative stress in brain is mediated by excessive activation of NMDA receptors and support the idea that oxidative stress can play a role in the mechanism of ammonia toxicity.

Liver contains both glutamine synthetase and glutaminase but the enzymes are localized in different cellular segments. This ensures that the liver is neither a net producer nor consumer of glutamine. The differences in cellular location of these two enzymes allows the liver to scavange ammonia that has not been incorporated into urea. The enzymes of the urea cycle are located in the same cells as those that contain glutaminase. The result of the differential distribution of these two hepatic enzymes makes it possible to control ammonia incorporation into either urea or glutamine, the latter leads to excretion of ammonia by the kidney.

When acidosis occurs the body will divert more glutamine from the liver to the kidney. This allows for the conservation of bicarbonate ion since the incorporation of ammonia into urea requires bicarbonate. When glutamine enters the kidney, glutaminase releases one mole of ammonia generating glutamate and then glutamate dehydrogenase releases another mole of ammonia generating alpha-ketoglutarate. The ammonia will ionizes to ammonium ion (NH_4^+) which is excreted. The net effect is a reduction in the pH. So, synthesis of glutamine also reduces the total free ammonia level circulating in the blood; therefore, a significant increase in blood glutamine concentration can signal hyperammonemia.

Marked brain damage is seen in cases of failure to make urea via the urea cycle or to eliminate urea through the kidneys. The result of either of these events is a buildup of circulating levels of ammonium ion. Aside from its effect on blood pH, ammonia readily traverses the brain blood barrier and in the brain is converted to glutamate via glutamate dehydrogenase, depleting the brain of alpha-ketoglutarate. As the alpha-ketoglutarate is depleted, oxaloacetate falls correspondingly, and ultimately TCA cycle activity comes to a halt. In the absence of aerobic oxidative phosphorylation and TCA cycle activity, irreparable cell damage and neural cell death ensue.

In addition, the increased glutamate leads to glutamine formation. This depletes glutamate stores which are needed in neural tissue since glutamate is both a neurotransmitter and a precursor for the synthesis of gama-˜aminobutyrate (GABA); another neurotransmitter. Therefore, reductions in brain glutamate affect energy production as well as neurotransmission. Additional untoward consequences are the result of elevations in neural glutamine concentration. Glial cell (astrocytes) volume is controlled by intracellular organic osmolyte metabolism. The organic osmolyte is glutamine. As glutamine levels rise in the brain the volume of fluid within glial cells increases resulting in the cerebral edema

The strong response of liver glutaminase to pH and the fact that the reaction can supply metabolites for urea synthesis suggest a possible regulatory role of glutaminase in ureagenesis. It was also found that the activity of rat liver glutaminase is strongly affected by variation in the Mg2+ concentration within the approximate physiological range of activators. A rise in the Mg2+ concentration stimulates glutaminase by increasing the apparent affinity of the enzyme for its positive modifer phosphate. Since Mg2+ stimulates glutaminase, as does a rise in pH, by increasing the apparent affinity of the enzyme for phosphate, it reduces the

inhibitory effect of a decrease in pH and/or phosphate concentration over a physiologically relevant range.

Glutaminase activity in intact mitochondria from liver is activated by polyamine spermine, as indicated both by increased glutamate production from glutamine and by increased respiration with glutamine as sole substrate. It was found that spermine is effective in the presence of physiological concentrations of Mg2+ and suggested that spermine may be a physiological activator of hepatic glutaminase.

Magnesium deficit and NMDA- induced neurodegeneration

Studies have demonstrated that Mg^{2+} can protect against NMDA- induced neurodegeneration, brain injury, and convulsions. Mg^{2+} competes with calcium at voltage-gated calcium channels both intracellularly and on the cell surface membrane. Mg^{2+} is capable of blocking NMDA receptors both intracellularly and extracellularly. Mg^{2+} also enhances mitochondrial buffering of raised intracellular free calcium ions and prevents release of intracellular calcium stores from endoplasmic reticulum. NMDA receptor channel is additionally blocked by phencyclidine (PCP).

An important consequence of NMDA receptor activation is the influx of Ca^{2+} into neurons. Overstimulation of the NMDA receptor as well as other excitatory amino acid receptors results in neurotoxicity and neuronal injury. These receptors are considered as the final common pathway for many acute and chronic neurologic conditions. So, excessive NMDA receptor stimulation is thought to be an important factor in neuronal cell damage, mediated by excessive calcium entry into the cell.

Activation of NMDA receptors requires binding of both glutamate and the co-agonist glycine for the efficient opening of the ion channel. D-Serine, recently appreciated as the endogenous ligand for the glycine site of the glutamate NMDA receptor. D-serine coactivates postsynaptic NMDA receptors together with glutamate. D-Serine is formed by serine racemase (enzyme), which directly converts L-serine to D-serine. Inhibitors of this enzyme should reduce NMDA neurotransmission and might be therapeutic in stroke and other conditions associated with glutamate excitotoxicity. Treatment of astrocytes with the calcium ionophore as well as with compounds that augment the intracellular calcium levels such as glutamate or kainate led to an increase in the amount of D-serine present in the extracellular medium. These results suggest that there might be a glutamatergic-mediated regulation of serine racemase (SR) activity by intracellular Ca2+ concentration.

Under normal conditions of synaptic transmission, the NMDA receptor channel is blocked by Mg^{2+} sitting in the channel and only activated for brief periods of time. Under pathological conditions, however, overactivation of the receptor causes an excessive amount of Ca^{2+} influx into the nerve cell, which then triggers a variety of processes that can lead to necrosis or apoptosis. The latter processes include Ca^{2+} overload of mitochondria, resulting in oxygen free radical formation and activation of caspases, Ca^{2+}-dependent activation of neuronal enzyme nitric oxide synthase (NOS), leading to increased nitric oxide (NO) production and the formation of toxic peroxynitrite. For example energetically compromised neurons become depolarized because in the absence of energy they cannot maintain ionic homeostasis; this depolarization relieves the normal Mg^{2+} block of NMDA receptor-coupled channels because the relatively positive charge in the cell repels positively-charged Mg^{2+} from the channel pore. Hence, during periods of ischemia and in many neurodegenerative diseases, excessive stimulation of glutamate receptors is thought to occur.

Elevations in extracellular glutamate are not necessary to invoke an excitotoxic mechanism. Excitotoxicity can come into play even with normal levels of glutamate if NMDA receptor activity is increased, e.g., when neurons are injured and thus become depolarized (more positively charged); this condition relieves the normal block of the ion channel by Mg^{2+} and thus abnormally increases NMDA receptor activity. When glutamate and glycine bind and the cell is depolarized to remove Mg^{2+} block, the NMDA receptor channel opens with consequent influx of Ca^{2+} and Na^{+} into the cell.

Polyamines block the NMDA receptor channel in a voltage- dependent manner at higher concentrations. However, endogenous polyamines may not modulate the NMDA receptor in vivo in the brain. The NMDA receptor is on the extracellular surface of the cell, spermidine and spermine are usually found intracellularly. Thus, polyamines may not act physiologically on NMDA receptors except under pathological conditions. Protons suppress NMDA receptor activation, and polyamines, such as spermine, relieve the proton block.

Polyamines can confer to the mitochondria an important role in the regulation of the free cytoplasmic Ca2+ concentration in the cell and of the free Ca2+ concentration in the mitochondrial matrix. Stimulation of mitochondrial Ca2+ uptake by spermine was inhibited by Mg2+ in a concentration – dependent manner.

H**yperammonemia** plus **hypomagnesaemia „simultaneous“ action**

There is the possibility that these mechanisms have a strong influence on CNS, especially in ruminants, and that the **BSE has its roots in a more common nutritional problem**. This alternative „ ammonia- magnesium“ theory is based on the chronic Mg-deficiency potentiated by hyperammonemia in ruminants. So, there is the main idea of this review; to show the hypomagnesemia plus hyperammonemia „simultaneous“ action on the ruminant tissues (the CNS and liver tissue, especially). As a typical example; the ryegrass staggers is showed in ruminants. So, various clinical symptoms can be observed because the nervous system controlling both voluntary and unvoluntary muscles is affected (Mg and Ca disturbances). It seems, that during the chronic hypomagnesemic disease, the heavy weather changes (cold- rainy, windy...) or nutrition (high intake of crude protein...) stress - these episodes of acute abruptions, may accelerate the nervous, like to „BSE“ disease. If the BSE is involved; a longer- chronic action of corresponding biochemical changes in the blood (CSF) is necessary, to rise irreversible neurodegenerative changes.

Introduction

Bovine spongiform encephalopathy (BSE) was initially recognized in cattle in the UK in 1986; there is good information that it had not occurred before then. Epidemiological research led to the **conclusion that the bovine agent had originated from the scrapie** agent, which had been present in sheep in the United Kingdom for at last 200 years. However, it is presumed**, but will likely never be proven,** that the scrapie agent jumped species and moved into cattle when sheep offal (the leftover parts of butchered animals) was included in **meat and bone meal (MBM**)- protein supplements fed to cattle.

In response to the emergence of BSE, **epidemiological studies were started in April 1987**. The initial objective was to obtain detailed data from a study of 200 cases. These

showed the pattern of BSE cases to be **typical of an extended common source epidemic** (i.e. an epidemic involving many individual, independent disease outbreaks, which can each be traced back to a common source). No evidence was found of cattle to cattle transmission, nor of any common exposure to specific pharmaceutical products or pesticides (explicitly including organophosphorous compounds). Nor was there **any evidence that the disease was simply genetic**. The possible direct transmission of the **scrapie agent from sheep to cattle, by either direct or indirect contact, was an untenable hypothesis** as there were no sheep present on around 20% of the farms affected with BSE.

The only **common feature of all the cases of BSE investigated** was the use of commercially produced **compound feed containing meat and bone meal (MBM)** (**Wilesmith**, Wells, Cranwell & Ryan 1988 Vet. Rec. 123, 638-644). This conclusion was further supported by the fact that the incidence **of BSE in dairy herds was much greater than in beef suckler herds,** closely matching the use of compound feeds in these two types of herd. Subsequently a formal case-control study of calf feeding practices and MBM inclusion in proprietary concentrates provided substantiating evidence (**Wilesmith**, Ryan &Hueston 1991 Res Vet Sci 52 325-331). These conclusions are from the „Department for Environment Food and Rural Affairs“ (DEFRA; 25 October 2005) about the BSE pathogenesis (http://www.defra.gov.uk/animalh/bse/science-research/diagnos.html).

BSE belongs to the family of diseases known as the **transmissible spongiform encephalopathies (TSE´s).** These diseases are caused by similar uncharacterized agents that produce spongiform changes in brain. In the next decades, a series of **experiments, many led by professor Prusiner**, demonstrated that prion protein (PrP) actually is present in healthy animals, but in a different form from the one found in diseased animals. While many of Prusiner colleagues have come to accept the once heretical prion theory, **most say it still faces some crucial unanswered questions.**

A decade or so ago, the Nobel prize for medicine was awarded to a scientist who had an idea so radical that it was condemned as heresy by his peers. Rather than blame conventional agents such as viruses and bacteria for a series of baffling "spongiform" brain disorders like Creutzfeldt-Jakob Disease (CJD), scrapie and BSE, **Stan Prusiner proposed that a novel type of infectious agent was responsible**. Prusiner began his long journey to this breakthrough in 1972 after one of his patients died of dementia resulting from CJD. Now a **professor at the University of California in San Francisco, he named the culprit the "prion" - "proteinaceous infectious particle"**. Unlike viruses, bacteria or parasites, a prion is an infectious protein that contains no genetic material.

When he suggested the idea, it was greeted with disbelief since it marked the only lifeform that could multiply without a gene. Scientific ridicule was heaped on Prusiner's head, but in 1997 his dogged persistence paid off and his Nobel prize citation described how "an unwavering Prusiner continued the arduous task to define the precise nature of this novel infectious agent". Abnormal prions are thought to enter the body through food or cuts to set off a chain reaction: the infectious, abnormally shaped prion causes a domino effect, converting normal forms of the protein into abnormal proteins, creating deposits that cause irreversible brain damage.

Because these prion diseases have such long incubation times, it has taken an age to study them in detail and there is still a lot we don't understand. **But even today, and almost a decade after Prusiner's Nobel prize, findings still challenge his hypothesis so that, at best, it seems incomplete and, at worst, it may even be wrong.** One recent example came from **Dr Martin Jeffrey at the Veterinary Laboratories Agency. His team studied 50 sheep** to see what happened when they ate food contaminated with the spongiform disease

scrapie. The team monitored the passage of half a gram of liquified brain containing millions of abnormal prions.

They were thought to pass undigested through the gut wall into specialised lymphoid tissue called Peyer's patches, where they multiplied before spreading to the central nervous system and on to the brain. But his team **reports in the Journal of Pathology** how the prions did not go to Peyer's patches as expected, but were digested or vanished into the lymph nodes. Separate experiments show that abnormal prions can easily be digested by sheep stomach juices, so even if an animal ingested large quantities of infected feed, hardly any abnormal prions would survive. In the three sheep that did develop scrapie after being injected with diseased tissue, abnormal prions began accumulating in the Peyer's patches 30 days later, even though all the prions from the original gut injections had long gone.

When Dr Jeffrey looked into this, he found that the prions were being formed afresh in the patches. Because the disease was triggered by liquefied sheep brain, the study raises the possibility that an unidentified agent caused the infection which, a month later, triggered the Peyer's patches to make the abnormal prions. This will remain only conjecture until the infectious agent is identified, **but the work shows that the prion hypothesis "is not completely satisfactory", says Dr Jeffrey**. To further undermine the link between prions and spongiform disease, his team has shown that the prions do not seem to build up into clumps of sufficient size and in the right place in animals to link with the symptoms of spongiform disease.

Working with Dr Bruce Chesebro of the Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, Dr Jeffrey found damaged areas in scrapie brains where there were no prions. **Dr Chesebro's own experiments have raised questions**. He exposed two groups of six-week-old mice to different strains of scrapie. Within 150 days of being inoculated with the natural form of scrapie prion protein, all 70 mice in the control group showed signs of infection: twitching, emaciation and poor co-ordination. But in GM mice that made a prion protein that does not anchor to cells, he found clumps of abnormal protein in the brain, brain damage, but no disease. "The mice didn't get sick. That's very significant. The dense accumulations of scrapie plaque in the brain resembled the plaque seen in Alzheimer's, but it wasn't toxic." These findings once again raise the **possibility that the abnormal proteins are a consequence of the disease process, rather than a cause**. (Interestingly, a similar argument is raging over the protein deposits linked with Alzheimer's disease.)

The most fundamental issue of all was raised by the Nobel prizewinner **Prof Kurt Wuthrich of the Swiss Federal Institute of Technology, Zurich**: he pointed out that researchers have failed to produce spongiform disease using laboratory-made prions, the only real way to eliminate the possibility that another agent might be responsible.

That challenge seemed to have been met last year in the journal Cell, in an experiment by **Prof Claudio Soto at the University of Texas Medical Branch at Galveston.** His team took prions from infected hamsters and placed them in test tubes containing healthy brain proteins. When the healthy proteins had been largely transformed into prions, the samples were diluted over and over again and the process repeated, until the only remaining prions were presumably those that had been newly generated in the test tubes. These were then injected into the brains of healthy hamsters, which died less than six months after inoculation. But, say the critics, extraordinary claims need extraordinary evidence. The **infectivity was tiny and there are questions over how Prof Soto purified the prions and whether a non-prion disease agent could have remained after dilution**. Prof Soto has confirmed his results in other species, and using more stringent conditions. "Indeed, we have a couple of papers currently under review showing that we can generate infectious prions starting from what is estimated to be one single molecule of infectious prion," he says. But although he believes the evidence for prions is overwhelming, he admits that it is "a minor possibility" that "there

might be another component necessary for infectivity, including a possible nucleic acid (DNA or RNA genetic material)".

Dr Surachai Supattapone, from Darmouth University in New Hampshire, has repeated the same study using purified protein in which, presumably, no nucleic acids are present and presented his results in March at a conference in Saint Moritz. "I can't give too much detail at this point, but I think that our studies with purified protein cannot rule out a second component," says Dr Supattapone. **Another central tenet of the Prusiner hypothesis** is that a single prion protein can give rise to different strains of disease with varying infectivity and other properties, each reflecting different shapes of the prion protein.

This seemed to be confirmed in work on yeast by a team led by Dr Jonathan Weissman at the University of California, San Francisco, and Dr Chih-Yen King at Florida State University. But **yeast prions "are quite distinct from mammalian prions in spite of the similar names**", commented Dr Chesebro, who remains unconvinced.

And when his British collaborator, Dr Jeffrey, looked at the effects **of prion shape in sheep, he found that the shape can vary**, depending on which sort of cell it inhabits, even though it produces the same strain of disease in mice.

