Journal of Neuroscience Research 00:00–00 (2014)

Brain Infection With *Staphylococcus aureus* Leads to High Extracellular Levels of Glutamate, Aspartate, γ-Aminobutyric Acid, and Zinc

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Staphylococcal brain infections may cause mental deterioration and epileptic seizures, suggesting interference with normal neurotransmission in the brain. We injected *Staphylococcus aureus* into rat striatum and found an initial 76% reduction in the extracellular level of glutamate as detected by microdialysis at 2 hr after staphylococcal infection. At 8 hr after staphylococcal infection, however, the extracellular level of glutamate had increased 12-fold, and at 20 hr it had increased >30-fold. The extracellular level of aspartate and γ-aminobutyric acid (GABA) also increased greatly. Extracellular Zn^{2+}, which was estimated at ~2.6 μmol/liter in the control situation, was increased by 330% 1–2.5 hr after staphylococcal infection and by 100% at 8 and 20 hr. The increase in extracellular glutamate, aspartate, and GABA appeared to reflect the degree of tissue damage. The area of tissue damage greatly exceeded the area of staphylococcal infiltration, pointing to soluble factors being responsible for cell death. However, the N-methyl-D-aspartate receptor antagonist MK-801 ameliorated neither tissue damage nor the increase in extracellular neuroactive amino acids, suggesting the presence of neurotoxic factors other than glutamate and aspartate. In vitro staphylococci incubated with glutamine and glucose formed glutamate, so bacteria could be an additional source of infection-related glutamate. We conclude that the dramatic increase in the extracellular concentration of neuroactive amino acids and zinc could interfere with neurotransmission in the surrounding brain tissue, contributing to mental deterioration and a predisposition to epileptic seizures, which are often seen in brain abscess patients.

Key words: brain abscess; staphylococci; MK-801; Zn^{2+}; glutamine

Bacterial brain infections that lead to abscess formation may be caused by *Staphylococcus aureus*, especially those that occur after intracranial surgery or trauma (Luby, 1992; Goodkin et al., 2004; Carpenter et al., 2007). Early symptoms of bacterial brain infections include mental deterioration and seizures (Yang and Zhao, 1993; Kilpatrick, 1997; Kao et al., 2003; Roche et al., 2003; Dahlberg et al., 2014), suggesting interference with normal neurotransmission. Glutamate is the major excitatory neurotransmitter in the mammalian brain (Traynelis et al., 2010). In vitro, staphylococci have been shown to metabolize glutamate avidly (Sinha and Chatterjee, 1967; Wu and Bergdoll, 1971). Therefore, staphylococci that enter the brain could affect neurotransmission by using glutamate and other neuroactive amino acids in the extracellular fluid as metabolic substrates. However, brain infection with *S. aureus* causes abscess formation, which entails cell death and loss of brain tissue. Therefore, in cases of *Staphylococcus*-mediated cellular damage, spilling of intracellular neuroactive amino acids into the extracellular fluid could occur.

The purpose of this study was to characterize early changes in the amino acid composition of the extracellular fluid after focal staphylococcal brain infection. We found a biphasic change in the extracellular level of glutamate; an initial reduction was followed by a dramatic and progressive increase in the extracellular glutamate concentration. The extracellular level of Zn^{2+}, an important allosteric inhibitor of glutamate receptors (Mott et al., 2008; Rachline et al., 2008) with neurotoxic potential (Sheline et al., 2000), also increased after injection of staphylococci. We further investigated staphylococcal

Contract grant sponsor: The Norwegian Chapter of the International League Against Epilepsy; Contract grant sponsor: The Norwegian Epilepsy Association

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Received 4 March 2014; Revised 6 May 2014; Accepted 1 June 2014

Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23444

metabolism of amino acids in vitro to shed light on in vivo findings.

MATERIALS AND METHODS

Animals

Male Wistar rats of 300–400 g body weight were from M&B (Ry, Denmark). Animals were caged in groups of five with free access to water and food for 3–4 weeks prior to experiments. The light–dark cycle was 12 hr, the ambient temperature was 20°C, and the air humidity was 50%. A pilot study had shown only small brain lesions 20 hr after injection of 10^5 or 10^6 *S. aureus* in 1 μl (n = 3 in each group). Therefore, 20 rats received 10^7 *S. aureus* intrastriatally, similar to previous studies (Himmelreich et al., 2005). Six rats were euthanized at 2 hr, 10 rats (of which 4 were treated with MK-801) were euthanized at 8 hr, and four rats were euthanized at 20 hr after injection of *S. aureus*. Thirteen rats received phosphate-buffered saline (PBS; NaCl 140 mmol/liter, NaH_2PO_4, 1 mmol/liter, pH 7.4) intrastriatally; nine were euthanized at 2 hr, and four were euthanized at 20 hr. after injection of *S. aureus*. Rats were used partly for microdialysis, partly for histological analysis, or both (see below for group sizes). Animals were acquired and cared for in accordance with the NIH *Guide for the care and use of laboratory animals* (NIH Publication No. 85-23).

S. aureus

S. aureus from The Culture Collection, University of Göteborg (catalog No. CCUG 46923), was cultured overnight (16 hr) on blood agar and suspended in PBS. The concentration was adjusted to 10^7 staphylococci/μl, as determined from culturing suspensions of bacteria on agar and optic density measurements at 600 nm.

