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Chapter 6

Pharmacological manipulation of neuronal ensemble activity by reverse microdialysis in freely moving rats: a comparative study of the effects of tetrodotoxin, lidocaine, and muscimol

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Abstract

To be able to address the question how neurotransmitters or pharmacological agents influence activity of neuronal populations in freely moving animals, the combidrive was developed. The combidrive combines an array of 12 tetrodes to perform ensemble recordings with a moveable and replaceable microdialysis probe to locally administer pharmacological agents. In this study, the effects of cumulative concentrations of tetrodotoxin, lidocaine, and muscimol on neuronal firing activity in the prefrontal cortex were examined and compared. These drugs are widely used in behavioral studies to transiently inactivate brain areas, but little is known about their effects on ensemble activity and the possible differences between them. The results show that the combidrive allows ensemble recordings simultaneously with reverse microdialysis in freely moving rats for periods at least up to 2 wk. All drugs reduced neuronal firing in a concentration dependent manner, but they differed in the extent to which firing activity of the population was decreased and the in speed and extent of recovery. At the highest concentration used, both muscimol and tetrodotoxin (TTX) caused an almost complete reduction of firing activity. Lidocaine showed the fastest recovery, but it resulted in a smaller reduction of firing activity of the population. From these results, it can be concluded that whenever during a behavioral experiment a longer lasting, reversible inactivation is required, muscimol is the drug of choice, because it inactivates neurons to a similar degree as TTX, but it does not, in contrast to TTX, affect fibers of passage. For a short-lasting but partial inactivation, lidocaine would be most suitable.

Introduction

Until recently, neurophysiological analysis of information processing in the brain was primarily based on the examination of firing activity of single cells during behavior, as measured with repetitive presentations of stimuli (Gerstein and Kiang, 1960). However, this could not provide an answer to the question of how information is represented by the pattern of activity distributed across a population of neurons. With the emergence of techniques to record large numbers of neurons simultaneously ("ensemble recordings"), it became possible to examine information coding at the level of cell populations (Wilson and McNaughton, 1993). However, an issue that has not been addressed thus far is how neurotransmitters influence the activity of these cell populations.

To gain more insight in the interaction between neurotransmitters or pharmacological agents and neuronal firing activity, we sought to develop a method in which drugs could be locally administered while performing ensemble recordings in freely moving rats. Because drugs should ideally be delivered with a constant concentration throughout the experimental session within the entire recording area, reverse microdialysis is preferred over either local injections, because with injections additional fluid is introduced into the brain, causing a change in pressure; or iontophoresis, with which only a very small area can be reached.

The combination of (reverse) microdialysis with extracellular electrophysiological recordings in vivo was initially applied in research concerning hypoglycemia, the pathophysiology of cerebral ischemia, and epilepsy. In those studies, performed in freely moving or anesthetized animals, a single recording electrode for monitoring the electroencephalography was glued next to the dialysis probe (Vezzani et al., 1985; Sandberg et al., 1986; Ludvig et al., 1992), inserted in the inflow tubing (Obrenovitch et al., 1991), or placed within the proximity of the dialysis probe (Tossman et al., 1985). Later, microdialysis/electrode devices were developed that were suitable to perform single-unit recordings in freely moving rats, cats, and monkeys (Dudkin et al., 1996; Ludvig et al., 1994, 2000; Sakai and Crochet, 2000). The devices used in rats consisted of a fixed microelectrode array positioned next to a guide in which the dialysis probe was fitted (Ludvig et al., 1994; Brazhnik et al., 2004). Although these studies did not present data concerning the effect of perfusion per se on neuronal activity, the results did show the possibility of recording single units and influencing their activity by drug administration. A recent study in anesthetized animals in which reverse microdialysis was combined with intracellular recordings furthermore demonstrated that effects of dialysis on the membrane properties, excitability, and ongoing synaptic activity of neurons in the vicinity of the probe are minimal (West et al., 2002). Hence, this suggests that the technique of reverse microdialysis is suited to be combined with a multitetrode array (Gothard et al., 1996) to conduct local pharmacological interventions during ensemble recordings.

To this end, the combidrive was developed, a multitetrode array consisting of a circular row of 12 individually movable tetrodes and two reference electrodes surrounding a movable microdialysis probe. Unlike the already existing recording devices, this design should allow use for several weeks after implantation, because the dialysis probe can be replaced if necessary. Furthermore, the electrophysiological recordings are performed with a multitetrode array instead of single electrodes, keeping the advantages of tetrodes in isolating single units and yielding high numbers of cells (McNaughton et al., 1983; Recce and O'Keefe, 1989; Gray et al., 1995). During assessment of combidrive functioning, several issues were addressed, including whether firing activity of neurons in the prefrontal cortex would be affected by perfusion per se and whether the dialysis probe could be replaced without loss of recording capacity. The combidrive was applied in a comparative study of three drugs that all exert an inhibitory effect on neuronal activity but that differ in mechanism of action and physical-chemical properties, namely, the sodium channel blockers lidocaine and tetrodotoxin and the $GABA_A$ agonist muscimol. Although these drugs are widely applied in behavioral studies to transiently inactivate selective brain areas (Albert and Mah, 1973; Brioni et al., 1989; Ivanova and Bures, 1990), little is known about the dynamics of their inhibitory effect in relation to population activity in awake animals. For example, these drugs are known to differ in duration of the inhibitory effect (Boehnke and Rasmusson, 2001), but it remains unclear to what extent neurons within a population and the neuronal population as a whole respond to the various drugs. Furthermore, a comparison of the effects of the drugs on neuronal activity within freely moving animals has not been made until now.

Materials and Methods

Subjects

All experiments were approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences, and they were carried out in agreement with Dutch Law (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC). Data were collected from four male Wistar rats (Harlan CPB, Horst, The Netherlands), weighing 360 to 425 g at the time of surgery. Animals were housed in standard type 4 macrolon cages. They were weighed, handled daily, and kept under a reverse 12-h light/dark cycle (dimmed red light at 7:00 AM) with free access to food and water (standard rat chow; Hope Farms, Woerden, The Netherlands). After surgery, animals were housed individually in a larger cage (1 x1 x 1 m) under the same conditions.