Similar observations that prion shape changes do not alter the strain of the disease have also been **reported by Prof Laura Manuelidis of Yale Medical School, who concluded that many facts "are discordant with the prion hypothesis"** in a review in the journal Viral Immunology. Prusiner's idea does not fulfil the classic criteria formulated by Robert Koch in 1884 to link an agent to a disease, says Prof Manuelidis. "Not a single one of Koch's proven postulates of infection are fulfilled by prion proteins." Such is the hold of the prion hypothesis over the scientific establishment, she says, that **"this evidence (or lack of evidence) led one dominant prion proponent to question the use of Koch's postulates".** There is even evidence for viral particles, although she says this has been ignored. "It has also been obvious for a long time that abnormal prion protein is the consequence of infection, but not the causal agent," she says. "You might say that abnormal prion protein lacks the dynamite for weapons of mass destruction, though it certainly has a lot of rhetoric inside it. Those natural truths are not defined by popular vote or cabal." She is also disturbed by the hostility faced by those who question the prion idea and says she has seen the good work of others trashed by the traditional weapon of choice in scientific disputes - anonymous peer review. "At issue, unfortunately, is public health." (HIGHFIELD, 2006).

Prions have been responsible for an entire century of tragic episodes. **Fifty years ago, kuru decimated the population of Papua New Guinea**. Then, iatrogenic transmission of prions caused more than 250 cases of Creutzfeld-Jakob disease (CJD). More recently**, transmission of BSE to humans caused a widespread health scare**. On the other hand, the biology of prions represents a fascinating and **poorly understood phenomenon**, which may account for more than just diseases and may represent a **fundamental mechanism of crosstalk between proteins**. The two decades since Stanley Prusiner´s formulation of the protein-only hypothesis have witnessed spectacular advances, and yet some of the **most basic questions in prion science have remained unanswered** (AGUZZI and POLYMENIDOU, 2004). Prion infectivity is typically restricted to the central nervous and lymphatic systems of infected hosts, but **chronic inflamation can expand the distribution of prions**. SEEGER et al (2005) tested whether chronic inflammatory kidney disorders would trigger excretion of prion infectivity into urine. They found, that **urine may provide a vector for horizontal prion transmission**, and inflammation of excretory organs may influence prion spread.

There are no published references to date in which intake of crude protein (and potassium) high enough to lead to a state of hyperammonemia (and hypomagnesemia) during the incubation period of the BSE. Therefore there is the main idea of this review; **to show the hypomagnesemia plus hyperammonemia „simultaneous" action on the ruminant tissues (the CNS and liver tissue, especially). As a typical example; the ryegrass staggers is showed in ruminants**. For example in Britain , the crude protein may reach to over 300 g/ kg dry matter in young, heavily- fertilized ryegrass. Also, perennial ryegrass feeding must be considered in the differential diagnosis of hypomagnesemia. So, the **various clinical symptoms can be observed because the nervous system controlling both voluntary and unvoluntary muscles is affected (Mg and Ca disturbances).** It seems, that during the chronic hypomagnesemic disease, the heavy weather changes (cold- rainy, windy...) or nutrition (high intake of crude protein...) stress - these episodes of acute abruptions, may accelerate the nervous, like to „BSE" disease. **If the BSE is involved; a longer- chronic action of corresponding biochemical changes in the blood (CSF) is necessary, to rise irreversible neurodegenerative changes.**

Ryegrass feeding and the spongiform encephalopathy

However, to date still the accepted cause of the human vCJD disease is that BSE spread from cattle to humans by the **consumption of infected beef.** However, the evidence that supports this is very thin. Here is one important **example** from the United Kingdom **that the BSE is not an „infectious" disease** (see publications; MOORBY et al., 2000; DEWHURST et al.,2000; MOORBY et al., 2000a);

MOORBY,J.M.- DEWHURST,R.J.- TWEED,J.K.S.- DHANOA,M.S.: Aspects of the metabolism of dairy cows during the incubation period of bovine spongiform encephalopathy. Vet.Rec., 147, 2000; 409-412

DEWHURST,R.J.- DEWHURST,R.J.- MOORBY,J.M.- DHANOA,M.S.- EVANS,R.T.- FISHER,W.J.: Effects of altering energy and protein supply to dairy cows during the dry period. 1. Intake, body condition, and milk production. J.Dairy Sci., 83, 2000: 1782-1794.

MOORBY,J.M.- DEWHURST,R.J.- TWEED,J.K.S.- DHANOA,M.S.- BECK,N.F.G.: Effects of altering energy and protein supply to dairy cows during the dry period. 2. Metabolic and Hormonal Responses. J.Dairy Sci., 83, 2000a: 1795-1806.

This experiment was conducted **using diets and other conditions typical of northwestern Europe**, under well defined conditions of husbandry and nutrition. The effect of altering the amount of protein and energy over the final 6 wk of the dry-period diet and during the first 21 wk of the subsequent lactation was investigated, in 47 dairy cows. **Perennial ryegrass** silage was used **ad libitum**; final 6 wk of the dry-period diet and during lactation plus a concentrate with high crude protein (CP) level (22.5%) was fed. Blood samples were taken each week before calving, and during weeks1,3,5,7,13,17, and 21 of lactation.

During lactation daily total dry matter (DM) intake was ca 17.4 kg; the **content of CP (N x 6.25) was ca 20% during first 12 weeks**, and ca 17.5% of CP in the diet DM, to the 22 wk of the lactation period. So, very high CP concentrations in the diet used, and high levels of plasma urea-N (38- 43 mM) were found during lactation. But high also during dry period (30- 36 mM) when in one experimental group was a low dietary CP concentration(ca 11 %).

No clinical metabolic disorders were recorded. However, after the collection of the last blood sample (21 wk of lactation), **six of the 47 animals (so, 13 per cent!) developed**

clinical signs of BSE (later histopatologicaly confirmed). Although when they were sampled it was not known that they were incubating the BSE.

My conclusions: **long-term dietary CP surpluss, significantly higher than the norm** (NRC, 2001; if about daily 30 kg of milk production was recorded; so, only 15% of CP in DM was needed) during 21 wk of lactation period and mostly in cows during 6 wk of dry period. So, there hyperammonemia plus hypomagnesemia action on the animal tissues (CNS and liver, especially) can be found. If the BSE is involved; a long-chronic action is necessary to rise irreversible neurodegenerative changes. It seems that there is the similarity between the **individual susceptibility in the hypomagnesaemic „indicator cows" and the „BSE cows**".

In addition, from **older australian literature** is well known that in cases of **protracted ryegrass staggers of sheep and cattle;** cerebellar lesions involving Purkinje cell axons... were found (MASON, 1968). These lesions consist of eosinophilic homogenous swellings in the cerebellar layer, generally located in groups rather than randomly distributed and with the tendency to be more numerous adjacent to the Purkinje cell layer. In cerebellums with a high density of torpedoes, swollen neuronal elements were sometimes detected in the cerebellar molecular layer. These swellings appear to be Purkinje cell dendrites…

In general, **axons with torpedoes were myelinated at least over part of their traceable length**, offten on both sides of the swelling. Total encasement with myelin was often demonstrable about small swellings, whereas larger ones sometimes had only vestiges remaining. The myelin sheath, however, remained intact both about the bulb and the empty retraction space. The myelin sheats would appear to remain relatively unaffected **about degenerating axons**, **vacuoles were observed in some torpedoes**. It appears that the longer the disturbing syndromes has been present, the greater the likelihood of finding these axonal changes, for example in protracted ryegrass staggers in sheep and cattle. Therefore the lesions described are not regarded as pathognomonic of protracted ryegrass staggers but probably arise from a number of factors, which may include disturbed neuronal metabolism, neuronal exhaustion and repeated anoxic insults (MASON, 1968).

MASON,R,W.: Cylinder degeneration associated with ryegrass staggers in sheep and cattle. Austral.Vet. J., 1968: 44: 428

Why the BSE epidemic occured in the United Kingdom, especially ?

Therefore, the **feeding of MBM to cattle per se may not be the main cause of BSE**. However, by excessive feeding of MBM, especially in dairy cows, a state of hyperammonemia can be achieved and hypomagnesemia can also be initiated. When this article about „ryegrass toxicity" on the CNS of cows was published**; it was prompted to air my views in studies of CNS neurotoxities by nutritional causes**. I reviewed about 200 papers on the CNS changes associated with BSE, and detected a possibility that these mechanisms have a strong influence on CNS, especially in ruminants, and that the **BSE has its roots in a more common nutritional problem** (HLÁSNÝ, 2001). This alternative „ ammonia- magnesium" theory is **based on the chronic Mg-deficiency potentiated by hyperammonemia in animals.**

This theory was introduced according to the well- known facts, that;

(1) Over the past 50 years **yields of many crops have increased roughly in proportion to the increase in nitrogen and potassium fertilizer** application- peaked in the mid-1980s, especially in the UK

(2) Also in the mid- 1980s (see the ARC, 1980; NRC, 1985; NRC, 1989), it is **a**

begining of the higher dietary protein recommendation including undegradable protein (MBM...) in dairy rations, especially in the UK

There are eyplanations from the research sources and the situation in the UK(period of the BSE incidence begining).

Ad 1/ Effects of high nitrogen and potassium fertilization; share of grassland and magnesium deficiency

A/ Research results;

Lush grass innately has an increased level of crude protein. This factor, **combined with increased use of nitrogenous fertilizers** in the soil, causes an increase in ammonia in ruminal fluid, leading to a decrease in the availability and **absorption of magnesium**. (MARTENS et SCHWEIGEL, 2000; URDAZ et al., 2003; FONTENOT et al., 1989).

It was been observed that application of nitrogen (N) fertilizer, which may increase K uptake by plants and/or decrease Mg utilization by livestock, often is associated with the occurence of tetany (FONTENOT et al.,l973).The highest **potassium (K) level in a dairy cows TMR resulted in reduced plasma Mg** and reduced milk yield (FISHER, et al, 1994; KHORASANI et al, 1997; FREDEEN, et al, 1995). Because Na deficiency causes an increase of K concentration in saliva and ruminal fluid, Na deficiency should be considered a potentially important risk factor. The most prominent signs of hypomagnesemia are excitations and muscle cramps, which are closely correlated with the Mg concentration in the CSF. It is suggested that the clinical signs are caused by spontaneous activation of neurons in the CNS at low Mg concentrations, which leads to tetany.

While non-ruminants absorb Mg primarily from the small intestine, ruminants are able to absorb much of their Mg requirement from the rumen. In fact, the reticulum and rumen can account for up to 80% of the Mg absorption along the entire digestive tract (REMOND, et al, 1996). . Probably, the nutrient having the **greatest adverse effect on Mg absorption is an excess of K in the ration**, as shown by at least four sheep experiments (GRACE, et al, 1988; YANO, et al, 1990; DALLEY, et al, 1997; WACIRAPAKORN, et al, 1996). The response was similar in goats. Increasing the dietary K concentration from 0.78 to 3.4% reduced Mg absorption from 29.8 to 22.1%. (SCHONEWILLE et al, 1997).

The crude protein intake of ruminants grazing young grass fertilized by nitrogenous fertilizers, was **increased by approximately 25-35% in Britain (HEAD and ROOK, 1955**). As this protein is readily fermentable, it leads to increased intraruminal ammonia concentrations up to 30- 70 mmol/l (MARTENS and RAYSSIGUIER, 1980). Ammonia absorption from the rumen is linearly related to ruminal ammonia concentration between 3 and 18 mmol/l (BODEKAR et al., 1990) and is normally dotoxified in the liver to urea. It appears that **ruminal ammonia may contribute to decreased Mg absorption** under the circumstances which may be encountered during grazing. In the rumen of sheep, quanite ($MgNH_4PO_4$) formation is seen to occur at pH 6.2 – 7.2 with ruminal ammonia concentration in the range of 40 mmol/l and depresses the available amount of magnesium (Axford et al., 1982).

It was found that the effect of ammonium ions on magnesium absorption was **greater in the bovine than the ovine rumen** (GABEL and MARTENS, 1986). It has been found that only a small proportion of any flock or herd will suffer clinical hypomagnesaemia (BUTLER, 1963). A genetic factor for magnesium absorption has also been suggested. However, the phenomenon of individual susceptibility to grass tetany within a dairy herd of similar animals, the so-called indicator cows, still requires a convincing explanation, (DUA and CARE, 1995).

Magnesium is a nutrient required for all animals, but it is **especially critical for ruminants**. A physiological deficiency of Mg results in hypomagnesemic tetany. Typically, **only female ruminants are affected**, and the disturbance usually occurs during the early stages of lactation (FONTENOT et al., 1989). The experiments demonstrated that **younger cows are better able to mobilize Mg** from the body reserves than older cows (Van MOSEL, et al, 1990).

Magnesium is an essential mineral with many physiologic and biochemical functions. Surprisingly, **Mg homeostasis is not regulated** by a hormonal feedback system, but simply depends on inflow (absorption) from the gastrointestinal tract and outflow (endogenous secretion, requirement for milk production, uptake by tissues) (MARTENS et SCHWEIGEL, 2000).

Magnesium solubility declines sharply as ruminal pH rises above 6.5. Grazing animals tend to have **higher ruminal pH because the high content of nitrogen and potassium** positively correlate, in ryegrass especially (HLÁSNÝ, 1990). Also, there is the stimulation of salivary secretions associated with grazing heavily fertilized .

Highly fertilized young herbage is characterized by a high content of crude protein (CP) and a high rate and extent of degradation of CP causing high concentrations of ammonia-N in the rumen (van VUUREN et al., 1986) and substantial N losses via urinary excretion.

MAYLAND et al. (1976) found that higher N concentration in forages gives higher contents of **higher fatty acids (HFA)**. It is likely that higher concentrations of the HFA in the forage decrease the availability of feed Ca and Mg; by the formation of **insoluble Ca and Mg soaps** in the gastrointestinal tract (WIND et al. 1966). Also, if P content in young spring pasture is high and high rumen P is produced, at such high levels, even at the acidic pH of the rumen, precipitation of guanite ($MgNH_4PO_4 . 6H_2O$) may begin to occur (AXFORD et al., 1982).

Literature data from 1940s-1950s suggested, that **HFA might affect Mg and Ca utilization** (BROUWER et al.,l943: BROUWER,l944: DUEL, l955). More recent experimental work confirmed this hypothesis; Increasing amounts of HFA added to the rations of ruminants resulted in a lower apparent availability or in a reduction of the serum Mg levels (KEMP et al.,l966: WILSON et al.,l969). MOLLOY et al.(l973) found that HFA in the herbage of New Zealand grass-clover pastures were positively correlated with plant N**.**

Higher levels **of added fat in dairy cow rations** can react with minerals such as Ca and Mg resulting in the formation of Ca and Mg soaps, possibly leading to reduced mineral availability (RAHNEMA et al, 1994; PANTOJA et al, 1997). Increasing fat intake decreased the apparent absorption of Mg. Ca absorption was affected more by type of fat, with unsaturated fats causing the greater decrease in absorption.

The adverse effect of the **formation of insoluble Mg soaps on Mg absorption** is supported by research indicating that the **rumen is an important site of net Mg absorption** (GRACE and MACRAE,l972: GRACE et al.,l974: KEMP et al., l973: TOMAS and POTTER,l976).

Adequate amounts of **fermentable carbohydrates** are important in maintaining serum magnesium levels, since magnesium solubility and the absorptive surface area of rumen papilla both improve with availability of short chain fatty acids and lowered rumen pH (MARTENS et SCHWEIGEL, 2000).

Lush pastures often high in nonprotein nitrogen are relatively low in readily fermentable carbohydrates. The ability of the ruminal microbes to incorporate the nonprotein nitrogen into microbial protein is exceeded and ammonia and ammonium ion build up in the rumen increasing ruminal pH. When high grain rations are fed ruminal fluid pH is often below 6.5

and magnesium solubility is generally adequate. This may explain why magnesium in **concentrates is generally more available then the magnesium in forages** (NRC, 2001).

Grazing experiments (BARTLETT, 1958) on pastures containing different amounts of clover and similarly finding in the field conditions (HLASNY, 1991) have shown higher serum Mg levels in cows grazing on the clover rich sward. A **predominately grass sward provides a lower Mg concentration** in the forrage consumed by the animals, even although in a grassy sward heavy N dressing mostly increase the Mg content in the grass slightly. Although the Mg concentrations between grass species may differ considerably, **legumes herbs contain more Mg than grasses** (BAKER and REID,l970: HLASNY,l989).

Fertilization with acid-forming fertilizers results in increased soil acidity. Substantial evidence exists that this may result in a differential loss from the soil of K, Ca and Mg (ADAMS et al.,l967). In practice, the effect is enhanced because K losses may be replaced by fertilization **whereas Ca and Mg losses may be ignored because of lack of effect on yield**.

Grass tetany remains a significant cause of cow deaths every year. Blood samples were taken from cows in a commercial dairy herd experiencing grass tetany to determine the effects of **supplementing the ration with 22.5 grams Mg per day** (CONTRERAS, et al, 1992). That is equal to 1.5 ounces of MgO daily. Initial blood serum Mg averaged 1.29 mg/dl. This increased to 1.92 mg and 2.16 mg on days 11 and 44 after supplementation, respectively. By 7 days after supplementation ended the blood serum Mg had dropped to 1.7 to 1.9 mg. This is **good evidence that blood Mg can be an indicator of Mg status in deficient animals**.

Stress may lead to clinical hypomagesemia, since **sympathetic nervous system activation (cool and cold marine climatic regions)** causes an epinephrine release resulting in a decreased plasma magnesium concentration (MARTENS et SCHWEIGEL, 2000; HOFF et al., 1993).

