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Slow wave sleep is accompanied by release of certain amino acids in the thalamus of cats

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To determine whether EEG synchronization in sleep has a metabolic equivalent, we investigated state-dependent changes in extracellular concentrations of amino acids. *In vivo* microdialysis studies were performed in the ventroposterolateral (VPL) nuclei of the thalamus of cats during natural slow wave sleep (SWS), waking (W) and carbachol-induced paradoxical sleep (PS) like episodes. About two-fold increases in aspartate, glutamate, asparagine, glycine, alanine and γ-aminobutyric acid (GABA) were observed in SWS compared with control samples collected in W, but serine increased to 487 ± 211%. In the PS-like state, glutamine increased and GABA decreased. These results suggest changes in intracellular processes reflected by amino acid release in the thalamus, specific to slow wave generation in EEG during natural sleep.

Key words: Amino acids; Microdialysis; Release; Thalamus; Slow-wave-sleep

Introduction

The idea that physiological sleep has a neurochemical equivalent has been raised by several authors. The sleep-regulatory effects of uridine and glutathione suggest involvement of intracellular detoxification processes and glial nursing of neurones in sleep controlling mechanisms.[1,2]. We assume that sleep is an altered biochemical state of the brain whose function is energy restoration or intracellular reparation. However, changes in glucose uptake were found to reflect the average firing rate of neurones during sleep,[3] suggesting no sleep-specific state in energy production of cells.

EEG slow waves in sleep are generated by rhythmic calcium spike genesis in the thalamus[4] that not only elevates intracellular free Ca^{2+} but could also modify membrane potential-dependent transporters.[5,6] Transport of glutamate and some other amino acids (alanine and glutamine) plays an important role in metabolic coupling between glial cells and neurones.[7] Thus the extracellular concentrations of amino acids could reflect state-dependent neurochemical changes in the brain during EEG synchronization. Because of the relatively poor time resolution of the dialysis technique, we used the carbachol-induced paradoxical sleep (PS) model.[8]

Materials and Methods

Animals were treated in accordance with local ethical rules of the institute which are concord with the Guidelines on the Use of Living Animals in Scientific Investigations 1984. Cats ($n = 6$, weight 3–4 kg) were anaesthetized with Nembutal® (40 mg kg^{-1}) and placed in a stereotaxic frame. Dental screw electrodes (AB Dentatus) were implanted into the skull above the frontal cavity to record eye movements. Similar screws were placed above the frontal, parietal and occipital cortices for EEG recording. Two multistrand stainless steel wires were placed into the neck muscles for recording electromyograms indicating muscular atonia in PS. A stainless steel guide cannula was implanted at 5 mm above the coordinates of dorsal cell group of tegmental gigantocellular field (FTG) (P: 4 mm, L: 1.2 mm, V: –8.5 mm). Following insertion of a 1 μl Hamilton syringe into the guide cannula, carbachol was microinjected at the location from where it induced a PS-like state.[8]

Microdialysis probes were made as described earlier.[9] Briefly, a hollow fibre (Travenol™, cut off: 5000 D, o.d. 0.2 mm, length 3 mm) was adjusted into a 27-gauge stainless steel tubing. Glass capillaries pulled from Jancons glass tubing were used for inlet and outlet of the probe. The glass capillaries were guided by stainless steel tubes which were connected to plastic tube connectors. This design of probes allowed application of optimally shaped microdialysis devices and disconnection of the animals after experimental sessions. The probes were placed into the ventroposterolateral thalamic nuclei (VPL) bilaterally (A: 10 mm, L: 7.5 mm, V: 0–3 mm, according to the atlas of Berman). The final position of the probes was reached within 20 min to reduce tissue damage. Artificial cerebrospinal fluid (ACSF), containing 140 mEq Na^+, 3 mEq K^+, 1.2 mEq Ca^{2+},

2 mEq Mg^{2+} and 144 mEq Cl^- was applied for perfusion. ACSF solution was made of amino acid-free, non-pyrogenic water and a sample of the ACSF was tested by high performance liquid chromatography (HPLC) in each experiment. To avoid contamination of the samples, disposable syringes were used for perfusion. The perfusion rate was 1 μl min^{-1}. Internal volumes of probes and the outleading tubes were calibrated by an electrochemical technique as described earlier[9] for synchronization of sample collection with sleep stages (Fig. 1).

