

# Spatial and temporal parameters of cortical inactivation by GABA

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## Abstract

Inactivation by GABA is a powerful tool for studying the function of specific cortical regions. It is especially useful in electrophysiology, because inactivation is reversible within short time periods, and because the extent of the inactivated region can be accurately controlled. Iontophoresis of GABA inactivates neurons up to 300  $\mu\text{m}$  around the micropipette. Pressure injection of GABA inactivates neurons further away, but the spatial and temporal characteristics of inactivation by this method have been poorly studied. In order to address this question, we built devices made of micropipettes and microelectrodes glued at various distances. We experienced that repetition of small injections of 100 mM GABA inactivate cortex in a more homogenous way than bolus injections. Diffusion of GABA after pressure injection does not seem to follow a point spread diffusion model as in the case of iontophoresis: GABA probably goes up along the micropipette shaft, and the volume of inactivation has an ellipsoidal form. In order to precisely determine the extent of the inactivated region, we built a mathematical model to fit the experimental data of inactivations obtained above and below the pipette tip. The model provides estimates of the inactivated region for volumes smaller than 60 nl of GABA 100 mM. Limits of inactivation are between 250 and 500  $\mu\text{m}$  lateral to the tip of the pipette. The geometry of inactivation is difficult to predict beyond 60 nl and it seems hazardous to try to inactivate neurons beyond 800  $\mu\text{m}$  with pressure injections of GABA 100 mM. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Reversible inactivation; GABA; Pressure injection; Microiontophoresis

## 1. Introduction

By inactivating specific brain regions it is possible to test their contribution to the function of a network of interconnected areas or to behavior. For example, when combined with electrophysiology, inactivation techniques are important tools to assess the contribution of specific connections to the responses of recorded neurons. There are numerous techniques of inactivation of brain tissue. They differ by their spatial and temporal characteristics: some act on small regions (a few hundred of microns), others are more diffuse (a few millimeters); some have long-lasting effects (hours), others are reversible within a small time period (minutes). The choice of an inactivation technique depends therefore on the spatial and temporal constraints of the object of the study.

For example, if one wants to inactivate brain tissue for a long period, muscimol, an agonist of the neurotransmitter GABA, can be used. Inactivation can last several hours, and recovery takes more than 4 h when administered by iontophoresis (Grieve and Sillito, 1991). The extent of the muscimol inactivation is a few hundred microns wide with iontophoresis, and it can go beyond 3 mm with pneumatic microinjections (Martin et al., 1993). This method is well suited for studying the role of specific structures in behavior, like that of subcortical nuclei in sleep and behavior (Lin et al., 1989; Sallanon et al., 1989; Sastre et al., 1996). However, muscimol is not well suited for electrophysiological studies where single unit recording is required, because of the long recovery. When testing a neuron several hours after injection, it is likely that the unit would be lost or that the neuron responsiveness would have changed. The requirement of testing neurons both before and after the inactivation is however essential if

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one wants to link changes in the responses of neurons to inactivation of a brain region. A fast recovery would be therefore an advantage.

Inactivation by cooling offers a good solution to the constraints of extracellular single cell electrophysiology since it provides periods of inactivation of controllable duration, and since the recovery of inactivated neurons is fast (Lomber et al., 1994). The size of the inactivated region depends on the cooling probe: from a few millimeters (Bénita and Condé, 1972) to a few centimeters (Girard and Bullier, 1989). A potential drawback of this method is that cooling probes create a temperature gradient that affects brain regions located a few millimeters away (Girard and Bullier, 1989; Lomber et al., 1994). The cooling method is therefore best used when the cooled region and the region under study are further apart than 10 mm (Girard and Bullier, 1989).

If one wants to study neighbouring regions, there are other methods, widely used in electrophysiology, which provide more localized and short acting inactivations; these methods consist in injecting short acting anaesthetics, cobalt or magnesium chloride or GABA.

Local anaesthetics like lidocaine or lignocaine provide short acting and precisely localized inactivations (Malpeli and Schiller, 1979; Maunsell et al., 1990; Casanova et al., 1992; Martin et al., 1993), and are therefore widely used in electrophysiological studies (Schiller et al., 1979; Malpeli et al., 1981; Maunsell et al., 1990; Molotchnikoff and Hubert, 1990; Casanova et al., 1992; Alonso et al., 1993; Martin et al., 1993; Porter and Izraeli, 1993). A potential drawback of this method is the possible inactivation of en passant fibers: one should therefore be careful in the interpretations when the inactivated region contains or borders fibers from other regions that can interact with the recorded neuron.

When such a risk exists, one could preferentially inject substances that spare en passant fibers, as cobalt and magnesium chloride or GABA. Toxicity of cobalt chloride prevents however repeated injections (Malpeli, 1983), and magnesium chloride produces inactivations that are only partially reversible (Maunsell et al., 1990).

Among all these candidates to inactivation, GABA is one of the best that makes possible repeated, short acting and precisely localized inactivations without affecting en passant fibers, and is therefore well suited for electrophysiological studies, even when the regions of study are very close to each other. Two modes of injection have been used: microiontophoresis and pressure injection. Microiontophoresis of GABA was used for example to study the role of horizontal connections between orientation columns in the visual cortex (Eysel et al., 1988, 1990; Crook et al., 1991; Crook and Eysel, 1992; Crook et al., 1996) or the role of specific layers in the visual cortex (Grieve and Sillito, 1991; Allison et al., 1995). Pressure injection of GABA was used for the

study of interlaminar connections in the visual cortex (Bolz and Gilbert, 1986), of different subdivisions in the LGN (Nealey and Maunsell, 1994), or to test the function of a cortical area or a subcortical structure (Chevalier et al., 1985; Martin et al., 1993; Dias et al., 1995).

A comprehensive study of inactivation by GABA microiontophoresis has already been done (Herz et al., 1969), but there is no systematic study of the spatial and temporal characteristics of the effect of pressure-injected GABA. It is clear however that iontophoresis and pressure injection differ by their spatial characteristics:

Herz et al. studied GABA iontophoresis inactivation in the range 12–300  $\mu\text{m}$ . Even high dosages of GABA were not always sufficient to inactivate neurons beyond 200  $\mu\text{m}$ . We tried GABA iontophoresis and found it impossible to inactivate neurons located 380  $\mu\text{m}$  away from the iontophoresis pipette, even with passing a 400 nA current during more than 20 min.

On the contrary, pressure injection seems to be better suited for inactivating large regions, as Chevalier and his collaborators (Chevalier et al., 1985) could inactivate neurons 600  $\mu\text{m}$  away from the injecting pipette. However, the limits of action of GABA are not precisely known, although Nealey and her collaborators (Nealey and Maunsell, 1994) have shown that GABA was not able to inactivate neurons 1 mm away from the pipette.

In this article we provide information concerning the geometry of the inactivated region around the injection GABA pipette. We also present an optimal strategy to inactivate homogeneously a region of a given extent while preserving a rapid recovery of function.

## 2. Materials and methods

Experiments were performed on four cats. Cats were initially anaesthetized by an intramuscular injection of ketamine hydrochloride (Imalgene, 15 mg/kg) and chlorpromazine (Largactil, 1 mg/kg). An intravenous catheter was placed in the cephalic vein, and an endotracheal tube was positioned. During surgery, the animals were anaesthetized by repeated intravenous injections of 0.1–0.2 ml of alphadolone and alphaxolone (Saffan). During recording, they were paralyzed by a continuous infusion of pancuronium bromide (10 mg/kg  $\text{h}^{-1}$ , in a solution of lactate Ringer and glucose 5%), artificially ventilated with  $\text{N}_2\text{O}/\text{O}_2$  (70%/30%), and anaesthetized by adding 0.2–0.8% halothane. The end-tidal  $\text{CO}_2$  level and the heart rate were monitored and maintained at proper levels. The animal head was held by a device sealed to the skull with screws and dental cement. A craniotomy was performed above areas 17–18, and the dura matter was incised for the penetration

of the microelectrodes (recording) and micropipettes (microinjections). The nictitating membranes of both eyes were retracted and the pupils dilated by corneal application of 1% atropine. Refractive lenses were used to focus the eyes at a distance of 1.14 m from a tangent screen. Recording microelectrodes were tungsten-in-glass type (Merril and Ainsworth, 1972) with 15–20 microns tips and 0.5–4.0 M $\Omega$  impedance, providing multi-unit as well as single-unit recording of cortical neurons. Window discriminators (Neurolog) isolated single units from multi-unit activity. Spike activity was recorded with a PC-based system (CED 1401 interface and Spike 2 software). Receptive field was plotted and moving visual stimuli were displayed at the preferred orientation to maintain spike activity.