B/ The situation in the United Kingdom

Ba/ The report from the british journal „Animal Science“;

HEMINGWAY,R.G.: The effect of changing patterns of fertilizer applications on the major mineral composition of herbage in relation to the requirements of cattle: a 50 – year review. Animal Science, 69, 1999; 1-18)

Highest nitrogen fertilizers consumption in the world (period of the BSE incidence begining), in **England and Wales, especially (1983-1988)**: This author´s summary indicates that in the UK there was the „intention“ to use the high N- fertilization (and K-fertilization) for intensive silage production, especially. The effect of changing patterns of fertilizer applications on the major mineral composition of herbage in relation to the requirements of cattle: a 50 – year review“; showed that the long time research about the NPK fertilization in the UK has been sumarized For example in 1983- 1988 period, in England and Wales; higher rates were used for intensive silage production; 201 kg (nitrogen), 15 kg (phosphorus) and 53 kg (potassium) per ha. In contrast recommended applications (MAFF, 1994) were much higher 340 kg N, 18 kg P and 25 kg K per ha for grazing and 380 kg N, 40 kg P and 260 kg K per ha for intensive silage. Later, nitrogen application rates to grass have progressively declined. Increasing environmental issues and the present interest in organic farming and low input systems indicate that these trends will continue in the UK. Present overall fertilizer use for grazing on dairy farms is about 170 kg N, 10 kg P and 20 kg K per ha (HEMINGWAY, 1999).

Bb/ The report from british book „Animal Nutrition“:

McDONALD,P.- EDWARDS,R.A.- GREENHALGH,J.F.D.: Animal Nutrition (4th edition), 1988: 543 pages

In Britain perennial ryegrass is the most important species of sown pastures, but Italian ryegrass (Lolium multiflorum), timothy (Phleum pratense), cocksfoot and the fescues (Festuca spp.) are also common. The composition of the dry matter (DM) of pasture is very variable: for example, the **crude protein (CP) may range from as little as 30 g/ kg in very mature herbage to over 300 g/kg in young, heavily- fertilized grass**.In temperate areas having a reasonably uniform distribution of rainfall, grasses grow and mature relatively slowly and can thus be utilized at an early stage of growth when their nutritive value is high. Varieties within a species generally differ to only a small degree in nutritive value, if the comparison is made at the same stage of growth, but differences between comparable species may be larger. A classical example for the temperate grasses is the difference between British varieties of perennial ryegrass (Lolium perenne) and of cocksfoot or orchard grass (Dactylis glomerata). **At the same stage of growth the cocksfoot variety, S.37, is 0.05- 0.06 units lower in dry matter digestibility (CP, especially) than the ryegrasses, S.23 and S.24 (McDONALD et al., 1988).**

In Britain, the small area of lucerne grown is harvested mainly for artificial drying or made into silage. Lucerne varieties are distinguished by the time of flowering, and under UK conditions early flowering types are recommended.These varieties usually flower in the second week of June, but to obtain a cut with an acceptable digestibility, the crop should be **first harvested at the early bud stage (end of May**), when the expected digestible organic matter content would be 620- 640 g/ kg DM, and subsequently cut at 6 to 8 – week intervals to give values 560- 600g/ kg DM.

The digestibility of the organic matter is one of the main factors determining the nutritive value of forage, and this may be as high as 0.85 in young spring pasture grass and as low as 0.50 in winter forage. The digestibility decreases as plants mature, the relationship is complicated by there being a **spring period of up to a month during which the herbage digestibility remains fairly constant. This period has been described as the „plateau“ (McDONALD et al., 1988).**

Bc/ The report from „european journal“ Livestock Production Science **(the explanation about the „high share of grassland“ in the UK):**

LEE,J.: Forages. Livestock Production Science, 19, 1988: 13-46

In Eastern European countries, such as Poland and Czechoslovakia, much of the grassland area comprises natural meadows and haylands receiving comparatively low levels of fertilizer application. Grassland output at farm level in the U.K., France, the F.R.G. and Ireland is 4500- 8000 kg DM/ha , whereas in Benelux and Denmark it is 8000- 12000 kg DM/ha, which reflects intensive pasture use at farm level. **Rainfall and the available water capacity of the soil are major yield determinants, with output in the U.K., for example ranging from 6000-14000 kg DM/ha under intensive fertilization**. However, there are notable exceptions such as Benelux, wchich although characterized by the highest pasture yields in Europe, has a comparatively **low share of grassland in total ruminant feed composition (50- 55%) – compared with Ireland (97%), U.K. (83%), France (71%).**

The permanent grazings of the Mediterranean zone are subject to severe moisture stress with annual production being limited to about 1000 kg DM/ ha. However, in this climatic zone, **irrigated legume and legume/ grass swards are capable of outputs of 20000 kg DM/ ha** (LEE, 1988).

Conclusion

The highest share of grassland in total ruminant feed composition is in Ireland (97%) and in U.K.(83%). H**igh intake of grasses in ruminants; available water capacity, high N (and K-fertilization by animal excrements), cool and cold marine climatic region; these circumstances are ideal for the subclinical (chronic) hypomagnesaemia in Britain-Ireland ruminants, especially.**

Bd/ The report from the british journal „The Veterinary Record";

McCOY, M.A.- GOODALL,E.A.- KENNEDY,D.G.: Incidence of hypomagnesaemia in dairy and suckler cow in Northern Ireland. Vet.Rec., 132, 1993: 537

McCOY,M.A.- RICE,D.A.- WRIGHT,A.- KENNEDY,D.G.: Use of time- lapse video equipment to determine the efficasy of commercial magnesium blocks in cattle. Vet.rec., 135, 1994: 209-210

In britain veterinary journals (period; 1985-1995), there is only one information (article as survey) about the cow hypomagnesaemia testing (McCOY et al., 1993)- from Northern Ireland. There clotted blood samples submitted under the Brucellosis Eradication Scheme were used for this survey published in The Veterinary Record; **513 dairy and 1266 suckler cow herds were sampled** during the grazing season from March to November 1991 (to February 1992- suckler cows). It was found; **serum blood Mg below 0.8 mmol/l in 14.1 of dairy and in 33.9% of suckler cows. The peak of hypomagnesaemia incidence; in both dairy and suckler herds occurred in the period from March to June,** coinciding with the period of peak milk production. In addition , in 8.2% of suckler cows – the blood Mg below 0.6 mmol/l was found !

One year later, report from McCOY et al. (1994) describes a novel method to evaluate the most popular commercially available hardened magnesium blocks – as oral mineral Mg-supplement in cattle feeding. There are following informations (from their survey- article); about the evidence **of Mg- oral supplementation changes in the cattle of UK**:

Proof No 1: In early 1980s (1983; published in „Outlook on Agriculture"), it was stated that in most circumstances there is no safe alternative to providing extra dietary magnesium (Mg), **with 30 g of available Mg per lactating cow per day being an average target**.

So, there is the evidence about the Mg-deficit in britain cows in 1980s yet, because - high Mg-supplementation was recommended (for example; in a cow with 20 kg dry matter (DM) intake; 30 g only as extra dietary Mg = 0.15% Mg of DM of the dairy ration

Proof No2: The incidence of hypomagnesaemia in adult cows remains high in Northern Ireland. An increasingly popular method of supplying additional dietary magnesium is by allowing cattle free access to hardened, magnesium- rich blocks or licks.

So, there is the evidence that the problem about Mg-deficit in britain cows; not only continued to **1993/94 period ,but at this time (period) there is increased „preventive" interest to cow Mg supplying .**

Proof No 3: Five commercial hardened Mg-blocks were tested in non-pregnant heifers with the intention to achieve intake of 30 g as extra dietary Mg; per animal and day (during experiment, it was found; 4.4 g, 58.3 g, 8.7 g, 49.2 g, 15.0 g Mg- on 1,2,3,4,5 types of Mg blocks, resp.). The present study shows that available commercial Mg-blocks; have considerable variation in palatability, Mg-content and Mg-intake of the hardened Mg rich blocks – as it was concluded by authors.

However, there is evidence; if some of commercial Mg-blocks (type 2 and 4) are used , **very high intake of Mg was achieved.**

Proof No 4: Many magnesium rich blocks are available, but unfortunately little is known about their relative effectiveness. There is a need for commercially available blocks to by evaluated before being put on the market. The use of time-lapse video recording (equipment)

provides an **additional method of carrying out such quality control** (McCOY et al.,1994).

Conclusion

The begining of the „special“ interest to monitoring of intake Mg-blocks by cattle (1993/94); there is evidence of higher interest of Mg-supplying in the UK. So together, finally; there is evidence that in **1993/94 period; it seems that in the UK it was the begining about; an increasingly popular method of supplying additional dietary magnesium-** preferably be given as an oral mineral supplement rather than by attempting to modify the mineral content of herbage- by the soil Mg fertilization.

Ad 2/ The begining of the higher dietary protein recommendation including undegradable protein (MBM...) in dairy rations

A/ Research results;

Several new theoretical protein systems have been proposed during the mid 1970s to the mid 1980s. Four new U.S. systems have been proposed. These systems was called (a) „Burroughs system“ (BURROUGHS et al., 1975a; 1975b); (b) „Satter system“ (SATTER, 1982); (c) „Chalupa system“ (CHALUPA, 1980), (d) „Cornell system“ (FOX et al., 1982; Van SOEST et al., 1982) sumarized in „Ruminant Nitrogen Usage“ (NRC, 1985). As reported by the USDA, average production per cow in the United States reported in 1975 was 10,360 lbs as compared to 14,213 lbs in 1988.

Two new European systems- the ARC (1980) system in Great Britain and the PDI grele systém in France (VÉRITÉ et al., 1979)- are official proposals within each country. KAUFMANN (1979) has proposed a system in Germany, and LANDIS (1979) in Switzerland. DANFAER (1979) has outlined many factors in a model of protein utilization from Denmark. **All of the new systems consider the dietary intake crude protein to be divided into undegradable dietary protein (UDP) and degradable dietary protein (DRP) fractions.**

The extensive interrelationships of protein and energy supply have been the topics of considerable discussion in 1982-1984 (KAUFMANN,1982; VERITÉ et al., 1982; JOURNET et al., 1983; OLDHAM, 1984). These „concepts“ are more fully discussed in the National Research council **(1985) publication „Ruminant Nitrogen Usage“ (**http://www.nap.edu/books/030903597X/html/). Large excess of dietary protein may decrease the energy supply because excess protein must be deaminated to ammonia and, for the most part, transformed back into urea for excretion. Each gram of N excreted as urea in urine represents a theoretical excretion of 5.41 kcal. So, excessive N from DIP affects glucose metabolism, as observed in a number studies.

Conclusion: There is a **begining about the „necessity“ of undegradable protein (UDP) in dairy rations and there are other relationships;**

The inability of the cow to consume adequate energy in early lactation , coupled with the feeding of protein that is too degradable, **will produce excess ammonia in the rumen and high blood and milk concentrations,** but the protein concentrations in milk will be low. **As milk production per cow increases**, it becomes more and more important that dietary protein escape degradation in rumen fermentation(KAUFMANN, 1982).

This lower milk protein concentration is due to a reduced bacterial crude protein supply caused by a lack of ruminally fermented energy. On the other hand, cows that consume adequate energy but excess degraded intake protein (DIP) or intake protein (IP) produce **excess ammonia in the rumen, high blood and milk urea concentrations**, and normal protein concentrations (more than 3.2 percent) in milk (KAUFMANN, 1982).

B/ The situation in the United Kingdom

Ba/ The explanation from professor John Webster about the grossly overstimated requirement of cattle for UDP (ARC, 1980)

In 1980 a technical committee of the Agricultural Research Council (ARC) produced „The Nutrient Requirements of Ruminant Livestock". This publication **first drew attention of the U.K. feeding industry to the specific need for undegradable dietary protein (UDP)** i.e. a dietary source of essential amino acids which escapes degradation in the rumen and so can augment the supply of amido acids. It became clear that **animal proteins were a particularly rich source of UDP**, not only because they contained a near ideal balance of amino acids but also because they were highly undegradable,; i.e. the **majority of the protein in ingredients such as fishmeal** and MBM, escaped degradation in the rumen and so could provide the extra amino acids thought necessary for high milk yields in lactating dairy cows, especially.

However, the ARC (1980) publication, according to professor John Webster **grossly overstimated the requirement of cattle for UDP**. This is because they calculated supply of microbial protein from the rumen from experiments with sheep fed at or close to the maintenance level of nutrition. More productive high yielding dairy cows eat at least three times as much food as an animal fed for maintenance only. In these circumstances, retention time of digesta in the rumen is greatly reduced and a **much higher proportion of microbial protein passes out of the rumen than that predicted by ARC (1980)**. This is recognized in modern feeding systems such as the Metabolizable Protein System now in general use in the U.K. (WEBSTER, 1992; ALDERMAN et al.,1993). Indeed computer- based ration formulations based on the current formulae within the Metabolize protein system very seldom predict a deficiency of UDP. In other words, improvements in our understanding since 1980 reveal that the cow can operate at optimal biological and economic efficiency without recourse to protein of animal origin. **I feel we have anticipated this**, says professor John Webster, former President of both The Nutrition Society and the British Society for Animal Science (www.bseinquiry.gov.uk/files/ws/s439.pdf).

Bb/ Statement of Ben GILL ; former chairman of the Livestock and Wool Committee of the NFU; he says:

„I have been involved in the emerging story of BSE since the NFU first learnt of the existence of a nine cattle in July 1987. I was vice chairman and chairman of the Livestock and Wool Committee of the NFU (1986-1991). Feed compounds used for feeding cattle, farmers may buy compound feed from feed producers. The **actual ingredients used will vary from time to time and from producer to produce**r. These are commercial decisions taken by the feed producers. For example, protein could amongst others be generated by soya bean meal, or from processed meat and bone meal. At the time that feed producers switched from soya bean meal to meat and bone meal, there would have been no restrictions on them doing so.

A **farmer buying compound feed would not know what ingredients had been used to provide protein**. He would not know if the protein source in the compound was soya bean meal or meat and bone meal. The arguments for a declaration of ingredients are well rehearsed in an NFU paper prepared in March 1983. **The absence of ingredient listing meant that farmers buying compound feedstffs would not know whether or not the feed included meat and bone meal,** and if so, whether it was bovine or ovine meat and bone

meal… The alternative to purchasing compound feeds is for a farmer to purchase the individual ingredients (referred to as „straights“) and to prepare feed compounds himself…“ (www.bseinquiry.gov.uk/files/ws/s047.pdf).

Conclusion

In the UK the second half of the 1970s and beyond, farmers were being encouraged to increase their milk production. However, where „unpalatable„ MBM was fed in cows?

Bc/ Statement of Richard SIBLEY, chairman of the BCVA BSE Group, he says:

„The British Cattle Veterinary Association is a specialist division of the British Veterinary Association. **We have 1600 members of whom 1000 are practising veterinary surgeons working with cattle** in farm animal veterinary practices. It was the inquiring minds and instinctive concerns of some veterinary surgeons that led to them to refer the first BSE cases to such organisations as the Veterinary Investigation Service who investigated further.

Such referral was **voluntary and based upon a relationship between the private practice and the local VI Centre**. Not all practices submit samples or refer cases to their local VI Centre. **Private laboratories** provide a similar and competitive service. We now know that most BSE cases occur as a single sporadic case and **many of these would not have been referred to any VI Centre**. Submission of cases and samples was based on a voluntary commercial decision of the attending veterinary surgeon. It may be that the original geographical recorded incidence of this disease was influenced by the vigilance and activity of veterinary surgeons in those areas and the relationship they had with their local VI Centre.

The Scientific **paper published in the Veterinary Record of October 1987 was the first large scale publicity that the profession received.** The surveillance of BSE up to June 1988 and indeed the surveillance of any non-notifiable disease is based upon the vigilance and inquisitiveness of the farmer and attending veterinary surgeon, **with the voluntary referral to a VI Centre. The system is informal and haphazard** and is based upon a relationship between the practice and the VI Centre that **may or may not exist**.

The relationship is often dependent on personal contact, commercial expediency and local availability rather than the wider ramifications of national disease surveillance. The **epidemiological studies of the first cases that concluded** that an agent was transmitting the disease contained in meat and bone meal (MBM) provided the key to control. The control measures introduced were logical and rational as well **as inspirational**. **Veterinary practitioners are scientists who meet practical disease control challenges on a daily basis.** Such veterinary surgeons have much to offer in terms of BSE surveillance and control, including determining practical policies. However, there was scant opportunity for their voices to be heard with little formal consultation or involvement with policy making…“.(www.bseinquiry.gov.uk/files/ws/s421.pdf).

Conclusion

Why submission of BSE cases and samples was based on a voluntary commercial decision of the attending veterinary surgeon?

National Research Council's; Nutrient Requirements of Dairy Cattle, 7th Revised Edition (January 2001)

The previous edition was released in 1989. One of the main differences is the size of the latest publication. The 2001 edition has 224 more pages than the 1989 publication. There are changes about the lowering of protein requirements; in early lactation especially.