Construction of the Combidrive and Microdialysis Probes

The combidrive presented here was custom-built at the Netherlands Institute for Neuroscience (Royal Netherlands Academy of Arts and Sciences, Amsterdam, The Netherlands), and it was adapted from the multielectrode drive array ("hyperdrive") as described by Gothard et al. (1996). The bundle of this hyperdrive, which contains 12 individually movable tetrodes and two reference electrodes, was modified to fit a microdialysis probe in the center. This resulted in a design in which the dialysis probe is surrounded by a circular row of 12 tetrodes and two reference electrodes, with a distance of 550 μm between the surface of the dialysis probe and the center of a tetrode. The total weight of the combidrive was 32 g, the height (without guide tube for the probe) was 4.4 cm, and the diameter at the site where the connectorboard was attached was 3.3 cm (Fig. 1, A to C).

Tetrodes were constructed as described by Gray et al. (1995). In brief, four nichrome wires (diameter, 13 μm; Kanthal, Palm Coast, FL) were twisted together, and a microbundle was formed by melting the polyimide coating with a heat gun. Electrode tips were goldplated with the use of a gold cyanide solution (Select Plating, Meppel, The Netherlands) to achieve an impedance range of 0.1 to 1.0 MΩ.

To be able to reach all subareas of the prefrontal cortex (including the ventral part), concentric dialysis probes with a length of 8 cm were constructed from two pieces of fused silica (0.075 mm i.d., 0.150 mm o.d.) that were inserted into a wider piece of fused silica (0.320 mm i.d., 0.430 mm o.d.) (Aurora Borealis Control, Schoonebeek, The Netherlands). Both pieces protruded approximately 1 cm on one side of the outer fused silica to serve as inlet and outlet, which were both protected by 25-gauge needles. On the opposite side, the part of the probe entering the brain, one piece of the inner fused silica protruded 3 mm, whereas the other piece remained 1 cm inside. A Hospal AN69 membrane (0.240 mm i.d., 0.320 mm o.d.) with a recovery over the membrane of about 10 to 15% was closed with (two-part epoxy) glue and fitted over the inner and in the outer fused silica. An exposed length of approximately 2 mm was used for dialysis. To fit the dialysis probe in the combidrive, a 7-cm-long stainless steel guide tube (0.5 mm i.d., 0.9 mm o.d.) was inserted along its central axis. To attach the dialysis probe to this guide tube, a coupling nut (2.5 mm o.d.) was glued to the outer fused silica just beneath the 25-gauge needles; together with the thread on the guide tube, this nut formed a screwed joint. By means of a worm-gear transmission, the guide tube can be lowered and raised, resulting in the downward and upward movement of this guide. Whenever the tube is maximally lowered, it fits in the central channel of the bundle, and it does not exceed the length of the bundle. Hence, the guide tube is prevented from entering the brain, whereas in this position the probe has reached its maximal depth in the brain. Replacement of the probe is achieved by raising the probe out of the brain and removing it from the guide, after which a new probe can be inserted and lowered again.
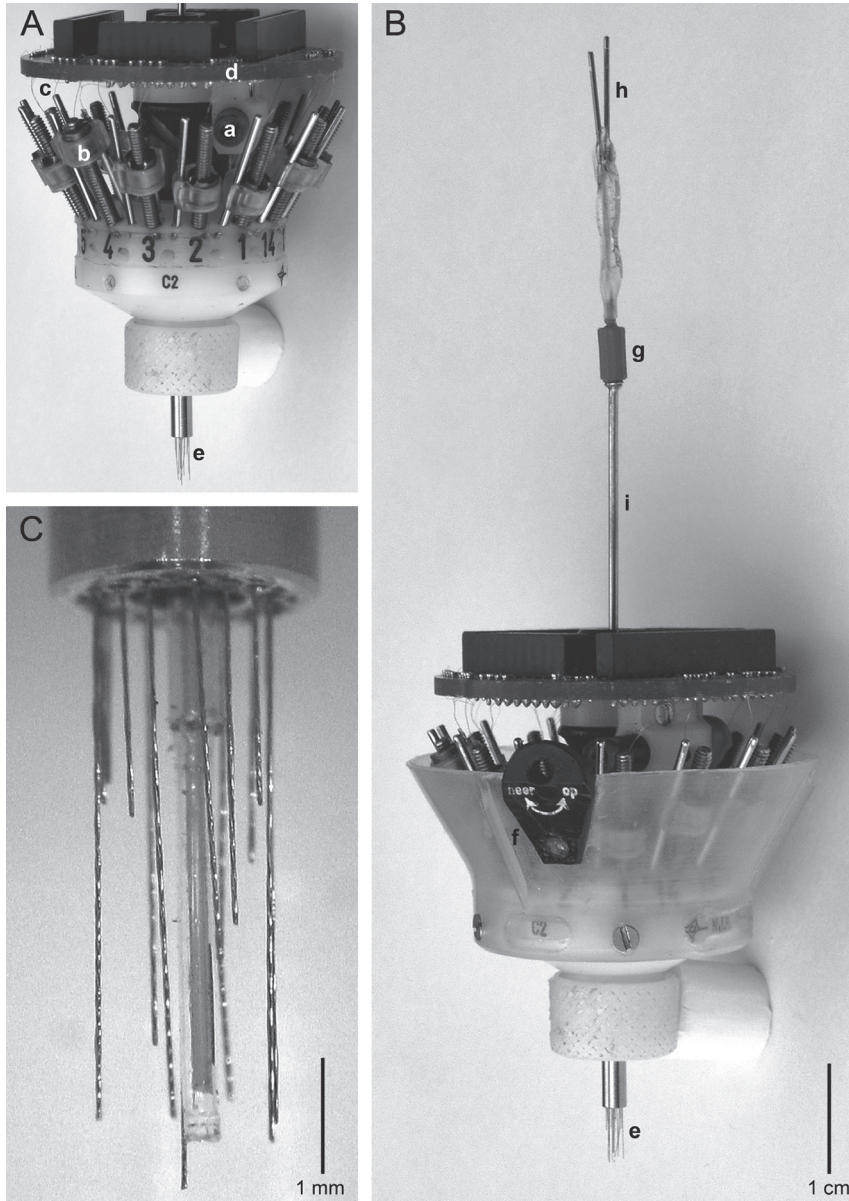
Fig. 1. The combidrive (design based on Gothard et al., 1996). A, close-up of the combidrive without its protective covers: screw that drives the upward and downward movement of the guide for the microdialysis probe (a), one of 14 drive screws that allow individual (vertical) movement of the tetrodes (b), individual wires of a tetrode (c) connecting to the printed circuit board (d), and bundle through which tetrodes and microdialysis probe exit the combidrive (e). B, overview of the entire combidrive showing the bundle (e), protective cover (f), screwed joint connecting the microdialysis probe to the guide (g), inlet and outlet of the microdialysis probe (h), and guide for the microdialysis probe (i). C, magnification of the bundle showing 14 tetrodes, surrounding the microdialysis probe.