Intrastriatal Injection: Microdialysis

For intracerebral injection of *S. aureus*, male Wistar rats were anesthetized with an intraperitoneal injection (per kilogram body weight) of fentanyl citrate 0.1 mg, fluanisone 5 mg, and diazepam 2.5 mg. The skin over the skull was anesthetized with a subcutaneous injection of 50 μl of lidocaine, 10 mg/ml. The animals were mounted in a stereotactic frame, the skull was exposed through a skin incision, and a hole was drilled 3 mm laterally and 1 mm anteriorly to bregma. One microliter of a suspension containing 10^7 *S. aureus* in PBS was injected 5 mm ventrally from dura into the striatum, with an injection rate of 0.3 μl/min. Two minutes after completion of the injection, the cannula was withdrawn slowly over a duration of 15 sec. Control animals received 1 μl of PBS intrastriatally. While animals were under anesthesia, a microdialysis probe (CMA/11; CMA Microdialysis, Solna, Sweden) with membrane lengths of 3 mm was inserted immediately (n = 6), 6 hr (n = 6), or 18 hr (n = 4) after injection of staphylococci. The probe was placed according to the same parameters as for the injection cannula. Control animals received the microdialysis probe either immediately (n = 9) or 18 hr (n = 4) after injection of PBS. Sampling of dialysis fluid began 30 min after insertion of the microdialysis probe, but the reported data are from samples taken 2 hr after insertion of the probe. The microdialysis solution contained (in mmol/liter) NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.4, and NaH_2PO_4 1, pH 7.3. Flow rate was 3 μl/min. Four animals that received 10^7 *S. aureus* intrastriatally were treated with the N-methyl-D-aspartate (NMDA) receptor blocker MK-801, 1 mg/kg intraperitoneally, at the time of injection of staphylococci. The dose was repeated at 3 and 6 hr after injection of staphylococci (Gerriets et al., 2003).

The dialysates were collected in 30-min fractions (90 μl per fraction) in Eppendorf tubes (Hamburg, Germany) containing 10 μl sodium azide (300 mmol/liter) to inhibit bacterial growth and α-aminoadipate (200 μmol/liter) as an amino acid concentration standard. Dialysis was performed for 2 hr. Body temperature was monitored with a rectal thermometer and maintained at 37°C with a heating lamp. Anesthesia was maintained for the duration of the experiment by repeated intraperitoneal injections of fentanyl citrate, fluanisone, and diazepam. After completion of microdialysis, the animals received a lethal injection of pentobarbital intraperitoneally and, when deeply anesthetized, were perfusion-fixed transcardially with 4% phosphate-buffered formaldehyde. Brains were removed and stored in 4% formaldehyde at 4°C before being embedded in paraffin, which was done within 2 days.

Microdialysis probe recovery experiments in vitro used the same probe, microdialysis solution, and flow rate as described above by inserting the microdialysis probe in microdialysis solution supplemented with amino acids at 1, 5, 10, 50, 100, 500, and 1,000 μmol/liter and Zn^{2+} at 1, 3.3, 10, 33, and 100 μmol/liter. Recovery was calculated as concentration in the dialysate in percentage of the dialyzed solutions.

Brain Histology and Immunohistochemistry

Perfusion-fixed brains were embedded in paraffin, and sections were cut coronally at a thickness of 5 μm. Sections were mounted on glass slides, and serial sections were stained with hematoxylin and eosin (H-E) or Gram stain; the latter was done as described by Engbæk et al. (1979). Briefly, sections were passed to water and immersed in crystal violet 1% for 2 min, rinsed in water, immersed in iodine/potassium iodide (1:2) 5% in water for 5 min, and subsequently rinsed in water to remove the iodine. Excess water was removed with filter paper. Sections were differentiated in acetone until stain stopped running and were then rinsed in water before immersion in basic fuchsin 1% for 5 min, followed by rinsing in water. Sections were next immersed in formaldehyde 2% and acetic acid 1% (Gallego's solution) for 5 min before being rinsed in water and dried on filter paper. Sections were differentiated by dipping three times in acetone, then three times in 0.1% picric acid in acetone, and then three times in acetone before xylene treatment and mounting.

For immunohistochemistry, mounted sections were incubated with primary antibodies against staphylococcal α-hemolysin or enterotoxin A (rabbit polyclonal antibodies; Sigma, St Louis, MO), NeuN (mouse monoclonal; Merck Millipore; Darmstadt, Germany), synaptophysin (mouse monoclonal; Dako, Glostrup, Denmark), glial fibrillary acidic protein (GFAP; rat monoclonal; Life Technologies, Carlsbad, CA), activated caspase 3 (rabbit polyclonal; Cell Signaling Technologies, Danvers, MA), or glutamate transporter GLT (rabbit polyclonal;