Cats were trained to stay and sleep in their narrow recording cage (18 × 22 × 50 cm) for 2 weeks before implantation. Following implantation, sample collection was started after 1 day recovery to ensure the disappearance of anaesthetic from the cat. Feeding was accomplished 1 h before the recording session. After starting perfusion, 45 min was allowed for establishment of a steady flow in the dialysis system. At least two samples were collected in wakefulness (W) before sleep. At the end, one more sample was collected in W. The average concentrations of amino acids in these samples were used as baseline. The changes in concentrations in slow wave sleep (SWS) were expressed in the percentage of this baseline. One day later, 9 μg of carbachol in 0.5 μl ACSF was injected slowly (over 8–10 s) into the FTG after collecting baseline samples in W and some samples in SWS. Anatomical location of the microinjection was verified on Nissl-stained sections at the end of the experiment. PS-like stage samples were collected when muscular atonia and rapid eye movements (REM) dominated the polygraphic record. The amino acid concentrations of all previously collected samples from W and that following carbachol were compared because of the relatively low dispersion of data collected in desychronized EEG. We applied analysis of variance (ANOVA) for statistical analysis; data are reported as means ± s.d.

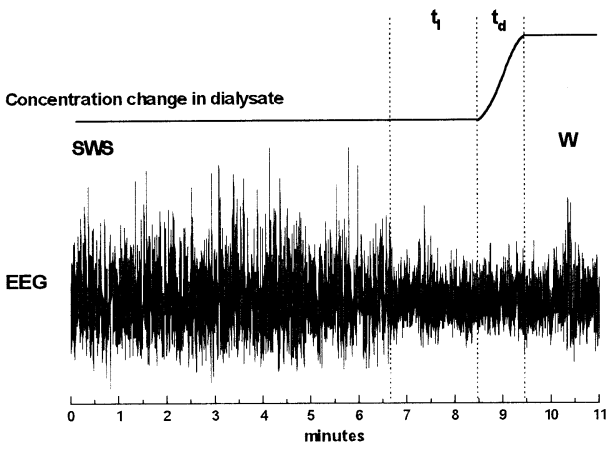

FIG. 1. The relationship between concentration changes in a probe *in vitro* and changes in EEG during transition from slow wave sleep (SWS) to waking (W). Concentration change was generated at time 0; t_l is the latency of the concentration ramp appearing at the end of the outleading tube; t_d is the duration of the concentration ramp induced by diffusion in the outlet tube at 1 μl min^{-1} flow rate. Comparison of the electrochemical calibration of the probe to the EEG allowed a real estimation of time resolution of microdialysis.

Detection of low concentrations of amino acids from dialysis samples can be achieved by precolumn derivatization of primary amino acids with ortophthalaldehyde (OPA). The derivatization reaction was performed at pH 10.5 in the presence of mercaptoethanole. OPA-derivatized amino acids were detected with 305–395 nm excitation and 430–470 nm emission filters of a fluorescent detector. Because of the instability of OPA derivatives, the HPLC technique was automatized on a Pharmacia AminoSys Chromatograph System specialized for amino acid analysis. Detection limits for amino acids were 0.5–5 pmol in 10 μl.