Surgery and Electrophysiology

Animals were anesthetized with 0.08 ml/100 g Hypnorm i.m. (0.2 mg/ml fentanyl, 10 mg/ml fluanison) and 0.04 ml/100 g of Dormicum s.c. (5 mg/ml midazolam), and then they were mounted in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). After the incision, additional local anesthesia (10% Xylocaine spray; Astra Hässle AB, Mölndal, Sweden) was applied to the skull as well. Body temperature was maintained at 37.5°C using a heating pad. After exposure of the cranium, six small holes were drilled into the cranium to accommodate surgical screws; one screw served as ground. Another larger hole was drilled over the prefrontal cortex in the left hemisphere (the center of the hole was 3.6 mm anterior, 3.2 mm lateral to bregma according to Paxinos and Watson, 2005). The dura was opened, and the bundle of the combidrive was lowered onto the exposed cortex, after which it was anchored to the screws with dental cement. To protect the brain from the dental cement, the hole was first filled with a silicone elastomer (Kwik-Sil; WPI, Sarasota, FL). Immediately after surgery, all tetrodes were advanced 1.5 mm into the brain, whereas the reference electrodes were lowered 1 mm. The microdialysis probe was slowly lowered into the brain (5 mm below cortical surface) over a time course of 30 min. Over the course of the next 3 days, tetrodes were gradually lowered until they were within range of the dialysis membrane, after which experiments started. Electrophysiological recordings were performed using a Cheetah recording system (Neuralynx, Tucson, AZ). Signals from the individual leads of the tetrodes were passed through a low noise unity-gain field-effect transistor preamplifier, insulated multiwire cables, and a fluid-enabled 72-channel commutator (Dragonfly Inc., Ridgeley, WV) to digitally programmable amplifiers (gain, 5000 times; band-pass filtering, 0.6–6.0 kHz). Amplifier output was digitized at 32 kHz and stored on a Windows NT station. A 1-ms data sample was taken whenever the signal crossed a preset voltage boundary, so that the width of a recorded spike was captured in 32 data points.

Pharmacological Agents and Fluid Connections

Muscimol hydrobromide and lidocaine hydrochloride were obtained from Sigma Chemie (Deisenhofen, Germany). Tetrodotoxin (TTX) was obtained from Tocris Cookson Inc. (Bristol, UK). For muscimol and TTX, stock solutions of 1.00 mM in Milli-Q water (Millipore Corporation, Billerica, MA) were made and stored at -80°C; they were further diluted with phosphate-buffered artificial cerebrospinal fluid (aCSF), containing 143 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.26 mM NaH_2PO_4, and 1.74 mM Na_2HPO_4, pH 7.4. Lidocaine was dissolved in aCSF before every experiment. All solutions were controlled for osmolality (micro-osmometer, model 3300; Advanced Instruments, Norwood, MA; allowed range, 270–290 mOsM) by correcting the amount of NaCl in the aCSF based on the Merck index for sodium equivalents, and, if necessary, adjusted for pH (allowed range, 7.2–7.6).

A Univentor 801 microinfusion syringe pump (Univentor, Zejtun, Malta) was used to pump the solution through 167-cm-long polyetheretherketone tubing (0.51 mm o.d., 0.13 mm i.d.; Aurora Borealis Control) that ran via one channel of a quartz-lined dual-channel swivel (Pronexus, Skärholmen, Sweden) and the central channel of the commutator toward the inlet of the dialysis probe (flow rate, 2 μl/min). All connections were made of polyvinylchloride tubing (0.38 mm i.d.). Switching between the different solutions was done by detaching the polyetheretherketone tubing and connecting it to a second, pressurized syringe pump. Between sessions all tubing was rinsed with Milli-Q water and methanol.

Experiments

Control Experiments

At the start of an experimental day, the animal was connected to the system, and the flow-through dialysis probe was checked. To record new neurons during each recording session, tetrodes were lowered with increments of 40 μm under continuous perfusion of aCSF. Once the tetrodes were lowered, the animal was placed in the recording chamber (40 x 37 x 41.5 cm) and left for at least 1 h to stabilize unit recordings, after which the actual recording started. The recording chamber was placed in a sound attenuated and electrically shielded box, fitted with a motion detector to monitor locomotor activity of the animal. Control experiments were performed to determine whether the design of the combidrive actually allowed electrophysiological recordings during reverse microdialysis and whether aCSF perfusion per se would influence firing activity of single units. To this end, recordings of baseline firing activity were made during a 20-min period with no flow, followed by the pump being switched on (referred to as "pump switch") and an aCSF perfusion period of 20 min.

Furthermore, to examine the effect of replacement of the probe on the activity of the surrounding neurons, the dialysis probe was changed in a separate session under continuous recording of neuronal activity. Probe replacement was done by hand over a time course of 60 min (for both raising and lowering 30 min).

Pharmacological Interventions

After finishing the control experiments, the effects of several drugs on neuronal firing were examined. One experimental session was carried out per day with a single drug, with each drug tested at least two times in at least two animals. The general procedure within these recording sessions was to record baseline neural activity for 20 to 30 min during aCSF perfusion, followed by perfusion of various drug concentrations for 30 min each. Cumulative concentration-effect curves were made for all drugs, consisting of 0.074, 0.74, 7.4, and 74 mM (0.002–2%) solutions for lidocaine (2% is the highest

concentration of lidocaine within the physiological range for which osmolality could be controlled); 1.0, 3.0, 10.0, and 30.0 μM solutions for muscimol; and 0.01, 0.03, 0.1, 0.3, and 1.0 μM solutions for TTX. The session ended with a washout period during which aCSF was perfused. This period was variable in duration for the various drugs, because the (qualitative) criterion to end washout was the recovery of neural firing as visible on the oscilloscope (not necessarily back to baseline firing rate). Whenever the dialysis probe was clogged, it was replaced, with a maximum of two new probe insertions per animal to prevent extensive tissue damage. After the insertion of a new probe, recordings continued the next day to allow the tissue to recover.

In addition, because the recording sessions in which drug perfusions occurred were long-lasting (up to 6 h), control sessions of a similar duration were performed, either with or without aCSF perfusion. These control sessions served to exclude the possibility that observed changes in neuronal firing could be ascribed to natural changes in the firing activity of single units, instead of being the result of the drug perfusions.

