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Neuropeptide changes and neuroactive amino acids in CSF from humans and sheep with neuronal ceroid lipofuscinoses (NCLs, Batten disease)

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Abstract

Anomalies in neuropeptides and neuroactive amino acids have been postulated to play a role in neurodegeneration in a variety of diseases including the inherited neuronal ceroid lipofuscinoses (NCLs, Batten disease). These are often indicated by concentration changes in cerebrospinal fluid (CSF). Here we compare CSF neuropeptide concentrations in patients with the classical juvenile CLN3 form of NCL and the classical late infantile CLN2 form with neuropeptide and neuroactive amino acid concentrations in CSF from in sheep with the late infantile variant CLN6 form.

A marked disease related increase in CSF concentrations of neuron specific enolase and tau protein was noted in the juvenile CLN3 patients but this was not observed in an advanced CLN2 patient nor CLN6 affected sheep. No changes were noted in S-100b, GFAP or MBP in patients or of S-100b, GFAP or IGF-1 in affected sheep. There were no disease related changes in CSF concentrations of the neuroactive amino acids, aspartate, glutamate, serine, glutamine, glycine, taurine and GABA in these sheep.

The changes observed in the CLN3 patients may be progressive markers of neurodegeneration, or of underlying metabolic changes perhaps associated with CLN3 specific changes in neuroactive amino acids, as have been postulated. The lack of changes in the CLN2 and CLN6 subjects indicate that these changes are not shared by the CLN2 or CLN6 forms and changes in CSF concentrations of these compounds are unreliable as biomarkers of neurodegeneration in the NCLs in general.

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The neuronal ceroid lipofuscinoses (NCLs, Batten disease), are a group of genetically distinct human inherited neurodegenerative diseases, each with a number of disease causing mutations (see www.ucl.ac.uk/ncl). Common clinical features include blindness and seizures culminating in premature death (Goebel et al., 1999). Characteristic pathologies and ultrastructure of each form of these lysosomal storage diseases have been well described, along with the genetics and biochemical characteristics (Goebel et al., 1999; Haltia 2003, 2006; Jalanko and Braulke 2009). Genetic advances leading to better diagnosis have lead to a much greater appreciation of the incidence of these diseases, including a rapid rise in the number of CLN6 cases diagnosed (e.g. Cannelli et al., 2009).

Four NCL associated genes, *CLN1, CLN2, CLN5* and *CTSD (CLN10)* code for soluble lysosomal proteins (Vesa et al., 1995; Sleat et al., 1997; Siintola et al., 2006; Sleat et al., 2005) while others encode putative membrane proteins of unknown function. *CLN3* probably encodes a lysosomal or endosomal membrane protein (Ezaki et al., 2003; Kyttälä et al., 2004; Fossale et al. 2004), *CLN8* an endoplasmic reticulum-Golgi protein (Lonka et al., 2004), *CLN6* an endoplasmic reticulum resident protein (Gao et al., 2002; Heine et al., 2004; Mole et al., 2004) and *CLN7* a putative membrane protein (Siintola et al., 2007).

Profound neurodegeneration associated with severe brain atrophy is a common feature of the NCLs as is the accumulation of specific proteins in fluorescent lysosomally derived organelles. Subunit c of mitochondrial ATP synthase is stored in most forms, and the sphingolipid activator proteins, SAPs A and D in the infantile and congenital forms (Goebel et al., 1999; Haltia 2003, 2006). NCLs also occur in animals. Each major form is represented by a murine analog (Cooper et al., 2006), and large animal models are a valuable complement. Most studied is the naturally occurring CLN6 form in New Zealand South Hampshire sheep, caused by low CLN6 mRNA concentrations (Tammen et al., 2006). These sheep are maintained under standard pastoral conditions free of any neuroactive drugs and have a large human-like complex CNS, useful for neuropathological studies and the assessment of potential therapies. Studies of affected sheep indicated a close association between glial activation and subsequent neurodegeneration but not with storage body accumulation (Oswald et al., 2005; Kay et al., 2006). Glial activation has also been noted in murine models (Mitchison et al., 2004; Cooper et al., 2006; Kielar et al., 2007; Partanen et al., 2008) and in other lysosomal storage diseases. Studies of interneuron changes in the affected sheep showed that these followed the pattern of glial activation, and that location and connectivity, not phenotype, determines GABAergic interneuron survival (Oswald et al., 2008).