Amino acids were separated by Chrompack MicroSphere reversed phase columns (100 × 4.6 mm), filled with 3 μm, C18 spherical packing material. Eluent A was 0.1 M phosphate buffer, pH 7.2; eluent B contained 70% acetonitrile in eluent A and adjusted to pH 7.2 with phosphoric acid. The gradient profile was: 0% B at 0 min, 15% B at 10 min, 25% B at 25 min, 50% B at 35 min. The column was equilibrated for 10 min in 100% B and for 10 min in 100% A. The separation of glutamate, aspartate, asparagine, serine, glutamine, threonine, glycine, histidine, alanine, taurine, γ-aminobutyric acid (GABA), arginine, tryptophan, methionine, leucine, isoleucine and lysine was achieved. Chromatograms were evaluated by PE Nelson 2000 software. External standards of 10 μM amino acids were injected after every ten samples. Measuring GABA in this full-scale amino acid analysis is very difficult because it is difficult to separate GABA from tau and the low concentration of GABA easily makes its measurement unreliable in less sophisticated HPLC systems than we used. After analysing 500 samples on a column, detection of GABA became impossible so the column had to be changed. All of our amino acid chromatograms contained a clearly distinguished GABA peak using freshly installed columns.

Results

Cats had almost undisturbed sleep–wake cycles in the recording cage. The distribution of sleep stages was 17.2 ± 3.7% W, 12.3 ± 2.0% PS and 70.4 ± 11.2% SWS ($n = 6$). Changing sample containers induced only few seconds of desynchronization in SWS. The average duration of SWS episodes was 16.8 ± 5.7 min, which allowed collection of 10 μl samples in SWS. Cats were kept in W before and after sleep by the experimenter applying clicks to wake up the cat when

slow waves appeared in the EEG. Injection of 9 μg carbachol in 0.5 μl volume into the FTG induced muscular atonia and REM after a 28 ± 3.8 min ($n = 6$) latency. It was interrupted by some movements showing awakenings. Animals were able to wake up but they preferred to stay in a sleeping position on the floor. Microinjection of carbachol initially evoked contralateral head movements, followed by a PS-like stage which lasted for 3–4 h, allowing enough time for sample collection.

Extracellular concentrations of amino acids in samples collected from VPL were very consistent and there was little dispersion of data in W. The molar concentrations of amino acids in the thalamic perfusate were 1.7 ± 0.03 μM aspartate, 6.6 ± 0.3 μM glutamate, 1.5 ± 2.0 μM asparagine, 10.3 ± 1.5 μM serine, 61.3 ± 3.4 μM glutamine, 8.7 ± 0.6 μM glycine, 4.2 ± 0.7 μM threonine, 3.9 ± 0.4 μM alanine, 17.8 ± 0.7 μM taurine, 0.7 ± 0.2 μM GABA ($n = 22$). In SWS, amino acid content of dialysates increased significantly with the exception of glutamine (Fig. 2, top). There was no increase in methionine, leucine or tryptophan. The most robust elevation was found in serine (487 ± 211%). It is also interesting that the increases in aspartate and glutamate were nearly twofold, but the s.d. of these increases were small. The degree of elevation in asparagine, serine, threonine, alanine and taurine were more variable (Fig. 2, top).

Carbachol-induced PS-like state did not change the extracellular availability of amino acids significantly compared with W (Fig. 2, bottom), with the exception of glutamine and GABA. Glutamine increased and GABA decreased, which is opposite to the change found in SWS ($p < 0.005$).

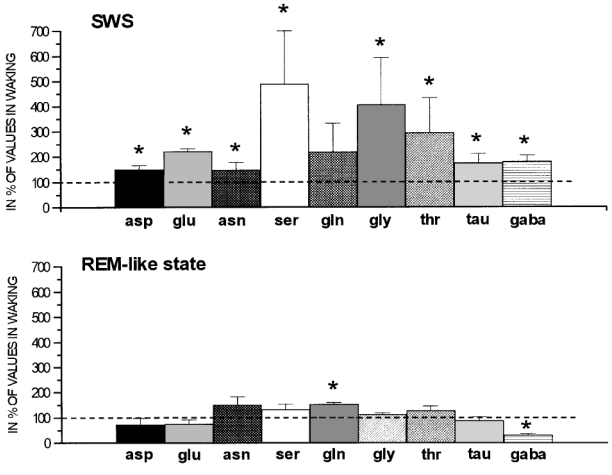

FIG. 2. Increase in amino acid content in the thalamic extracellular space during slow wave sleep (SWS) is shown on the bar graph at the top; the dotted line indicates 100% established on the basis of control values in waking (W). *Significant changes ($p < 0.005$). The bar graph at the bottom presents the changes in amino acids during the carbachol-induced rapid eye movement-like state. The dotted line indicates 100% calculated from the values obtained in waking before injection of carbachol.