Effects of injections of various volumes of GABA were observed at several distances from the injecting micropipette. In order to make these observations, recording microelectrodes were glued at known distances from the GABA pipette.

Injection pipettes were made from borosilicate glass capillaries without filament (Clark Electromedical Instruments, GC100-10, 0.58 mm I.D.). They were pulled in a vertical electrode puller and broken to obtain a tip of 20–45  $\mu$ m outer diameter (about 10–25  $\mu$ m inner diameter)<sup>1</sup>. Only micropipettes with a sharp and 10–50  $\mu$ m long bevel were selected. The bevel facilitated penetration in the cortex without indentation.

To build the compound microelectrode-micropipette, the injection pipette was bent at about 10 mm of the tip and approached with micromanipulators from the microelectrode. Special care was taken to keep both tips parallel, as described previously (Akaoka et al., 1992b). Direct measurement of the distance between the pipette and microelectrode tips was made with a small telescope (Specwell M820-S) equipped with a graticule of 50  $\mu$ m precision. Devices were built with different distances between the pipette and the electrode tips, ranging from 50–1500  $\mu$ m. The pipette and microelectrode tips were adjusted to lie in a plane orthogonal to the axis of the pipette (called the 'Z axis' further on).

<sup>1</sup> We failed to find a relationship between the aperture size of the pipette and the facility to inject GABA. Great variability was observed from one pipette to another with the same characteristics, or from one penetration to another with the same pipette. We even observed sometimes some variability of the relation between the pressure and the volume of injected GABA from one site to another with the same pipette. This could be due to partial clogging of the pipette. Some pipettes got completely blocked up, and could be cleared only by greatly increasing the pressure. In our most recent experiments we use pipettes with 30–35  $\mu$ m outer diameter and 30–40  $\mu$ m of bevel: this opening is large enough to prevent clogging, even after several days of implantation in the cortex, but the risks of leakage are still low. Such risks exist if one uses long tubing (20–30 cm) filled with GABA as we do in our present experiments. These risks can be prevented by positioning the glass capillary that contains the air/GABA interface at the same height as the tip of the pipette.

Distance in Z was also varied for the study of the geometry of inactivation. We built devices with either one pipette and one or two microelectrodes, or with two pipettes and one or two microelectrodes. When a second microelectrode was used, it was bent by heating as was done for the pipette. The elements were glued at 5–10 mm above the tip with *bis*-acryl-composite dental glue (Protomp II), and the compound was reinforced with glued glass barrels, as shown in Fig. 1. Distances between all the glued elements were checked under a microscope (40 or 4 microns precision, depending on the distance to measure).

Pipettes were filled by aspiration (Akaoka et al., 1992b) just prior to the experiment with GABA 100 mM dissolved in a solution of artificial CSF (NaCl 150 mM, Glc 10 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.2 mM, KCl 3 mM, CaCl<sub>2</sub> 1.25 mM, MgSO<sub>4</sub> 1 mM, pH = 7.2). Pipettes were connected to a pneumatic picopump (WPI, PV800). Pressure was maintained at 20 PSI. The duration of the injection (called 'puff') could be controlled within a millisecond precision. Amounts of injected GABA were adjusted by varying either the puff duration (usually in the range 100–300 ms), or the number of successive puffs. Delivery rate of puffs was about 2 IPS (Impulses Per Second). Estimations of the resulting quantity of injected GABA were made by direct measurements of the meniscus displacement of GABA in the pipette, using the small telescope equipped with the 50  $\mu$ m precision graticule. As the inner diameter of the pipette is 0.58 mm, a 50  $\mu$ m displacement of the meniscus corresponds theoretically to a volume of 13.2 nl of GABA. When the relation between the parameters of injection and the observed injected volume was constant, at a given site, delivery of smaller quantities of GABA could be estimated by extrapolation.

Two methods of injection were used: bolus injection and repeated injections. Injections in both cases were made of small puffs repeated at a frequency of 2 IPS. It has been previously observed that a 30 nl injection applied at once leads to significant tissue distortion (Akaoka et al., 1992b). To prevent this phenomenon,

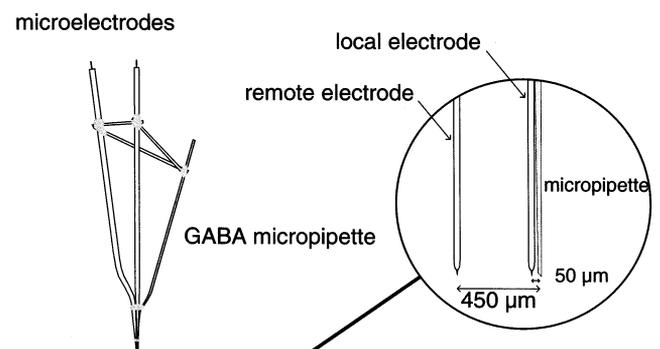


Fig. 1. Drawing of a two microelectrodes/one micropipette compound device.

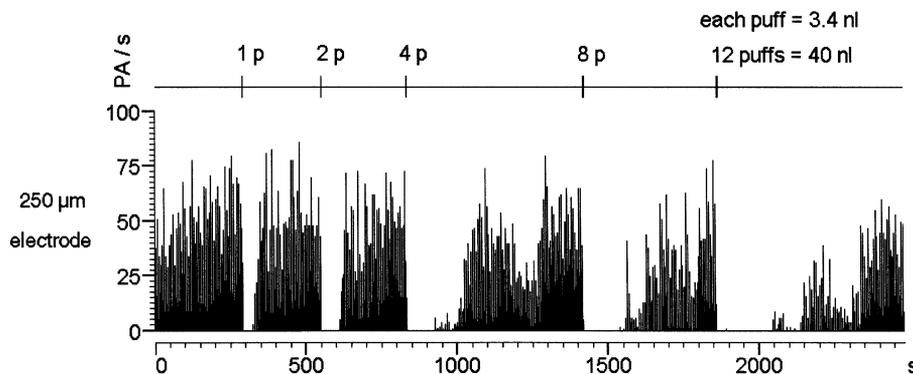


Fig. 2. Example of inactivation at 250  $\mu\text{m}$  from the GABA micropipette. This continuous recording is obtained with a device made with one micropipette and one microelectrode at 250  $\mu\text{m}$  distance. Spike events are plotted within 1 s bins. Injections of GABA 100 mM through the micropipette are progressively increased from 3.4 nl (value estimated by extrapolation) up to 40 nl by increasing the number of puffs ( $p$ ). Inactivation time increases with the volume of injected GABA. Spiking activity does not return immediately to its control frequency after injections larger than 10 nl, but it increases progressively. Recovery time also increases with the amount of injected GABA.

we increased the injected volume by increasing the number of puffs rather than the duration of a single puff. Each single puff was adjusted to be hardly detectable (less than 13 nl).

Attention was focused on multi-unit activity, i.e. a group of two to five neurons recorded by the microelectrode. Once the mean visually evoked spike activity was stabilized, small quantities of GABA were injected into the cortex, and the effect on the mean spike discharge was observed<sup>2</sup>. The threshold volume for inactivation was the minimal volume of GABA that inactivated the recorded neurons. Determination of temporal dynamics of GABA inactivation was done by comparing the durations of inactivation for neurons recorded near and far away from the GABA pipette. Inactivation duration was defined as the time when activity less than 5% of control was recorded by the electrode while the neurons were stimulated by a moving bar on the screen. Recovery durations were also measured. Recovery duration was defined as the period separating the first return of activity and visual responses similar to those recorded before the GABA (mean response superior to 75% of the control). Even if no effect was observed, an arbitrary recovery time was allowed (15–30 min).

### 3. Results

#### 3.1. Temporal dynamics of GABA inactivation

There are two important periods when studying the effect of inactivation of a region on another region: when neurons are totally inactivated and when they have recovered the activity of the control period. As the

probability of losing the isolation of the recorded neuron or of facing changes in the responsiveness level increases with time, recovery time should be kept as short as possible.

The duration of total inactivation depends on the amount of bolus injected GABA. Fig. 2 shows recordings of a small group of neurons obtained with an electrode 250  $\mu\text{m}$  away from the tip of the GABA pipette. As one increases the amount of GABA, by increasing the number of successive puffs, the duration of inactivation increases. Recovery duration depends also on the volume of injected GABA.