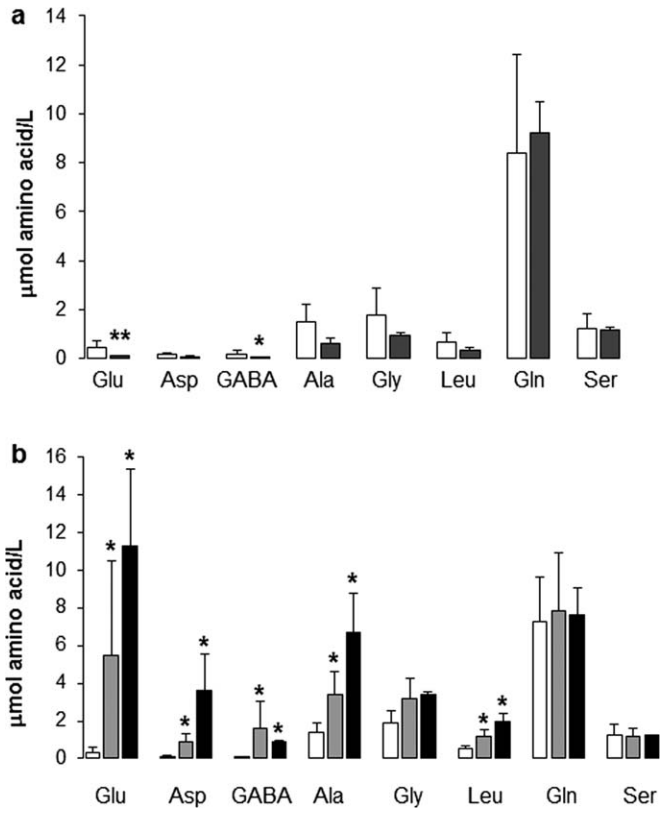

Fig. 1. Levels of amino acids in microdialysate fluid after injection of *S. aureus* into rat striatum. Anesthetized Wistar rats received an intrastriatal injection of 10^7 *S. aureus* in 1 μl PBS. Microdialysis of the striatum was performed with rats under anesthesia. **a:** Levels of amino acids 2 hr after injection of PBS (open bars; n = 6) or staphylococci (gray bars; n = 6). **b:** Levels of amino acids 8 hr (gray bars; n = 6) or 20 hr (black bars; n = 4) after injection of staphylococci; open bars represent amino acid levels 20 hr after injection of PBS (n = 4). Values are not corrected for probe recovery, which for glutamate was 2.7 ± 0.2% in vitro. Data are micromoles amino acid per liter, mean + SD values; $\star P < 0.05$, $\star\star P < 0.01$, Mann-Whitney U test or Kruskal-Wallis one-way ANOVA on ranks.

a generous gift from Dr. N.C. Danbolt, University of Oslo, Norway). Primary antibody binding was detected with secondary peroxidase-conjugated species-specific antibodies (Sigma). Counterstain was hematoxylin. The sections were evaluated by a neuropathologist (E.-A.A. or J.M.). The area of the lesion at the injection site was measured by planimetry as previously described (Hassel et al., 1994).

In Vitro Exposure of *S. aureus* to Amino Acids

To assess the ability of staphylococci to metabolize glutamate and other amino acids, staphylococci (5×10^7) were incubated at 37°C in 500 μl of the Krebs' solution that was used for microdialysis (see above), which was saturated with oxygen and supplemented with glucose, 3 mmol/liter, lactate, 0.75 mmol/liter, and all proteinogenic amino acids plus GABA and taurine at 10 micromol/liter, except for glutamine (400 μmol/liter) and glycine (20 μmol/liter), to mimic the extracellular fluid of the brain (Lada and Kennedy, 1996; Kanamori and Ross, 2004; Eide and Stanisic, 2010). Incubation solutions (500 μl) without bacteria were incubated as described above in parallel with those that contained bacteria. Incubation media were sterile filtered prior to use. In some experiments, glutamate, 10 μmol/liter, GABA, 10 μmol/liter, glutamine, 400 μmol/liter, glucose, 3 mmol/liter, or glutamine + glucose, 0.4 and 3 mmol/liter, respectively, were the only energy substrates present. Incubation media were harvested at 0, 1, or 4 hr after incubation (n = 4 per time point). The bacteria were precipitated by centrifugation at 5,000*g* for 15 min, and the supernatants were analyzed with respect to glucose and lactate or mixed 1:1 with α-aminoadipate (10 μmol/liter) for the analysis of amino acids (or with α-aminoadipate 500 μmol/liter for the analysis of glutamine) by high-performance liquid chromatography (HPLC; see below). Amino acid and glucose concentrations in media without bacteria that had been incubated at 37°C for 0 or 4 hr were the same.

Analysis of Amino Acids, Lactate, and Glucose

Amino acids were analyzed in microdialysates and incubation media by HPLC and fluorescence detection after precolumn derivatization with o-phthaldialdehyde (Dahlberg et al., 2014), using an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA). Quantification was done using α-aminoadipate as an internal concentration standard. Glucose and lactate were measured in incubation media by reflectance spectrophotometry (Kodak DT60 II; Kodak, Rochester, NY).

Zn^{2+} Measurement

Microdialysates were pooled in triplicates (total volume 270 μl, corresponding to 90 min of sampling time 1 hr after insertion of the microdialysis probe) and diluted in 6 ml ultrapure HNO_3 0.5% (vol/vol; Merck, White House Station, NJ; distilled in house). Zn^{2+} was analyzed on an inductively coupled plasma mass spectrometer (Thermo X-series II ICP-MS; ThermoFisher Scientific, Waltham, MA). Zn^{2+} reference solutions (TM 23.4 and TMDA 61.2; Analytical Reference Material, Environment Canada, Gatineau, Quebec, Canada) were analyzed in addition to standards made in house. Blanks (ultrapure HNO_3 0.5%) were repeatedly analyzed between samples to control for background contamination. The detection limit was calculated as 10 times the standard deviation of the blank Zn^{2+} concentration and was 1.5 nmol Zn^{2+}/liter. The correlation between calculated concentration standards (0–10 μmol/liter) and measured values was excellent, with r = 1.0 ($P = 3 \times 10^{-10}$). Values obtained with fresh microdialysis buffer (<10% of sample values) were subtracted from sample values.

Statistical Analysis

Data on metabolite and Zn^{2+} concentrations and areas of tissue damage are presented in absolute values, mean ± SD. It should be realized that the amino acids and Zn^{2+} in the microdialysates are diluted by the dialysis fluid, so their concentrations in the dialysates are lower than their concentration in the extracellular fluid. Statistical analysis was by one-sample tests, Mann-Whitney U-test, Kruskal-Wallis one-way ANOVA on

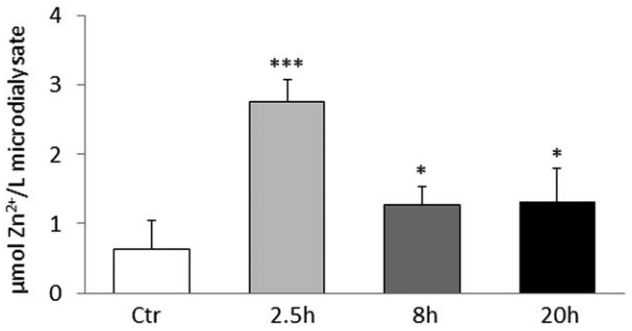

Fig. 2. Levels of Zn^{2+} in microdialysis fluid after injection of *S. aureus* into rat striatum. Anesthetized Wistar rats received an injection of 10^7 *S. aureus* in 1 μl PBS into the striatum. Microdialysis was performed for 90 min, ending 2.5, 8, or 20 hr after injection of staphylococci (light gray, dark gray, and black bars; n = 6, 6, and 4, respectively). Control levels of Zn^{2+} represent value 1–2.5 hr after injection of PBS (open bar; n = 9); this value was the same as that at 20 hr. Values are not corrected for probe recovery of Zn^{2+}, which was 23 ± 3% in vitro. Data are micromole Zn^{2+} per liter, mean + SD values. $\star P < 0.05$, $\star\star\star P = 0.001$, one-way ANOVA, Dunnett's method for multiple comparisons.

ranks, or ANOVA with Dunnett's method as appropriate. $P < 0.05$ was considered significant.