Neuropeptides have been suggested to play a role in NCLs. Insulin-like growth factor-I (IGF-1) was reported to restore neurite outgrowth in neuron cultures from English setter dogs with a CLN8 form (Dunn et al., 1994; Katz et al., 2005), and GABAergic neurons at risk in murine CLN8 *(mnd)in vivo* (Cooper et al., 1999). Low cerebrospinal fluid (CSF) IGF-1 concentrations have been associated with neuropathology in patients with the infantile CLN1 form (Riikonen et al., 2000). The product of *CLN2*, tripeptidyl peptidase I, (Vines and Warburton, 1999) removes Gly-Pro-X triplets from the N-termini of peptides (McDonald et al., 1985) and a crucial role in the processing of other neuropeptides has been suggested (Kopan et al., 2004; Warburton and Bernardini, 2002).

A number of theories for the molecular mechanism(s) of neurodegeneration involve neuroactive amino acids. These include a perturbation in carnitine and trimethyllysine, based on the trimethyllysine content of the stored subunit c (Katz et al., 1997) but subsequently discounted because normal vertebrate subunit c is trimethylated (Buzy et al., 1996; Chen et al., 2004). A defect in arginine transport has been suggested in the CLN3 form (Ramirez-Montealegre and Pearce, 2005), as well as disturbances of glutamate, glutamine and γ-aminobutyric acid (GABA) following autoimmune blocking of glutamic acid decarboxylase (Chattopadhyay et al., 2002; Pearce et al., 2003; Pears et al., 2005). Qualitative changes in brain glutamate, N-acetyl-aspartate, creatine and glutamine concentrations were noted in a bioinformatics analysis of a murine *CLN8* model (Griffin et al., 2002). Other suggestions include a primary role for changes in catecholamine metabolism and dopamine concentrations (Weimer et al., 2007), and in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor activity in CLN3 (Kovács et al., 2006; Kovács and Pearce, 2008). A qualitative metabolomic study of CLN6 affected South Hampshire sheep suggested an increase in glutamine and decreases in glutamate, N-acetyl aspartate and GABA in regions of the brain (Pears et al., 2007). However these changes followed the course of glial activation and neurodegeneration.

Higher concentrations of neuroactive amino acids reported in other neuropathies include increased glutamate concentrations in CSF in amyotrophic lateral sclerosis (Spreux-Varoquaux et al., 2002) and HIV (Ferrarese et al., 2001). Increases of neuropeptides reported include neuron specific enolase (NSE), glial fibrillary acidic protein (GFAP), S-100b and myelin basic protein (MBP) with brain damage in hydrocephalus (Beems et al., 2003), of GFAP and S100b in multiple sclerosis (Petzold et al., 2002), and GFAP in progressive encephalopathies in children (Ehlers et al., 1994).

In addition to implications for theories of pathogenesis, any measurable disturbances in amino acids or neuropeptides in CSF would provide a valuable biomarker for diagnosis and monitoring the effectiveness of attempted therapies (Verbeek et al., 2003). This study describes the measurement of indicted neuropeptides in patients with late infantile CLN2 and juvenile CLN3 forms as well as in sheep with the CLN6 form and neuroactive amino acids in those sheep.

Methods

Patients

Informed parental consent was obtained for the lumbar punctures from the patients described below.

Patient 1, the oldest son of healthy non-consanguineous parents suffered from loss of visual acuity from the age of two, mild learning difficulties and an epileptic seizure in the year before referral to the Department of Pediatric Neurology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, when aged 8. Juvenile NCL (Batten-Spielmeyer-Vogt disease), suspected on clinical grounds, was confirmed by homozygosity for the common 1 kb deletion mutation in *CLN3*. During follow-up the disease showed a progressive course with blindness, further cognitive and motor decline, and seizures.

Patient 2, younger brother of patient 1, was diagnosed with the juvenile form by detection of the 1 kb deletion at the age of 4 years. He seemed completely healthy at that time, and his visual acuity was normal. CSF was collected when he was 5.3 years old.

Patient 3, a 6-year-old asymptomatic boy, was also diagnosed by homozygosity for the 1 kb deletion mutation in *CLN3* because of an older affected sibling. Shortly thereafter symptoms became apparent. CSF was collected when he was 6.5 years old.