Fig. 3 shows a summary of all the data collected with the 250  $\mu\text{m}$  electrode-pipette device. Inactivation and recovery clearly depend linearly on the volume of injected GABA, recovery being slightly longer than inactivation (the slope of the regression line is slightly larger).

To increase the inactivation time at a given distance from the injecting pipette, one can repeat small injections of GABA over time (each time spike activity starts to recover) instead of increasing the volume of a bolus injection of GABA. The bolus injection method is compared with the repeated injection method (Fig. 4A and B), using the same device as in Fig. 2 and Fig. 3. An injection of about 40 nl of GABA totally silences neurons during 3 min; recovery lasts more than 4 min. If approximately the same volume of GABA is injected within three successive series of four puffs, the inactivation period lasts four times that of the bolus method. Despite this large increase in inactivation time, recovery period lasts only 10 min, slightly more than twice that observed with the bolus method. Using equations of the regression lines of Fig. 3, one can calculate that to inactivate neurons recorded at 250  $\mu\text{m}$  for 12 min with a single injection, we would need 115 nl of GABA, instead of the 36 nl of GABA needed with repeated injections. Recovery would last 15 min, instead of 10

<sup>2</sup> The different volumes were not always tested at the same cortical site, and identical volumes of GABA were usually tested in different sites with the same pipette.

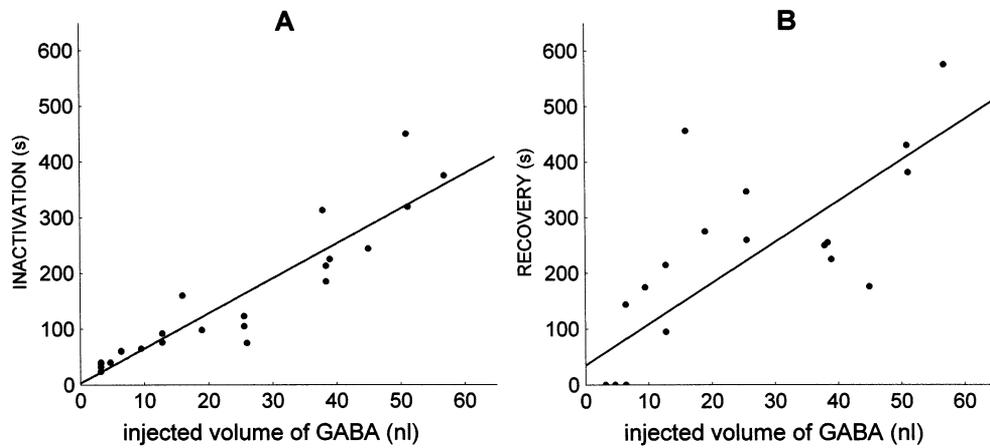


Fig. 3. Summary of the results obtained with the electrode located 250  $\mu\text{m}$  from the GABA micropipette. (A) Inactivation time is plotted as a function of the injected volume of GABA 100 mM. Inactivation and volume are significantly correlated (Pearson product-moment correlation,  $r = 0.93$ ,  $p < 0.05$ ). Equation of the regression line is:  $\text{inactivation} = 2.72 + 6.26 \cdot \text{vol}$ . (B) Recovery time is plotted as a function of the injected volume of GABA 100 mM. Recovery and volume are significantly correlated (Pearson product-moment correlation,  $r = 0.79$ ,  $p < 0.05$ ). Equation of the regression line is:  $\text{recovery} = 35.07 + 7.35 \cdot \text{vol}$ .

min. The method of repeated injections is therefore useful to improve the efficiency of inactivation and to shorten the recovery.

It should be noticed that intervals between injections increase with repetitions: the inactivation time lasts longer each time one repeats an injection, although the same volume of GABA is injected. After each repeated injection of 12 nl of GABA, inactivation lasts respectively, 1 min 45 s, 3 min 15 s and 7 min. There is therefore a potentialization effect of repeated injections.

Eighteen inactivation-experiments were performed with the repeated injection method, at 250, 420, 430, 450, and 470  $\mu\text{m}$  from the GABA pipette. In all the cases, there was a potentialization. There was also a better efficiency of the inactivation with repetitions than with the bolus injection method.

Another advantage of the repeated injection method is a more homogenous inactivation of the brain tissue. Fig. 5 illustrates this point. Neurons are recorded 50 and 450  $\mu\text{m}$  away from the GABA pipette (see Fig. 1). On Fig. 5A, about 32 nl of GABA inactivate all the neurons recorded by the two electrodes during 5 min and 2 min 40 s, respectively. Inactivation period is therefore not spatially homogenous: it lasts longer near the pipette than away from it. However, repetitions of small injections of GABA (Fig. 5B) inactivate neurons recorded by both electrodes during the same time (about 12 min). Recoveries are comparable near the pipette and away from it.

In conclusion, in order to homogeneously inactivate a given region of cortex for a given time and with a rapid recovery, one has to find the threshold bolus volume of GABA that inactivates this region, and then repeat at increasing intervals this threshold injection for the desired period of inactivation.

### 3.2. Spatial characteristics of GABA inactivation

We first studied the maximal lateral extent of the inactivated region. It was never possible to inactivate neurons recorded 1.5 mm away from the pipette, even with bolus injections of more than 1  $\mu\text{l}$  of GABA. We were able to inactivate neurons located 940  $\mu\text{m}$  away from the pipette, but only twice in a series of 13 trials obtained in five different sites. Inactivation at such a distance was therefore not reproducible. Some explanations of these observations will be proposed in the discussion.

When one wants to inactivate small structures of the brain, it is essential to know precisely the geometry of the affected region. When inactivating specific layers of cortex (Bolz and Gilbert, 1986), or specific laminae of the LGN (Malpeli et al., 1981; Maunsell et al., 1990), one should be careful to spare the neighbouring layers or laminae. It is usually supposed that a substance injected in the cortex occupies a spherical volume centered at the tip of the pipette (Nicholson and Phillips, 1981; Nicholson, 1985). However, it is easily verifiable that a liquid pushed from a pipette goes up along the pipette, by capillarity, and forms a droplet that is centered above the tip of the pipette. We could suppose that a similar asymmetry is created when injecting GABA into the cortex. To test this point, we used special devices configured to have two microelectrodes, one located above, and one below the tip of the pipette (Fig. 6A). Distances between each electrode and the pipette are similar. Fig. 6B presents multiunit activity recorded by the two corresponding electrodes. Injection of about 47 nl of GABA leads to a long lasting total suppression of the activity of all the neurons recorded by the upper electrode, but multi-unit activity recorded

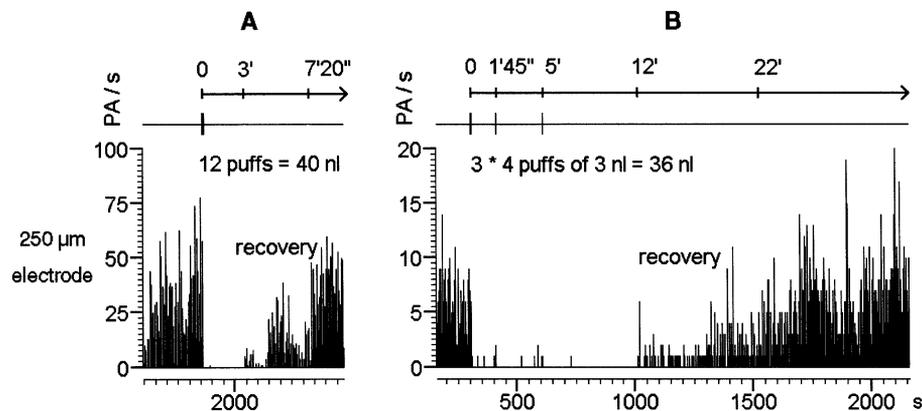


Fig. 4. Bolus injection method versus repeated injections method. The recordings are obtained with the same device as in Figs. 2 and 3. (A) Bolus injection method: inactivation and recovery are measured after one single bolus injection of 40 nl of GABA 100 mM (the right part of Fig. 2 is reproduced). The 40 nl bolus injection is performed with a series of 12 puffs at 2 IPS frequency (Section 2). Inactivation lasts 3 min and recovery of mean spike discharge takes 4 min 20 s. (B) Repeated injection method: 12 nl of GABA 100 mM is injected (= 4 puffs) at time 0 and each time spike activity begins to come back. Three injections of 12 nl were done, for a total amount of 36 nl injected GABA. Inactivation lasts 12 min, four times the 3 min inactivation time obtained with a 40 nl bolus injection. Recovery takes 10 min.

by the lower electrode is only briefly reduced. Obviously, the neurons recorded by the upper electrode were much more affected by GABA, meaning that GABA probably goes up along the pipette. A substance injected into the cortex occupies therefore a volume that can be better estimated as ellipsoidal rather than spherical, with the center of the ellipsoid being above the tip of the pipette.