RESULTS

Extracellular Amino Acids After Intracerebral Injection of Staphylococci

Two hours after injection of staphylococci into the striatum, the extracellular concentration of glutamate, as reflected in the microdialysis fluid, was reduced by 76% compared with the control level ($P = 0.007$; Fig. 1a). The level of GABA also was reduced ($P = 0.03$) and was not different from zero in *Staphylococcus*-infected striatum. The levels of aspartate and alanine tended to decrease but without being significantly different from control levels ($P = 0.07$ and 0.05, respectively).

Eight hours after injection of staphylococci, the extracellular concentration of glutamate was 12 times the control value (Fig. 1b). The levels of aspartate, GABA, alanine, and leucine also were increased.

Twenty hours after injection of staphylococci, the extracellular concentration of glutamate had increased to 32 times the control value (Fig. 1b); the concentration in the dialysate was 11 ± 4 μmol/liter, whereas the value for glutamine was 8 ± 1 μmol/liter. The latter value was similar to control (7 ± 2 μmol/liter). The levels of aspartate and GABA were 38 times the control values; the concentration of GABA in the dialysate was 0.91 ± 0.04 μmol/liter. The concentration of alanine and leucine had increased to approximately four times the control values. In contrast, the extracellular concentrations of glutamine and serine remained similar to control values (Fig. 1b).

To determine whether glutamate toxicity played an important part in brain cell damage and the ensuing rise in the extracellular concentration of amino acids, four animals were treated with the noncompetitive NMDA receptor antagonist MK-801 (1 mg/kg body weight) at 0, 3, and 6 hr after injection of staphylococci. No differences in the extracellular level of amino acids were found in this group at 8 hr after injection. The levels of glutamate and GABA were 5.6 ± 3.4 μmol/liter and 2.3 ± 1.6 μmol/liter, respectively, similar to the values of 5.5 ± 5.0 μmol/liter and 1.6 ± 1.4 0 μmol/liter, respectively, found in animals that did not receive MK-801 treatment.

In vitro probe recovery was linear over a 1–1,000 μmol/liter amino acid concentration range, giving the following recoveries: glutamate 2.7% ± 0.2%, aspartate 3.6% ± 0.4%, GABA 4.2% ± 0.6%, and glutamine 3.5% ± 0.7%. The other amino acids had similar recoveries.

Extracellular Zn^{2+} After Intracerebral Injection of Staphylococci

The concentration of Zn^{2+} in the dialysis fluid was approximately 0.6 μmol/liter in control animals at both 2 and 20 hr after injection of PBS (0.6 ± 0.4 and 0.6 ± 0.2 μmol/liter, respectively). Two hours after injection of staphylococci, the concentration of Zn^{2+} had increased to 430% of the control value (Fig. 2). At both eight and twenty hours after injection of staphylococci, the concentration of Zn^{2+} was 200% of control values.

The in vitro probe recovery for Zn^{2+} was linear in the 1–10 μmol/liter range, being 23% ± 3%. This range covered the concentrations found in vivo. Correcting the dialysate concentration of Zn^{2+} (~0.6 μmol/liter) for the probe recovery would give an extracellular concentration of Zn^{2+} in the control situation of ~2.6 μmol/liter. With higher concentrations of Zn^{2+} in the dialyzed fluid, recovery dropped; at Zn^{2+} concentrations of 33 and 100 μmol/liter, recovery was 8.4% and 2.3%, respectively.

Histological Changes in Staphylococcal Brain Infection

Two hours after injection of staphylococci into striatum, foci of Gram-positive bacteria could be seen. No neuronal cell death, leukocyte infiltration, or tissue damage other than the trace caused by the injection cannula could be seen at this time point. Apart from the presence of staphylococci, this picture was similar to that seen in controls.

Twenty hours after injection of staphylococci, a sharply demarcated area of tissue damage was evident; the damaged area included most of the striatum and extended into the overlying cortex (Fig. 3a). In the area of tissue damage there was a loss of neuronal cell bodies (Fig. 3b), in contrast to the normal-looking striatum of control animals (Fig. 3c). In the central portion of the lesion, numerous polymorphonuclear leukocytes were present, many of which seemed to contain, or to be covered by, Gram-positive staphylococci (Fig. 3d, inset); here, the tissue appeared loose and disintegrated. In the lesion area there was a marked reduction in the immunoreactivity for the neuronal nuclear protein NeuN (Fig. 3e). In contrast, immunoreactivity for the synaptic vesicle protein synaptophysin did not appear to be reduced (Fig. 3f).