Data Analysis

Single units were isolated by off-line cluster cutting procedures (BBClust/MClust-3.0). Before a cluster of spikes was accepted as belonging to a single unit, several parameters were checked visually, namely, the averaged waveforms across the four leads, the cluster plots showing spike parameter distributions such as peak amplitudes across the four dimensions, the autocorrelogram, and the spike interval histogram. Because the absence of spike activity during the refractory period (2 ms) is indicative of good isolation, units of which the autocorrelogram and the spike interval histogram revealed any activity during this period were removed from the analysis.

Control Experiments

For the statistical analysis, only single units were included that had a baseline firing rate of at least 0.1 Hz and that were active throughout the entire recording session. To determine the effects of aCSF perfusion on neuronal firing activity, the normalized firing rate was calculated in blocks of 5 min. The final 5-min block of the baseline period served as control (100%) value. The final 5-min block of both conditions (i.e., baseline without flow versus aCSF perfusion) was compared using a repeated measures ANOVA ($p<0.05$) with time (block) as within-subject variable (SPSS for Windows, version 12.0.1; SPSS Inc., Chicago, IL). A possible effect of the pump switch was assessed in a similar manner, although in this case the final 5-min block of the baseline condition was compared with the first 5-min block of the aCSF perfusion period. If indicated by Mauchly's test of sphericity, a Huynh-Feldt correction was applied to adjust the number of degrees of freedom. To examine whether the replacement of the probe influences recording capacity, the number of single units recorded before and after replacement of the probe was compared.

Pharmacological Interventions

Single units that were removed from the analysis included neurons with a baseline firing rate less then 0.1 Hz and units that exceeded the 99% confidence interval of the mean firing rate of the population (i.e., 3 standard deviations from mean baseline firing). Furthermore, neurons that did not show any activity in the last 30 min of the washout period were discarded; this was done to exclude cells that stopped firing for reasons other than drug perfusion. To examine drug effects, the final 5-min block of each perfusion period was compared with the activity in a 5-min block at the corresponding time point during the aCSF control sessions. A repeated measures ANOVA ($p<0.05$) was performed with group (i.e., drug and aCSF) as the between-subjects variable and time as the within-subjects variable. Whenever a group or interaction effect was found, additional *t* tests were performed to determine which 5-min blocks were different. To examine possible effects of time within groups, an ANOVA with repeated measures was performed over the separate groups as well ($p<0.05$); whenever a time effect was found, a simple (first) contrast was performed to examine which block differed from baseline firing activity. Based on the number of comparisons, the α-value was adjusted by a Bonferroni correction. Furthermore, the number of degrees of freedom was adjusted by a Huynh-Feldt correction when indicated by Mauchly's test of sphericity. Based on firing rate and valley width, putative interneurons were initially separated from pyramidal cells. However, due the small amount of interneurons recorded per drug (muscimol, 12, lidocaine, 1; and TTX, 2), all data were pooled.

To assess whether the observed drug responses for the different drugs were identical over all sessions, which were spaced across non-consecutive days and were recorded in different animals, a repeated measures ANOVA ($p<0.05$) was performed over all sessions with sessions as the between-subjects variable.

A comparison of the relative reduction of firing activity between the three drugs was made by comparing the cumulative reduction at the highest concentration. Two-sample Kolmogorov-Smirnov tests for equality of distribution were used to assess whether each of the drugs caused different degrees of inhibition.

Histology

The final position of the tetrodes was marked by passing a 10-s, 25-μA current through one of the leads of each tetrode to induce a lesion and initiate gliosis. After 24 h, the animal was perfused transcardially using a 0.9% saline solution followed by 10% formalin. After removal from the skull, the brain was stored in 10% formalin for several days before sectioning. Brain sections (40 μm) were cut using a vibratome, and they were Nissl-stained to identify the location of the probe and to reconstruct the tracks of the tetrodes and their final position.

Results

Histology

Histological verification of the positions of both tetrodes and microdialysis probe showed that the recording sites and probes in all animals were located in the orbital and lateral areas of the prefrontal cortex. The placement of the probe in each of the four animals is shown in Fig. 2A, together with a representative section (Fig. 2B) showing the location of the probe and the endpoint of three different tetrodes (Paxinos and Watson, 2005). In two animals where the probe was replaced once, the extent of tissue damage was comparable with that of the animals in which a single probe was inserted.
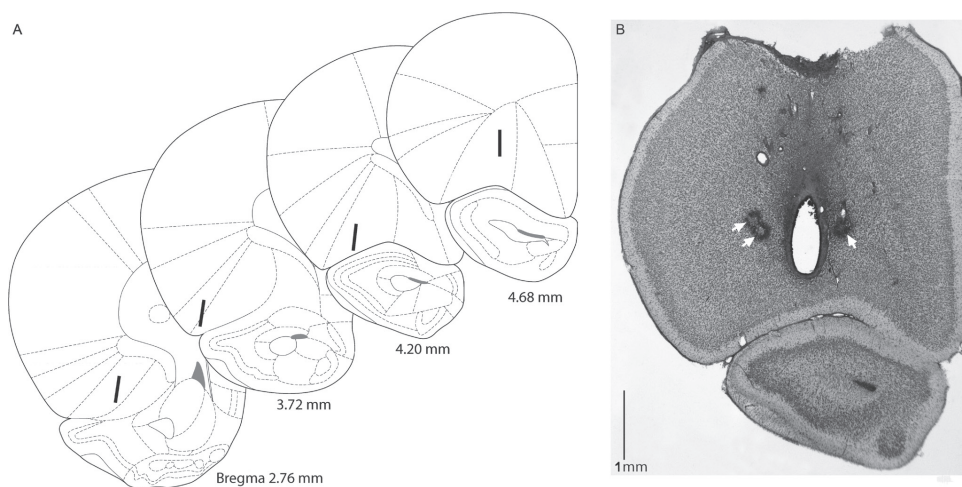

Fig. 2. Localization of the microdialysis probe and tetrode recording sites in the four animals (A), and a representative histological slide showing probe location and three marked endpoints of different tetrodes (indicated by a white arrow) (B). The white area in the center of the section marks the location of the probe tip. Recordings in all rats were localized in the prefrontal cortex, primarily the orbital and lateral areas (Paxinos and Watson, 2005).