Patient 4, who had a severe, progressive neurodegenerative disorder from the age of 2.5 years, was referred to the Department of Pediatric Neurology, Radboud University Nijmegen Medical Centre at the age of 6 years. Based on the clinical features and characteristic histological abnormalities of a muscle biopsy, the CLN2 late infantile form, (Jansky-Bielschowsky disease) was diagnosed and confirmed by demonstrating tripeptidyl-peptidase I deficiency in leukocytes and cultured skin fibroblasts caused by a homozygous mutation (c.509-1G>C) in *CLN2*. CSF was obtained when he was 6.6 years old.

Reference samples

Reference values were determined in CSF obtained by lumbar puncture from patients at the Department of Pediatric Neurology aged below 15 years who had normal CSF cell counts, haemoglobin, bilirubin, total protein, lactate, glucose and no oligoclonal IgG bands.

Sheep

Sheep were maintained under standard New Zealand pasture conditions on a University research farm and all animal procedures carried out according to NIH guidelines and the New Zealand Animal Welfare Act, 1999. The CLN6 affected South Hampshire flock was maintained by crossing affected rams with heterozygote ewes, and affected lambs identified using a discriminatory c.822G>A polymorphism in the *CLN6* gene (Tammen et al., 2006). Homozygously affected sheep behaved normally and showed no clinical signs of disease until progressive vision impairment commenced at around 1 year of age. At 18-24 months of age affected sheep were completely blind but otherwise in good health. Sheep heterozygous for the gene defect, genetically very similar to affected sheep, but free of NCL symptoms, were used as controls.

Cerebrospinal fluid (CSF) collection

CSF was taken from the cistern magnum of sheep anaesthetised with intravenous ketamine/valium after overnight fasting; cellular material removed by centrifugation at 10,000 rpm, 1 min, and samples free of blood contamination snap frozen within 3 min of collection and stored at −130°C.

Neuropeptide assays

Analysis of neuron specific enolase (NSE) and S-100b was performed using a quantitative luminometric immunoassay on a Liaison analyser (DiaSorin, MP Products, Amersfoort, The Netherlands). The detection limit of the NSE assay was 0.04 μg/l; the assay was linear to 200 μg/l and the inter-assay coefficient of variation was 5.3 % while the S-100b assay was linear to 30 μg/l, the detection limit was 0.02 μg/l and the inter-assay coefficient of variation was 11% (Beems et al., 2003; Abdo et al., 2004).

GFAP was determined using an enzyme-linked immunoassay (ELISA) (van Geel et al., 2002). CSF myelin basic protein (MBP) concentrations were analyzed by ELISA (DSL, Webster, Texas), which was linear up to 10 μg/l with an inter-assay variation coefficient of <10%. CSF tau was analyzed by using the Innotest hTau assay (Innogenetics, Gent, Belgium that was linear to 1,200 pg/l with an inter-assay variation coefficient of <6.0%.

IGF-1 was quantified using a non-extraction ELISA (Diagnostic Systems Laboratories, TX, USA) verified as suitable for sheep by comparison with an extraction radioimmune assay (GroPep IGF-1 RIA, Adelaide, Australia). CSF was pre-treated to dissociate IGF-1 from

binding proteins and 20μl aliquots assayed in duplicate in a sandwich-type ELISA. Optical densities were quantified in a Metertech plate reader and Tablecurve 2D software used to construct the 0 to 600μg/l standard curve which spanned the detection limit, 4μg/l. All samples were assayed in a single assay.

Amino acid analysis

Free amino acid concentrations in CSF were measured by high performance liquid chromatography (HPLC) on an Agilent 1100 series HPLC after pre-column derivatisation using o-phthaldialdehyde (OPA) and 3-mercaptopropionic acid (Sigma, St.Louis, MO, USA) (Heems et al., 1998). A C18 Kingsorb (Phenomenex, Ca, USA) 150 × 4.60mm 3μcolumn was used with a solvent gradient of 0.01M Na_2HPO_4 with 0.8% tetrahydrofuran, pH 7.5, (solvent A) and acetonitrile, 40%, methanol, 40% and solvent A, 20% (solvent B) and fluorescent detection at λ_{Ex} 335 and λ_{Em} 440 nm.