In order to find the parameters of this ellipsoid, a systematic study was performed by positioning the pipettes at different distances in  $X$  and  $Z$  from the electrode (which has the coordinates  $X=0$ ,  $Z=0$ ; Table 1). Thirteen different configurations were tested. For each pipette, threshold volumes for inactivation were measured at different distances by the method described below:

A series of bolus volumes of GABA was tested, and total inactivation times of the recorded neurons were measured. In order to compare the results obtained with the different devices, we chose to calculate by extrapolation the volume of GABA needed to inactivate the recorded neurons during 60 s. The threshold inactivation volumes of Table 1 are the extrapolated volumes of GABA that produce 60 s inactivation. The results obtained with an electrode located 770  $\mu\text{m}$  away from the pipette are plotted on Fig. 7. Injections of GABA smaller than 100 nl do not inactivate neurons recorded by the electrode 770  $\mu\text{m}$  away. Beyond 100 nl, inactivation time is linearly related to the volume of injection. It was therefore possible to calculate by linear extrapolation the volume of GABA needed to inactivate the neurons during 60 s. The threshold volume is here 111 nl for 60 s inactivation.

In order to determine the geometry of the inactivated region, we created a model that fits the data presented in Table 1. This model relates the volume of inactiva-

tion to the distances of inactivation that we measured. It estimates also the way GABA occupies the extracellular space.

All the data of Table 1 were not used in the model: pipette 1 was excluded because the threshold volume was too small to be calculated with enough precision. Pipettes 5 and 7 had only two measurements, so the extrapolation for finding the threshold could not be reliable. Pipettes 12 and 13 gave contradictory results (Section 4). As all the threshold volumes kept in the model are lower than 60 nl, this model only applies to injections smaller to 60 nl.

### 3.2.1. Description of the model

A substance injected from a micropipette into the brain may either form a cavity or it may infiltrate the extracellular space (Nicholson, 1985; cf Fig. 1 of his paper); if it infiltrates the extracellular space without any tissue distortion, then the injected substance occupies a volume about five times larger than if it creates a cavity, and this prior to any diffusion (extracellular or pore space fraction,  $\lambda$ ;  $\lambda = 0.21$ , Nicholson and Phillips, 1981). We recorded neurons 50  $\mu\text{m}$  away from the pipette when injecting for example approximately 32 nl of GABA (see Fig. 5). If 32 nl of GABA formed a cavity in the tissue, it would enclose the electrode: the cells would be pushed about 100  $\mu\text{m}$  away from the pipette tip in the direction of the electrode<sup>3</sup>, so we should not have been able to see any recovery of neuron activity. Since we did, we could assume that there is little tissue distortion. This is in good agreement with previous observations (Akaoka

<sup>3</sup> A total of 32 nl corresponds to a sphere of radius = 200  $\mu\text{m}$ . The value of 100  $\mu\text{m}$  is founded with an ellipsoidal model, as described below.

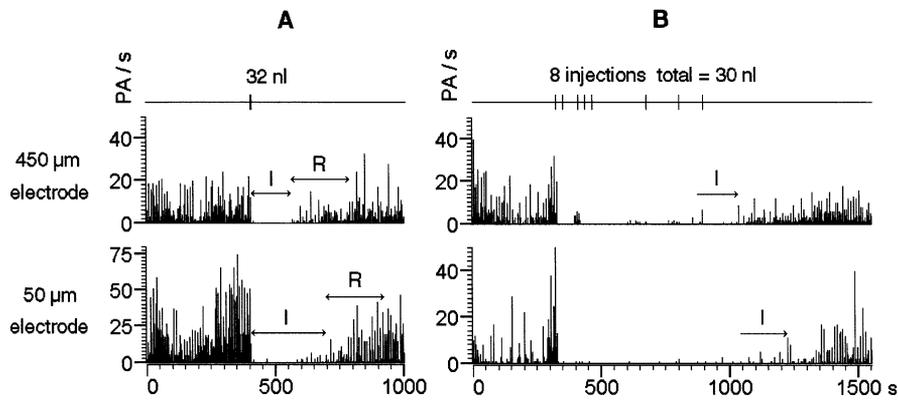


Fig. 5. Spatial homogeneity of GABA inactivation: Bolus injection versus repeated injections method. Recordings are obtained with the device drawn on Fig. 1. Two electrodes record neurons at 450  $\mu\text{m}$  (upper traces) and 50  $\mu\text{m}$  (lower traces) distance from the tip of the GABA pipette. A. Bolus injection of 32 nl of GABA 100 mM. Inactivation (I) lasts 2 min 40 s at 450  $\mu\text{m}$  (upper trace) and 5 min at 50  $\mu\text{m}$  (lower trace). Recovery (R) takes 4 min in both cases. B. Injection of 30 nl of GABA 100 mM achieved with eight repetitions. Inactivation (I) lasts 12 min near the pipette and 14 min at 450  $\mu\text{m}$ . Recovery takes only 2 min for both electrodes.

et al., 1992b) showing that the shapes of spikes recorded very close to the pipette were not altered after an injection of 50 nl, if injected by repeated pressure pulses, as we did in our experiments (Section 2).

The injected GABA occupies therefore the extracellular space and diffuses into the cortex following its concentration gradient. Nicholson and collaborators have described equations of diffusion of a substance into brain tissue (Nicholson and Phillips, 1981; Nicholson, 1985). However, GABA is recaptured very efficiently (Dingledine and Korn, 1985), and we could not apply these equations. For simplification we distinguish  $V_e$  and  $V_i$ .  $V_e$  is the volume of GABA in the extracellular space: it is the volume that GABA occupies in the brain tissue at the moment of injection. The injection is considered as instantaneous.  $V_i$  is the whole volume of brain tissue containing GABA of concentration sufficient for 60 s inactivation.

For each pipette, we can calculate  $V_e$  from  $V_p$ , the threshold inactivation volume for each pipette, given in Table 1.

$$V_e = (1/\lambda) * V_p \text{ with } \lambda = 0.21 \quad (1)$$

We suppose from the data of Fig. 6 that the GABA injected in the cortex occupies an ellipsoidal volume, as GABA probably goes up along the pipette.  $V_e$  is therefore the volume of the ellipsoid E of GABA in the extracellular space. E is defined by its three radii. As we suppose that injection is isotrope in the plane orthogonal to the pipette axis, we consider only the height  $h$  (along the Z axis) and the width  $w$  (along the X axis) of E (cf Fig. 8).

$$V_e = 4/3 * \pi * w * w * h \quad (2)$$

$V_i$  is the volume of inactivated cortex in which the concentration of GABA is sufficient to inactivate neurons for 60 s. The limits of E have the concentration of

100 mM at the time of injection. Then GABA diffuses, and concentration decreases as one goes away from E, until the threshold inactivation concentration  $C_i$  is reached.  $d$  is the distance between E and the volume of concentration  $C_i$ .  $d$  depends only on the concentration of injected GABA, not on the volume of injected GABA. For each pipette, we should find therefore  $w$  and  $h$  (depending on  $V_p$ ) so that:

$$V_i = 4/3 * \pi * (w + d) * (w + d) * (h + d) \quad (3)$$

The position of the center of the ellipsoid E is located at the distance  $p$  above the tip of the pipette (Fig. 8).  $Z_p$  is the Z coordinate of the tip of the pipette (Table 1);  $Z_e$  is the Z coordinate of the center of E.

$$Z_e = Z_p + p \quad (4)$$

To explain that  $Z_e$  is above  $Z_p$ , we suppose that a minor fraction of the injected GABA goes up along the pipette for an unknown distance of  $2 * p$  (Fig. 8: black region around the pipette). We suppose that the more GABA is injected, the higher it goes along the pipette.  $p$  is therefore proportional to the injected volume.

$$p = k * V_p \quad (5)$$

Then GABA infiltrates the extracellular pore space in a homogenous way and occupies the ellipsoidal volume  $V_e$  (Fig. 8). We make the hypothesis that the form of the ellipsoid of GABA is determined by the following equation (cf Fig. 8):

$$h = w + p \quad (6)$$

$V_p$  is given by Table 1. Eq. (1) gives  $V_e$ . If we knew  $k$ , equation Eq. (5) would give  $p$ . From Eq. (2) and Eq. (6) we can therefore calculate  $h$  and  $w$ .