During early lactation (0-70 days postpartum) milk production increases rapidly, peaking at 4 to 6 weeks after calving. Protein content is critical **during early lactation; rations may** need to contain 19% of more crude protein (ENSMINGER et al., 1990). For example, the same high protein level is recommended in turkeys- in animals with highest protein requirements from animals (NRC,1994). In growing young turkeys (age; 11 to 14 weeks) there is the recommendation (http://www.nap.edu/books/0309048923/html/) 19 percent of protein of diet (90% dry matter). Almost the same situation is in „monogastric" young rapidly growing pigs allowed ad libitum diet of 90% dry matter (NRC, 1998)- average weight in range 15 kg (20.9% of crude protein) and 35 kg (18% of CP) (http://darwin.nap.edu/books/0309059933/html).

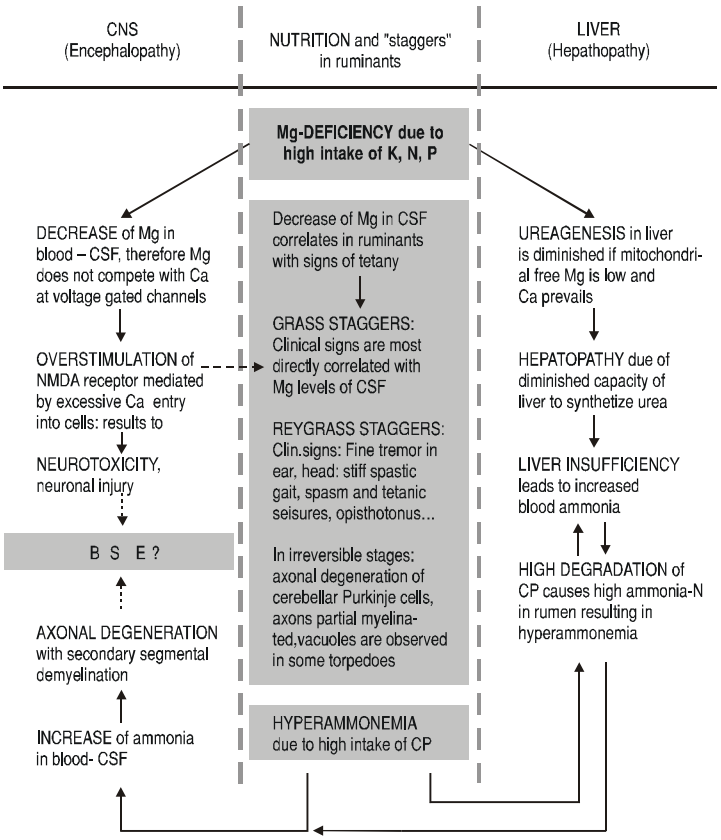
Almost the same high protein recommendations (18.8 % dry matter) are from McCULLOUGH (1994) to dairy rations of high producing „supercows"- **during the „all" lactation.** However, according to the **NRC (1989) this high protein level is recommended only during first three weeks (0-21 days postpartum)** after calving. So, above mentioned recommendation were **„overdosed" in dairy practice**. Recent research , **during 1990s resulted to decrease of protein** content in dairy cows – compared NRC (1989) and NRC (2001)- (http://www.nap.edu/catalog/9825.html).

Dairy cow:600-680 kg body weight							
	Lactation				Early lactation		Dry pregnant
Milk yield (kg/day)	25	35	45	55	25	35	
Degradable protein – „DP" (%):							
NRC,1989	8,8	9,7	10,4	10,4		9,7	-
NRC,2001	9,5	9,7	9,8	9,8	10,5	10,3	9,9
Undegradable protein- „UDP" (%):							
NRC,1989	5,4	5,7	6,0	6,3		7,2	-
NRC,2001	4,6	5,5	6,2	6,9	5,4	5,6	3,2
Crude protein – „CP"- (%):							
NRC,1989	15,0	16,0	17,0	17,5		**19,0**	12,0
NRC,2001	14,1	15,2	16,0	16,7	15,9	**15,9**	13,1

If rumen NH_3 levels are excessively high, the NH_3 is absorbed into the blood and either recycled or excreted in the urine as urea. Excess NH_3 in the portal system can readily pass through the liver and enter the arterial system. Brain tissue rapidly extracts NH_3 from the arterial blood. Within the critical blood concentration range, it appears that the **animal cannot detoxify ammonia fast enough to keep ahead of absorption from the stomach (rumen)**. In other words, the urea and glutamine- synthesizing mechanisms are saturated. Ammonia then builds up in bloodstream (hyperammonemia) and more and more NH_3 accumulates in tissue cells. The CNS is first to malfunction because it has a large requirement for energy. Behavioral and nervous signs do seem to appear first, cellular energy and respiration deficits probably cause ultrastructural damage and the degenerative changes (BOOTH and McDONALD, 1988). Therefore **at elevated blood concentrations ammonia is toxic on the central nervous system**.

So, I described a Czech alternative „ammonia- magnesium“ BSE theory (March, 2001)- see the Bulletin of Research Institute of Cattle Breeding in Rapotín , Czech Republic (see Fig 1) see also this text reprinted in international journal „Feed-Mix“(2002) (http://www.agriworld.nl/feedmix/headlines.asp?issue=3).

Fig. 1 Nervous diseases and connections with nutrition in ruminants



CNS: Central nervous system • CSF: Cerebrospinal fluid • CP: Crude protein
NMDA: "N-Methyl-D-Aspartate" receptor.

Generally, it seems that diseases of the nervous system in ruminants are in some connections with the nitrogen– magnesium metabolism. The surpluss of nitrogen and potassium intake can have association with hyperammonemia complicated with subclinical hypomagnesiemia, and the neurodegeneration may be involved. These mechanisms should also be studied in bovine spongiform encephalopathy (BSE) which can be on these mechanisms based. The epidemiological studies show, that BSE occurence to this date is **only in „western countries“ where significantly higher NPK fertilization is applied**. And also there **high protein content in dairy rations** was recommended, because high milk production was wanted.

This study is based on **my experiences from West Virginia University (WVU), t**here hypomagnesemia and high blood urea nitrogen (BUN) levels were found, in connection with high nitrogen and potassium intake in grazing ewes. I participated at the grazing trial; it has

been as a cooperative grant between West Virginia University and the USDA-ARS-NAA Appalachian Soil and Water Conservation Research Laboratory, Beckley, WV (CRIS No.l932-23330 - 00l- 00D) on a project entitled" **Improved Forage and Sheep Production Efficiency**".

About the BSE, most basic questions in prion science have remained unanswered. However, above described **„BSE magnesium- ammonia theory" is supported by more comprehensive relationships** in connection with the situation in the United Kingdom; see following research findings.

Some similarities; calcium, ammonia and TSEs

A/ The toxicity of the normal cellular prion protein is dependent on the NMDA receptor- associated Ca2+ channel activation

The evidence used to support the hypothesis that variant Creutzfeldt-Jacob disease (vCJD) is caused by eating meat from cattle infected with BSE is unconvincing. It is postulated (STOCKDALE, 2002**)** that the morphological **symptoms of both diseases are caused by high concentrations of intracellular calcium (Ca^{2+}).**

MULLER et al.(1993) incubated rat cortical cells with the scrapie prion protein (PrPSc). At concentrations of 3 ng/ml of PrPSc and higher, the viability of the cells decreased significantly after a 12-h incubation period. PrPSc did not affect the viability of astrocytes. Drugs known to block NMDA receptor channels, such as memantine, prevented the effect of PrPSc. They concluded that **antagonists of the NMDA receptor – channel complex (i) abolish the PrPSc – induced neuronal injury in vitro**, and (ii) display no influence on the usynthesis and/or the processing of PrionSc.

The PrPSc, as well as its peptide fragment, PrP106–126, are toxic on neuronal cells, **resulting in cell death by an apoptotic, rather than necrotic mechanism**. In vitro, the **toxic effects displayed by PrPSc and its peptide fragment can be blocked by antagonists of *N*-methyl-D -aspartate (NMDA) receptor channels**, like memantine. Also Flupirtine, a non-opiod analgesic drug, which is already in clinical use, was found to display in vitro a strong cytoprotective effect on neurons treated with PrPSc or PrP106–126. This drug acts like a NMDA receptor antagonists, but does not bind to the receptor (MULLER et al., 2000).

Cellular prion protein (PrP) is associated with regulation of intracellular free calcium levels through an interaction with voltage-sensitive calcium channels (WHATLEY et al., 1995) . The PrPC is a normal physiological protein, especially present in the central nervous system, including that of the human, with functions that are little known as yet. Locating the PrPC meant being able to identify which places in the central nervous system the prions operate. MOLERES and VELAYOS (2005), described the presence and location of the cellular prion protein (PrPC) in the brain of the rat. The findings enabled the research team to establish **that the PrPC is a protein involved in the neuronal metabolism of calcium.** Moreover, the existence of neurones without PrPC and surrounded by perineuronal nests breaks with the hypothesis, to date, that the disappearance of such nests – a special form of extracellular matrix – is a primary event in the course of spongiform encephalopathies; rather it is secondary event. According to these researchers' observations, the loss of these nests and consequent neuronal death are due to the damage produced after the appearance of the prions in the brain, where they act upon such perineuronal nests, amongst other structures.

The PrP 106–126, a synthetic peptide, represents a suitable model for studying the pathogenic role of the PrP^{Sc}, retaining, in vitro, some characteristics of the entire protein, such as the capability to aggregate in fibrils, and the neurotoxicity. THELLUNG et al. (2000) reported that the PrP 106–126 induces proliferation of cortical astrocytes, as well as degeneration of primary cultures of cortical neurons or of neuroectodermal stable cell lines (GH_3 cells). In particular, these two opposite effects are mediated by the same attitude of the peptide to **interact with the L-type calcium channels: in the astrocytes, the activity of these channels seems to be activated by PrP 106–126**, while, in the cortical neurons and in the GH_3 cells, the same treatment causes a blockade of these channels causing a toxic effect.

The infectious prion protein (PrPSc) is the etiological agent of transmissible neurodegenerative conditions such as scrapie or CJD. Its fragment 106-126 **(PrP 106-126) has been reported to maintain most of the pathological features of PrPSc**. This prion protein fragment directly stimulates the **proliferation of astrocytes via an increase in intracellular Ca2+** through the L-type voltage-sensitive calcium channels (FLORIO et al., 1996). However, there are two protein syntetic fragments; beta-amyloid 25-35 (betaA 25-35) and PrP 106-126. The toxicity of both peptides involves Ca2+ uptake through voltage-sensitive Ca2+ channels but **only PrP 106-126 toxicity involves the activity** of NMDA receptors (BROWN, D.R. et al., 1997).

Studies of the intracellular free calcium concentration revealed an alteration of the maximal increase of intracellular calcium concentration with depolarization in the **prion protein-deficient mice ($Prnp^{0/0}$) mouse Purkinje cells.** These data provide strong evidence (HERMS et al.,2001) that Ca^{2+}-activated K^+ currents in $Prnp^{0/0}$ mice **are reduced due to an alteration of intracellular calcium homeostasis.**

B/ Brain pathological changes in EAAs toxicity and TSE infection; cyclic form of the NAAG in murine and bovine CNS

The cytoprotective effects of the antagonists of the NMDA receptor-channel complex – see more of details in „Pathophysiology of transmissible spongiform encephalopaties“ (SCALLET et al., 2003). There are **similarities between brain pathological changes caused by excitatory amino acids (EAAs) toxicity and by TSE infection**. Neurons mediating the neuroendocrine functions of the hypothalamus as well as Purkinje cells of the cerebellum are **targets for damage by TSEs and EAAs**. Both EAAs and TSE agents **cause astrocytosis.** TSEs commonly feature swollen astrocytes either containing abnormal prion proteins (PrPSc) or located immediately adjacent to PrPSc deposits in the neuropil. Antigenic properties of the PrPSc may stimulate the release of nearby microglial and astrocytic cytokines, resulting in dysfunction and damage to elements of the neuropil. Such swolen astrocytes also commonly occur following exposure to a number of other neurotoxicants such as EAAs. Early neuropatological changes in scrapie involve primarily astrocytes and nerve terminals, but not nerve-cell bodies or spongiform changes. These data suggest that the initial events in the pathophysiology of TSEs may be the astrocytic response to PrPSc accumulation, leading to NOS/free radical damage to nearby nerve terminals.. They suggest that the end-stage neuropathology produced by TSEs has many of the characteristics of EAA-mediated neuronal necrosis, a well-established final common pathway of neurodegeneration from a variety of neurotoxicants. The cytoprotective effects of the antagonists of the NMDA receptor-channel complex such as memantine (1-amino-3,5- dimethyladamantane; MEM), MD-ADA (1-N-methylamino-3,5- dimethyladamantane) or dizolcipine will be considered (SCALLET et al., 2003).

The temporal **relationship between BSE and vCJD** only coincidentally supports the notion that BSE caused vCJD, and as such is not strong evidence.The evidence other than this comes from research **using mouse models** and analysis of subtypes of abnormal prion protein. This supporting evidence is related to four papers published in high-ranking journals (BROWN, 2001). **Aspartates and glutamates are both excitatory amino acids**. When excessive amounts of these are present in the brain they act as a neurotoxin destroying brain cells. The cyclic form (N-Acetylsuccinimidylglutamate[(asu)NAAG]), of the peptide N-acetylaspartylglutamate (NAAG) in which the aspartyl residue is linked to glutamate via the alpha- and beta-carboxylates, was identified and **quantified in the murine and bovine CNS**. In the **rat, the highest concentrations of (asu)NAAG were detected in the spinal cord** (1.83 +/- 0.15 pmol/mg of wet tissue weight) and brainstem (1.16 +/- 0.08 pmol/mg wet weight). The (asu)NAAG levels progressively increased from week 2 to month 12 after birth. In **bovine spinal cord**, the contents of (asu)NAAG and NAAG were **comparable with these of murine** in gray and white matter as well as in the dorsal and ventral horns (BROVIA et al., 1996). Data from cerebellar neurons and glia in primary culture confirmed the predominance of neuronal synthesis and glial uptake of NAA, leading to the hypothesis that while neurons synthesize NAA for NAAG biosynthesis, glia may take it up from the extracellular space. These results are consistent with the glutamate–glutamine cycle greatly favoring uptake of glutamine into neurons and glutamate by glia and suggest that NAA availability may be rate-limiting in the synthesis of NAAG by glia under some conditions (GEHL et al., 2004).

C/ Two mechanisms in TSEs

The PrPc (prion protein cellular) is a naturally occurring protein found in cells of central nervous system and other tissues. When the PrPc molecule refolds into an aberrant shape it **becomes pathogenic** and causes Transmissible Spongiform Encephalopathy (TSE). The disease- associated form of the normally occurring prion protein is designated as protease resistant protein (PrP^{Res}) or PrP Scrapie (PrP^{Sc}). **PrPc is a cell membrane glycoprotein particularly abundant in the synapses**. Prion diseases are characterized by the replacement of the normal PrPc by a protease-resistant, sheet-containing isoform that is pathogenic. A prion is a unique particle that **contains no nucleic acid** and differs from bacteria, viruses, fungi, viroids and plasmids. Prions are resistant to inactivation by most procedures, such as heat or ionizing ultraviolet radiation, that destroy most biological agents. Prions also are **resistant to enzymes such as protease** that quickly break down normal proteins. Pathology, **in prion diseases, develops only in the brain**. No other organ is affected. Early on, neurons develop intracytoplasmic vacuoles. As the disease progresses, vacuolization becomes more pronounced and, microscopically, the cortical neuropil develops a spongy appearance, hence the term spongiform encephalopathy. Advanced cases show neuron loss, gliosis (astrocytosis), and brain atrophy. So, as a result prions multiply, are not broken down by proteases and accumulate in brain tissue, where damage results by one of **two mechanisms**: (1) accumulation of the abnormal form of the protein itself causes the damage **(„vacuolization**"); (2) the loss of function of normal protein results in cell death **(„astrocytosis"**).

Cerebellar atrophy is usually severe. The different appearance of the molecular and granular layers in the cerebellum should be noted. The **differences are due to different cell types**. The granular layer contains mainly Golgi cells and granule cells, whereas the molecular layer contains mainly parallel fibres and dendrites of Purkinje and Golgi cells. Purkinje cell axonal swellings, also termed „torpedoes". **Axonal torpedoes are within the granular layer of the cerebellum.** Unlike most cerebellar degenerations, there is more pronounced loss of granular neurons than Purkinje cells. In some cases, prion proteins precipitate as amyloid plaques**. Gliosis is mostly present in the molecular layer** of the

cerebellar cortex. In some cases, prion proteins precipitate as amyloid plaques. Recent data point to synapses as principal targets of abnormal PrP deposition. Moreover, impairment of glomerular synapses and attenuation of parallel fiber pre-synaptic terminals on Purkinje cell dendrites is a cardinal consequence of abnormal PrP metabolism in CJD. Accumulation of synaptic proteins in the soma and axonal torpedoes of Purkinje cells suggests additional impairment of axonal transport (FERRER, 2002).

