Research report

Neurochemical changes and laser Doppler flowmetry in the endothelin-1 rat model for focal cerebral ischemia

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Abstract

Generalized neurotransmitter overflow into the extracellular space, after cerebral ischemia, has been suggested to contribute to subsequent neuronal death. This study aims to investigate the striatal release of the neurotransmitters dopamine (DA), glutamate (Glu) and gamma-aminobutyric acid (GABA) by means of microdialysis, in a rat model for focal transient cerebral ischemia. Ischemia was induced by the application of 120 pmol endothelin-1 (Et-1), adjacent to the middle cerebral artery (MCA) in freely moving rats. Ischemia produced a large increase in extracellular striatal DA concentrations (2400%), Glu (5500%) and GABA (800%) concentrations. Laser Doppler flowmetry in anaesthetized rats, indicated that the blood flow within the striatum decreased by 75±11%. The period of sustained drop of blood flow, was dose-dependently related to the concentration Et-1 injected. Histological analysis of brain slices, taken from anaesthetized and conscious animals, indicated a 500 pmol dose of Et-1 was required to produce a similar infarct in anaesthetized rats to a 120 pmol dose of Et-1 in freely moving rats. The immediate drop in striatal blood flow, and the prompt increase of extracellular DA, after the micro-application of Et-1, were quite striking. This suggests that the DA release, rather than the Glu overflow may be the primary event initiating the cascade of processes ultimately leading to cell death and neurological deficits. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Several mechanisms have been proposed to explain the cell death following an ischemic insult, such as increased excitotoxicity, calcium overload, free radical formation, immune activation, inhibition of protein synthesis, and changes in gene expression [31]. Several labs have investigated alterations in levels of extracellular amino acids after global [2,13,1] and focal permanent ischemia [15,4] in anaesthetized animals. There are only a few reports of neurotransmitter changes after transient focal cerebral ischemia in freely moving animals [17,26]. We therefore aimed at studying the time profile of the biochemical responses to a reversible focal cerebral ischemia in freely moving rats. The changes of extracellular dopamine (DA), glutamate (Glu) and gamma-aminobutyric acid (GABA) were monitored in the striatal nuclei, ipsi- as well as contralateral to the focal application of Et-1. Et-1 injection was used to induce the transient ischemic insult. The biochemical parameters were chosen to be monitored for the following reasons: (1) a temporary overload of Glu can cause excitotoxic damage; (2) the metabolisation of DA to quinones in ischemic conditions may be harmful; and (3) in contrast, an increase of GABAergic inhibition may be indicative for a potential protective role.

In clinical practice, transient ischemic injury is most frequently observed in the vascular territory of the middle cerebral artery (MCA). Hence, to simulate the clinical situation as closely as possible, we selected the Et-1 induced vasoconstriction of the MCA, a model that was first described by Sharkey and co-workers [34]. Et-1 is a

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peptide consisting of 21 amino acids and is one of the most potent vasoconstrictors discovered to date [40]. Et-1 produces contractile responses that are characteristically long lasting and resistant to wash-out [40]. In order to monitor the dose-dependent effects of Et-1 on the actual cerebral blood flow, laser Doppler flowmetry was performed as well. These measurements allowed us to gain information on the blood flow time profile, occurring in the area of the transient ischemia.

The rather well-defined localization of the infarcted zone after the insult, the good reproducibility of the model and the ability to perform the experiments on conscious, freely moving animals, make the Et-1 model attractive for fundamental and pharmacological investigations without the need to use anaesthetics.

2. Materials and methods

All protocols for animal experiments described in this study were performed according to the 86/609/EEC directive, and were approved by the Ethics Committee on Animal Experiments of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel (Belgium).

2.1. Surgery and probe positioning

Male albino Wistar rats were used in our experiments, weighing between 250 and 300 g. Twenty-four hours prior to the experiment, rats were anaesthetized and placed onto a stereotaxic frame (David Kopf Stereotaxic Instruments, model 1404). During the surgical procedure, body temperature was kept at 37°C, using a heating blanket.

Three types of animal experiments were performed.