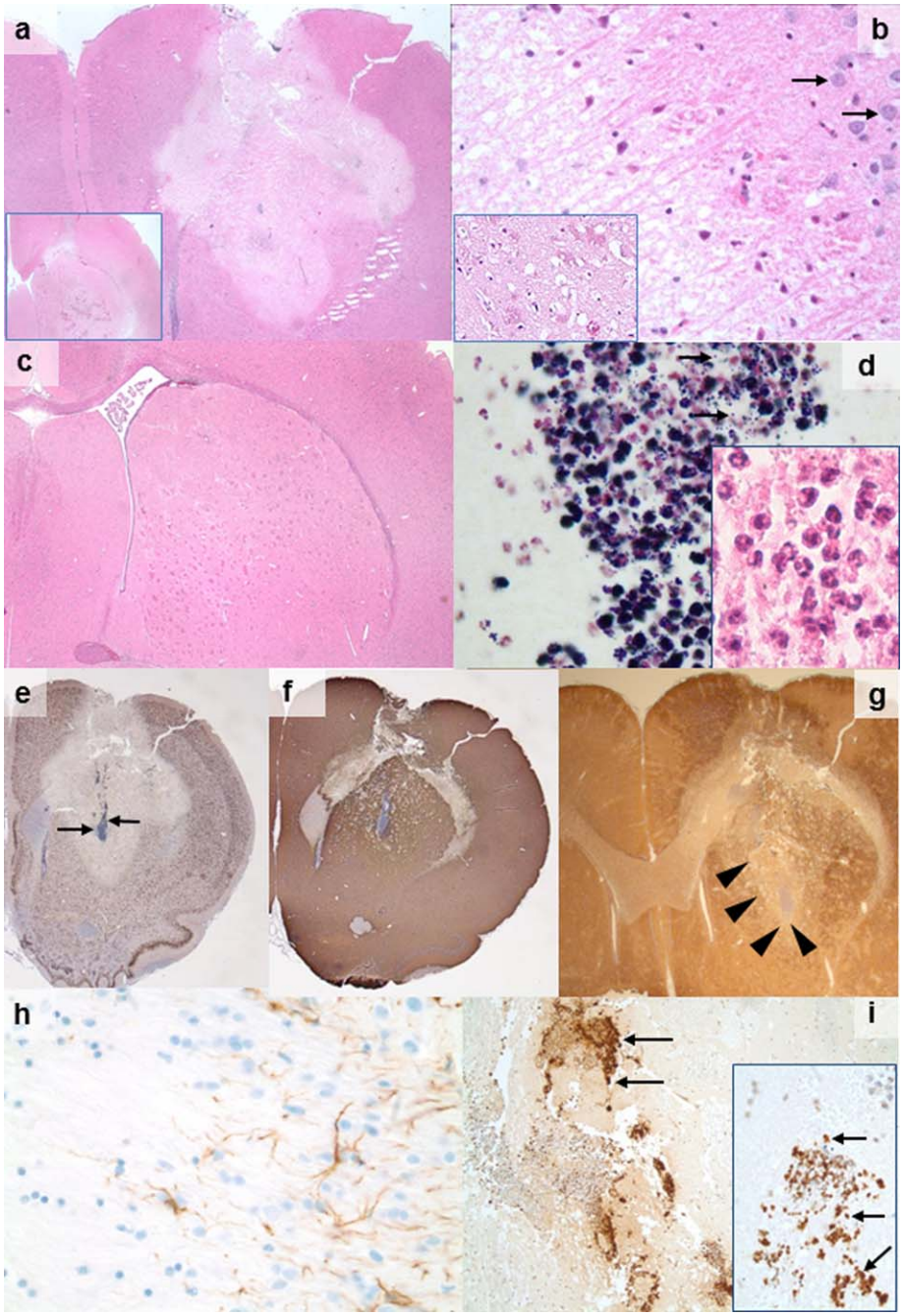

Fig. 3. Brain lesions after focal injection of *S. aureus* into rat striatum. Wistar rats received 10^7 staphylococci in 1 μl PBS into the striatum. **a:** At 20 hr after injection, a large demarcated area of edema and necrosis is seen (H-E; ×10). **Inset:** At 8 hr after injection, the lesion is extensive but less sharply demarcated than at 20 hr after injection. **b:** At 20 hr the border between damaged and histologically normal striatum is sharp, and in the damaged area neurons are absent (arrows point to normal-appearing neurons outside the lesion area; H-E; ×100). **Inset:** At 8 hr after injection, neurons in the periphery of the lesion are shrunken and damaged but still visible. **c:** Histologically normal striatum 20 hr after injection of 1 μl PBS. **d:** Twenty hours after injection, staphylococci (arrows) are present in the central portion of the lesion, which is heavily infiltrated with polymorphonuclear leukocytes, many with adherent or phagocytosed staphylococci (Gram stain; ×100). **Inset:** At 8 hr after injection of staphylococci, infiltrating polymorphonuclear leukocytes are present (H-E; ×100). **e:** Immunoreactivity for neuronal marker protein NeuN (dark punctate staining; ×5) is reduced in the lesion area 20 hr after injection of staphylococci. Note that the lesion area (large pale area without NeuN staining) is much larger than the area of staphylococcal infiltration (arrows). **f:** Immunoreactivity for synaptophysin is preserved 20 hr after injection of staphylococci (×5). **g:** Immunoreactivity for glutamate transporter GLT (lesion area indicated by arrowheads; ×10) 20 hr after injection of staphylococci. **h:** Lack of glial fibrillary acidic protein labeling of astrocytes (brown filaments; ×100) in corpus callosum is evident in the lesion area (left part of image) compared with the unlesioned area (right part of image) 20 hr after injection of staphylococci. **i:** Immunoreactivity for staphylococcal α-hemolysin (×20) and enterotoxin A (**inset**) coinciding with bacteria (arrows) 20 hr after injection, identifying the bacteria as staphylococci. Note a gradient of brownish color representing α-hemolysin spreading from the bacteria (arrows in Fig. 3i).

Immunoreactivity for the glutamate transporter GLT (Fig. 3g) and GFAP was also reduced in the lesion area (Fig. 3h).