Control Experiments

During all experiments in the recording chamber, animals did not show any sign of distress due to the implanted combidrive or the attachment to the recording equipment, and were able to behave normally. The effect of aCSF perfusion and pump switch on firing activity of neurons was examined in a single session in one rat in which 23 cells were recorded, 17 of which passed the criteria for statistical assessment. Mean firing rates ranged between 0.11 and 3.83 spikes/s. No effects of the aCSF perfusion or pump switch on the baseline firing activity of these cells were found [baseline: firing rate

(average±S.E.M.), 1.00±0.27 Hz; pump switch: 0.99±0.26 Hz; $F(1,16)=0.598$, $p=0.451$; and aCSF perfusion: 0.71±0.21 Hz; $F(1,16)=0.292$, $p=0.597$].

The probe was replaced under continuous recording, during which a total number of 59 single units was recorded Examination of the spike waveforms before and after the probe change suggested that 48 U (81%), recorded before the probe replacement started, were still present when replacement was finished and the new probe had been inserted. Furthermore, 8 U (14%) stopped firing during probe movement and they were lost, whereas 3 U (5%) newly occurred during the probe change. Hence, a total number of 56 U were recorded before and 51 U were recorded after replacement of the probe. Fig. 3, A and B, shows an example of neuronal activity before and after the probe was raised.
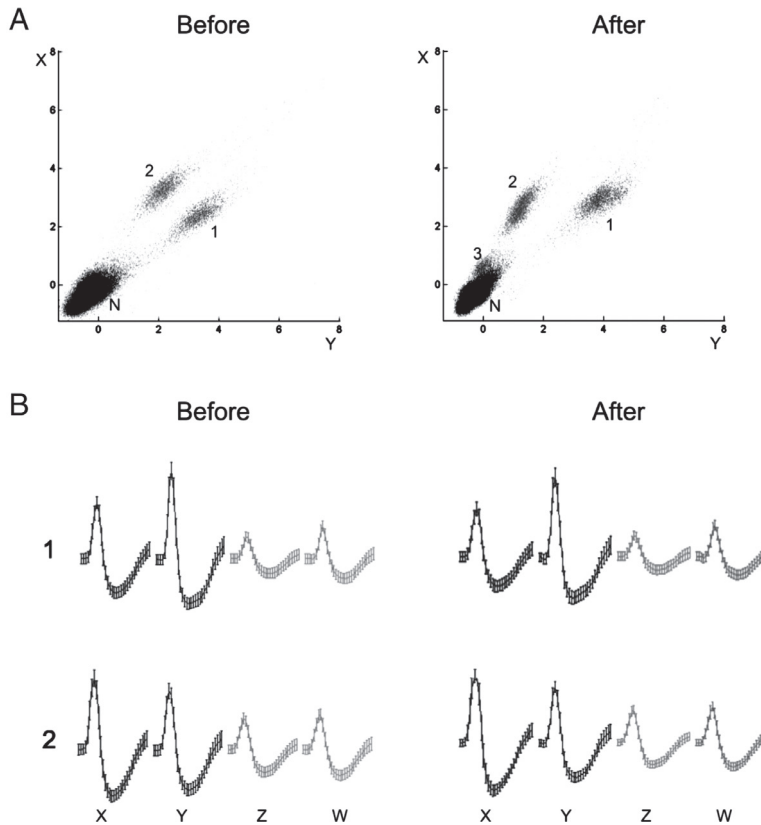

Fig. 3. Plots showing peak amplitudes of neuronal activity as recorded on a single tetrode before and after probe movement. A, before movement of the probe, two different clusters (1 and 2) representing two different single units (B) were recorded, which were still present when probe movement was finished as suggested by similarity in waveform and lack of major shifts in the cluster plots. However, a possible new cluster occurred (cluster 3), which was still close to the noise (N) (not shown in B). B, average waveforms across the four leads of the tetrode of clusters 1 and 2.

Pharmacological Interventions

During the recordings with drug perfusions and the aCSF control sessions (n = 15), a total number of 348 single units was recorded. No effect of drug perfusion on the motor activity of the animals was observed. During the four aCSF control sessions, 69 single units were recorded, of which 29 passed the criteria for the statistical analysis.

During four sessions with muscimol perfusion, a total number of 161 neurons was recorded, of which 92 were statistically assessed (Fig. 4). A main effect of time [$F(3.586,426.731)=9.969$, $p=0.000$] and group [$F(1,119)=14.002$, $p=0.000$] was found, as well as a group x time interaction [$F(3.586,119)=5.999$, $p=0.000$]. An additional t test revealed a significant reduction of neuronal firing activity compared with the aCSF control during perfusion of the 10 and 30 µM muscimol solutions (10 µM: $t=$-2.560, $p=0.000$; and 30 µM: $t=$-4.148, $p=0.016$) but not at the end of the washout (Fig. 5A). Furthermore, analysis of the individual groups showed a main effect of time for muscimol [$F(2.420,220.201)=37.209$, $p=0.000$] but not for the aCSF control: firing activity during perfusion of 3, 10, and 30 µM muscimol solutions and at the end of the washout (after 3 h) was significantly reduced compared with baseline.