2.1.1. Microdialysis in freely moving animals

Stereotaxic placement of the probe in the striatum and the cannula in the proximity of the MCA occurred under anaesthesia, using a mixture of 25 mg/kg of ketamine and 5 mg/kg of diazepam. The skull was locally anaesthetized using 50 µl 0.1% Xylocaine®. Burr holes were made in the skull to expose the dura. Microdialysis guides (CMA Microdialysis, Stockholm, Sweden) were inserted in the striata: A+1.2 mm, L±2.4 mm, V+2.8 mm and ipsilateral in the proximity of the MCA: A–0.3 mm, L+5.6 mm, V+7.0 mm. The stereotaxic coordinates were determined according to the Paxinos and Watson atlas [30] and are relative to bregma. Once the guides were correctly positioned they were fixed with two anchor screws in the skull and dental cement. All rats received an intraperitoneal injection of the analgesic Ketofen® post-operatively. Through the guides in the striata, a microdialysis probe (CMA 12 Microdialysis, Stockholm, Sweden) with 3 mm membrane length and outer diameter of 0.52 mm was inserted. Through the guide in the proximity of the MCA, a cannula of 0.25 mm outer diameter was positioned. The animals were allowed to recover from the surgery overnight and had free access to water and food. The probes in the striata were continuously perfused with a Ringer’s solution (147 mM NaCl, 4 mM KCl, and 1.1 mM CaCl₂) at a flow rate of 2 µl/min using a CMA/100 microdialysis pump (CMA Microdialysis, Stockholm, Sweden). The next day, sampling was started from the striata of the freely moving rats. Dialysates were collected every 20 min in vials containing 10 µl of an anti-oxidant solution (0.02 M HCl, 0.2% Na₂S₂O₃, 0.02% Na₂EDTA). After the collection of six dialysate samples under baseline conditions, Et-1 was administered via the cannula near the MCA in the awake and freely moving animal, using a micro injection pump (BAS Bioanalytical Systems, IN, USA) at a flow rate of 1 µl/min. Dialysate sampling from the striata continued for at least 3 h after the ischemic insult.

2.1.2. Laser Doppler flowmetry in anaesthetized animals (LaserFlo blood perfusion monitor 403A, fiber optic probe P336387)

The local cerebral blood flow was determined using two different protocols of anaesthesia: A group of animals received a combination of ketamine (25 mg/kg) and diazepam (5 mg/kg). A second group was anaesthetized by halothane (1% in nitrous oxide: oxygen 70:30). Besides the type of anaesthesia, the experimental set-up was identical. The cannula for Et-1 injection was placed in the proximity of the MCA as described above. For the striatal placement of the laser probe we used coordinates R+1.2, L+2.4 and V+5.8, relative to bregma.

2.1.3. Histological investigations

The experimental protocol regarding the surgical procedure was unaltered, except that no microdialysis guides and probes were implanted in striatum. This enables us to evaluate the extension of the damage per se over successive days. The day after the surgery, a group of conscious animals received a dose of 120 pmol of Et-1, while another group of anaesthetized rats received either 120 pmol Et-1 or 500 pmol Et-1. These doses were selected from an earlier study, using diffusion-weighted magnetic resonance imaging (MRI). This study showed that about a four-fold higher Et-1 dose was required in anaesthetized rats compared to conscious rats, to induce an infarct of the same size [22].

All these animals were also tested behaviourally. The functional impairment and improvement was assessed using a simple limb stepping test.

4. The systemic blood flow was recorded in anaesthetized rats from the femoral artery. For this, an incision was performed in the left groin. The femoral artery was cannulated with a polyethylene 50 catheter. Arterial blood pressure was measured using a pressure transducer connected to the catheter. At the end of the experiment, the catheter was removed, the vessel was ligated and the skin sutured.

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2.2. Materials

DA, Glu, GABA, Et-1, o-phtalaldehyde and iodoacetamide were obtained from Sigma (St Louis, MO, USA). β-mercapto-ethanol and tertiair-butylthiol were obtained from Janssen Chimica (Beerse, Belgium). Sodium metabisulphite, Na$_2$EDTA and solvents of gradient grade quality were purchased from Merck (Darmstadt, Germany). The non-selective Et-1 antagonist, PD 142893, was obtained from RBI (Natick, MA, USA). All aqueous solutions were prepared in fresh water purified by a Seralpur Pro 90 CN system (Belgolabo, Overijse, Belgium) and filtered through a membrane filter (pore size 0.2 μm).