Eight hours after injection of staphylococci, a lesion was clearly visible, but the border between damaged and normal tissue appeared less distinct (Fig. 3a, inset), and the tissue generally appeared less damaged than at 20 hr after injection. In contrast to the situation at 20 hr after injection of staphylococci, neurons were present in the periphery of the lesion, but they appeared shrunken and damaged (Fig. 3b, inset); in the central part of the lesions, neurons could not be seen, but infiltrating polymorphonuclear leukocytes (Fig. 3d, inset) and foci of staphylococci were present. In none of the animals were apoptotic cells seen; nuclear fragmentation was absent and immunohistochemical staining for activated caspase was negative in both striatum and corpus callosum (not shown). These morphological changes were highly similar among animals within the same experimental group.

The area of the lesion at the level of the injection site at 8 hr after injection of staphylococci was 7 ± 2 mm^2; at 20 hr after injection it was similar, 9 ± 2 mm^2. In animals that had been treated with MK-801 during 8 hr of staphylococcal infection, it was 8 ± 3 mm^2, which was not different from untreated animals. The area of the staphylococcal clusters at 8 hr after injection of staphylococci was 1.2 ± 0.5 mm^2, $17\% \pm 10\%$ of the area of the lesion (difference from lesion area: $P = 4 \times 10^{-4}$), so the extent of tissue damage was far greater than the area of bacterial clusters, as previously described by Kielian (2004). This was evident also at 20 hr after injection (Fig. 3e). At this time point, the staphylococci were to some extent located on or inside leukocytes (Fig. 3d).

Presence of Staphylococcal Exotoxins in Brain Lesions: α-Hemolysin and Enterotoxin A

Staphylococcal exotoxins α-hemolysin and enterotoxin A could be visualized by immunocytochemistry in the *Staphylococcus*-infected brains (Fig. 3i). The staining for both toxins coincided with the presence of staphylococci, confirming the staphylococcal identity of the injected bacteria. Staining for α-hemolysin was evident even at some distance from the staphylococci, which probably represented diffusion of soluble toxin away from the bacteria (Fig. 3i).

In Vitro Metabolism of Amino Acids by *S. aureus*

We incubated staphylococci in an O_2-saturated medium with glucose, 3 mmol/liter, lactate, 0.75 mmol/liter, and amino acids at 10 μmol/liter, except glutamine (400 μmol/liter) and glycine (20 μmol/liter), to mimic the extracellular fluid of the brain (Ronne-Engström et al., 1995; Lada and Kennedy, 1996; Kanamori and Ross, 2004; Eide and Stanisic, 2010). The levels of aspartate, serine, glycine, and lactate fell 80–90% within 4 hr of incubation (Fig. 4a). In contrast, the level of glutamate increased by 80% and 121% of the initial value at 1 and 4 hr after incubation, respectively ($P < 10^{-5}$; Fig. 4a). The reduction in lactate level confirmed that culture conditions were aerobic (Sun et al., 2012). The level of alanine had dropped 18% by 4 hr after incubation ($P = 3 \times 10^{-5}$). The level of lysine and arginine dropped >70% ($P < 2 \times 10^{-3}$; Fig. 4b), as did the level of branched-chain amino acids and tryptophan, which reached almost zero within 4 hr of incubation ($P < 2 \times 10^{-3}$; Fig. 4c). The level of tyrosine was reduced as the level of phenylalanine increased. The levels of glucose and glutamine were significantly reduced by 4 hr after incubation (by 11% and 8%, respectively; $P < 2 \times 10^{-4}$). The levels of GABA, asparagine, and methionine were not significantly changed (Fig. 4b,c). At the end of the 4-hr incubation period, the optic density of the cultures was $143\% \pm 2\%$ of the initial value ($P = 6 \times 10^{-6}$), whereas the number of colony-forming units was $143\% \pm 23\%$ of the initial value (not significantly different).

To determine whether staphylococci could consume glutamate, we incubated bacteria with glutamate, 10 μmol/liter, as the only energy substrate. Under these conditions, the level of glutamate decreased to 14% at 1 hr and to 5% at 4 hr (Fig. 4d) after incubation, confirming the ability of staphylococci to metabolize glutamate. When glutamine and glucose were the only energy substrates present (at 0.4 and 3 mmol/liter, respectively), the level of glutamate increased from zero to approximately 8 μmol/liter at 1 hr after incubation and declined somewhat thereafter (Fig. 4d). When only glutamine or glucose was present in the Krebs solution, formation of glutamate did not exceed 0.7 μmol/liter at any time point, pointing to the combination of the two substrates as possible precursors for the glutamate that was formed when staphylococci were incubated in a mixture of amino acids and glucose (Fig. 4a). When GABA, 10 μmol/liter, was the only energy substrate present, its concentration remained unchanged throughout the 4-hr incubation with staphylococci (not shown).

DISCUSSION

Biphasic Change in the Extracellular Glutamate After Staphylococcal Infection

In this study we found a biphasic change in the extracellular concentration of glutamate in the brain after focal staphylococcal infection. In the initial phase, the level of glutamate dropped; at later stages, there was a progressive and dramatic increase in the extracellular levels of glutamate, aspartate, GABA, and other amino acids. Twenty hours after injection of staphylococci, the extracellular concentration of glutamate was probably at least 400 μmol/liter. In the microdialysate, the concentration of glutamate was similar to that of glutamine (11 ± 4 vs. 8 ± 1 μmol/liter, respectively); glutamine has previously been found to be ~400 μmol/liter in the extracellular fluid in rat brain (Kanamori and Ross, 2004). The tortuosity of the extracellular space of the brain influences the recovery of solutes by microdialysis, so in vitro recovery tends to be greater than recovery in the brain itself