Hyperammonenia and neuronal toxicity

The original clinical and pathological description of the disorder (increased brain ammonia concentration) by ASBURY et al (1963) has been ample corroborated (DYCK et al., 1971: FORNO and ALSTON, 1967) and it appears to be;

- a primary axonal degeneration with secondary segmental demyelination
- the segmental loss of myelin is consequent to an abnormality in the axon cylinder, which probably reflects a metabolic failure of the perikarion.....

Similar cerebellar lesions; degenerating axons myelinated at least over part of their traceable length…. were found **in protracted ryegrass staggers in sheep and cattle** (MASON, 1968), probably arised from a number of factors, which may include disturbed neuronal metabolism, neuronal exhaustion and repeated anoxic insults.

A/ Ornithine cycle, urea synthesis and the elimination of excess ammonia

The hepatic urea cycle (ornithine cycle) is the major route for disposal of waste nitrogen generated chiefly from protein and amino acid metabolism. In the same context, low-level synthesis of certain cycle intermediates in extrahepatic tissues also makes a small contribution to waste nitrogen disposal. **Two moles of waste nitrogen are eliminated with each mole of urea excreted.** A portion of the cycle is mitochondrial in nature; mitochondrial dysfunction may impair urea production and result in hyperammonemia. Overall, activity of the cycle is regulated by the rate of synthesis of *N*-acetylglutamate (NAG), the **enzyme activator that initiates incorporation of ammonia into the cycle.**

During catabolism of amino acids, large amounts of NH_4 and HCO_3 (in addition to CO_2) are formed in humans, one mol of each per day is formed when the daily protein consumption is 100 g (AITKINSON and BOURKE, 1984). In mammals the major metabolic pathway responsible for the removal of these two products is the synthesis of nontoxic urea in the liver. This activity named as the "ornithine cycle" gives constant intrahepatic ammonia (the sum of NH_3 and NH_4) concentration, must be carefully controlled for several reasons. Entry of ammonia into the systemic circulation must be prevented, because at plasma concentrations higher than 50 uM the compound is toxic central nervous system. On the other hand, it is important that ammonia is not completely converted into urea, because ammonia is also an essential metabolite in a number of vital metabolic processes, such as the synthesis of nonessential amino acids. Thus it is important that the intrahepatic concentration of ammonia is kept within certain limits. Obviously, ammonia, bicarbonate, and aspartate are the direct substrates for the ornithine cycle.

Since the discovery of the ornithine cycle, urea synthesis has generally been considered as a mechanism for the elimination of excess ammonia. However, ATKINSON and BOURKE (1984) have stressed the importance of the ornithine cycle as the only mechanism by which the body can get rich of HCO_3 ion that is produced in large amounts during amino acid

metabolism. In contrast NH4 ion also produced during amino acid catabolism, can be eliminated not only as urea but can also be excreted as such in the urine, with glutamine as the nontoxic N carrier between the tissues and the kidneys. Amino acid catabolism produces NH_4 cations and HCO_3 anions in nearly equal amounts. This is illustrated by the complete oxidation of alanine:

ALANINE + 3 O_2 ..... $2CO_2$ + HCO_3 + NH_4 + H_2O

Synthesis of urea results in a net consumption of NH_4 and HCO_3 ions in equal amounts:

2 NH_4 + 2 HCO_3 ..... Urea + CO_2 + 3 H_2O

This process can be seen as an ATP- dependent H ion pump that transfers H ion from the very weak acid NH_4 cation to HCO_3 anion (ATKINSON and BOURKE, 1987).

Thus the sum of the two processes, amino acid catabolism and urea synthesis does not result in accumulation of their NH_4 cation or HCO_3 anion, and the systemic pH is not affected. However, in metabolic acidosis that is not the result of renal insufficiency, HCO_3 anion is retained by the body to the extent that NH_4 cation is excreted in the urine and can be used to the neutralize the excess of H cations in the blood. Thus the function of urea synthesis is to stabilize the concentration of HCO_3 anions, not to decrease it (ATKINSON, 1986). Inhibition of urea synthesis in metabolic acidosis leads to retention of HCO_3 anion to compensate for the excess of H cations. The concentration of HCO_3 must also be kept constant: although a small amount of HCO_3 (2.5- 5.0 % of the total amount produced by amino acid catabolism) can leave the body via the urine the remainder can only be eliminated via synthesis of urea (AITKISON and BOURKE, 1984).

B/ Ammonia toxicity is mediated by the NMDA type of glutamate receptors

Previous experiments suggested that ammonium toxicity could be mediated by the NMDA type of glutamate receptors. To assess this hypothesis MARCAIDA et al.(1992) tested if MK-801, a specific antagonist of the NMDA receptor, is able to prevent ammonium toxicity. The remarkable protection afforded by MK-801 indicates that **ammonia toxicity is mediated by the NMDA receptor** (MARCAIDA et al., 1992).

The aim of the work of MINANA et al (1995) was to assess whether perinatal hyperammonemia impairs the function of NMDA receptors in rats and whether this impairment affords protection against acute ammonia toxicity and glutamate and NMDA neurotoxicity. Their results indicate that **exposure to ammonia during the prenatal and lactation periods results in long-lasting impairment of NMDA receptor function**. This would be the reason for the delayed protection afforded by exposure to low ammonia levels against acute ammonia toxicity in animals and against glutamate and NMDA toxicity in neuronal cultures.

Ammonia is a main factor in the pathogenesis of hepatic encephalopathy, acute **ammonia toxicity is mediated by activation of NMDA receptors**. FELIPO et al. (1998) have tried to identify intracellular events involved in the process of neuronal death. It is known that the rise of Ca2+ is an essential step. Glutamate (Glu) leads to depletion of ATP; some compounds (e.g. carnitine) prevent Glu-induced neuronal death without preventing ATP depletion: additional events are required for neuronal death. Glu induces activation of Na+/K+-ATPase, which could be involved in the toxic process. Inhibitors of protein kinase C, calcineurin or nitric oxide synthase prevent Glu toxicity. FELIPO et al. (1998) results indicate that Glu toxicity can be prevented at different steps or by activating receptors coupled to the

transduction pathways interfering with the toxic process. Agents acting on these steps could prevent excitotoxicity in vivo in animals.

During ammonia intoxication, NMDA receptors are excessively stimulated, resulting in a larger influx of Ca^{2+} than usual into neurons. This would elicit a cascade of reactions and eventually lead to neuronal cell death. **How does ammonia cause excessive activation of NMDA receptors?** It has been shown that NH_4^+ induced depolarization in cultured rat cortical astrocytes (ALLERT et al., 1998). This ammonia-induced depolarization could also take place in neuronal membranes and **result in removal of Mg^{2+} that normally blocks the NMDA receptor channel,** leading to excessive activation of the NMDA receptor (FELIPO and BUTTERWORTH, 2002).

It was shown (KOSENKO et al., 1999) that MK-801, an antagonist of NMDA receptors prevents ammonia-induced changes in superoxide dismutase, glutathione peroxidase and catalase. Ammonia intoxication also induces a depletion of glutathione and an increase in lipid peroxidation. Both effects, as well as ammonia-induced increase in superoxide formation are prevented by MK-801. These results indicate that **ammonia-induced oxidative stress in brain is mediated by excessive activation of NMDA receptors** and support the idea that oxidative stress can play a role in the mechanism of ammonia toxicity (KOSENKO et al., 1999).

C/ Urea production in the liver is controlled by magnesium

Both, chronic and acute liver insuficiencies are associated with increased blood ammonia levels. Hyperammonemia in cirhosis is the result of a diminished capacity of the liver to synthesize urea and to a decrease in glutamine synthetase (KAISER et al., 1988**). At elevated concentrations ammonia is toxic to the central nervous system** (ADAMS and FOLEY, 1953). The normal value for fasting arterial blood ammonia in humans is less than 50 uM/l (COODER and PLUM, 1987). The brain ammonia to blood ammonia concentration ratio is in the range of 1.5 : 1 to 3 : 1. This ratio is maintained by a complex interaction of blood flow, the pH difference across the blood- brain barrier, and a balance between enzymatic removal and synthesis.

The urea level in blood of animals is closely related to rhythmic intake of dietary protein (EGGUM, 1970) and is strictly reversely proportional to the biological value of the dietary protein (MUNCHOW and BERGNER, 1968). The plasma urea level and its urinary excretion also shows circadian rhythmic changes in man (KANABROCKI et al., 1973). In dairy cattle the meal time - dependent circadian rhythm of blood urea is small (ERBERSDOBLER et al., 1980). Since urea is mostly excrected by the kidneys, the blood urea is increased in renal failure. However, in healthy animals the **serum urea level is primarily affected by their feeding regime**. The protein intake (IDE et al., 1966) and particularly the ratio of protein to energy affect the urea levels in blood and milk of cows (PAYNE et al., 1970).

The hepatic urea cycle is the major route for disposal of waste nitrogen generated chiefly from protein and amino acid metabolism. The brain must expend energy to detoxify and to export the ammonia it produces. This is accomplished in the process of producing adenosine diphosphate (ADP) from adenosine triphosphate (ATP) by the enzyme glutamine synthetase, which is responsible for mediating the formation of glutamine from an amino group. Synthesis of glutamine also reduces the total free ammonia level circulating in the blood; therefore, a **significant increase in blood glutamine concentration can signal hyperammonemia.**

The CNS is most sensitive to the toxic effects of ammonia. Many metabolic derangements occur as a consequence of high ammonia levels, including alteration of the metabolism of important compounds, such as pyruvate, lactate, glycogen, and glucose. **High ammonia levels also induce changes in *N*-methyl D-aspartate (NMDA)** and gamma-aminobutyric acid (GABA) receptors and causes downregulation in astroglial glutamate transporter molecules (ROTH, 2006).

A high rate and extent of degradation of crude protein causing high concentrations of ammonia – N in rumen results in hyperammonemia, because of diminished capacity of liver to synthetise urea in ornithine cycle. Of **prime importance in the control of carbamoyl-phosphate synthese activity in ornithine cycle;** is the mitochondrial concentration of N-acetylglutamate, a compound that is indispensable for enzyme activity. In addition to the absolute concentration of mitochondrial N-acetylglutamate, the **concentration of liver mitochondrial free Mg^{2+} may be relevant,** since binding N-acetylglutamate to carbamoyl-phosphate synthase is dependent on this action (MEIJER, 1985).

The function of activity changes in carbamoyl- phosphate synthetase, via the well-documented alterations in the intramitochondrial concentration on N-acetylglutamate, is to buffer the intrahepatic ammonia concentration rather than to affect urea production per se. At constant concentration of ammonia the rate of **urea production is entirely controlled by the activity of carbamoyl- phosphate synthetase** (MEIJER et al., 1985). In other words; N-acetylglutamate synthetase is a mitochondrial matrix enzyme which catalyses the synthesis of N-acetylglutamate, which activates carbamoyl-phosphate synthetase (CPS), which initiates the first step of urea synthesis from ammonia. Regulation of CPS activity depends upon the levels of N-acetylglutamate. In cases of homozygous deficiency of CPS, the ability to fix waste nitrogen is completely absent, which results in increasing levels of free ammonia with the attendant effects on the CNS. Overall, **activity of the cycle is regulated by the rate of synthesis of N-acetylglutamate, the enzyme activator of CPS (which also is a mitochondrial enzyme) which initiates incorporation of ammonia into the urea cycle.**

Liver contains **both glutamine synthetase and glutaminase** but the enzymes are localized in different cellular segments. This ensures that the liver is neither a net producer nor consumer of glutamine. The differences in cellular location of these two enzymes allows the liver to scavange ammonia that has not been incorporated into urea. The enzymes of the urea cycle are located in the same cells as those that contain glutaminase. The result of the **differential distribution of these two hepatic enzymes** makes it possible to control ammonia incorporation into either urea or glutamine, the latter leads to excretion of ammonia by the kidney.

When acidosis occurs the **body will divert more glutamine from the liver to the kidney**. This allows for the conservation of bicarbonate ion since the incorporation of ammonia into urea requires bicarbonate. When glutamine enters the kidney, **glutaminase releases one mole of ammonia generating glutamate and then glutamate dehydrogenase releases another**

mole of ammonia generating alpha-ketoglutarate. The ammonia will ionizes to ammonium ion (NH_4^+) which is excreted. The net effect is a reduction in the pH (KING, 2006).

Marked **brain damage is seen in cases of failure to make urea via the urea cycle** or to eliminate urea through the kidneys. The result of either of these events is a buildup of circulating levels of ammonium ion. Aside from its effect on blood pH, **ammonia readily traverses the brain blood barrier and in the brain is converted to glutamate** via glutamate dehydrogenase, depleting the brain of alpha-ketoglutarate. As the alpha-ketoglutarate is depleted, oxaloacetate falls correspondingly, and ultimately TCA cycle activity comes to a halt. In the absence of aerobic oxidative phosphorylation and TCA cycle activity, irreparable cell damage and neural cell death ensue.

In addition, the **increased glutamate leads to glutamine formation**. This depletes glutamate stores which are needed in neural tissue since glutamate is both a neurotransmitter and a precursor for the synthesis of gama-˜ aminobutyrate (GABA); another neurotransmitter. Therefore, **reductions in brain glutamate** affect energy production as well as neurotransmission. Additional untoward consequences are the result of elevations in neural glutamine concentration. **Glial cell (astrocytes) volume is controlled** by intracellular organic osmolyte metabolism. The organic osmolyte is **glutamine. As glutamine levels rise** in the brain the volume of fluid within glial cells increases resulting in the cerebral edema (KING, 2006).

The apparent effect of pH on the afinity of glutaminase for phosphate was found (SZWEDA and ATKINSON, 1989). The strong response of liver glutaminase to pH and the fact that the reaction can supply metabolites for urea synthesis suggest a possible regulatory role of glutaminase in ureagenesis. It was also found (SZWEDA and ATKINSON, 1990), that **the activity of rat liver glutaminase is strongly affected by variation in the Mg2+ concentration within the approximate physiological range** of activators. A rise in the Mg2+ concentration stimulates glutaminase by increasing the apparent affinity of the enzyme for its positive modifer phosphate. Since **Mg2+ stimulates glutaminase**, as does a rise in pH, by increasing the apparent affinity of the enzyme for phosphate, it reduces the inhibitory effect of a decrease in pH and/or phosphate concentration over a physiologically relevant range (SZWEDA and ATKINSON, 1990**).**

Glutaminase activity in intact mitochondria from rat liver is activated by spermine, as indicated both by increased glutamate production from glutamine and by increased respiration with glutamine as sole substrate. It was found (KOVACEVIC et al., 1995) that **spermine was effective in the presence of physiological concentrations of Mg2+**. Autors suggest that spermine may be a physiological activator of hepatic glutaminase.

D/ Ammonia plays a key role in contributing to the astrocytic dysfunction of the HE

Hyperammonemia is a key factor in the pathogenesis of hepatic encephalopathy (HE) as well as other metabolic encephalopathies. Excess ammonia is toxic to the brain resulting in deleterious effects, by both direct and indirect mechanisms, on cerebral metabolism and neurotransmission. Acute HE results in increased brain ammonia (up to 5 mM), astrocytic swelling, and altered glutamatergic function. This high level of **brain ammonia is a key factor in the pathogenesis of central nervous system dysfunction** in acute and chronic liver failure. The nature and severity of the central nervous system disorder mainly depend upon the degree and acuteness of the onset of hyperammonemia (FELIPO and BUTTERWORTH, 2002).

Because **hyperammonemia is thought to contribute to the pathogenesis of hepatic encephalopathy**, IZUMI et al.(2005) examined the effects of ammonia on ATP levels, neuronal morphology, and synaptic function in rat hippocampal slices - indicating that **ammonia impairs neuronal function via altered metabolism and untimely NMDA receptor activation.** Their results suggest that **L-carnitine and NMDA receptor antagonists have the potential to preserve neuronal function during hyperammonemia.**

Ammonia is a neurotoxic substance which accumulates in brain in liver failure and it has been suggested that **ammonia plays a key role in contributing to the astrocytic dysfunction characteristic of hepatic encephalopathy**. In particular, the effects of ammonia may be responsible for the reduced astrocytic uptake of neuronally-released glutamate and high extracellular glutamate levels consistently seen in experimental models of hepatic encephalopathy. CHAN et al. (2000) found that the reduced capacity of astrocytes to reuptake glutamate following ammonia exposure may result in compromised neuron-astrocyte trafficking of glutamate and could thus contribute to the **pathogenesis of the cerebral dysfunction characteristic of hyperammonemic syndromes such as hepatic encephalopathy.**

The role of ammonia in the glutamatergic dysfunction demonstrated in HE is supported with a **positive correlation between extracellular brain concentrations of glutamate and arterial ammonia concentrations** in ALF in rats (MICHALAK et al., 1996). In addition, using mild hypothermia as a treatment in rats with ALF, extracellular brain glutamate concentrations were normalized concomitantly with a lowering of brain ammonia (ROSE et al., 2000).

Acute hyperammonemia results in **alterations of mitochondrial and cellular energy** function resulting from **ammonia** -induced inhibition of the tricarboxylic acid cycle enzyme α-ketoglutarate dehydrogenase and by activation of the **NMDA** receptor. Antagonists of this receptor prevent acute ammonia-induced seizures and mortality and prevent acute ammonia-induced changes in mitochondrial **calcium** homeostasis and cellular energy metabolism. Acute hyperammonemia also results in decreased activities of free radical scavenging enzymes and again, free radical formation due to ammonia exposure is prevented by NMDA receptor (FELIPO and BUTTERWORTH, 2002).