2.3. Drug treatment

To provoke the ischemic insult, the vasoconstrictor Et-1, dissolved in Ringer’s solution, was injected into the proximity of the MCA. In awake animals, the Et-1 doses varied from 43 to 360 pmol and were administered for 6 min at a flow rate of 1 μl/min. From this dose-dependency study, a dose of 120 pmol/6 μl Et-1 was selected for further work in freely moving rats.

The nature of the laser Doppler experiments required the use of anaesthetized animals. The doses of Et-1 used in these experiments varied from 180 to 1000 pmol Et-1.

Control experiments for both histology and microdialysis were performed with intrapiriformal Ringer’s injection.

Presumably, to antagonize and confirm the mechanism of action of Et-1, PD 142893 was used. This non-selective Et-1 antagonist (Et$_{1β}$) was administered via the cannula for the Et-1 injection in conscious rats at a dose of 500 pmol/20 μl in the 20-min collection period prior to the Et-1 injection. To exclude a direct action of Et-1 on striatal transmission, PD 142893 was administered in the same concentration but during 1 h via the microdialysis probe in the striatum in another set of experiments. The 60-min perfusion with the antagonist was started 30 min before Et-1 injection.

2.4. Sample analysis

Microdialysates were divided for parallel analysis of DA, Glu and GABA. For the analysis of DA, reversed phase ion-pair microbore liquid chromatography (LC) with electrochemical detection was used [36]. Chromatographic conditions and precolumn derivatisation procedures for the analysis of Glu and GABA, have been previously described in detail [35]. For Glu, reversed phase microbore LC with gradient elution and fluorescence detection was used. Precolumn derivatisation was performed with o-phtalaldehyde/β-mercaptop-ethanol. The method for GABA consisted of reversed phase microbore LC with isocratic elution and electrochemical detection. The two-step pre-

2.5. Behavioural observation

To evaluate the behaviour of the animal, we tested a group of animals daily, for 1 week, using the ‘limb stepping test’ as described by Olsson et al. [29]. Those animals were also used for histological screening. In case of akinesia, the limbs contralateral to the infarct were not used or showed retardation in reflexes.

2.6. Histology

In parallel with the microdialysis studies, identical groups of rats were tested in order to verify the ischemic lesion size and to follow up the development of the damage. Rats were killed on successive days, starting from day 1 until day 7, in order to monitor the evolution of the damage. The rat brain was fixed for histological analysis by perfusion with a 4% buffered formalin solution (NaH$_2$PO$_4$.H$_2$O, Na$_2$HPO$_4$. H$_2$O, formaldehyde 40%; pH=7). Briefly, the animals were anaesthetized with Nembutal® and placed in a supine position, allowing thoracotomy. A catheter was inserted via the apex into the aorta, the right atrium was incised, and saline solution was infused until the perfusate from the right atrium became bloodless. Then, the 4% formalin solution was infused. The animals were decapitated immediately after the perfusion fixation and the brain was removed. Before sectioning, the brain was kept for more than 24 h in formalin solution (4%). Sections of 100 μm were made using a vibratome (MA752 Motorised Advance Vibroslice, Campden Instruments, UK). The sections were stained with cresylviolet and were examined by light microscopy (Zeiss, Stemi 2000-C, Zeiss Belgium). The images of these sections were digitalized with a camera (Sony DXC, Sony Belgium), connected to the microscope. A computer program (‘NIH image 1.41’) quantified the damaged zone on the digital images. Every 600 μm, the damage was measured and multiplied with the interspace. The sum of these data results in a total volume of damage, expressed as mm$^3$. These measurements were done blindly.

2.7. Statistical analysis

The results, shown in the Figs. are the mean dialysate concentrations in % (mean±s.e.m). Data were not corrected for the recovery across the membrane of the microdialysis probes. The basal value was taken as the mean of six stable dialysate concentrations obtained in basal conditions, i.e. before drug administration. Data were analysed with a one-way analysis of variance (ANOVA) for repeated measures, and Fisher’s protected least significance difference (Fisher’s PLSD) post-hoc tests ($α=0.05$). The same statistical analysis method was used to evaluate
the laser Doppler experiments. A $t$-test was used to evaluate the differences between the quantitative histological data, $\alpha=0.05$.