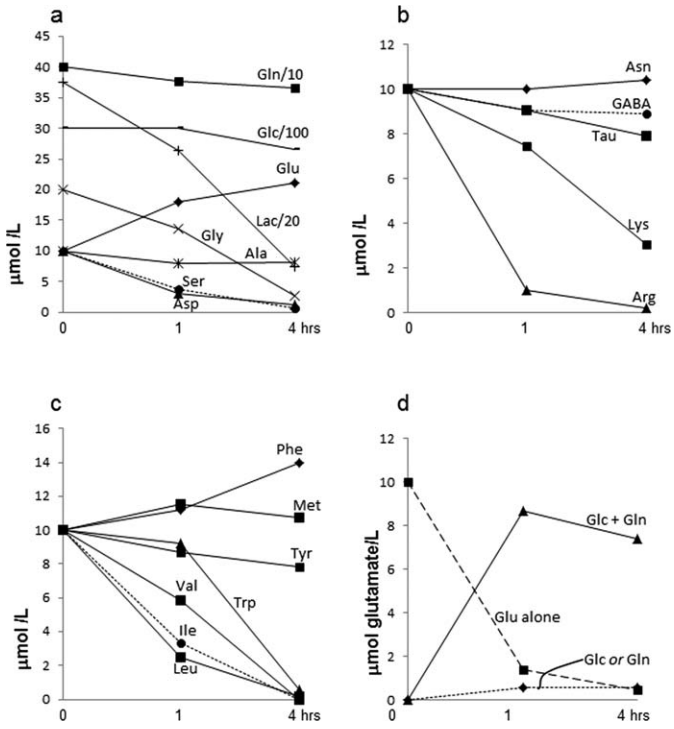

Fig. 4. Metabolism of amino acids, glucose, and lactate by *S. aureus* in vitro. Staphylococci (5×10^7) were incubated at 37°C in 500 µl of an oxygenated Krebs' solution containing amino acids at 10 µmol/liter, except for glutamine (400 µmol/liter), glycine (20 µmol/liter), glucose, 3 mmol/liter, and lactate, 0.75 mmol/liter, to mimic concentrations in the extracellular fluid of the brain. Cultures were harvested at 0, 1, or 4 hr after incubation. **a:** Glucose, lactate, and some amino acids closely related to energy metabolism (glutamate, aspartate, glycine, alanine, and glutamine). Glc/100, Lac/20, and Gln/10 refer to values for glucose, lactate, and glutamine being divided by 100, 20, and 10, respectively. Note that glutamate increases at 1 and 4 hr after incubation. **b:** GABA, taurine, asparagine lysine, and arginine. **c:** Branched-chain amino acids, phenylalanine, tyrosine, tryptophan, and methionine. At 4 hr after incubation, all values were different from the initial values ($P < 2 \times 10^{-3}$) except those for GABA, asparagine, phenylalanine, and methionine (one-way ANOVA, Dunnett's method for multiple comparisons). **d:** Reduction in the concentration of glutamate (dashed line), with time, when glutamate (10 µmol/liter) was the only energy substrate present in the Krebs' solution. Formation of glutamate (solid line) when glucose and glutamine (3 and 0.4 mmol/liter, respectively) were the only energy substrates present. When glucose or glutamine was the only energy substrate present, the concentration of glutamate did not exceed 0.7 µmol/liter at any time point (dotted line represents values obtained with glutamine alone). Data are micromoles amino acid per liter; mean values. SD values were 1–3% of mean values and are not shown; n = 4 per time point.

(Benveniste et al., 1989). Correcting for the recovery of glutamate of 2.7% found in vitro in our study would give an extracellular glutamate concentration at 20 hr after injection of staphylococci of ~400 µmol/liter (11 µmol/liter × 100%/2.7%), which could be an underestimation, although destruction of cell membranes during necrosis would be expected to cause an increase in the interstitial volume fraction and a decrease in tortuosity, reducing the magnitude of that underestimation.

Both a decrease and an increase in the extracellular levels of glutamate, aspartate, or GABA could interfere with normal excitatory and inhibitory neurotransmission in the brain. Similarly, changes in the extracellular concentration of Zn^{2+}, an inhibitor of both glutamate and $GABA_A$ receptors (Westbrook and Mayer, 1987; Mott et al., 2008; Rachline et al., 2008; Sensi et al., 2011), could affect normal neurotransmission, so changes in the extracellular concentration of neuroactive amino acids and Zn^{2+} could contribute to the alteration in mental state that is frequently observed in brain infection caused by staphylococci and other bacteria (Yang and Zhao, 1993; Kilpatrick, 1997; Kao et al., 2003; Roche et al., 2003; Dahlberg et al., 2014). The increase in the extracellular concentration of glutamate and aspartate could lead to excessive activation of glutamate receptors, which, together with Zn^{2+}-mediated inhibition of $GABA_A$ receptors, could contribute to the seizure tendency that is another frequent symptom of abscess-causing brain infections (Yang and Zhao, 1993; Kilpatrick, 1997; Kao et al., 2003; Roche et al., 2003). High extracellular concentrations of glutamate, aspartate, and Zn^{2+} could act in conjunction with other seizure-inducing factors. For instance, interleukins 1β and 17 are produced in *Staphylococcus*-infected brain (Kielian and Hickey, 2000; Holley and Kielian, 2012). Recent studies suggest important roles for these interleukins in seizure generation (Ravizza et al., 2008; He et al., 2013).

The extracellular concentration of Zn^{2+} changed in a manner different from that of glutamate. In the initial phase, when the level of glutamate was reduced, the level of Zn^{2+} was increased fourfold. Because Zn^{2+} is a negative allosteric modulator of both NMDA- and kainate receptors (Mott et al., 2008; Rachline et al., 2008), it is possible that the increase in extracellular Zn^{2+} contributed to a reduction in glutamate receptor activation in the initial phase of staphylococcal brain infection. Zn^{2+} is located in synaptic vesicles in glutamatergic nerve terminals in the striatum and other brain areas (Mengual et al., 1995), and glutamate and Zn^{2+} are typically coreleased (for review see Tóth, 2011). Therefore, the increase in extracellular Zn^{2+} during a reduction in the level of glutamate was unexpected. However, a dissociation between Zn^{2+} and glutamate release has been observed in the hippocampus during exploratory behavior in the rat (Takeda et al., 2006), suggesting that the synaptic release of Zn^{2+} and glutamate to some extent may be regulated independently of one another, which could explain the observed increase in extracellular Zn^{2+} during a phase of reduced extracellular levels of glutamate in this study.

