The use of in vitro preparations of the isolated amphibian central nervous system in neuroanatomy and electrophysiology

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Abstract

In the present study an isolated preparation of the complete anuran central nervous system (CNS) is described which can be kept alive for several days and allows tracing, immunohistochemical and electrophysiological studies. A simple perfusion chamber is being used in which the isolated CNS preparation is superfused with oxygenated Ringer. The use of an isolated CNS has many advantages including: (1) virtually all areas are easily accessible at the same time without having the problem of blood vessels that hinder access; (2) large lesions and massive tracer applications are possible without survival problems of the animal, and tracers will not be translocated by blood circulation; (3) since pulsations caused by the pressure changes of blood circulation do not occur, intracellular recordings are comparatively easy and stable; and (4) this approach offers the possibility of working on the same brain for several days by storing the preparation in a refrigerator overnight at low temperatures, thus allowing extensive utilization of a single preparation and reduction in the number of experimental animals required. Some applications to the anuran auditory system illustrate that the isolated anuran CNS is well-suited for a variety of neuroanatomical and physiological techniques.

Keywords: Amphibian; Isolated brain preparation; Tract-tracing technique; Intracellular recording; Intracellular staining; Auditory pathway

1. Introduction

Many isolated preparations of the central nervous system (CNS) have been developed and their success often depends on special features of the preparation that enable it to survive outside the body or on elaborate support strategies that reduce the effects due to absence of a blood supply. A factor that enhances viability of isolated CNS preparations is a decreased need for oxygen and metabolic substrates and this can be achieved in some cases by cooling mammalian preparations but is often found naturally in non-mammalian species.

Among such species are the lamprey and freshwater turtles. The lamprey spinal cord is thin, has no intrinsic blood vessels, and is oxygenated directly from the cerebrospinal fluid (CSF). The brain stem has intrinsic blood vessels, but is probably also oxygenated to a large extent from the CSF (Brodin and Grillner, 1990). Rovainen (1967a,b) took advantage of these favourable conditions by developing an in vitro preparation of the lamprey nervous system. The isolated spinal cord can be maintained in vitro for periods of 2–3 days at a temperature around 7–10°C (Wallén et al., 1985). The brain stem is somewhat more sensitive but can often be maintained during a similar period of time (Brodin and Grillner, 1990). The turtle brain has an unusual resistance to anoxia (Lutz et al., 1985; Hounsgaard and Nicholson, 1990). In vitro preparations of isolated parts of the CNS, especially of the telencephalon (e.g., Connors and Kriegstein, 1986; Kriegstein and Connors, 1986; Larson-Prior et al., 1991) and of an isolated cerebellum-brain stem-spinal cord preparation (Keifer and Houk, 1989; Keifer et al., 1992; Sarrafizadeh and Houk, 1994), are increasingly being used for combined tracing and electrophysiological studies as well as for pharmacological manipulations of the turtle brain.
For most mammalian tissues it is not possible to maintain adequate physiological integrity without perfusing the vascular system with some form of blood substitute, usually oxygenated artificial CSF. An example of such a preparation is the isolated guinea-pig brain (Llinás et al., 1981; Mühlethaler et al., 1993). Isolated neonatal CNS or brainstem-spinal cord preparations, however, can be kept alive without perfusion of the vascular system. An in vitro brainstem-spinal cord preparation is available for studies on the central control of respiration and locomotion (e.g., Smith and Feldman, 1987). An isolated CNS preparation of the newly born South American opossum, Monodelphis domestica, is a very attractive model for studies on the development and regeneration of synaptic interactions (e.g., Nicholls et al., 1990; Møllgard et al., 1994).

In vitro preparations of the amphibian CNS are increasingly used in physiological studies. Various approaches have been described including the use of a superfused preparation of Xenopus laevis embryos (e.g., Kahn and Roberts, 1982; Roberts and Clarke, 1982; Roberts et al., 1986), brain slices (Holohean et al., 1990), brainstem preparations (Schmidt, 1976; Schaffer, 1982; Cochran et al., 1987; Straka and Dieringer, 1993; Atzori and Nistri, 1994; Dicke and Roth, 1994; McLean et al., 1995), and combined spinal cord-muscle preparations (Sagawa et al., 1986; Wheatley and Stein, 1992). So far, the use of isolated CNS preparations for tracing experiments in anurans has been restricted to a simple in vitro horseradish peroxidase (HRP) technique in which perfused and subsequently fixed brains are used (McCormick and Braford, 1984; González and Muñoz, 1979), and the cobalt-labeling procedure for fixed brains (e.g., Székely and Galliyas, 1975; Görcs et al., 1979). Straka and Dieringer (1991) used an isolated brainstem-spinal cord preparation for HRP tracing.