As  $d$ , the distance between E and the threshold inactivation limit, should be constant, the distances between the ellipsoids E of GABA and the recording

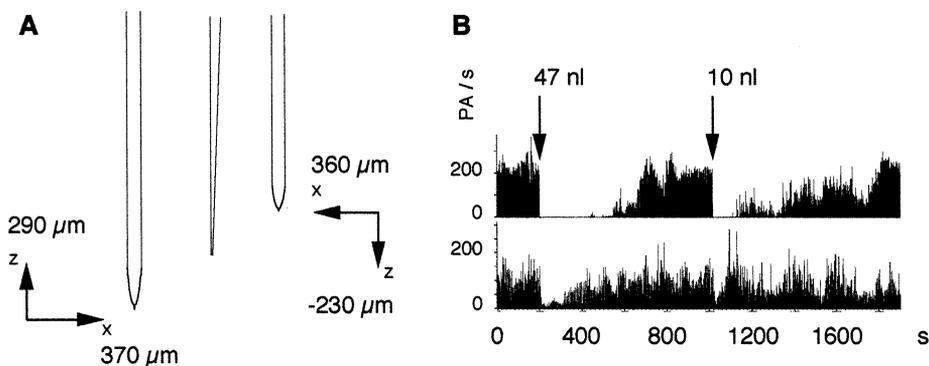


Fig. 6. Anisotropy of pressure injected GABA inactivation. (A) Drawing of a device made from one pipette and two electrodes. Distances between the pipette and microelectrodes are given along the  $X$  and  $Z$  axis (cf Table 1 for a definition). One electrode (the lower electrode) is located under the tip of the pipette:  $z = 290 \mu\text{m}$ . The other one (the upper electrode) is located above the tip of the pipette:  $z = -230 \mu\text{m}$ . Distances between each tip of the electrodes and the tip of the pipette are very close to each other ( $d = (x^2 + z^2)^{1/2}$ ;  $d = 470 \mu\text{m}$  for the lower electrode and  $d = 430 \mu\text{m}$  for the upper electrode). (B) Example of a recording obtained with the device drawn in A after two bolus injections of GABA 100 mM. The upper trace shows multi-unit activity recorded with the upper electrode. The lower trace shows multi-unit activity recorded with the lower electrode. Inactivation is more efficient above than below the pipette tip.

electrode should be the same for all threshold volumes: we made this hypothesis to find the parameter  $k$ , on which  $p$ , and therefore  $h$  and  $w$  depend.  $k$  minimizes  $s$ , the S.D. of the distances between the ellipsoid for each pipette and the electrode. We found  $k$  that minimizes  $s$  using the Matlab FMIN function, which uses a Simplex search method.  $k = 7.1$ ;  $d = 235 \mu\text{m}$ ;  $s = 10.5 \mu\text{m}$  (max – min =  $29 \mu\text{m}$ )

The solution of this optimisation is given in Fig. 9: We have plotted the planar projections of the ellipsoids  $E$  of GABA (which occupies the extracellular space, as in Fig. 8) for the different pipettes. The coordinates are those of Table 1. Distances between the ellipsoids and the microelectrode are clearly similar.

In order to have an idea of the precision of the parameter  $k$  that we have found, we calculated a 95% confidence interval using bootstrap statistical methods (Efron, 1982; Efron and Tibshirani, 1993). This interval is bias corrected (Jackknife method).

$$6.8 < k < 8.5$$

In Fig. 10 we plotted the planar projection of  $E_{50}$  and of the volume of brain tissue ( $V_i$ ) inactivated during more than 60 s after an injection of 50 nl of GABA 100 mM.  $V_i$  was calculated with  $k = 7.1$ ,  $d = 235 \mu\text{m}$ , + or  $-s = 10.5 \mu\text{m}$ . We plotted as well the ellipsoids obtained with the limits of the confidence interval of  $k$ ,  $k = 6.8$  and  $k = 8.5$ .

Fig. 11A gives the volume of inactivated brain tissue as a function of the injected volume of GABA 100 mM. Fig. 11B, C and D give the limits of inactivation measured from the tip of the pipette, as a function of the injected GABA volume. Distances of inactivation are given respectively lateral to, above and below the tip of the pipette.

## 4. Discussion

Pressure injection of GABA provides inactivation of a volume of brain tissue with great precision in both spatial and temporal dimensions, if a precise experimental procedure is followed. We found that repetitions of small injections of GABA inactivated brain tissue in a more homogenous way than when bolus injections were used.

### 4.1. Procedure of inactivation

In order to design the procedure of inactivation, two parameters have to be known: the volume of each repeated injection, and the moments of repetitions. One procedure for estimating these parameters is to glue a microelectrode at the distance of the GABA injection pipette that corresponds to the limit of the region that one wishes to inactivate.

#### 4.1.1. Volume of injection

The volume of each injection will be the threshold volume that inactivates the neurons recorded by the microelectrode. In other terms, the choice of the volume of GABA to inject depends on the volume of cortex that one wishes to inactivate.

The relationship between the volume of injected GABA and the volume of inactivation is not simple: it depends on the way GABA occupies brain tissue. Even when repeating brief pressure pulses of GABA to prevent tissue distortion, one can not avoid that some GABA might go up along the pipette by capillarity. This determines the form of the volume that GABA occupies in the extracellular space, and therefore the volume of inactivated brain tissue.

Table 1  
Summary of the data

Pipette	Figures	$X_p$ ( $\mu\text{m}$ )	$Z_p$ ( $\mu\text{m}$ )	Distance ( $\mu\text{m}$ )	$V_p$ (nl)	No. of measurements	Kept in model
1	2–3–4	250	0	250	<3	24	No
2	6	360	–230	430	4	19	Yes
3		420	0	420	8.8	15	Yes
4	1–5	440	80	450	11.3	5	Yes
5		520	0	520	26	2	No
6		340	–680	760	26	17	Yes
7		430	–500	660	30	2	No
8		480	40	480	36	17	Yes
9		400	–750	850	36	20	Yes
10		510	–480	700	49	5	Yes
11	6	370	290	470	59	29	Yes
12		770	0	770	111	10	No
13		600	–30	600	135	20	No

We use cartesian coordinates in the plane passing by the micropipette and the microelectrode.  $Z$  axis is along the axis of the microelectrode;  $X$  axis is along the direction normal to the microelectrode axis (Fig. 6A). The reference ( $X=0$ ,  $Z=0$ ) is the tip of the microelectrode.  $X_p$  and  $Z_p$  are the coordinates of each pipette. ‘Distance’ is the distance between the tip of the microelectrode and the tip of the pipette:  $\text{distance}=(X_p^2+Z_p^2)^{1/2}$ .  $V_p$  is the volume of GABA that inactivates the neurons recorded by the microelectrode during 60 s. We also indicate the number of measures from which the threshold volume has been calculated. The last column states whether the values were kept for the model.

We found equations and parameters that fit very well the threshold inactivation volumes (all inferior to 60 nl) measured with eight microelectrodes located at different distances from the GABA injecting pipette. We could therefore estimate the amount of GABA needed to inactivate a given volume of cortical tissue.

As the inactivation volume has a theoretical ellipsoidal form, we gave the curves of distances of inactivation as a function of the injection volume in three directions: above, below and lateral to the tip of the pipette (Fig. 11). These curves allow one to predict the volume of GABA necessary to inactivate the neurons recorded by the microelectrode.

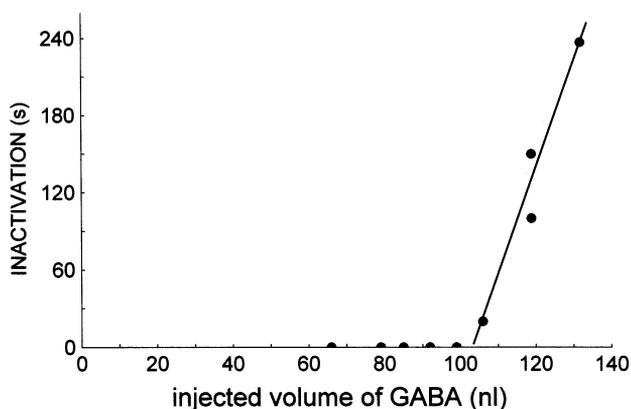


Fig. 7. Summary of the results obtained with an electrode located 770  $\mu\text{m}$  from the GABA pipette: determination of the 60 s inactivation threshold. Inactivation duration is plotted against the volume of GABA 100 mM. The regression line is calculated only for volumes superior to 100 nl. Its equation is:  $\text{inactivation} = -866 + 8.35 \cdot \text{vol.}$  ( $r = 0.97$ ). We calculate that 111 nl of GABA 100 mM inactivate the neurons recorded by this electrode for 60 s. A total of 111 nl is the 60 s inactivation threshold.