Standards (1mM) for aspartate, glutamate, serine, glutamine, glycine and taurine were made up in 0.1N HCl. Standard curves for aspartate, serine, glutamate and taurine made up in pooled CSF were constructed over the range of 0 to 100 μM, glycine from 0 to 200 μM and glutamine from 0 to 1000 μM. The pooled CSF was run as a blank and automatically subtracted during sample analysis. Retention times for aspartate, glutamate, serine, glutamine, glycine, taurine and GABA were approximately 3.2, 4.9, 6.6, 7.2, 8.2, 10.3, and 11.0 minutes respectively. The inter- and intra-assay coefficients of variation for aspartate, glutamate, serine, glutamine, glycine, taurine and GABA were 16.6, 9.1, 10.0, 13.9, 19.2, 27.3, 22.0% and 17.6, 9.8, 5.7, 5.5, 10.2, 6.3, 6.9% respectively. The detection limits for aspartate, glutamate, serine, glutamine, glycine, taurine and GABA were 1.8, 0.2, 12.7, 159, 3.5, 0.4 and 0.2 μM respectively.

Results

Brain specific proteins in CSF

There was a sharp and progressive increase in the CSF concentrations of NSE in the juvenile CLN3 patients, the concentration in the 8 year old affected child being 25.4 μg/l, over fourfold that of his younger brother and well in excess of the reference range, as was the NSE concentration in the intermediately aged case (Table 1). There was also a marked increase in the tau concentrations in the CLN3 symptomatic cases, to over two-fold the top of the reference range. In contrast GFAP and MBP concentrations showed no disease related changes and remained in the reference range, while the S-100b concentration in the more advanced case approached the top of the reference range.

This sharp disease related increase in NSE concentration was restricted to the CLN3 form. No such increase was observed in CSF from the advanced late infantile CLN2 patient (Table 1) nor the CLN6 affected sheep (Table 2). S-100b concentrations were not elevated in CSF from the CLN2 patient nor affected sheep. Sheep CSF NSE concentrations declined with age, from high concentrations in the 5-10 month old affected and control animals. GFAP concentrations in affected sheep CSF were similar to controls and became too low for the sensitivity of the assay as the sheep grew older (Table 2). Occasional extreme values were recorded. One young affected sheep yielded an S100 concentration five times the control value while another yielded an NSE concentration seven times the norm. These high values were specific in that all the other brain specific protein and amino acid CSF concentrations in these sheep were normal. They were omitted from the calculations of the means in Table 2. Including them did not result in means statistically different from normal.

Sheep CSF IGF-1 concentrations were measured in a separate experiment in 14 month old affected animals, already showing impaired vision. The mean concentration ± sem of nine affected sheep, 12.4 ± 3.6 μg/l was not different from that of 38 controls, 11.1 ± 1.7 μg/l.

Amino acid concentrations in sheep CSF

No significant differences were found in the mean concentrations of the amino acids measured in CSF from affected and control sheep of similar ages, even at advanced disease (Table 3). Glutamine concentrations were highest, in the range of 630 -1010μM, serine next highest, around 65 μM, glycine around 20 μM, aspartate, glutamate and taurine concentrations were mainly below 5μM and GABA concentrations were the lowest, 2μM. Whereas the concentrations of the other five amino acids were unaffected by age, GABA concentrations in both the affected and control 18-32 month old sheep were only a third of the concentrations in the 5-10 month old sheep. Two normal adult sheep showed unexpectedly high values for a single amino acid, one a high glutamate concentration and another high glycine concentration, 6x and 9x the means of the remaining concentrations. These extreme values were omitted from the calculation of the groups means (Table 3). Including them did not result in means statistically different from the mean affected values.

Discussion

The only disease related changes noted in this investigation were increases in the CSF concentrations of the brain specific proteins NSE and tau restricted to the CLN3 symptomatic cases, particularly the advanced case (Table 1). This is in spite of the extensive neurodegeneration in all the forms of NCL studied, indicating that CSF concentrations of these brain specific peptides or neuroactive amino acids are not useful as general biomarkers of these diseases.

There is widespread loss of neurons from CLN2 affected brains and from CLN6 affected sheep brains (Oswald et al., 2005). NSE is a marker for sheep neurons (Chiocchetti et al., 2004) and has been quantified in sheep serum (Fujii et al., 2004), so it is surprising that NSE concentrations were not elevated in CSF from these cases (Tables 1 and 2). It could be that the neuronal component proteins are rapidly cleared from the parenchyma in the lymph and never appear in the CSF or that the rate of CSF turnover is such that concentrations fail to rise above normal.