In the present study an isolated preparation of the complete anuran CNS is described which can be kept alive for several days and allows tracing, immunohistochemical and electrophysiological studies. A simple perfusion chamber (modified after Schaffer, 1982) is being used in which the isolated CNS preparation is superfused with oxygenated Ringer. The use of an isolated CNS has many advantages. Some applications on the anuran auditory system will show that the isolated CNS is well-suited for a variety of neuroanatomical and physiological techniques.

2. Material and methods

Various anuran species were tested for the applicability in vitro: Rana temporaria, R. perezi, Bombina orientalis, Discoglossus pictus, and X. laevis. Animals were deeply anesthetized with tricaine methanesulphonate (MS 222) and cooled to a body temperature of 5°C. The heart was exposed by rapid thoracotomy in order to perfuse the animal transcardially with approximately 40 ml iced Ringer solution (75 mM NaCl, 25 mM NaHCO₃, 2 mM CaCl₂, 2 mM KCl, 0.5 mM MgCl₂, 11 mM glucose) that had been oxygenized with carbogen (95% O₂, 5% CO₂) to a pH of 7.3 (Straka and Dieringer, 1993). Head and vertebral column were then severed and the skin removed to avoid the spread of cutaneous toxins. The following preparation was carried out under a dissecting microscope in a dish the bottom of which was coated with Sylgard (Dow Corning) and covered with ice-cold Ringer solution. The skull was fixed with stainless-steel needles stuck into the Sylgard layer. The complete brain and spinal cord were isolated by a dorsal or ventral approach by removing the overlying bony tissue of the skull and the vertebrae. The nerves were cut with microscissors, special care was taken with the cranial nerves and especially the branchlets of the auditory nerve.

After isolation which took approx. 10–15 min, the CNS was transferred into another dish with fresh iced Ringer. Subsequently, the pituitary gland, the dura mater, and the choroid plexus were removed to facilitate oxygen diffusion into the tissue. For ethical reasons, the telencephalon was removed (Mühlethaler et al., 1993). The application of tracer substances usually followed immediately. In case of intracellular studies, the CNS was isolated 1 day prior to electrophysiological recordings and was stored overnight at 6°C in oxygenated Ringer solution.

2.1. Tracing techniques

Two different approaches, crystalline application and the injection of aqueous solutions, were used for the application of tracers. These were horseradish peroxidase (HRP, Sigma), biocytin (N*-biotinyl-L-lysine, Sigma), biotinylated dextran amines with molecular masses of 3 kDa (BDA, D-7135) or 10 kDa (BDA, D-1956), and various 10 or 3 kDa lysine-fixable dextran amines conjugated to different fluorochromes, to either tetramethylrhodamine (RDA, D-1817 and D-3308, respectively) or fluorescein (FDA, D-1820 and D-3306, respectively). All dextran amines were purchased from Molecular Probes (Eugene, OR). For massive application of tracers, e.g., after hemisections or in case of superficial application sites, the substances were applied as small crystals dried onto the tips of glass microelectrodes or sharp tungsten needles. Since all substances tested are hydrophilic, the application area had to be rather dry to avoid undesirable spread of the dissolving tracer. A reduction of spread was achieved by taking the brain out of the Ringer solution for a short moment, and rapidly positioning the crystal with the aid of a micromanipulator. Immediately after the application, the brains were submerged again into the Ringer solution that was subsequently changed several times.

For the application of the dextran amine-coupled fluorescent dyes into deeper brain areas, we tested the pressure injection of aqueous solutions (approx. 5–10% substance in distilled water) via glass microelectrodes that were broken down to tip diameters of 10–30 μm and attached to
a Hamilton syringe. The electrodes were then positioned with a micromanipulator and 1–10 μl of the solution were injected. With this approach, the injection speed turned out to be a crucial parameter since rapid injection (more than 1 μl per min) resulted in leakage of the substance along the penetration track.