It should be kept in mind that the application domain of this model is only for volumes of injection in the range 10–60 nl.

#### 4.1.2. Injection repetitions

After verification of the threshold volume of inactivation, repetitions of the same injection of GABA should occur each time the spike activity recorded by the microelectrode starts to recover, and this during all the desired period of inactivation. Only a microelectrode located at the limit of the zone that one wishes to inactivate can provide precise information about the times when repetitions of injections should occur.

If one does not have a control microelectrode, the following procedure of inactivation could be used ‘blindly’ with a good chance of creating a homogenous region of inactivation, for inactivations up to 400 or 500  $\mu\text{m}$  lateral to the tip of the pipette. In order to inactivate the neurons recorded at these distances of the pipette during more than 10 min, it was necessary to inject a suprathreshold volume of GABA<sup>4</sup>, and then to repeat injections of the same volume of GABA about 1 min 30 s, 3 min 30 s and 6 min after the first injection.

#### 4.1.3. Recovery

It is necessary to know when inactivated neurons have recovered their control activity, in order to decide when one should test the neurons of the region of interest.

Recovery time would be about as long as inactivation time. Waiting twice the time of inactivation, we always

<sup>4</sup> For example, a suprathreshold injection was about 13 nl of GABA for the pipette 3 of table 1, which was separated from the microelectrode by 420  $\mu\text{m}$  and for which the extrapolated threshold inactivation volume was 8.8 nl.

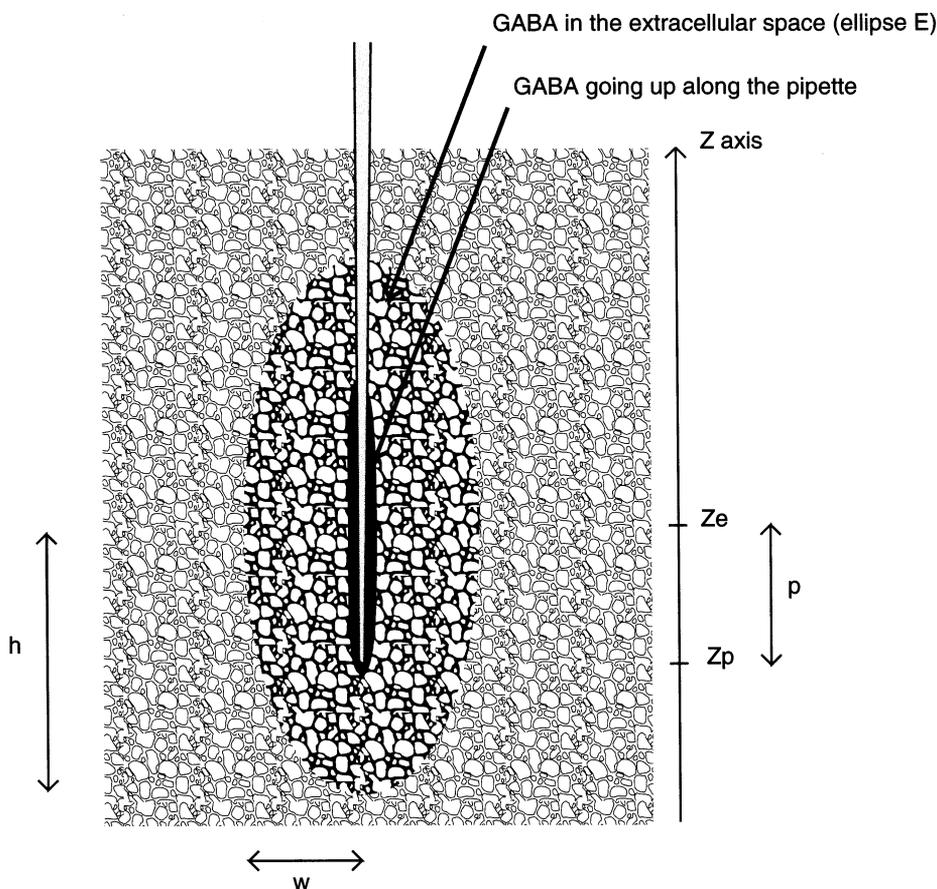


Fig. 8. Modelization of the dynamics of pressure injected GABA; drawing of a pipette into the cortex. As one pushes GABA from a micropipette, a minor fraction of it goes up along the pipette by capillarity (black filled ellipse around the pipette). Then GABA infiltrates the extracellular pore space (black pore ellipse). The distance between the limits of infiltration and the closest source of GABA is about the same everywhere on the ellipse E of GABA. In other terms, we replace a point spread diffusion model (Nicholson, 1985) by a line spread diffusion model.  $Z_c$  is the Z coordinate of the center of the ellipse E.  $Z_p$  is the Z coordinate of the tip of the pipette.  $Z_c = Z_p + p$ .  $2*w$  is the width of E,  $2*h$  the height of E.  $w$  is the lateral distance between the pipette and E; as only little GABA goes up along the pipette,  $h$  is also an approximation of the distance between the GABA going up along the pipette and E. Therefore,  $h = w + p$ .

have good functional recoveries in our present experiments. We never have to wait more than 30 min after the end of the total inactivation.

We have therefore perfected a GABA inactivation method very well suited to electrophysiological studies.

#### 4.2. Extension to other structures

It should be reminded that all these experiments have been done in the cat visual cortex, and that generalization of these results to regions other than cortex should be done with precaution. Neurons often have large dendritic arborisations, which can extend a few millimeters away from the cellular body. It has been shown, in some cases, that synapses located on distal dendrites can be more effective than proximal synapses (Akaoka et al., 1992a). Even very small injections of GABA may therefore inactivate some neurons very far away from the injection pipette. As a consequence, one should be aware of the dendritic organisation of neurons of the studied regions.

When studying the effect of inactivation of a cortical area on another cortical area, this is not a problem, as dendrites restrict themselves to a given area. However, direct inactivation of remote neurons cannot be excluded when studying interactions within a cortical area, for example long range connections of the visual cortex. Eysel and collaborators (Eysel et al., 1988) did indeed observe a possible direct inactivation of neurons within area 17 of the cat 1.45 mm away from the iontophoresis pipette (cf the Fig. 7 of their paper). As GABA cannot diffuse so far away, this effect could be mediated by long range dendrites.

However, we did not observe such long distance inactivations in our experiments: this could be due to the fact that we recorded multi-unit activity, and that we could miss the inactivation of individual neurons. Nevertheless, even if it is the case, such distant inactivations should not be frequent. This is perhaps due to the columnar organisation of dendrites in the cortex, and to the high concentration of GABA receptors near the soma (Freund et al., 1983).

### 4.3. Limits of the model

The limits of the model are reached for volumes larger than 60 nl, when one wants to inactivate neurons further than 500  $\mu\text{m}$  lateral to the tip of the pipette. The model predicts that it is not possible to inactivate the neurons located 600 or 770  $\mu\text{m}$  lateral to the tip of the pipette. However, data from Table 1 contradicts this prediction. But the data collected with the pipettes 12 and 13 of Table 1 are not consistent (and were therefore excluded of the model): the threshold volume is higher for pipette 13 than for pipette 12, although the second one is further away from the recording electrode. Other paradoxical results obtained with a pipette located 940  $\mu\text{m}$  from the electrode have already been mentioned. We propose several reasons to explain these contradictory results. All these reasons start from the same argument: injection of GABA was considered in the model as instantaneous; GABA was injected and then diffused in the tissue. This simplification is however difficult to follow for injections of volumes as large as 50 or 100 nl, which do take time (a few seconds, up to 10 or 15 s) because of the repetition of brief pressure pulses.