3. Results

The effects of different doses of Et-1, injected adjacent to the MCA, on the DA, Glu and GABA dialysate levels in ipsilateral striatum are given in Fig. 1. These data represent the values obtained in the collection period following Et-1 injection. Application of Et-1 had no effects on the levels at the contralateral side (control-side), (data not shown). Also the injection of 6 $\mu$l Ringer’s in the proximity of the MCA, had no effect on the extracellular transmitter levels (Fig. 1).

From this dose-dependency study in freely moving rats, 120 pmol Et-1 was selected for further experiments. The microdialysis experiments performed with 120 pmol Et-1 caused significantly elevated extracellular striatal DA, Glu and GABA levels. DA levels increased by 2412% (from $2.20 \pm 0.15$ nM up to $53.06 \pm 9.53$ nM, $n=20$). For Glu, increases of 5508% (from $0.64 \pm 0.03$ $\mu$M up to $35.25 \pm 4.57$ $\mu$M, $n=20$), were noticed. GABA basal levels increased by 789% (from $0.038 \pm 0.007$ $\mu$M up to $0.300 \pm 0.019$ $\mu$M, $n=20$). Fig. 2 illustrates the time course of the transmitter changes after the Et-1 injection (120 pmol). Higher doses, i.e. 360 pmol Et-1, resulted in severely disturbed behaviour and prevented us from working in freely moving animals. Lower doses, such as 43 pmol, induced no apparent behavioural alterations and the histological sections did not reflect reproducible ischemic damage.

Systemic blood pressure was controlled in anaesthetized animals with a femoral catheter, no significant changes were observed during the experiments.

![Graph](https://via.placeholder.com/150)

Fig. 2. The effect of 120 pmol Et-1 on extracellular dopamine (DA), glutamate (Glu) and gamma-aminobutyric acid (GABA) levels in the striatum of freely moving rats. Et-1 was administered in the middle of collection period 2. Results are expressed in % (mean±s.e.m.), $n=20$. All dialysates were sampled under perfusion with Ringer’s solution. One-way ANOVA for repeated measures followed by Fisher’s PLSD post-hoc tests were used ($\alpha=0.05$).
Laser Doppler flowmetry experiments were performed on anaesthetized animals (ketamine/diazepam) using different doses of Et-1 in order to study the dose dependency. Since in previous MRI experiments, performed in our laboratory, it was shown that a four-fold higher dose of Et-1 was required to obtain a similar histological outcome in anaesthetized rats versus the dose used in conscious rats, we focussed on those Et-1 applied doses. Our laser Doppler findings were verified by histological studies (see below). Laser experiments with 180 pmol Et-1 resulted in a decreased (lower than 75%) blood flow for less than 10 min \( (n=4) \). A dose of 500 pmol Et-1 caused a decrease in blood flow of 75±11% for more than 30 min \( (n=5) \). Using 1000 pmol Et-1, a similar decrease was obtained which lasted for more than 1 h \( (n=4) \). Using halothane as anaesthetic induced a similar profile at this dose \( (n=2) \) (Fig. 3).

The blood flow measurements were expressed as relative changes (100% = 32 ml/min*100 g) since the quantification of the flow and the flow changes by this measurement technique and the underlying mathematical algorithm have been questioned [7].

Behavioural observations showed a temporary contralateral rotation of the animal immediately after the 120 pmol injection of Et-1. Using the limb stepping test, all animals showed retardation in the use of their contralateral paws (hind- and forepaw). This clear effect disappeared almost completely in 5 days.

Histological analysis showed an edema developing at day 1 and lasting for 3 days. The infarction was localized in the striatum, overwhelming towards the cortex (Fig. 4a). Fig. 4b shows quantitative data of the ischemic brain damage, 24 h after the insult. In conscious rats, 120 pmol Et-1 \( (n=11) \) was used; in anaesthetized animals, 500 pmol Et-1 \( (n=4) \) was injected. No significant difference in ischemic damage was observed between the freely moving group \( (66.18±15.15 \text{ mm}^3, \text{ mean±S.D.}) \) and the anaesthetized group \( (75.52±10.95 \text{ mm}^3, \text{ mean±S.D.}) \). However, the ischemic damage observed after applying 360 pmol Et-1 \( (151.88 \text{ mm}^3, n=1, \text{ due to the high mortality rate using this dose}) \) in conscious rats was clearly larger.