A total number of 71 single units was recorded during five recording sessions with lidocaine perfusion. Of these neurons, 44 were statistically assessed. A main effect of time [$F(2.914,206.861)=10.251$, $p=0.000$] and a group x time interaction was found [$F(2.914,206.861)=3.947$, $p=0.009$]; the post hoc t test revealed a significant reduction in neuronal firing activity compared with the aCSF control during the perfusion of 74 mM lidocaine solution ($t=$-2.457, $p=0.016$) but not at the end of the washout (Fig. 5B). The analysis of the individual groups showed a main effect of time for lidocaine [$F(1.551,66.681)=16.137$, $p=0.000$] but not for the aCSF control; compared with baseline, neuronal firing activity was significantly reduced during perfusion of 74 mM lidocaine and at the end of the washout (after 30 min).
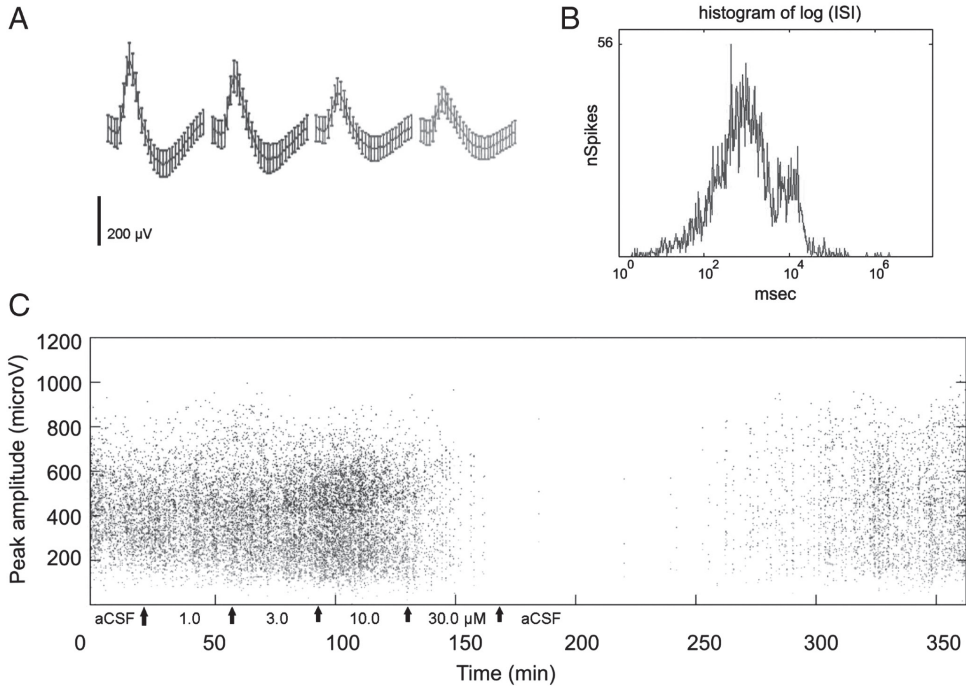
Fig. 4. Example trace of the activity of a single unit during muscimol perfusion. A, average waveform across the four leads of the tetrode (width, 1 ms). B, graph showing the distribution of the spike intervals (ISI). C, activity of the neuron during the cumulative perfusion of aCSF and 1, 3, 10, and 30 μM muscimol. During perfusion of 30 μM, activity gradually disappeared, and it recovered during the washout period when aCSF was perfused. Horizontal axis, time (minutes); vertical axis, peak amplitude across the four different leads (microvolts). The arrow indicates entrance in the brain.

During two sessions in which TTX was perfused, a total number of 47 single units was recorded, of which 20 were used for the statistical assessment. A main effect of time was found [$F(4.058,190.711)=8.194$, $p=0.000$], as well as a group x time interaction [$F(4.058,190.711)=2.879$, $p=0.023$]. An additional t test showed a significant reduction in neuronal firing activity compared with the aCSF control during the perfusion of the 0.1, 0.3, and 1.0 μM solutions and at the end of the washout period (0.1 μM: $t=-2,569$, $p=0.013$; 0.3 μM: $t=-2.249$, $p=0.036$; 1.0 μM: $t=-2.249$, $p=0.033$; and washout: $t=-3.262$, $p=0.003$) (Fig. 5C). Analysis of the individual groups showed a main effect of time for TTX [$F(1.638,31.121)=14.297$, $p=0.000$] but not for the aCSF control. During TTX perfusion, firing activity was significantly reduced during the 0.1, 0.3, and 1.0 μM TTX applications and at the end of the washout period (after 2.5 h) compared with baseline.
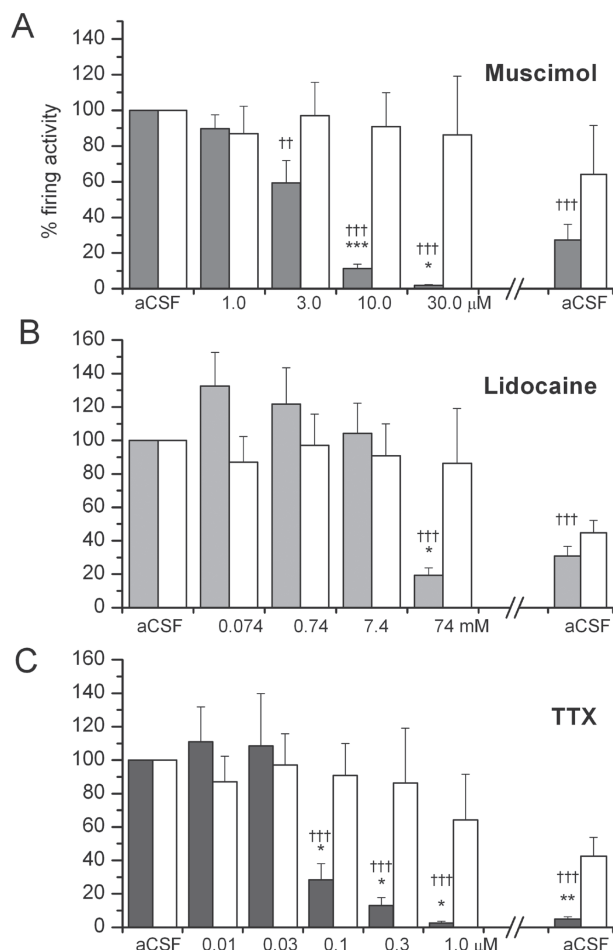
Fig. 5. Cumulative concentration-effect curves for muscimol (A; $n=92$), lidocaine (B; $n=44$), and TTX (C; $n=20$). The normalized firing activity during aCSF control (white bars) and drug (gray bars) perfusion is depicted across concentrations (mean±S.E.M.). The washout periods were 3 h (A; muscimol), 30 min (B; lidocaine), and 2.5 h (C; TTX). †, significant difference compared with baseline firing activity. *, significant difference between groups (aCSF control and drug evaluated at a similar time point). Three symbols, $p<0.000$; two symbols, $p<0.01$; one symbol, $p<0.05$. Horizontal axis, condition of perfusion; vertical axis, normalized firing activity.

These results indicate that, in addition to the aCSF perfusion period of 20 min that was used in the control experiments, longer aCSF perfusion periods that were performed to serve as control for the drug perfusions did not significantly affect firing activity either, because no time effect was found, in contrast to the drug perfusions. It can be concluded that duration of perfusion does not affect stability of neuronal activity, but it should be noted that firing activity of the population tended to decrease during these

aCSF control sessions. A possible explanation for this could be the biological state of the animals, for example, a diminished arousal due to the fact that they stayed for 6 h in the same recording environment.

The replaceable dialysis probe allowed the use of animals for repeated recording sessions, because the probe could be changed when needed; in two animals, the probe needed replacement once for both animals after the fourth recording session. For the other two animals, no probe replacement was needed.