The brain must expend energy to detoxify and to export the ammonia it produces. This is accomplished in the process of producing adenosine diphosphate (ADP) from adenosine triphosphate (ATP) by the enzyme glutamine synthetase, which is responsible for mediating the formation of glutamine from an amino group. **Synthesis of glutamine also reduces the total free ammonia level circulating in the blood; therefore, a significant increase in blood glutamine concentration can signal hyperammonemia.**

E/ Hyperammonemia leads to calcium- dependent glutamate release from astrocytes

Excess ammonia is toxic to the brain resulting in deleterious effects, by both direct and indirect mechanisms, on cerebral metabolism and neurotransmission. Hepatic encephalopathy (HE) resulting from acute liver failure (ALF) disorder; mainly depend upon the degree and acuteness of the onset of hyperammonemia.

As ammonia exceeds normal concentration, an increased disturbance of neurotransmission and **synthesis of both GABA and glutamine occurs in the CNS**. A **correlation between arterial ammonia concentration and brain glutamine** content in humans has been described. Moreover, brain content of glutamine is correlated with intracranial pressure. In vitro data also suggest that direct glutamine application to astrocytes in culture causes free radical production and induces the membrane permeability transition phenomenon, which leads to ionic gradient dissipation and consequent mitochondrial dysfunction. However, the true mechanism for neurotoxicity of ammonia is not yet completely defined. The pathophysiology of hyperammonemia is that of a CNS toxin that causes irritability, somnolence, vomiting, cerebral edema, and coma that leads to death.

Irrespective of the underlying cause, the clinical picture is relatively constant. This implies that the pathophysiologic mechanism, focusing on the CNS, is common to all individuals with hyperammonemia. The normal process of removing the amino group present on all amino acids produces ammonia. The a-amino group is a catabolic key that protects amino acids from oxidative breakdown. Removing the a-amino group is essential for producing energy from any amino acid. Under normal circumstances**, both the liver and the brain generate ammonia in this removal process**, contributing substantially to total body ammonia production. The **urea cycle is completed in the liver, where urea is generated from free ammonia.**

The biologic requirement for tight regulation is satisfied because the capacity of the hepatic urea cycle exceeds the normal rates of ammonia generation in the periphery and transfer into the blood. Hyperammonemia never results from endogenous production in a state of health. **The CNS is most sensitive to the toxic effects of ammonia**. Many metabolic derangements occur as a consequence of high ammonia levels, including alteration of the metabolism of important compounds, such as pyruvate, lactate, glycogen, and glucose. **High ammonia levels** also induce changes in NMDA and gamma-aminobutyric acid (GABA) receptors and **causes downregulation in astroglial glutamate transporter molecules**.

Over the past 10 years, there has been an increasing body of evidence demonstrating that **ammonia toxicity is involved in alterations of glutamatergic synaptic regulation which is implicated in the pathophysiology of HE in ALF.** The **total soluble ammonia level** in a healthy adult with 5 L of circulating blood is **only 150 mcg, in contrast to approximately 1000 mg of urea nitrogen** present. Because urea is the end product of ammonia metabolism, the disparity in blood quantities of the substrate and product illustrates the following 2 principles:

- The central nervous system (CNS) is protected from the toxic effects of free ammonia.
- The metabolic conversion system that leads to production of urea is highly efficient.

An individual is unlikely to become hyperammonemic unless the conversion system is impaired in some way. In older individuals, the impairment is more often the consequence of a diseased liver. **However, a growing number of reports address adult-onset** genetic disorders of the urea cycle **in previously healthy individuals.**

An acute exposure to ammonia, **resulting in cytosolic alkalinization** (pH action), **leads to calcium-dependent glutamate release from astrocytes**. A deregulation of glutamate release from astrocytes by ammonia could contribute to **glutamate dysfunction consistently**

observed in acute HE (ROSE et al., 2005). A rapid increase in ammonia results in an increase in pH_i (intracellular alkanization) in all cell types, including astrocytes. It is commonly known that **ammonia (NH4+/NH3) application** induces an increase in pH_i in many different cell systems. This alkaline shift is simply due to the rapid permeation of the gaseous NH_3 into the cytosol and the subsequent formation of a new NH4+/NH3 equilibrium according to the Henderson-Hasselbach equation rendering the cytosol alkaline (MARCAGGI and COLES, 2001). It has been also demonstrated that **intracellular alkalinization is accompanied with an increase in $(Ca^{2+})_i$** in cultured acinar cells (SPEAKE and ELLIOT, 1998), in enthothelial cells (DANTHULURI et al., 1990), in pituitary cells (SHORTE et al., 1991), and in neurons (MIRONOV and LUX, 1993). Furthermore, ammonia-induced intracellular alkalinization has been demonstrated to increase $(Ca^{2+})_i$ in microglia initiating Ca^{2+} release from thapsigargin-sensitive stores (MINELLI et al., 2000).

Hypomagnesaemia and neuronal toxicity

One process of neuronal death is called excitotoxicity. It appears to involve sustained elevations of intracellular calcium levels. **Impairment of neuronal energy metabolism may sensitize neurons to excitotoxic cell death. Calcium in cells is tightly regulated and mostly** unrelated to necessary dietary calcium**. However, a high content of calcium in the ration increases the magnesium requirements of the animal. The** lower the magnesium level **in the animal ration (and** in the tissue cells**); the more marked is „**calcium effect excitotoxicity**". It can be also accentuated; a** low temperature raises magnesium requirements.

The experiments demonstrated (Van MOSEL, et al, 1990) that **younger cows are better able to mobilize Mg from the body reserves than older cows.** Also as well known, older especially high milk yielding cows have hepatopathy in connection with high dietary protein intake.

A/ Glutamate triggers neuronal excitotoxicity when NMDA receptors are activated

Glutamic acid **(Glutamate** –„Glu") as the major excitatory neurotransmitter in the mammalian CNS; acts **postsynaptically** at several receptor types named for their prototypic pharmacological agonist. **One of these receptors, the NMDA receptor, is required for many forms of the process known as long-term potentiation**, a model of the synaptic basis of memory (COLLINGRIDGE and LESTER, 1989).

Activation of NMDA receptors **requires binding of both glutamate and the co-agonist glycine** for the efficient opening of the ion channel which is a part of this receptor. In addition, a third requirement is **membrane depolarization**. A positive change in transmembrane potential will make it more likely that the ion channel in the NMDA receptor will open **by expelling the Mg2+ ion that blocks the channel** from the outside.

NMDA receptor is also crucial to a **process known as exitotoxicity, in which excessive release of glutamate** leads to over- excitation of neurons producing neuronal injury and death (CHOI, 1988). Because **excitotoxicity has been implicated in many disorders** (acute neurological insults- e.g. Status epilepticus; chronic neurological disorders- e.g. Huntington´s

disease; metabolic disorders- e.g. hyperammonemia), development of NMDA receptor antagonists has become a major area of pharmacological investigation (CHOI,1988; CHOI et al.,1988; CHOI and ROTHMAN, 1990; LIPTON, 1993).

Glutamic receptors play a vital role in the mediation of excitatory **synaptic transmission**. This process is the means by which cells in the brain (**neurons**) communicate with each other. An electrical impulse in one cell causes an **influx of calcium ions** and the subsequent release of a chemical neurotransmitter (e.g. glutamate). The transmitter diffuses across a small gap between two cells (the **synaptic cleft**) and stimulates (or inhibits) the next cell in the chain by interacting with **receptor** proteins. The specialised structure that performs this vital function is the **synapse** and it is in the synapse that the ionotropic glutamate receptors are generally found. The ionotropic receptors themselves are **ligand gated ion channels**, ie on binding glutamate that has been released from a companion cell, charged ions such as Na^+ and Ca^{2+} pass through a channel in the centre of the receptor complex. This flow of ions results in a **depolarisation** of the plasma membrane and the generation of an electrical current that is propagated down the processes (dendrites and axons) of the neuron to the next in line.

Glutamate-mediated synaptic transmission is critical for the normal functioning of the nervous system. Because of its role in synaptic plasticity, it is believed that **glutamic acid is involved in cognitive functions like learning and memory** in the brain. The ability of the nervous system to rapidly convey sensory information and complex motor commands from one part of the body to another, and to form thoughts and memories, is largely dependent on a single powerful excitatory neurotransmitter, glutamate. There are other excitatory neurotransmitters in the brain, but glutamate is the most common and widely distributed. Most neurons (and also glia) contain high concentrations of glutamate (~10 mM); after sequestration inside synaptic vesicles, glutamate is released for very brief amounts of time (milliseconds) to communicate with other neurons via synaptic endings (LIPTON and ROSENBERG, 1994) . Both the pre- and post-synaptic neurons at glutamic acid synapse have glutamic acid-reuptake systems which **quickly lower glutamic acid concentration**. In addition, a nanosensor to detect the release of glutamate by nerve cells was developed (a team at Stanford University). The sensor can currently be located only on the surface of cell so it can indicate glutamic acid activity only outside the cell (OKUMUTO *et al.*, 2005).

In the synaptic cleft glutamic acid binds to two type of receptors: **ionotropic and metabotropic** glutamic acid receptors. **Glutamate receptors** are composed of assemblies of ; **AMPA, NMDA and Kainate (KA)** subunits receptors. The AMPA receptor (AMPAR) is a non- NMDA-type ionotropic transmembrane receptor for glutamate that mediates fast synaptic transmission in the CNS. Its name is derived from its ability to be activated by the artificial glutamate analog AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid). AMPA is a specific agonist for AMPA receptor (AMPAR) , **AMPA mimics the effect of glutamate**. AMPARs are found in many parts of the brain and are the most commonly found receptor in the CNS.

NMDA receptors are **composed of assemblies of NR1 subunits and NR2 subunits**, which can be one of four separate gene products (NR2A-D). Expression of both subunits are required to form functional channels. The glutamate binding domain is formed at the junction of NR1 and NR2 subunits (hence the need for both subunits to be expressed). In addition to glutamate, the **NMDA receptor requires a co-agonist, glycine**, to bind to allow the receptor to function. The glycine binding site is found on the NR1 subunit . **The NR2B subunit also posesses a binding site for polyamines,** regulatory molecules that modulate the functioning of the NMDA receptor. At resting membrane potentials, **NMDA receptors are inactive**. This

is due to a **voltage-dependent block of the channel pore by magnesium** ions, preventing ion flows through it. Sustained activation of AMPA receptors by, for instance, a train of impulses arriving at a pre-synaptic terminal, depolarises the post-synaptic cell, releasing the channel inhibition and thus allowing NMDA receptor activation . Presence of the **glutamate receptor 2 (GluR2)** subunit prevents calcium influx through AMPA-receptor complexes. So, unlike GluR2-containing AMPA receptors, **NMDA receptors are permeable to calcium ions** as well as being permeable to other ions. Thus NMDA receptor activation leads to a calcium influx into the post-synaptic cells, a signal thought to be crucial for the induction of NMDA-receptor dependent LTP (**long-term potentiation**) and **long-term depression**-LTD.

Current flow through AMPA receptors containing GluR2 normally is carried largely by the movement of Na^+ from the extracellular face to the intracellular compartment; these receptors have very low Ca^{2+} permeability. However, **receptors that lack GluR2 are three to five times more permeable to Ca^{2+} than to the monovalent ions** (HUME et al., 1991), due to the absence of an arginine in the Q/R site. An asparagine residing in the homologous site of all NMDA receptor subunits confers high Ca^{2+} permeability on these receptors (WOLLMUTH et al., 1996); replacement of this asparagine with an arginine by site-directed mutagenesis produces NMDA receptors with very low Ca^{2+} permeability, similar to that of GluR2-containing AMPA receptors.

The ion channels coupled to classical NMDA receptors are generally the most permeable to Ca^{2+}. Excessive activation of the NMDA receptor in particular leads to production of damaging free radicals and other enzymatic processes contributing to cell death (LIPTON and ROSENBERG, 1994; LIPTON and NICOTERA, 1998).

In excess, glutamic acid (Glu) triggers a process called excitotoxicity, causing neuronal damage and eventual cell death, particularly when NMDA receptors are activated. This may be due to:

- High intracellular Ca2+ exceeding storage capacity and damaging mitochondria, leading to release of cytochrome and apoptosis,
- Glu/Ca^{2+}-mediated promotion of transcriptions factors for pro-apoptotic genes, or downregulation of transcription factors for anti-apoptotic genes.

Glutamate transporters exist in neuronal and glial membranes to remove excess glutamate from the extracellular space, thereby preventing a buildup of glutamate and the damage that such a buildup would cause (SHIGERI et al., 2004).

On the other hand a **hypofunction of glutamatergic neurons** has been hypothesized to **caused schizophrenia**. Findings of **reduced concentrations of glutamate** in the cerebrospinal fluid of patients with schizophrenia and the ability of glutamate-receptor antagonists to cause psychotic symptoms lend support to this hypothesis (TSAI et al., 1995). They also suggest that the therapeutic efficacy of neuroleptics may be related to increased glutamatergic activity. Evidence from histological and pharmacological challenge **studies indicates that NMDA receptor hypofunction may play an important role in the pathophysiology of schizophrenia**. The goal of RADANT et al.(1998) was to characterize effects of NMDA hypofunction further, as related to schizophrenia-associated neuropsychological impairment. They administered progressively higher doses of ketamine

to 10 psychiatrically healthy young men. They concluded that ketamine induces changes in recall and recognition memory and verbal fluency reminiscent of schizophreniform psychosis.

B/ Under pathological conditions depolarization of neurons relieves the normal Mg^{2+} block of NMDA receptor

Under normal conditions of synaptic transmission, the **NMDA receptor channel is blocked by Mg^{2+}** sitting in the channel and only activated for brief periods of time. **Under pathological conditions, however, overactivation of the receptor causes an excessive amount of Ca^{2+}** influx into the nerve cell, which then triggers a variety of processes that can lead to necrosis or apoptosis. The latter processes include Ca^{2+} overload of mitochondria, resulting in oxygen free radical formation and activation of caspases, Ca^{2+}-dependent activation of neuronal enzyme nitric oxide synthase (NOS), leading to increased nitric oxide (NO) production and the formation of toxic peroxynitrite ($ONOO^-$), and stimulation of mitogen-activated protein kinase p38 (MAPK p38), which activates transcription factors that can go into the nucleus and influence neuronal injury and apoptosis (BONFOCO, 1995; DAWSON et al., 1991; DAWSON et al., 1993; LIPTON et al., 1993; TENNETI et al., 1998; YUN et al., 1998; BUDD et al., 2000; OKAMOTO et al., 2002).

. **Increased activity of the enzyme nitric oxide synthase (NOS) is associated with excitotoxic cell death**. The neuronal isoform of the enzyme is physically tethered to the NMDA receptor and activated by Ca^{2+} influx via the receptor-associated ion channel, and increased levels of nitric oxide (NO) have been detected in animal models of stroke and neurodegenerative diseases. Apoptotic-like excitotoxicity is caused in part by excessive stimulation of the NMDA subtype of glutamate receptor . When activated, the **NMDA receptor opens a channel that allows Ca^{2+}** (and other cations) to move into the cell.

Elevations in extracellular glutamate are not necessary to invoke an excitotoxic mechanism. Excitotoxicity can come into play even with normal levels of glutamate if NMDA receptor activity is increased, e.g., **when neurons are injured and thus become depolarized** (more positively charged); this condition **relieves the normal block of the ion channel by Mg^{2+}** and thus abnormally increases NMDA receptor activity (ZEEVALK and NICKLAS, 1992).

When glutamate and glycine bind and the cell is depolarized to remove Mg^{2+} block, the NMDA receptor channel opens with consequent influx of Ca^{2+} and Na^+ into the cell, the amount of which can be altered by higher levels of agonists and by substances binding to one of the modulatory sites on the receptor. The two modulatory sites that are most relevant to this review are the magnesium (Mg^{2+}) site within the ion channel and an S-nitrosylation site located toward the N terminus (and hence extracellular region) of the receptor (LIPTON, 2004).

Energetically compromised neurons become depolarized **(more positively charged)** because in the absence of energy they cannot maintain ionic homeostasis**; this depolarization relieves the normal Mg^{2+} block of NMDA** receptor-coupled channels because the relatively positive charge in the cell repels positively-charged Mg^{2+} from the channel pore. Hence, during periods of ischemia and in many neurodegenerative diseases, excessive stimulation of glutamate receptors is thought to occur. These neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease**…, are caused by different mechanisms** but may share a final common pathway to neuronal injury due to the

overstimulation of glutamate receptors, especially of the NMDA subtype (LIPTON and ROSENBERG, 1994).