The extracellular concentration of Zn^{2+} in control animals was estimated at ~2.6 µmol/liter, based on a microdialysate concentration of ~0.6 µmol/liter and an in vitro probe recovery of 23%. Although this concentration could be an underestimate (see above), it is still 6-10 times higher than the concentration in cerebrospinal fluid (in man; Palm et al., 1983; Garty et al., 1995). This is similar to the situation for glutamate, which has been measured at approximately 10 µmol/liter extracellularly in the

brain (Eide and Stanisic, 2010; Sun et al., 2014) and below 1 μmol/liter in cerebroventricular fluid (Dahlberg et al., 2014). In this study, correcting the dialysate concentration of glutamate in control animals (~0.4 μmol/liter) for an in vitro probe recovery of 2.7% yields an extracellular concentration of glutamate in control animals of approximately 15 μmol/liter, which is similar to previous results in man (Eide and Stanisic, 2010). However, this concentration could be an overestimate if the microdialysis probe affects astrocytic glutamate transport, as has recently been suggested (Sun et al., 2014).

High Extracellular Concentration of Amino Acids Reflects Cell Damage

The increase in extracellular amino acids after staphylococcal brain infection probably reflected damage to neurons, with flooding of the extracellular space with amino acids that are concentrated intracellularly. GABA and aspartate are predominantly present in GABAergic nerve terminals in the striatum, and glutamate is concentrated in glutamatergic nerve terminals (Gundersen et al., 2001). The reduction in astrocyte function caused by the staphylococci, which could be inferred from the reduced immunoreactivity for glutamate transporter GLT and for GFAP, probably contributed to the increase in extracellular glutamate and aspartate, because astrocytes are a main sink for extracellular excitatory amino acids (Danbolt, 2001).

A recent study on brain abscesses in human patients showed that glutamate, aspartate, and several other amino acids reached millimolar values in pus, whereas GABA was absent (Dahlberg et al., 2014). Proteolysis was a likely cause of the high concentration of proteinogenic amino acids in pus in that study, which examined abscesses that were several days to weeks old. In the present study, cell damage with release of free intracellular amino acids, including the nonproteinogenic GABA, was the likely cause of high extracellular concentrations of amino acids, although the reduction in immunoreactivity for several proteins in the lesion area could suggest that some proteolysis already was taking place. The maintained immunoreactivity for synaptophysin at a time point when NeuN was severely reduced probably reflects differences in turnover of different proteins in the necrotic process.

The staphylococci themselves might also have been a source of glutamate, as could be seen in vitro in this study; staphylococci converted other metabolic substrates into glutamate. Glutamine and glucose were possible precursors for the glutamate, given that incubation of staphylococci with these two substrates in vitro supported formation of glutamate. Neutrophils, which were present in the brain at both 8 and 20 hr after injection of staphylococci, could also give off glutamate, which has been shown in vitro (Collard et al., 2002).

The initial drop in extracellular glutamate after intrastriatal injection of staphylococci is somewhat more challenging to interpret. It could have reflected metabolism of glutamate by the staphylococci. Such a mechanism is partially supported by the in vitro finding that the concentration of glutamate decreased rapidly when staphylococci were incubated with glutamate alone. However, when staphylococci were exposed to a mixture of amino acids and glucose that mimicked the extracellular fluid of the brain, the level of glutamate increased, in contrast to what was seen initially in vivo. It is possible that staphylococci metabolize glutamate differently in vivo and in vitro. Alternatively, mechanisms other than microbial metabolism might have been active, e.g., reduced glutamate release from neurons or increased cellular uptake of glutamate from the extracellular fluid. Support for such possibilities comes from the finding that the extracellular level of GABA, which was not a metabolic substrate for staphylococci in vitro, also decreased initially after intrastriatal injection of staphylococci. Previous studies have shown that staphylococcal proteins activate astrocytes, causing astrocytic formation of several inflammation mediators, including tumor necrosis factor (TNF)-α, within 1 hr after exposure (Esen et al., 2004). Activation of astrocytes with TNF-α has been shown to cause an increase in glutamate transport in vitro (Tilleux et al., 2009). Thus, injection of staphylococci per se might have stimulated astrocytic uptake of glutamate, causing the initial drop in extracellular glutamate seen in the present study.

The cell death caused by injection of staphylococci is not readily explained by direct contact between bacteria and brain cells, given that cell death was evident at some distance from the staphylococci. A similar observation was made earlier by Kielian (2004) and suggests the presence of a diffusible cytotoxic factor. A high extracellular concentration of glutamate and aspartate may cause neuronal cell death (for review see Arundine and Tymianski, 2004), but the lack of a neuroprotective effect of the NMDA receptor antagonist MK-801, together with the loss of astrocytic protein in the lesion area, suggests the presence of cytotoxic factors other than glutamate and aspartate. The staphylococcal exotoxin α-hemolysin, which is a pore-forming toxin that may cause lysis of eukaryotic cells (Menestrina et al., 2003) and which was formed in the brain in the present study, is one candidate for such a diffusible cytotoxic factor. Further studies are required to clarify the mechanism behind the death of brain cells in staphylococcal brain infection.

In conclusion, the present study shows that a staphylococcal brain infection causes dramatic changes in the extracellular concentration of neuroactive amino acids and zinc. These changes could interfere with normal neurotransmission in the surrounding brain tissue, contributing to mental deterioration and seizures in brain abscess patients.

ACKNOWLEDGMENTS

This study was supported by the Norwegian Chapter of the International League Against Epilepsy and the Norwegian Epilepsy Society.

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