Figure 6 shows the distribution of the reduction in firing rate of neurons within the population at the highest concentration for each drug. For both TTX and muscimol, firing of about 40% of the cells was abolished, whereas for lidocaine this was about 15% of the cells. A repeated two-sample Kolmogorov-Smirnov test revealed that the distributions of both TTX and muscimol were significantly different from lidocaine ($Z=2.242$, $p=0.000$ and $Z=3.380$, $p=0.000$, respectively), but they did not differ from each other ($Z=0.846$, $p=0.472$). This result indicates the relatively low homogeneity in neuronal response of the population to lidocaine compared with TTX and muscimol, as illustrated in figure 6. However, examination of the activity of individual neurons during drug perfusion revealed that for all drugs, including muscimol and TTX, variability in neuronal responses existed among neurons. Differences in the duration after which the firing activity of neurons decreased during perfusion were observed when neurons were recorded on different tetrodes and even when they were recorded on the same tetrode. This is illustrated in figure 7, in which responses of individual neurons during muscimol perfusion are shown that were recorded on either the same tetrode (Fig. 7A) or on different tetrodes (Fig. 7B).

Because drug sessions were spaced across non-consecutive days and they were recorded in different animals with different dialysis probes, the similarity between the observed neuronal responses for all different drugs over all sessions was examined as well. This revealed no group or interaction effect, meaning that the effect of a particular drug on neuronal firing activity was identical with different dialysis probes across all animals.
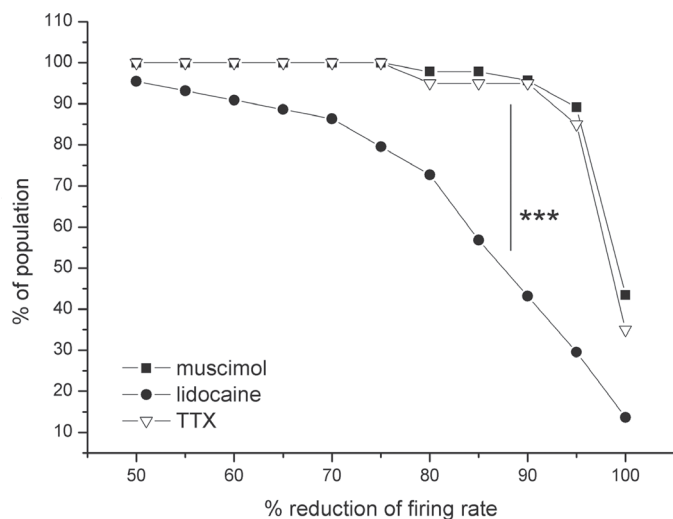
Fig. 6. Relative reduction in firing activity at the highest concentration for each drug. Depicted is the proportion of the population (*y*-axis) that shows the indicated reduction of firing activity (*x*-axis) for muscimol (*n*=92), lidocaine (*n*=44), and TTX (*n*=20). ***, significant difference in distribution, (Kolmogorov-Smirnov; *p*=0.000). The distributions of TTX and muscimol differ significantly from the distribution displayed by lidocaine, but they do not differ from each other.
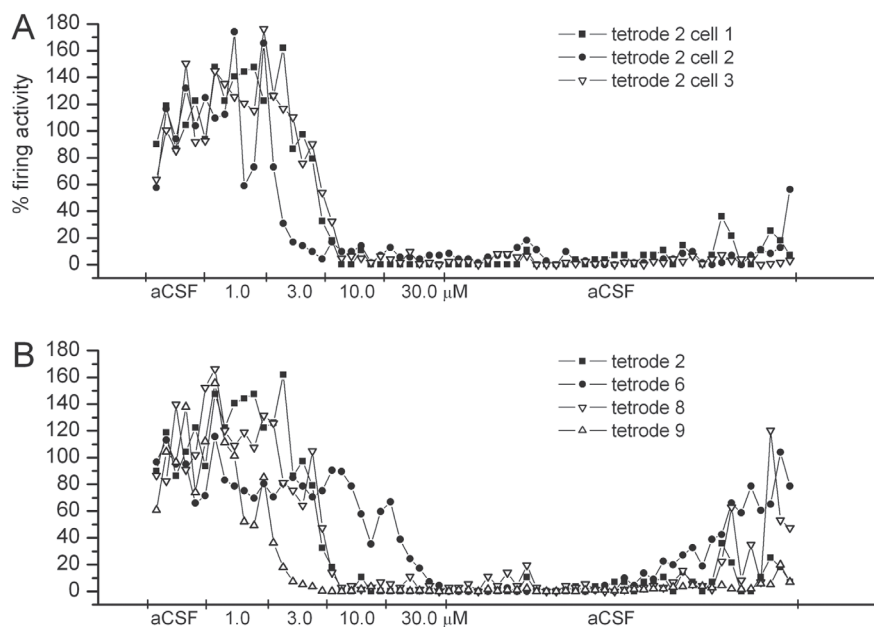

Fig. 7. Examples of different neuronal responses during perfusion of several muscimol concentrations within a single session. A, responses of three individual neurons recorded on the same tetrode. B, responses of single neurons that were recorded on different tetrodes. Horizontal axis, condition of perfusion; vertical axis, normalized firing activity.

Discussion

The present study demonstrated that the combidrive allows ensemble recordings simultaneously with reverse microdialysis in freely moving rats. Perfusion of the microdialysis probe with aCSF for several hours did not significantly affect the basal firing activity of single units. The concentration- dependent reduction in firing activity observed during the local administration of lidocaine, TTX, and muscimol showed that tetrodes are within the diffusion range of the probe. In addition, the probe could be used over extended periods, and it could be replaced. Similar pharmacological effects were obtained with multiple probes within a single animal, while the duration of periods during which recordings were made ranged between 7 and 13 days and included up to 10 recording sessions per animal. Based on these results, it can be concluded that the combidrive is suitable to be applied in behavioral studies, especially during more time demanding learning tasks.

The drugs tested showed differences in the extent to which they affected activity of the population and in speed of recovery. At the highest concentration used, muscimol and TTX caused a reduction of firing activity of 97.5 and 98%, respectively. Lidocaine showed the fastest recovery, but it also resulted in a smaller reduction of mean firing activity of the population, namely 80%.

Drug Perfusions

The concentration effect curve for lidocaine demonstrated a significant reduction in firing activity during perfusion of 74 mM (2% solution). Based on experiments in which a single 2% solution of lidocaine was perfused (to test probe functioning), the onset of the effect was determined at 5 to 6 min after brain entrance (data not shown), similar to the onset observed during recordings with the concentration effect curve. Neuronal activity (partially) recovered after 30 min, which is in accordance with previous findings after local injections (Albert and Madryga, 1980; Boeijinga et al., 1993; Tehovnik and Sommer, 1997) or reverse dialysis (Boehnke and Rasmusson, 2001).