To verify whether the effect of Et-1 was due to a direct action on the MCA, the effect of the non-selective Et-1 antagonist PD 142893 was examined. The Et-1-induced increases in extracellular DA, Glu and GABA release were completely prevented by pretreatment with 500 pmol PD 142893 administered in the piriform cortex (Fig. 5a). Administration of PD 142893 in the striatum was unable to antagonize the Et-1 induced neurotransmitter release, due to the provoked ischemic insult (Fig. 5b).

### 4. Discussion

A number of rat models for focal ischemia have provided valuable understanding of the pharmacology and pathophysiology of ischemic brain damage. The lack of an

![Fig. 3. Alterations in striatal cerebral blood flow after Et-1 injection in the piriform cortex. The administration of Et-1 at time 5' is indicated by an arrow. The values are expressed in % (mean±s.e.m.). Data were analysed by one-way ANOVA for repeated measures followed by Fisher’s PLSD post hoc tests. Only the first value that is significantly different from the baseline values, is indicated with *(a=0.05).*](image-url)
effective therapy suggests that current models may underestimate the processes occurring during ischemia and therefore newer models should be examined. The occlusion of the MCA is thought to be equivalent, at least in terms of volume damage produced by ischemia, to the one observed in humans [38]. One of the most widely used middle cerebral artery occlusion (MCAO) models is the intraluminal occlusion model, which has several advantages, including that there is no need of a craniectomy. The main disadvantage of this model however, is the unavoidable damage to the endothelial lining of the artery, which is occluded by a filament. This injury exacerbates by reperfusion and can: (1) initiate pathological processes in the vessel wall; (2) alter vascular reactivity; (3) alter blood–brain permeability; and (4) may provide a source of emboli, capable of occluding more distal vessels [23].

An alternative for the intraluminal occlusion model, is the Et-1 model, developed by Sharkey [34]; and applied by others [10–12,24,33]. The Et-1 model was selected for the present study because it can be performed in conscious, freely moving animals. Thus, it circumvents the use of anaesthesia with all its putative complications, such as the lack of body temperature regulation and a possible protective effect mediated by the anaesthesia itself.

This study shows a dose-dependent effect of the Et-1 induced vasoconstriction, consistent with the studies performed by Sharkey et al. [33]. These authors however showed the development of an infarct only using 60
pmol/3 μl Et-1, whereas we needed 120 pmol to obtain a reproducible infarct. Different rat strains and experimental procedures (e.g. different injection site) may explain this difference in concentration.

To prove that transient ischemia was indeed mediated via the Et-1 receptors on the MCA, the non-selective Et<sub>a</sub>/Et<sub>b</sub> receptor antagonist PD 142893 was applied. The Et-1 induced ischemia can be prevented via a direct blockage of endothelin receptors. The Et-1 receptor antagonist also produced a substantial and sustained blockage of the vasoconstriction, which suggests that Et-1 receptor antagonists may also be a possible therapeutic strategy for diseases associated with acute and sustained vasoconstriction [37,39].

To actually verify the drop in blood flow in the ischemic zone, laser Doppler flowmetry was performed. However, for practical reasons, this measurement could only be done on anaesthetized rats. Histological analysis indicated that,
to induce a comparable infarct size, a four-fold higher Et-1 dose was required in anaesthetized rats, compared with freely moving rats. The reason for this is not clear, but it may be related to a protective effect of the anaesthetic or to anaesthesia-induced changes in vascular reactivity. During vasoconstriction, laser Doppler flowmetry showed a 75% decrease of the blood flow, independently of the Et-1 dose. However, the duration of the decrease of blood flow was more sustained as the concentration of Et-1 was increased, ranging from 15 min to about 1 h. The histological studies indicated that the period of sustained decrease of blood flow should last for at least 20 min, in order to successfully induce a reproducible ischemic infarct. This is in agreement with other studies [1,13] that show a strong correlation between the ischemic duration and the subsequent degree of neurological damage.