#### 4.3.1. Eccentricity of the ellipsoids of GABA

Considering that injection is not instantaneous, the hypothesis that  $p$  depends linearly on the injected volume is not valid anymore. As a consequence, the eccen-

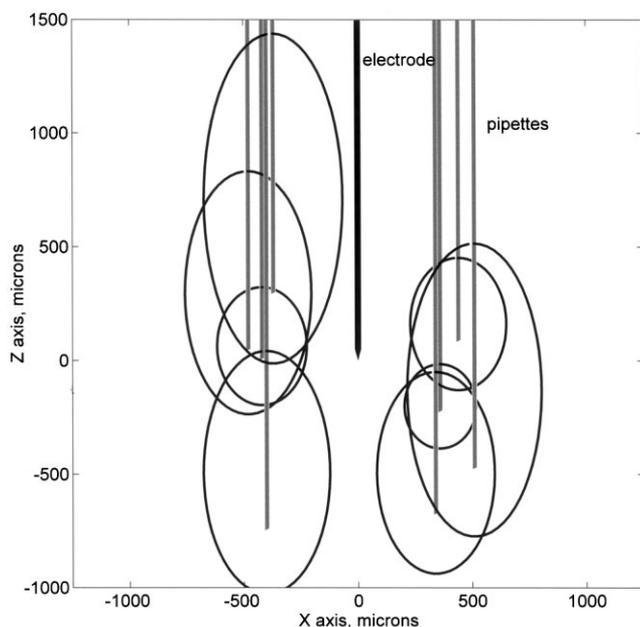


Fig. 9. Ellipses of the 60 s threshold volumes of GABA 100 mM for the eight pipettes kept in the model. Pipettes are plotted with respect to the recording electrode, following the coordinates given in Table 1. Ellipses are the ellipses of GABA in the extracellular space.  $\lambda = 0.21$ ,  $k = 7.1$ .  $k$  has been calculated for minimizing the distances between the ellipses and the electrode tip.

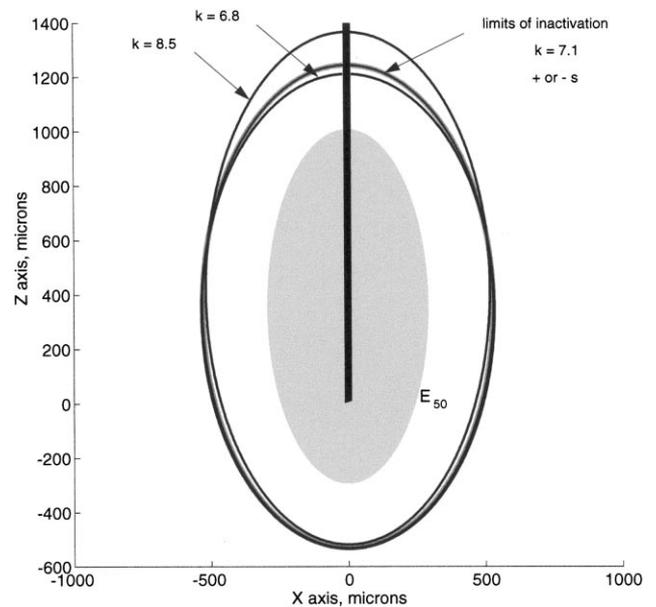


Fig. 10. Planar projection of the 60 s inactivated volume of brain tissue after a bolus injection of 50 nl of GABA 100 mM. The inner ellipse (light grey) is the ellipse of 50 nl of GABA in the extracellular space ( $E_{50}$ ) at the moment of injection. Then GABA diffuses into the tissue. Its concentration decreases as it goes away from  $E_{50}$ . Beyond 235  $\mu\text{m}$  away from  $E_{50}$ , concentration is too low to inactivate neurons during 60 s: the ellipse ( $k = 7.1$ ,  $d = 235 \mu\text{m}$ ) describes the limits of 60 s inactivation. In grey around this ellipse is a confidence interval for  $d$  ( $d - s$ ;  $d + s$ ;  $s = 10.5 \mu\text{m}$ ). The ellipses ( $k = 6.8$ ) and ( $k = 8.5$ ) are the ellipses of inactivated brain tissue for the limits of the confidence interval of  $k$ .

tricity of the GABA ellipsoids is overestimated for large volumes. Ellipsoids of GABA are therefore wider, and can inactivate neurons located further along the  $X$  axis, even at the level of the tip of the pipette.

#### 4.3.2. Recapture of GABA

When GABA injection lasts a few seconds, GABA starts to diffuse and is recaptured before the end of the injection. Recapture is very rapid (Dingledine and Korn, 1985): uptake mechanisms could probably remove 91% of the GABA in 4 s. Thus, at the end of injection, we never have the whole volume of injected GABA at the concentration of 100 mM.

If recapture during the injection of large volumes of GABA plays a significant role, the volume of inactivated cortex should be very sensitive to the parameters of delivery, such as the frequency of the puffs. We could confirm this sensibility with the pipette 10 of Table 1: we obtained 60 s inactivation when injecting 121 nl of GABA in 26 puffs at 1 IPS frequency, and 80 s inactivation with only 56 nl in 12 puffs, but at 2 IPS frequency (which we usually used). When doubling the frequency, we could reach the threshold of inactivation for some neurons with twice less GABA.

The volume of each puff should also be significant, as small puffs need to be repeated more often to achieve a

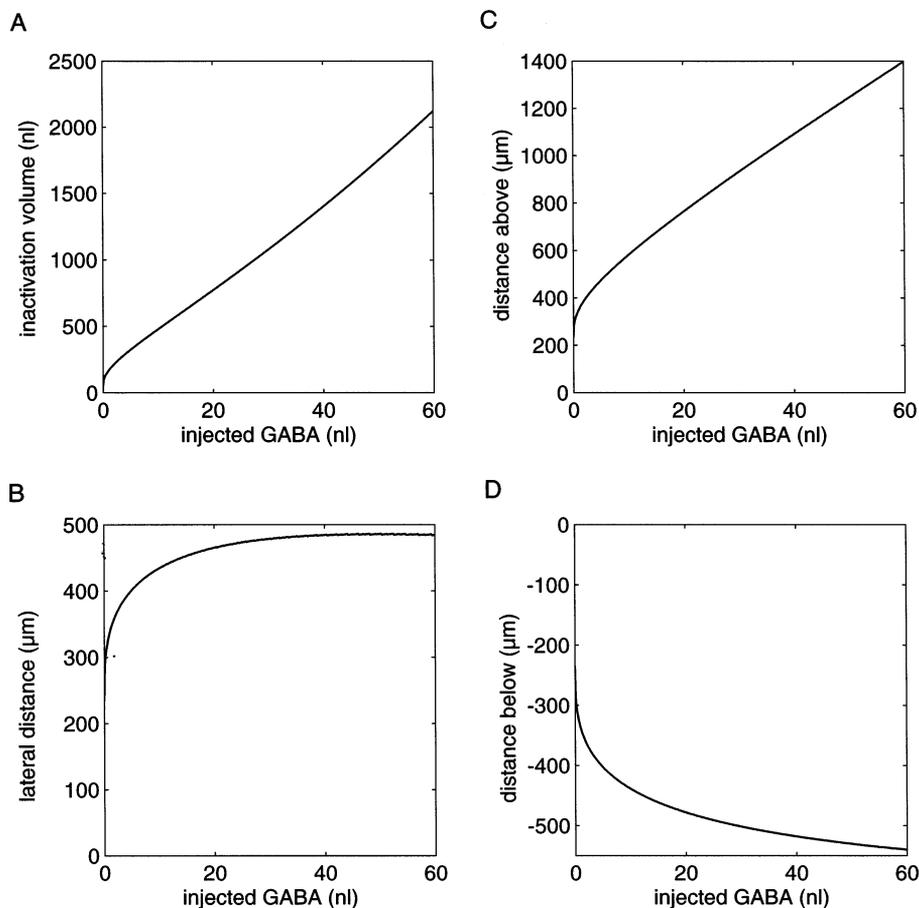


Fig. 11. (A) 60 s inactivation volume of brain tissue as a function of the volume of GABA 100 mM. (B, C, D) Distances of 60 s inactivation as a function of the volume of bolus injected GABA 100 mM. (B) The distance is measured from the tip of the pipette along the  $X$  axis (lateral distance). (C) The distance is measured along the  $Z$  axis between the tip of the pipette and the upper limit of 60 s inactivated brain tissue. (D) The distance is measured along the  $Z$  axis between the tip of the pipette and the lower limit of 60 s inactivated brain tissue.

large injection, which takes therefore more time. This seems to be the case for pipette 13, where the size of each puff was on average about twice as small as that of the average puff for pipette 12. More GABA was needed to reach the inactivation threshold of pipette 13, even if the distance was smaller than for pipette 12.