Compelling evidence supports contributions of glutamate receptor overactivation ('excitotoxicity') to neurodegeneration in both acute conditions, such as stroke, and chronic neurodegenerative conditions, such as amyotrophic lateral sclerosis. However, anti-excitotoxic therapeutic trials, which have generally targeted highly Ca(2+) permeable NMDA-type glutamate channels, have to date failed to demonstrate impressive efficacy (KWAK and WEISS, 2006). Whereas most AMPA type glutamate channels are Ca(2+) impermeable, an evolving body of evidence supports the contention that relatively unusual Ca(2+) permeable AMPA channels might be crucial contributors to injury in these conditions. These channels are preferentially expressed in discrete neuronal subpopulations, and their numbers appear to be upregulated in amyotrophic lateral sclerosis and stroke. In addition, **unlike NMDA channels, Ca(2+) permeable AMPA channels are not blocked by Mg(2+),** but are highly permeable to another potentially harmful endogenous cation, Zn(2+). The targeting of these channels might provide efficacious new avenues in the therapy of certain neurological diseases (KWAK and WEISS, 2006).

C/ Magnesium can protect against NMDA- induced neurodegeneration…

Glutamate and aspartate neurotransmitters produce their effects by interacting with specific receptors on the cell surface, the excitatory amino acid receptors (MONAGHAN et al., 1989). Five receptor subtypes have been identified. The most well characterized excitatory amino acid receptor are subtype is NMDA receptor which is permeable to Ca^{2+} (FAROOQUI and HORROCKS, 1991). **Overstimulation of the NMDA receptor as well as other excitatory amino acid receptors results in neurotoxicity and neuronal injury.** These receptors are considered as the final common pathway for many acute and chronic neurologic conditions (McDONALD et al., 1988). An important consequence of NMDA receptor activation is the influx of Ca^{2+} into neurons. **Excessive NMDA receptor stimulation** is thought to be an important factor in neuronal cell damage, **mediated by excessive calcium entry into the cell** (OLNEY, 1989: McMASTER et al., 1991). Studies have demonstrated that **magnesium can protect against NMDA- induced neurodegeneration, brain injury, and convulsions** in rats (McDONALD et al., 1990: WOLF et al., 1990).

Neuronal free calcium concentrations correlates with the likelihood of irreversible ischaemic cell death (EIMERL and SCHRAMM, 1994: CHOI, 1985), and free intracellular Ca increases may result from Ca entry via the NMDA ion channel and voltage - gated calcium channels, and release from endoplasmic reticulum and other intracellular stores. **Magnesium competes with calcium at voltage- gated calcium channels both intracellularly and on the cell surface membrane** (ISERI and FRENCH, 1984). It may thereby impede Ca-dependent presynaptic release of glutamate and prevent neuronal Ca overload via voltage-gated channels during ischaemia. Magnesium also enhances mitochondrial buffering of raised intracellular free calcium ions (FAVARON and BERNARDI, 1985), and **prevents release of intracellular calcium stores from endoplasmic reticulum.**

The NMDA receptor channel is additionally blocked by Mg^{2+} and phencyclidine (PCP). Protons suppress NMDA receptor activation, and polyamines, such as spermine, relieve the proton block. Magnesium is capable of blocking NMDA receptors both intracellularly and extracellularly (KUPPER et al., 1998).

Another important endogenous allosteric inhibitor of NMDA receptor activation is pH. The frequency of NMDA receptor channel openings is **reduced by protons over the physiological pH range,** with a midpoint at pH 7.4, such that at pH 6.0 receptor activation is suppressed nearly completely (NOWAK et al., 1984). This suggests that an ionizable histidine or cysteine may play a key role in receptor activation. One or more modulatory sites that bind **polyamines, such as spermine and spermidine, also are found on NMDA receptors**. Occupancy of one of the polyamine sites relieves tonic proton block and, thus, potentiates NMDA receptor activation in a pH-dependent manner (TRAYNELIS et al., 1995). At higher concentrations, however, **polyamines act on an extracellular site to produce a voltage-dependent block** of the ion channel and, thus, inhibit receptor activation.

The permeation pathway of NMDA receptors has a property that sets them apart from other conventional ligand-gated receptors. At hyperpolarized membrane potentials more negative than about −70 mV, the **concentration of Mg^{2+} in the extracellular fluid of the brain is sufficient** to virtually abolish ion flux through NMDA receptor channels even in the presence of the coagonists glutamate and glycine (NOWAK et al., 1984).

D/ Polyamines may act on NMDA receptors under pathological conditions

Polyamines such as spermine and spermidine can inhibit or potentiate the NMDA receptor in a concentration- dependent manner (ROCK and MacDONALD, 1992; WILLIAMS, 1997). At low micromolar concentrations, polyamines promote channel opening by two distinct mechanisms, referred to as „glycine dependent“ and „glycine independent“. The glycine dependent mechanism results from an increase in the affinity of the receptor for glycine in the presence of polyamines, while the glycine- independent effect increases the opening frequency of the channel with no change in the affinity of the endogenous agonists – glycine and glutamate (ROCK and MacDONALD, 1992). Polyamines block the channel in a voltage- dependent manner at higher concentrations. However, **endogenous polyamines may not modulate the NMDA receptor in vivo** in the brain. While the site of action on the NMDA receptor is on the extracellular surface of the cell, spermidine and spermine are usually found intracellularly. Thus, **polyamines may not act physiologically on NMDA receptors except under pathological conditions** (MUNIR et al., 1993). The endogenous modulator at this site **could be protons or magnesium** since their actions on NMDA receptors overlap with those of spermidine (PAOLETTI et al., 1995). **NMDA receptor activity is extraordinarily pH sensitive**, consistent with a physiological inhibition by protons (TRAYNELIS and CULL-CANDY, 1990). **Cerebellar NMDA receptors have different** single channel conductances than the majority of forebrain NMDA receptors (FARRANT et al., 1994).

LENZEN et al. (1986) investigated the effects of the spermine on the regulation of Ca2+ transport by subcellular organelles from rat liver, heart, and brain. Spermine stimulated Ca2+ uptake by mitochondria **but not by microsomes. The half maximally effective concentration of spermine (50 microM) was in the range of physiological concentrations of this polyamine in the cell. Spermidine was five times less effective. Putrescine was ineffective. The** stimulation of mitochondrial Ca2+ uptake by spermine was inhibited by Mg2+ in a concentration – dependent manner. **However, the diminished contribution of the mitochondria to the regulation of the free extraorganellar Ca2+ concentration could mostly be compensated for by microsomal Ca2+ uptake. It was concluded that** spermine is an activator of the mitochondrial Ca2+ uniporter and Mg2+ an

antagonist**. By this mechanism, the** polyamines can confer to the mitochondria an important role in the regulation of the free cytoplasmic Ca2+ concentration **in the cell and of the free Ca2+ concentration in the mitochondrial matrix (LENZEN et al.,1986).**

A dual effect of the polyamine spermine on Ca2+ uptake by isolated rat liver, brain and heart mitochondria could be demonstrated (LENZEN et al., 1992) by using a high-resolution systém for studying mitochondrial Ca2+ transport. **(1)** Depending on the experimental situation, spermine had an inhibiting or accelerating effects on mitochondrial Ca2+ - uptake rate, but invariably increased the mitochondrial Ca2+ accumulation. **(2)** Both effects were concentration- dependent and clearly discernible on the basis of their different kinetic characteristics. For mitochondria from all three tissues the half- maximally effective concentration for inhibition of the initial rate of Ca2+ uptake was aprox. 180 microM, whereas that for subsequent stimulation of Ca2+ accumulation was approx. 50 microM. **(3)** Acceleration of the initial uptake rate could be seen when the mitochondria were preloaded with spermine during a 2 min preincubation period and thereafter incubated in a medium without spermine. **(4)** When such spermine-preloaded mitochondria were incubated in a spermine- containing medium, the increase in Ca (2+)- accumulation capacity was maintained in spite of an unchanged rate of Ca2+ uptake. **(5)** Mg2+ interacted with the effects of spermine in a different manner, enchancing the initial inhibition of the rate of mitochondrial Ca2+ uptake and diminishing the subsequent stimulation of mitochondrial Ca2+ accumulation. **(6)** This **dual effect of spermine on mitochondrial Ca2+ transport resolves the apparent paradox that a polycationic compound can act as a stimulator of Ca2+ uptake** (LENZEN et al., 1992).

E/ Inhibitory effect of milimolar concentrations of Mg^{2+} on neurotransmitter release

Communication between nerve cells in the central nervous system (CNS) occurs at synapses, which are specialized sites **between the presynaptic nerve terminal and the postsynaptic cell**. In the vast majority of cases this communication involves the chemical substances termed neurotransmitters. The basic processes involved in chemical synaptic transmission in the CNS appear to be very similar to those occuring at peripheral and intervertebrate synapses in which the electrical signal in the presynaptic terminal is transduced into the secretion of a chemical signal that serves as the message to the postsynaptic cell. The first systematic analysis of postsynaptic potentials in the CNS was by ECCLES (1953), who recorded intracellularly from spinal motoneurons in the early 1950s.

Only **two types of synaptic potentials were recorded**. Activation of excitatory pathways evoked **excitatory postsynaptic potentials (EPSPs**) in which a very brief (at most a few miliseconds) increase in conductance to cations depolarized membrane. Activation of inhibitory pathways evoked **inhibitory postsynaptic potentials (IPSPs**) in which a brief (at most a few milliseconds) increase in ionic conductance, primarily to chloride ions, usually resulted in a hyperpolarization. The pharmacological and neurochemical studies made in clear that these **synaptic potentials are mediated by amino acids; glutamate for EPSPs and**

gama- aminobutyric acid (GABA) and glycine for IPSPs (CURTIS and JOHNSTON, 1974; KRNJEVIC, 1974).

In 1980s as the coupling mechanisms between receptor and ion channel have been defined, it has become clear that the great majority of neurotransmitters in the CNS act throufh coupling proteins to activate or block the action of voltage- dependent channels. The simplest kind of coupling that involves an intermediary **protein apparently occurs through a single protein (G protein), as first described in atrial cells of heart** (BREITWEISER and SZABO, 1985; PFAFFINGER et al., 1985; YATANI et al., 1987).

Receptor occupation leads to G protein activation by allowing the coupled G protein to exchange its bound GDP for GTP. The binding of GDP results in the dissociation of the G protein´s regulatory subunit (beta gama) from its catalytic subunit (alpha) (which is responsible for carrying out most of the G protein´s known intracellular activities). The G protein does not remain permanently activated because of its inherent guanosinetriphosphatase (GTPase) activity. The hydrolysis of GTP to GDP results in the reassociation of the beta gama-and alpha- subunits such that the G protein is now ready to be activated again by ligand occupation of the receptor (GILMAN, 1984; STRYER and BOURNE, 1986). There are **several species of G proteins originally named because of their ability to stimulate (Gs) or inhibit (Gi)** adenylate cyclase and Go- as a G protein is found in very high concentrations in brain ((GILMAN, 1984; STRYER and BOURNE, 1986).

In the central nervous system (CNS) **magnesium (Mg^{2+}) ion has two major functions**: the stabilization of synaptic connectivity and widespread enhancement of neurochemical enzymatic functions. The Mg^{2+} has been shown to **affect guanine nucleotide binding proteins (G proteins) in several ways: nanomolar concentrations of Mg^{2+}** are required for GPT-ase activity (GILMAN, 1987: HIGASHIJIMA et al., 1987), **micromolar concentrations of Mg^{2+}** are required for receptor mediated activation of G proteins (GILMAN, 1987: GIERSCHIK et al., 1988), **milimolar concentrations of Mg^{2+}** increase the affinity of various types of receptors for agonists, an effect thought to result from increased receptor- G- protein coupling (HULUME et al., 1983: BIRNBAUMER et al., 1990), voltage- dependent- Ca^{2+} channel (AUGUSTINE et al., 1987), and N-methyl - D - aspartate (NMDA) receptor operated ionic channel (CRUNELLI and MAYER, 1984: NOWAK et al., 1984). The **inhibitory effect of milimolar concentrations of Mg^{2+} on neurotransmitter release** have been already demonstrated by in vivo microdyalisis experiments (OSBORNE et al., 1991: OKADA et al., 1996, 1998).

Excitatory amino acids such as **glutamate and aspartate are major neurotransmitters** in the mammalian CNS. It is generally accepted that these aminoacids are primarilly responsible for normal excitatory synaptic transmission (WATKINS and EVANS, 1981). **In the 1980s great strides have been made toward better undersanding** the function of these neurotransmitters , in particular because of the application of voltage – and patch- clamp techniques to cultured neurons that express the receptors and because of the development of specific receptor antagonists (COLLINGRIDGE and LESTER, 1989; MAYER and WESTBROOK, 1987; MONAGHAN et al.,1989; STONE and BURTON, 1988). This surge in information has not only resulted in a detailed understanding of the currents that underlie the fast excitatory amino acid- mediated transmission at many central synapses but has unveiled **an exciting new receptor type, the N- methyl- D- aspartate (NMDA) receptor, the activity of which is gated in a unique manne**r both by ligand binding and by voltage.

Aspartate appeared to act at the NMDA receptor as well as at additional sites, since part of the aspartate response showed a Mg2+ sensitivity similar to that of the NMDA response (SEKIGUCHI et al.,1987). These results suggest that cerebellar Purkinje cell dendrites do posses NMDA channels, but their functional role was still unclear. The receptor pharmacology in cerebellum is apparently slightly different from other brain regions.

First, APV does not entirely block NMDA responses (CREPEL et al., 1983; SEKIGUCHI et al.,1987).

Second, aspartate seems preferentially to activate NMDA- like receptors, some of which mediate effects that are entirely blocked in 1 mM Mg2+ (SEKIGUCHI et al.,1987) and others at which NMDA may act as a competitive antagonist (CREPEL et al., 1983; KIMURA et al., 1985; SEKIGUCHI et al.,1987).

In neurons from spinal cord , 10 mM Mg2+ is required to block NMDA responses at potentials negative to 0 mV (MAYER and WESTBROOK, 1985). It may be that the **NMDA channel in Purkinje cells has a more extreme sensitivity to Mg2+ than that in other brain regions.**

F/ Astrocytes regulate neuronal Ca2+ levels through the Ca-dependent release of glutamate

The brain has two types of cells**; neurons and glia**. Neurons contain neurotransmitters, which are chemicals that trigger signals to pass messages. Until recently, neuroscientists believed neurons were the only brain cells transmitting message signals. **Glial cells (astrocytes)** were thought to serve only as support. Glia, once thought to simply provide structural support for their more important neuronal cousins, have been found, in the past

decade, to have a wide variety of important biological functions. One of the most important of these is to foster a proper chemical environment for neuronal function by **removing excess glutamate.** Why is this important? Because glutamate is a neurotransmitter, i.e., it can bind to receptors on the neuronal membrane and cause it to fire. Thus, glutamate is key to proper neurological functioning. Too much glutamate, however, is a problem, because it could cause neurons to work too hard, fatigue and die a premature death. This phenomenon is called **glutamate toxicity**.

From a structural perspective, the predominant glial cell of the central nervous system, the astrocyte, is positioned to regulate synaptic transmission and neurovascular coupling: the processes of one astrocyte contact tens of thousands of synapses, while other processes of the same cell form endfeet on capillaries and arterioles. The application of subcellular imaging of Ca^{2+} signaling to astrocytes now provides functional data to support this structural notion. Astrocytes express receptors for many neurotransmitters, and **their activation leads to oscillations in internal Ca^{2+}.** These oscillations induce the accumulation of arachidonic acid and the release of the chemical transmitters glutamate, D-serine, and ATP. The Ca^{2+} oscillations in astrocytic endfeet can control cerebral microcirculation through the arachidonic acid metabolites prostaglandin E_2 and epoxyeicosatrienoic acids that induce arteriole dilation, and 20-HETE that induces arteriole constriction. In addition to actions on the vasculature, the release of chemical transmitters from astrocytes regulates neuronal function. **Astrocyte-derived glutamate**, which preferentially acts on extrasynaptic receptors, can promote neuronal synchrony, enhance neuronal excitability, and modulate synaptic transmission. **Astrocyte-derived D-serine,** by acting on the glycine-binding site of the *N*-methyl-D-aspartate receptor, can modulate synaptic plasticity (HAYDON and CARMIGNOTO, 2006).

Results of a five-year study by Iowa State neuroscientists Vladimir Parpura and Philip Haydon (2000) give evidence supporting a relatively new theory about communications between brain cells. It was found that astrocytes can release glutamate in a calcium-dependent manner and consequently signal to adjacent neurons. Whether this glutamate release pathway is used during physiological signaling or is recruited only under pathophysiological conditions is not well defined. One reason for this lack of understanding was the **limited knowledge about the levels of calcium** (PARPURA et al., 1994) necessary to stimulate glutamate release from astrocytes and about how they compare with the range of physiological calcium levels in these cells. PARPURA et al. (1994) demonstrated an additional signalling pathway in which glutamate is released from astrocytes and causes an NMDA (*N*-methyl-d-aspartate) receptor-mediated increase in neuronal calcium. Thus, **astrocytes regulate neuronal calcium levels through the calcium-dependent release of glutamate**.