Widespread astrocytosis is a defining feature of all forms of NCL, being most extreme in the infantile form (CLN1). In CLN6 affected South Hampshire sheep astrocyte activation indicated by increased production of GFAP commences during late foetal life (Kay et al., 2006) and progresses with advancing disease (Oswald et al., 2005). It is not known whether the lack of GFAP in the CSF in the patients or sheep results from minimal GFAP escape into the CSF or its prompt removal. Similar observations in other neurodegenerative diseases (e.g. Petzold et al., 2002) support the conclusion that CSF GFAP has little diagnostic value (Verbeek et al., 2003). There was no disease association of CSF concentrations with the other astrocyte marker, S-100b. Of note were the high concentrations of S-100b seen in both control and affected sheep in the 5-10 month age bracket (Table 2).

No disease related changes were seen in any of the other potential markers or modulators of neurodegeneration. CSF concentrations of MBP, that has been associated with brain damage including hydrocephalus (Beems et al., 2003), remained normal in the CLN3 affected patients, as did concentrations of IGF-1 in affected sheep CSF. IGF-1 has been associated with neuronal survival (Broughton et al., 2007; Ozdinler and Macklis, 2006; Beck et al., 1995), concentrations in CSF from CLN1 patients were lower than normal (Riikonen et al., 2000), and IGF-1 was beneficial to canine CLN8 neuron cultures (Dunn et al., 1994; Katz et al., 2005), and

GABAergic neurons at risk in murine CLN8 *(mnd)in vivo* (Cooper et al., 1999). It was proposed that a lowering of IGF-1 bathing the brains may be involved in neurodegeneration in all the NCLs but this is not the case.

No significant changes were noted in the amino-acid concentrations in sheep CSF, either with disease or age (Table 3) and these were within the range of control values reported in humans (Corston et al., 1979;Gårseth et al., 2001;Peng et al., 2005;Rothrock et al., 1995). This contrasts with changes in CSF amino acid concentrations noted in other diseases including increased glutamate concentrations in ALS (Spreux-Varoquaux et al., 2002) and HIV dementia (Ferrarese et al., 2001), and significant changes in neuroactive compounds such as glutamate, taurine and glycine in patients with polyradiculoneuropathy (Gårseth et al., 2001).

Aspartate and glutamate are both excitatory neurotransmitters whereas GABA, glycine and taurine are inhibitory. Taurine also has a protective role in the brain (Saransaari and Oja, 2000) while glutamine serves as an energy source and is a precursor of glutamate and GABA. This indicates that postulated disturbances of glutamate, glutamine and GABA concentrations in CLN3 (Chattopadhyay et al., 2002, Pearce et al., 2003, Pears et al., 2005) or in murine *CLN8* (Griffin et al., 2002) are not part of a common neurodegenerative mechanism in the NCLs. It also confirms that relative increases in glutamine and decreases in glutamate, N-acetyl aspartate and GABA concentrations, seen in specific regions of the CLN6 affected sheep brain with advancing disease (Pears et al., 2007), are not reflected in concentrations of these amino acids in the CSF.

Some of the lack of response of various biomarkers of neurodegeneration in the CSF may be a result of molecular mechanisms present in the brain to ensure homeostasis. Recently it has been shown that activation of astrocytes, a response clearly seen in Batten disease (Oswald et al., 2005) leads to increased glutamate uptake (Vermeiren et al., 2005; Escartin et al., 2006) presumably to counter excitotoxic conditions, but apparently does not lead to an increase in CSF S-100b or GFAP. Furthermore, there is a mechanism in the choroid plexus to remove β-amyloid from the CSF (Crossgrove et al., 2005). It is possible that similar mechanisms may exist for other proteins such as NSE, S-100b and GFAP.

None of these caveats affect the conclusion that CSF concentrations of none of the potential biomarkers assayed, the neuropeptides NSE, tau, S-100b, GFAP, MBP or IGF-1, and the amino acids aspartate, glutamate, serine, glutamine, glycine or taurine, can be relied upon as general markers of neurodegeneration in different forms of Batten disease. There was no obvious reason for the isolated high CSF concentrations of S100, NSE, glutamate and glycine observed in particular animals (Tables 2 and 3). Presumably these result from unmonitored metabolic changes in these animals that are not obvious and are a cause for conservative use of these molecules as disease markers.