Discussion

Elevated extracellular concentrations of amino acids reported here could derive from neurones or glial cells or both. Microdialysis probes implanted in the extracellular compartment are not able to provide direct evidence about the origin of amino acids detected in the perfusate. Uneven distribution of some enzymes of the amino acid metabolic pathways, however, might give us some information about the origin of certain amino acids. Synthesis of glutamine is almost exclusively glial in the nervous system.[10] It is also clear that glutamate uptake is higher in glial cells than in neurones,[11] so that changes in glial glutamate can be a major source of extracellular glutamate. It is also difficult to estimate the contribution of synaptic overflow to the extracellular glutamate and aspartate concentrations. Because of the high estimated capacitance of glutamate transporters,[11] the synaptic release might be only a fraction of the net glutamate efflux determining the extracellular concentration of glutamate. At the present stage of understanding of amino acid metabolism and transport systems in the brain, we assue both glial and neuronal origin of extracellular amino acids.

The question arises of which major cellular processes generate the increase in extracellular amino acid concentrations. There is evidence supporting increased release of amino acids from cells during the compensatory volume decrease reaction[12,13] which usually follows swelling-induced volume increase. Amino acids are then released as osmolytes. In the brain, taurine is the major amino acid which is involved in volume control-related mechanisms.[14] Calcium influx triggers the volume decrease reaction;[15] the sleep-related increase in amino acid efflux could, therefore, be a result of changes in cell volume.

The extracellular ratio of glutamate and glutamine is controlled by a glutamate–glutamine shuttle which is based on high affinity glial uptake of glutamate and its conversion to glutamine. This is then returned to the neurones for synthesis of glutamate[7] because of the exclusively glial localization of glutamine synthase in the CNS.[10] In SWS, we found an increase in the extracellular ratio of glutamate and glutamine, suggesting SWS-related modification of the glutamate–glutamine shuttle. One of its major components could be the modulation of the glutamate transporter[16] by tonic hyperpolarization of thalamic cells in SWS.

Elevated extracellular amino acid concentrations were found in SWS but not in the carbachol-induced REM-like state. Changes in glutamine and GABA after carbachol microinjection was not a non-specific

result of the microinjection because we used a small volume which did not induced any non-specific effects in the original studies of Hobson *et al*.[8] This suggests that increases in amino acid release correlate with generation of slow waves, not with sleep in general. During slow waves, a transient calcium current flows into the cells, causing an extra calcium influx.[17] This calcium signal could activate calcium-dependent dehydrogenases of the citric acid cycle[18–23] which produce pyruvate, citrate and ketoglutarate – the major precursors of amino acid synthesis. If applicable, the metabolic explanation for elevated amino acid release in SWS is that citric acid cycle speeds up due to the calcium-dependent control of dehydrogenases. However, this takes 40–50 min[23] and the calcium dependency of dehydrogenases has not been confirmed in the nervous system.

Conclusion

The present finding suggests that slow vaves trigger amino acid release enhancement in thalamic cells, probably due to increased calcium influx into the cells. The increased release of amino acids during generation of slow waves supports the idea of a biochemical equivalent of sleep which is reflected by amino acid transport and distribution in the intra- and extracellular compartments of the brain tissue.

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General Summary
It is an old idea that sleep has a biochemical equivalent due to the altered metabolism in the brain. Recently, it was disclosed that generation of slow waves in the EEG is causally related to the calcium spike genesis in the thalamus. This enhanced calcium influx could result in changes in cellular metabolism in neurones and astrocytes. We assumed, that as in other tissues, an altered metabolic state of brain cells is reflected by amino acid release to the extracellular space. Using an *in vivo* microdialysis technique, we measured increase of neurotransmitter and non-transmitter amino acids in the thalamus of freely moving cats. Our findings advance the investigation of sleep related biochemical changes in the brain.