Compared with lidocaine, the effects of TTX and muscimol perfusion were stronger and more persistent. During perfusion of 0.1 μM TTX, firing activity was significantly reduced, followed by an even larger reduction during perfusion of 0.3 and 1.0 μM TTX. Activity partially recovered 2.5 h after perfusion was finished, but it was still significantly different from the aCSF control. For muscimol, firing activity was significantly reduced during perfusion of 10 μM compared with aCSF control. However, compared with baseline, perfusion of 3 μM already caused a significant reduction in firing activity. Activity partially recovered 3 h after end of drug perfusion.

The neuronal population did not respond homogeneously to the drugs. Perfusion of the highest drug concentrations of TTX and muscimol abolished firing activity of 40% of the cells, whereas for lidocaine this was 15% (Fig. 6). The fact that not all

neurons were fully responsive to the drugs, even when recorded on the same tetrode, might have been caused by the spatial location of recorded neurons with respect to the dialysis probe. Tetrodes record cells located within a region with an estimated radius of ± 65 µm (Gray et al., 1995), meaning that, under the assumption of a straight vertical descent, the recording area ranged between 485 and 615 µm from the dialysis probe. Although the exact location of the neurons cannot be reconstructed, cells located between the dialysis probe and tetrodes would be expected to respond faster and/or respond to lower drug concentrations than neurons located at the other side of the tetrodes, causing the cells within the population of neurons to respond differentially to the drugs (Fig. 7). However, based on the observed differences in the percentage of cells showing a certain reduction in firing between lidocaine compared with TTX and muscimol, a more likely explanation is that drug-specific differences, e.g., dissociation constants, determine the neuronal response. This is supported by Boehnke and Rasmusson (2001), who showed that, even with a lidocaine concentration of non-physiological osmolality (10%), neuronal activity could not be abolished.

The amount of single units showing no recovery at all after drug perfusion was largest for TTX, namely 36% (*n*=17); for lidocaine and muscimol, the number was 0 and 4% (*n*=7), respectively. Together with the difference in speed of recovery between TTX and lidocaine, this can be explained by the fact that TTX binding to sodium channels is stronger and longer-lasting than that of lidocaine binding (Hille, 1992). Therefore, it is expected that if washout was even more prolonged after TTX perfusion, these cells ultimately would have recovered their firing activity.

Although data concerning the effects of TTX, lidocaine, and muscimol on single units as applied by microdialysis is generally lacking, the results of this study are consistent with the few existing reports using related techniques. For example, Tehovnik and Sommer (1997) observed a lidocaine effect in monkey prefrontal cortex within 5 min after an injection at a distance of 1 mm from the electrodes, and they noted a recovery within 30 min. Boehnke and Rasmusson (2001) examined the effect of 10% lidocaine and 10 µM TTX on evoked potentials (evoked by stimulating the forepaw digits) at various distances from the microdialysis probe in raccoon somatosensory cortex. Recovery of activity at a distance of 0.5 mm from the dialysis probe required approximately 40 min after lidocaine application, but it was not observed within 2 h after TTX. The apparent discrepancy with the current study 30 of 47 (64%) neurons partially recovered from TTX when recorded with tetrodes positioned at 0.55 mm from the dialysis probe] can be explained by the fact that evoked potentials require higher levels of activity to be detected against a noisy background.

Edeline et al. (2002) reported a blocking effect of muscimol injections on neuronal firing activity in an area of drug diffusion of 2 to 3 mm, which was confirmed with autoradiography. This is consistent with the effect after 10 µM muscimol perfusion as

observed in the present study. Furthermore, the results are in agreement with Sakai and Crochet (2001), who observed a weak effect of 50 μM muscimol on single units in cat brain stem (distance between dialysis probe and electrodes, 1 mm), whereas concentrations of 100 or 500 μM caused an almost complete and complete suppression, respectively, of firing activity.

Diffusion of Drugs

One uncertain factor in the method concerns the spatiotemporal dynamics of drug diffusion. Diffusion can be calculated (Fick's law), but when the diffusion coefficient in brain tissue (D_{BR}) of a drug is unknown, only an estimate of the concentration delivered at the recording site can be made. Diffusion constants of most drugs have not been determined, although data for TTX (Zhuravin and Bures, 1991) and some neurotransmitters are available (Rice et al., 1985). Estimates can be calculated on the basis of diffusion constants in liquids and recovery data in vivo (Lindefors et al., 1989), which are relatively simple to measure, but the penetration into the brain tissue has to be determined experimentally for each individual drug, because factors such as binding to macromolecules and receptors and uptake into cells cannot be estimated from diffusion constants in liquids or recovery data (Benveniste, 1989). This was clearly demonstrated by the study of Boehnke and Rasmusson (2001), where TTX and lidocaine, despite comparable molecular weights, differed in spreading and time of duration of the effect. Alternative strategies for measuring effective spread are autoradiography (Edeline et al., 2002) or dual probes (Höistad et al., 2000). However, for the drugs tested in the current study, all available data indicated that they can bridge the 0.55 mm distance between microdialysis probe and recording tetrodes.

Overall, findings in this study demonstrated that both muscimol and TTX caused a comparable reduction in neuronal firing, whereas the effect of lidocaine was less strong. Hence, when during behavioral experiments the contribution of a brain area to learning is examined by inactivation of that particular area, and a longer lasting, but reversible inactivation is required, muscimol should be used. Firing activity was almost abolished for a longer period, and, although reduction of neuronal activity was comparable with TTX, muscimol has the advantage that it inactivates neurons locally, i.e., at the soma and the dendrites, whereas TTX also prevents the occurrence of action potentials in fibers of passage. Whenever an inactivation is required for a shorter period, lidocaine would be most suitable, although it should be taken into account that the population response was highly variable and not fully blocked. Regardless of the precise drugs used, the results also imply that behavioral studies relying on local injections should take into account the variability in the neuronal response to the drug, even in a confined region around the injection site.

Furthermore, the results demonstrate that the combidrive can be used to combine reverse microdialysis with ensemble recordings in freely moving animals and that it can be applied in future studies to examine how neurotransmitters exert their effect on the activity of neuronal populations during behavior. This provides information regarding the interplay between neurotransmitters and the activity of neuronal populations during, for example, processing of reward related information in behavioral learning tasks, and it would clarify which neurotransmitters are actually involved in these kinds of processes.

Acknowledgments