The results also suggest that cases of CLN3 are exceptions, changes in CSF NSE and tau concentrations reported here being mirrored by amino acid changes reported elsewhere and summarised in the introduction (Chattopadhyay et al., 2002; Pears et al., 2005; Ramirez-Montealegre and Pearce, 2005Kovács et al., 2006; Weimer et al., 2007; Kovács and Pearce, 2008). However it is unlikely that all these amino acid disturbances occur simultaneously. They may be restricted to certain disease causing mutations in *CLN3* rather than associated with all disease causing mutations in this gene. Also to be determined is if they are progressive markers of obvious neurodegeneration as suggested by the data here, in line with regional brain amino acid concentration changes in ovine CLN6, or are the consequence of underlying metabolic changes that cause the neurodegeneration.

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Table 1

Brain specific protein concentrations in the cerebrospinal fluid from patients with the CLN2 and CLN3 forms of Batten disease

BSP	Patient, disease, status and age (yrs) at lumbar puncture				Reference ranges [d]
	2 [a] CLN3, early symptomatic, 5.3	3 CLN3, symptomatic, 6.5	1 [a] CLN3, advanced symptomatic, 8	4 CLN2, end stage advanced, 6.6	
NSE (μg/l) [b]	5.9	14.6	25.4	3.0	<12.0 (99)
Tau (ng/l) [c]	225	639	580	-	<300 (66)
S-100b (μg/l) [b]	1.0	0.6	2.4	0.7	<2.5 (73)
GFAP (μg/l) [b]	0.5	0.4	1.0	1.5	<1.7 (67)
MBP (μg/l) [b]	-	0.2	0.2	0.7	<0.7 (134)

[a] Siblings.

[b] Immunoreactivity equivalent to human protein in μg/l.

[c] Immunoreactivity equivalent to human protein in ng/l.

[d] Number of samples determined in brackets.

Table 2

Brain specific protein concentrations in the cerebrospinal fluid from CLN6 affected sheep at different stages of the disease and age-matched controls (ng/l± sem)

	Disease stage			
	Aggressive neurodegeneration (5–10 months of age)		Advanced terminal disease (18 – 32 months of age)	
Marker	affected (n=6) [a]	control (n=12) [a]	affected (n=5) [a]	control (n=4) [a]
NSE [b]	1.1(0.2)[c]	0.6(0.1)	0.8(0.2)	0.2(0.1)
S100 [b]	46.2(13.0)[c]	34.3(4.6)	7.1(0.9)	4.0(0.7)
GFAP [b]	0.85(0.3)	0.7(0.1)	undetectable	undetectable

[a] n= the number of animals sampled.

[b] Immunoreactivity equivalent to human protein in μg/l (± sem).

[c] One affected animal sample had NSE and S-100b concentrations of 7.16ng/l and 236ng/l respectively and was excluded as an anomaly. Including it in the data did not result in a significant difference between the affected and control means.

Table 3

Amino acid concentrations in the cerebrospinal fluid from CLN6 affected sheep at different stages of the disease and age-matched controls (μM ± sem)

	Disease stage			
	Aggressive neurodegeneration (5 – 10 months of age)		Advanced terminal disease (18 - 32 months of age)	
Amino acid	affected (n=10)[a]	control (n=20)[a]	affected (n=5)[a]	control (n=4)[a]
aspartate	2.5(0.2) [b]	2.4(0.2)	2.5(0.3)	3.2(0.4)
glutamate	2.6(0.4)	2.3(0.2)	3.8(0.4)	2.5(0.25) [c]
serine	68.2(6.2)	65.6(2.5)	60.6(6.7)	67.5(7.0)
glutamine	748.8(61.6)[c]	635.6(19.1)	1008.3(139.5)	847.3(46.9)
glycine	24.1(3.1)[c]	23.4(2.4)	19.9(1.4)	21.8(2.6) [c]
taurine	3.4(1.0)	2.5(0.3)	3.0(0.2)	6.1(2.8)
GABA	0.96(0.09)	0.99(0.05)	0.31(0.03)	0.24(0.04)

[a] n= the number of animals sampled.

[b] μM ± sem.

[c] One control animal sample which had glutamate and glycine concentrations of 13.6μM and 199μM respectively was excluded as an anomaly. Including it in the data did not result in a significant difference between the affected and control means.