PARPURA and HAYDON (2000) demonstrated that the **astrocytic glutamate release pathway is engaged at physiological levels of internal calcium**. Consequently, the calcium-dependent release of glutamate from astrocytes functions within an appropriate range of astrocytic calcium levels to be used as a signaling pathway within the functional nervous system. So, the amount of calcium to be within the normal range, indicating that **astrocytes are part of the brain's communication network.**

The evidence obtained during the last few years has established a **new concept of the synaptic physiology**, the tripartite synapse, in which **astrocytes play an active role** by exchanging information with the synaptic elements (ARAQUE et al., 1999; CARMIGNOTO, 2000; AULD and ROBITABILE, 2003; NEWMAN, 2003). This concept is based on the demonstration that astrocytes display a form of **excitability based on intracellular Ca^{2+}**

variations (PASTI et al., 1997; VERKHRATSKY et al., 1998; HAYDON, 2001; NEDERGAARD et al., 2003), **respond to synaptically released neurotransmitters** (PORTER and McCARTHY, 1996; PASTI et al., 1997; GROSCHE et al., 1999; LATOUR et al., 2001; ARAQUE et al., 2002), and modulate neuronal excitability and synaptic transmission by releasing neuroactive substances through, at least some of them, **Ca^{2+}-dependent mechanisms** (ARAQUE et al., 1998a, 1998b; KANG et al., 1998; NEWMAN and ZAHS, 1998; ROBITAILLE, 1998; PARRI et al., 2001; BEATTIE et al., 2002; BROCKHAUS and DEITMER, 2002: NEWMAN, 2003; ZHANG et al., 2003; FIACCO and McCARTHY, 2004; LIU et al., 2004).

. Tne ability of astrocytes to release glutamate through a Ca^{2+}-dependent mechanism is well established (BEZZI et al., 1998, 2004; ARAQOE et al., 2000; PARPURA and HAYDON, 2000; PASTI et al., 2001; ZHANG et al., 2004). On the other hand the **ability of most neurotransmitters to increase astrocytic Ca^{2+} levels is firmly established** (PORTER and McCARTHY, 1997; VERKHRATSKY et al., 1998). Recent reports have shown that astrocytic receptor activation by exogenously applied transmitters **may have synergistic effects that increase the Ca^{2+} signal** (FATATIS et al., 1994; CORMIER et al., 2001; SUL et al., 2004). Ca^{2+} elevations in astrocytes stimulate the release of glutamate, which acting on presynaptic or postsynaptic receptors modulates synaptic transmission and neuronal excitability (ARAQUE et al., 1998a, 1998b; KANG et al., 1998; PARRI et al., 2001; PASTI et al., 2001; BROCKHAUS and DEITMER, 2002; FIACCO and McCARTHY, 2004; LIU et al., 2004).

Astrocytes, a subtype of glial cells, have numerous characteristics that were previously considered exclusive for neurons. One of these characteristics is a **cytosolic Ca2+ oscillation that controls the release of the chemical transmitter glutamate** and atrial natriuretic peptide. These chemical messengers appear to be released from astrocytes **via Ca^{2+}-dependent exocytosis.** Glutamate can be released from astrocytes, and several mechanisms have been proposed. Glutamate has been demonstrated to be an important signaling molecule for neuron-glia communication. **Astrocytes express receptors and transporters for glutamate** and recently have also been demonstrated to contain the protein machinery necessary to release glutamate by exocytosis through vesicles (BEZZI et al., 2004) and a fusion-related mechanism (ZHANG et al., 2004; KREFT et al., 2004). Overall, astrocytes have many characteristics that were previously considered exclusive for neurons and are therefore **actively involved in cell signaling by releasing glutamate**. Astrocytic glutamate release is calcium-dependent and can be triggered by any ligand that stimulates an increase in Ca2+, such as bradykinins (PARPURA et al., 1994), prostaglandins (BEZZI et al., 1998). **Even a spontaneous $Ca2+_i$ increase leads to glutamate release from astrocytes** (PASTI et al., 2001).

G/ Astrocytes as cellular elements involved in the information processing by the nervous system

Astrocytes establish rapid cell-to-cell communication through the release of chemical transmitters. The underlying mechanisms and functional significance of this release was, however, not well understood. BEZZI et al. (2004) identified an astrocytic vesicular compartment that is competent for glutamate exocytosis. After activation of metabotropic glutamate receptors, **astrocytic vesicles underwent rapid (milliseconds) Ca^{2+}-** and the vesicular SNARE protein (cellubrevin) -dependent exocytic fusion that was accompanied by glutamate release. These data document the existence of a **Ca^{2+}-dependent quantal**

glutamate release activity in glia that was previously considered to be specific to synapses (BEZZI et al., 2004).

Astrocytes in the brain form an intimately associated **network with neurons. They respond to neuronal activity and synaptically released glutamate by raising intracellular calcium concentration Ca2+,** which could represent the start of back-signalling to neurons**.** Glutamate has been demonstrated to be an important signaling molecule for neuron-glia communication. Astrocytes express receptors and transporters for glutamate and recently have also been demonstrated to **contain the protein machinery necessary to release glutamate by exocytosis through vesicles** (BEZZI et al., 2004) and a fusion-related mechanism (ZHANG et al., 2004; . KREFT et al., 2004).

Although cell culture studies have implicated the presence of vesicle proteins in mediating the release of glutamate from astrocytes, definitive proof requires the identification of the glutamate release mechanism and the localization of this mechanism in astrocytes at synaptic locales. To further determine whether vesicular exocytosis mediates calcium-dependent glutamate release from astrocytes, ZHANG et al.(2004), performed whole cell capacitance measurements from individual astrocytes and demonstrate an increase in whole cell capacitance, coincident with glutamate release. Together, these data allow to conclude that **astrocytes *in situ* express vesicle proteins necessary for filling vesicles with the chemical transmitter glutamate and that astrocytes release glutamate through a vesicle- or fusion-related mechanism**.

The synaptic control of the astrocytic intracellular Ca^{2+} is **crucial in the reciprocal astrocyte-neuron communication.** Using electrophysiological and Ca^{2+} imaging techniques in rat hippocampal slices, PEREA and ARAQUE (2005) investigated the astrocytic Ca^{2+} signal modulation induced by synaptic terminals that use glutamate and acetylcholine. Ca^{2+} elevations were evoked by glutamate released from Schaffer collaterals and by acetylcholine, but not glutamate, released by alveus stimulation, indicating that astrocytes discriminate the activity of different synapses belonging to different axon pathways. The Ca^{2+} signal was modulated bidirectionally by simultaneous activation of both pathways, being depressed at high stimulation frequencies and enhanced at low frequencies. The Ca^{2+} modulation was attributable to astrocytic intrinsic properties, occurred at discrete regions of the processes, and **controlled the intracellular expansion of the Ca^{2+} signal**. In turn, astrocyte Ca^{2+} signal elicited NMDA receptor-mediated currents in pyramidal neurons. Therefore, because astrocytes discriminate and integrate synaptic information, PEREA and ARAQUE (2005) proposed that **astrocytes can be considered as cellular elements involved in the information processing by the nervous system**.

H/ D-serine coactivates postsynaptic NMDA receptors together with glutamate; mediated by intracellular Ca2+

Other neurotransmitter is an amino acid D-serine. **Astrocyte-derived D-serine**, **by acting on the glycine-binding site of the *N*-methyl-D-aspartate receptor, can modulate synaptic plasticity** (HAYDON and CARMIGNOTO, 2006). This differs in structure from any known molecule in its class found in mammals and other higher animals. D-serine is what chemists call a right handed amino acid. Normally, amino acids have atoms that extend from the left side of the molecule. These L-amino acids, as they're called, are the rule in vertebrates, whose biochemistry is set up to deal with these forms. Some primitive organisms, however, notably

bacteria, have a mixture of both L-amino acids and their mirror images called D-amino acids. **But to find a D-amino acid in humans "is unprecedented**" (SNYDER, 2000).

Moreover, unlike dopamine, serotonin or other traditional nerve transmitters, **D-serine isn't secreted at nerve cell endings in the brain.** Instead, it comes from adjacent cells called astrocytes, which enclose nerve cells in the brain's gray matter like a glove. The current study adds conclusive evidence to the idea that D-serine (released from astrocytes) activates receptors on key nerve cells in the brain. Activating these NMDA receptors has long been linked with learning, memory and higher thought. NMDA receptors are also known culprits in stroke damage in the brain, and have become a focus for anti-stroke research (SNYDER and FERRIS, 2000).

Classic criteria for transmitters were based on the properties of acetylcholine but were markedly revised with the recognition of the catecholamines, serotonin, gama -aminobutyric acid (GABA), and other amino acid transmitters and neuropeptides. Nitric oxide and carbon monoxide are notably atypical, as they are not stored in synaptic vesicles, are not released by exocytosis, and do not act at postsynaptic membrane receptor proteins. D-Serine, recently appreciated as the **endogenous ligand for the glycine site of the glutamate NMDA receptor, overturns fundamental axioms of biology** as well as those of neuroscience. It is a D-amino acid, and it is synthesized and stored in glia rather than neurons. Released glutamate acts on receptors on the protoplasmic astrocytes closely apposed to the synapse to release D-serine, which **coactivates postsynaptic NMDA receptors together with glutamate**. D-Serine is formed by serine racemase (enzyme), which directly converts L-serine to D-serine. **Inhibitors of this enzyme should reduce NMDA neurotransmission** and might be therapeutic in stroke and other conditions associated with glutamate excitotoxicity (SNYDER and FERRIS, 2000).

These researchers also found **D-serine and serine racemase concentrated in astrocytes adjacent to NMDA receptors,** but less common or nonexistent in other neural tissues. For years, neuroscientists assumed that NMDA receptors could only be stimulated by a single neurotransmitter, an amino acid called glutamate. They **now know that two neurotransmitters are needed to stimulate the NMDA receptors**. D-serine was recently proposed by Hopkins scientists (Baltimore University) as the second, largely because microscope images of tagged D-serine show it's **physically near NMDA receptors in the synapse.** Also, knocking D-serine out with enzymes quickly stops NMDA receptors from being active.

COOK et al. (2002) found that divalent cations such as **calcium or manganese were necessary for complete serine racemase (SR) enzyme activity**, whereas the presence of chelators such as EDTA completely inhibited the enzyme. Moreover, direct binding of calcium to SR was evidenced using $^{45}Ca2+$. Treatment of astrocytes with the calcium ionophore as well as with compounds that augment the intracellular calcium levels such as glutamate or kainate led to an increase in the amount of D-serine present in the extracellular medium. These results suggest that there might be a **glutamatergic-mediated regulation of SR activity by intracellular Ca2+ concentration** (COOK et al. 2002).

Conclusions

Identified in 1986, BSE rapidly spread to affect UK herds although the incidence was very limited within individual herds. The source was assumed, on epidemiological grounds, to

be from commercial feed which contained rendered animal protein. Certainly, cases dramatically declined subsequent to the time **when the 'ruminant-derived protein ban' (MBM) came into force in 1988**, establishing this theory beyond reasonable doubt.

In 1995, two cases of CJD were reported in young people. By March 1996 that had risen to 10 cases. Neuropathological findings revealed the presence of large amyloid plaques in the brains of these unfortunate people, more akin to Kuru than sporadic CJD, and which was now **renamed variant CJD (vCJD)**. The TSE's have **raised considerable public concern with respect to the unknown extent of the infection in the food chain**, the possible transmissibility to humans and most particularly the relationship of BSE to vCJD.

The Blair government has warned that vCJD, caused by eating beef infected with „mad cow disease" (BSE) could claim many as 250,000 lives. This is double the previous estimate of 136,000 possible deaths and means that the government is now working on a „worst case scenario" of one in every 250 people in Britain dying from the disease. Putting the risk into context, microbiologist and leading CJD expert Dr Stephen Dealler said (www.wsws.org/articles/2000/nov2000/bse-n03.shtml) on average people in the UK had eaten 50 meals made from the tissue of an infected animal. At the moment the number of cases of CJD we are seeing are doubling every year. If the double for a long time **then the numbers are in millions**, if they double for just a few years then the numbers are in thousands….(Hyland, 2000).

However, circumstantial evidence linking the consumption of beefburgers by young people in support of the transinfection theory, whilst persuasive, **has never been proven, in that the putative 'infectious' burgers have never been identified, nor indeed fed, to experimental animals.** It is quite surprising that the one experiment that would confirm a **link between BSE and vCJD has not been carried out.** Groups supposedly more at risk such as farmers, vets, abattoir workers and butchers have not shown an increased risk of vCJD.

Scientific proof that BSE causes vCJD rests upon the fact that BSE was first in the United Kingdom diagnosed 10 years before vCJD was diagnosed. Therefore, the temporal relationship between BSE and vCJD **only coincidentally supports** the notion that BSE caused vCJD, and as **such is not strong evidence.**

The main **UK Government funders of research on BSE and the other TSEs** of animal and public health significance are Defra and the Biotechnology and Biological Sciences Research Council (BBSRC). The Department of Health (DH), the Medical Research Council (MRC) and the Scottish Executive also fund work on CJD and public health aspects of TSEs. Total Government spending on research into BSE, scrapie, CJD, and other TSEs was increased to over £37 million in 2002/03, with **around £240 million allocated in total since 1986.**

The working hypothesis for the origin of BSE is sheep scrapie (Wilesmith *et* al., 1988; Kimberlin, 1993; Kimberlin and Wilesmith, 1994; Kimberlin, 1996). This hypothesis arises from the original considerations with respect to the reason for **only the cattle population in the United Kingdom experiencing a major incidence of BSE** (Wilesmith and Wells, 1991). The risk factors originally identified were fourfold: a **large ratio of sheep to cattle population**, approximately 4: 1, larger than in any other country. However, *t*here has been no demonstration that feeding cattle meet and bone meal produced from sheep has led to the

development of BSE since introduction of the MBM is fact; the **assumption that the fall in incidence of BSE is due to this ban is no more than an assumption**… In addition, the source of the supposedly infected feed, however, **was never identified and the disease never reproduced experimentally** in this way. Also the limited nature of the infection, localised often to just one animal in a herd, was puzzling, the more especially since this could not be explained by host predisposition, as with sheep to scrapie, since no variant polymorphisms have been identified in cattle.

So, second alternative hypotheses of the origin of BSE have attracted considerable attention, **particularly those related to a possible cattle origin** (see Wilesmith *et* al., 1988; Kimberlin, 1993; Kimberlin and Whilsmith, 1994; Kimberlin, 1996). The major requirement for a cattle-origin hypothesis to be consistent with the start and subsequent development of the BSE epidemic is that a geographically widespread reservoir of infection was naturally maintained in the cattle population of the United Kingdom. However, the **cattle-origin hypothesis was not supported by the fact that there have been no major epidemics in other countries.**

Initially, the infectious prion was thought to be a modified scrapie prion which had crossed species- this was **dismissed by Lord Phillips (2000) on the ground that BSE differed essentially from scrapie** in disease-profile, incubation and transmissibility. The report states with confidence that "the BSE agent is not an unmodified form of scrapie. The Report of the BSE Inquiry (2000) concluded BSE probably originated from a novel source early in the 1970s, possibly a cow or other animal that developed disease as a consequence of a gene mutation, rather than rendering of sheep infected with "normal" scrapie.

In response, the UK Government asked **Professor Horn to lead a small team of scientists to look in greater detail at the origin of BSE** by pulling together all scientific understanding, including emerging findings, on the subject. Whilst differing in respect of the scrapie theory, the review team's findings (July 2001) do agree with many of those in the BSE Inquiry Report (October 2000). Although the Horn et al review at (http://www.defra.gov.uk/animalh/bse/publications/bseorigin.pdf) does not come to a firm conclusion about the origin of BSE, it does offer a **possible explanation as to why BSE first occurred in the UK.** This followed a rare sequence of events between 1970 and the 1980s: meat and bone meal was introduced into **the feed of dairy calves from the first or second week of age - young animals are thought to be more susceptible** to infection; and this, coupled with changes to the rendering process which may have affected the degree of infectivity, could have created a particular set of conditions which enabled the BSE agent to spread (IFST, October 2004).

From the outset, successive Institute of Food Science and Technology (IFST) Position Statements (October 2004) have pointed out **that in real life, simple "single cause -> single effect" relationships are rare**, and it seems quite possible, or even probable, that BSE developed as a result of a number of coincidental factors coming into play in a combined circumstance. It is doubtful whether it will ever be possible to prove either the origin of BSE or the precise combination of factors that led to its amplification and infection of cattle. **Debate about the origin of BSE in cattle is and will probably remain unresolved**, including the respective possible roles of recycling of scrapie infected sheep or possible low-level BSE-infected cattle, of imported MBM, of changes in rendering processes and the use of solvents, of the effect of organophosphates at sub-toxic levels, of the influence of trace

metals, of the possible role of exposure to bacteria showing cross-reactivity with nervous tissue…., as possible vectors (IFST, 2004).

The explanation rests on the unsatisfactory 'packet theory' of infection whereby single high titre doses were unevenly dispersed in the feed; **but this has never been confirmed** and is at odds with the known high infectivity of PrP^{Sc} affected tissues. Once again, and referred to by Lord Phillips, **other environmental factors may play an aetiological role.**

Despite extensive BSE research there is **much that is unanswered or mainly speculative** and it is time for a re-evaluation of the collated information, together with more recent investigations in this study- which have an important bearing on the pathogenesis on this unique class of diseases. So, really; other environmental factors may play an aetiological role**, for example „BSE ammonia- magnesium theory" can be the origin of the BSE epidemic in the United Kingdom.**