The longer the injection is—either because the size of the puff or the frequency of puffs—the more efficient is the recapture, and the larger is the volume of GABA needed to reach the inactivation threshold. However, it is not advised to increase the size of individual puffs or/and the frequency rate, because one could create tissue distortions, and probably change the characteristics of diffusion.

#### 4.3.3. Potentialization of inactivation

Another explanation can be provided for the inactivations observed at 600 and 770  $\mu\text{m}$ : as a large bolus injection is not instantaneous (2 puffs/s), it is somehow already a repetition of small injections. We know from the results about the temporal dynamics of GABA injections that repetition potentializes the du-

ration of the inactivation. The same phenomenon happens in the spatial domain: we observed that repeating an injection that is under the inactivation threshold for a given distance, without waiting for a sufficient recovery period, ends up inactivating the neurons recorded by the electrode. A possible mechanism underlying this potentialization could be a saturation of mechanisms of recapture and, or, of enzymatic degradation. If not eliminated, GABA would diffuse further (in terms of our model,  $d$  would increase with repetition of injections). Injection of large volumes should be therefore better modeled by a repetition of injections. Such a model is however difficult to implement because there are numerous influencing parameters, such as the precise volume of each individual puff, the speed of recapture and the nature of the mechanisms of saturation which could explain the potentialization.

Beyond 500  $\mu\text{m}$  in lateral distance, the relation between GABA volume and the distance of inactivation should be therefore determined for each case with glued microelectrodes. A limit exists however at about 800  $\mu\text{m}$  lateral distance.

#### 4.3.4. Homogeneity of the medium of diffusion

We made the hypothesis that brain tissue is homogeneous. However, we performed these experiments in the cat cortex, which has a thickness of about 1.5 mm. What happens when GABA is injected near interfaces such as white matter, pia or sulci? We repeated the experiments at various depths in the cortex, but we did not control for this parameter: some variability in the results (especially with the electrode located 940  $\mu\text{m}$  from the pipette) could probably be explained if we knew the position of the pipette relative to white matter and pia.

We think that injection and diffusion of GABA is greatly facilitated out of the cortex, as in pia or sulci. On the contrary, the resistance of the medium is higher in white matter, which acts as a barrier to diffusion: we used pipette 7 to test this barrier effect. Once we positioned the pipette and electrode on both sides of the white matter, another time we positioned the pipette in the white matter and the electrode in the cortex above. In both cases, injections of microliters of GABA could not inactivate the neurons recorded by the microelectrode located above the pipette. This phenomenon is a great advantage when one wants to test the functional connections of regions separated by only a few hundreds of microns of white matter, as the visual areas V1 and V2 of the macaque. We suppose that this barrier effect is due to the fact that GABA does not diffuse homogeneously in the white matter, but follows the fibers. A precise knowledge of the anatomy of the region than one wishes to inactivate is therefore needed.

#### 4.4. Suggestions to increase the inactivation volume

##### 4.4.1. GABA concentration

Diffusion is very sensitive to concentration, as shown by the published data: Bolz and Gilbert indicate that 100 nl of GABA 10 mM inactivate neurons located 350  $\mu\text{m}$  away from the pipette (Bolz and Gilbert, 1986); Nealey and collaborators inactivate neurons located 500  $\mu\text{m}$  away from the pipette with 100 nl of GABA 25 mM (Nealey and Maunsell, 1994). These low concentrations of GABA are however better suited to small volumes of inactivation, up to distances of 350  $\mu\text{m}$  from the pipette. Iontophoresis is a good alternative for such precise inactivations<sup>5</sup>. Inactivation is spherical with iontophoresis, as it is a point spread diffusion.

<sup>5</sup> If one prefers pressure injection to iontophoresis, it is possible to use our model for low concentrations of GABA, as long as volumes are inferior to 60 nl: the distance  $d$ , which is the only variable depending on the concentration, should be estimated for these low concentrations. Calculation of  $d$  would be performed with only one measure of threshold inactivation obtained with one electrode which distance to the injecting pipette is known along the  $X$  and  $Z$  axis.

In order to increase the volume of inactivated cortex, it is possible to increase the concentration instead of the volume of GABA. We tested pipette 4 with GABA 1 M: the threshold volume of inactivation was a few nanoliters, but not measurable with a sufficient precision. This range of volume is the one observed with GABA 100 mM and the pipette 1, 250  $\mu\text{m}$  away from the recording microelectrode. As we found that  $d$  is 236  $\mu\text{m}$  for GABA 100 mM, we suggest that  $d$  is about 440  $\mu\text{m}$  for GABA 1 M. It is therefore possible to inactivate neurons about 200  $\mu\text{m}$  further with GABA 1 M than with GABA 100 mM.

The drawback with increasing concentration of GABA is the recovery time: we were not always able to see recovery of the activity of the neurons recorded 50  $\mu\text{m}$  away from the pipette when injecting GABA 1 M. Recoveries were always very long. Such long recoveries are a problem for electrophysiological experiments. We therefore focused our attention on GABA 100 mM and did not make any exhaustive study of GABA 1 M injections.

##### 4.4.2. Multi-pipettes devices

To increase the volume of inactivated brain tissue without increasing recovery duration, one can also use several pipettes at the same time. We have chosen this solution to test the effect of the inactivation of monkey visual area V2 on the responses of visual area V1. We build compound devices with three pipettes and one to three microelectrodes. Pipettes are arranged in a triangular fashion. The distance between the pipettes is 1 mm. Fig. 12 shows the three pipettes as we position them in area V2. Ellipsoids of inactivation are plotted

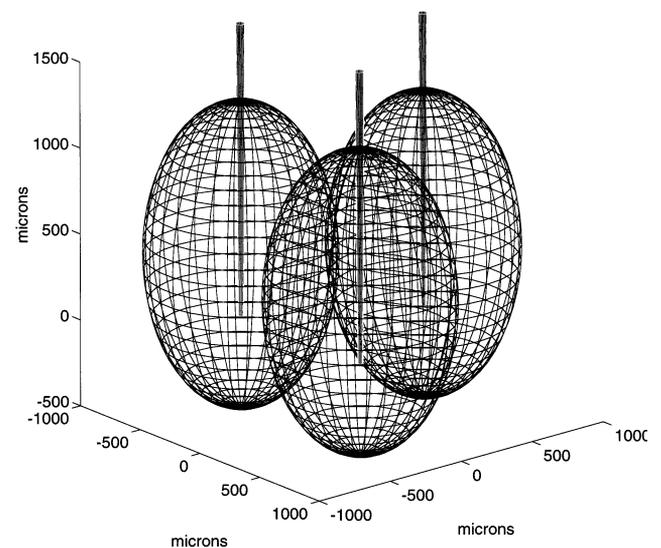


Fig. 12. 60 s inactivation volume obtained with simultaneous injections of 50 nl of GABA 100 mM in each of three pipettes. ( $z = 0$ ) = tip of the pipettes. The distance between the tips of the pipettes is 1 mm.

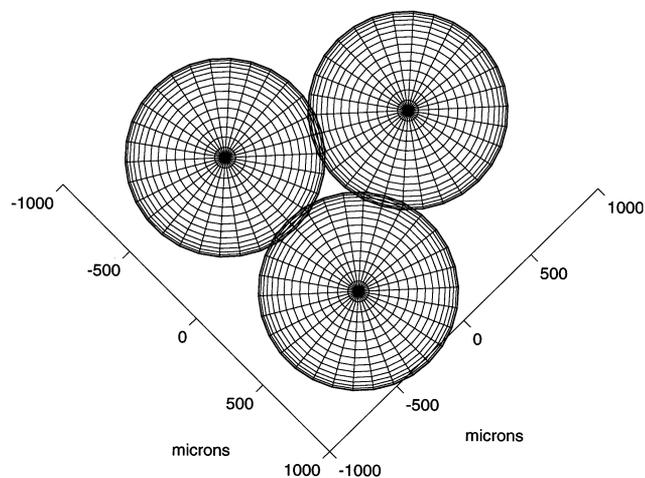


Fig. 13. Horizontal section of Fig. 12 showing the maximal lateral extent of inactivation. ( $z = 355 \mu\text{m}$ ).

for an injection of 50 nl in each pipette. Fig. 13 shows the maximal lateral extent of inactivation. Almost all the thickness of area V2 (1.5 mm) is inactivated within a diameter of about 2 mm. We could repeat many injections of GABA at the same site: pipettes remain usually at the same site in V2 for 1 or 2 days. Quality of inactivation and recovery do not decrease with time and repetition of injections.

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