The present microdialysis study shows that, upon Et-1 administration, an immediate increase of extracellular DA was observed, coinciding with the decline in striatal blood flow. There is also an associated increase in Glu and GABA, but their peak was reached with delay.

DA has been widely recognized as a potential neurotoxic transmitter during ischemia. It also has been demonstrated that DA depletion, either by pharmacological or surgical means, improves striatal outcome following cerebral ischemia [5,14]. The relevance of DA release to develop ischemic damage, is supported by the results obtained by Buisson et al. [3]. They showed that the striatum was protected when the substantia nigra was lesioned with 6-hydroxydopamine (6-OHDA) before the ischemic insult. Indeed, DA release appears to be a very sensitive indicator for sub-lethal ischemia, as extracellular levels become already maximal within the shortest period after the onset of the insult. Moreover, the striatal DA efflux during ischemia may be potentially hazardous to the neuronal survival since partial blood flow, and therefore oxygen delivery still persists, even in the ischemic core [41]. Reperfusion of the ischemic vascular bed may further exacerbate the injury by the increasing availability of oxygen to form free radicals.

To date it is thought that the major neurotoxic effects after an ischemic insult are produced by excessive Glu release [2]. It is generally believed that the opening of Glu-operated ion channels, excessive influx of Ca$^{2+}$ and Na$^+$ through Glu-gated ion channels, coupled to the release of Ca$^{2+}$ from intracellular stores, leads to calcium overload, which is a common activator of cell death [9,32]. Present study confirms that ischemia also provokes the release of inhibitory and/or modulatory neurotransmitters [26,27,19]. However, in comparison to the DA release, the excessive Glu release shows a delay, but lasts for longer. The delayed release of Glu, together with its low clearance, strengthens the idea that the events preceding the Glu rise may damage the cells to a certain extent and thereby initiate the Glu release. Activation of the ionotropic receptors enhances the intracellular concentration of Na$^+$, Ca$^{2+}$ and Cl$^-$. Hence, increased Glu levels can be responsible for the development of the edema [18,6]. The edema, observed immediately after the insult can last for up to 3 days and may also contribute to cell death by further increasing extracellular Glu concentrations [18,20]. Although Glu may not be the primary event initiating the cascade of harmful processes, there is an enormous body of pharmacological evidence indicating that Glu antagonists are neuroprotective in a number of stroke models [16].

The release of GABA exhibited a different profile. Ischemic efflux of excitatory and inhibitory amino acids was synchronous in onset [25,28]. However, in contrast to Glu or DA, the increase in GABA was much smaller and was very transient in nature, with the levels decreasing 40 min after infusion of Et-1. This may be of importance for further development of ischemic damage because GABA’s inhibitory effect is already lacking during the reperfusion phase, while in contrast DA, and especially Glu are still elevated.

In this study, upon Et-1 administration, it was observed that the neurotransmitter release time profile is dictated by the onset of occlusion. However, this time-profile seems to be independent of the duration of occlusion. On the other hand, the extent of the ischemic damage, depends on the Et-1 dose and congruently with the duration of occlusion, as evidenced by laser Doppler flowmetry and histology. These results are in agreement with observations reported by other investigators [1,8,13]. The data of the present study also show that the extent of the neurotransmitter release is Et-1 dose-dependent, while the time-profile is not.

In conclusion, transient focal cerebral ischemia was induced by the application of Et-1 adjacent to the MCA. Extracellular striatal DA concentrations increased simultaneously with the decline in local blood flow. Increases of Glu and GABA levels peaked with some delay. This suggests that DA and/or its metabolites, rather than Glu, is the trigger for the onset of ischemic damage. Although the release of all monitored transmitters occur in a short period (compared to the full development of the infarct) the trigger for ischemic damage seems to be set in this early phase. The observed cerebral edema may initiate secondary damage causing a gradual and accelerating rise in intracranial pressure and concomitant reduction of blood flow [21].

The good reproducibility and the ease of inducement of a transient ischemic insult in conscious animals make this Et-1 model attractive for further pharmacological studies. The model offers the opportunity to simulate the clinical situation in an animal model without the need to use anaesthetics.

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