2.2. Intracellular recordings of electrically evoked activity in the auditory pathway

During recording, the CNS was transferred into a perfusion chamber (modified after Schaffer, 1982) and fixed with stainless-steel pins to the Sylgard covered floor of the chamber (Fig. 1). The chamber was mounted on an X-Y table which allowed the CNS to be moved horizontally in relation to the recording electrode. The preparation was permanently superfused with freshly oxygenated Ringer solution. The flow rate was adjusted to 3–6 ml/min by a peristaltic pump (Masterflex Pump Controller) which ran through an ice container to maintain a superfusate temperature of 16°C (Straka and Dieringer, 1993). Temperature and pH or oxygenation, respectively, were monitored continuously.

Single branchlets of the auditory nerve were stimulated electrically with suction electrodes. Stimuli consisted of monophasic square pulses (200 μs duration, 1–50 μA, 0.5 Hz repetition rate) and were delivered via an isolation unit (WPI, stimulus isolator A 360) which in turn was controlled by an interval generator (WPI, digipulser series 1800). Recordings were made with glass microelectrodes with an impedance of 80–120 MΩ when filled with 1–2 M potassium acetate. After removal of the pia mater, electrodes were positioned stereotactically and lowered into the brain with a piezo-driven system (Marzhauser; PM 10-1). Recorded potentials were amplified (WPI, Cyto 720), digitized (Instrutech, VR100 A) and displayed on a personal computer screen for on-line control and stored on hard disk for off-line analysis.

For intracellular staining, electrodes were filled with a 3.5–4% solution of biocytin in 0.1–0.3 M potassium acetate. Due to the low molarity of the solution, the impedance of the electrodes was much higher (up to 500 MΩ), but this did not influence the recordings significantly. Staining of a neuron was achieved by applying a constant current (1–3 nA) with the iontophoresis unit of the intracellular amplifier for 1–10 min. The membrane potential was controlled every minute to ensure the intracellular position of the electrode. After iontophoresis, the electrode was immediately retracted out of the brain.

2.3. (Immuno)histochemical procedures

After extracellular application of the tracers or finishing the electrophysiological recordings the CNS was stored in Ringer at room temperature, the solution was changed several times and the pH was monitored to ensure sufficient oxygenation. Overnight, brains were put into freshly oxygenated Ringer (pH 7.3; approx. 300 ml) and the container sealed air-tight and kept at 6°C to slow down metabolism and oxygen consumption of the tissue. After 12 h at this temperature, the pH of the Ringer solution typically increased to 7.6–7.8. With this day/night protocol, transport times were usually 16–18 h for the 3 kDa BDA or fluorescent dextran amines, 20 h for biocytin, the 10 kDa BDA and the fluorescent dextran amines, and 45 h for HRP.

The subsequent steps were dependent on the tracer applied. In the case of HRP and biocytin, brains were fixed with 4% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffer (pH 7.4), or 4% paraformaldehyde in phosphate buffer (pH 7.4) for BDA. Brains were then embedded in gelatin, polyacrylamide or embedding medium (Reichert-Jung) for sectioning on a freezing mi-
crotome or in agar or polyacrylamide for sectioning on a vibratome. Section thickness was 20 \( \mu \text{m} \) if the sections were directly mounted onto slides after sectioning, and 40–50 \( \mu \text{m} \) if the following steps were carried out with free-floating sections. For the localization of biocytin and BDA, endogenous peroxidases were blocked by incubation in 0.5% \( \text{H}_2\text{O}_2 \) in phosphate buffer for 15 min. Sections were then rinsed several times, washed with 0.5% Triton X-100 for 10 min and subsequently incubated with streptavidin-coupled HRP (Amersham; dilution 1:100 in phosphate buffer) for 2 h. Like the tracer HRP, the streptavidin (biocytin)- or avidin-biotin (BDA)-coupled HRP was then visualized with the chromogen DAB following a modified protocol of Adams (1981); the peroxide was produced by a glucose-oxidase reaction (Shu et al., 1988). After the DAB procedure, sections were lightly counterstained with neutral red, dehydrated and coverslipped.

In the case of the fluorescent dextran amines, brains were fixed with 4% paraformaldehyde and sectioned on a freezing microtome (40 \( \mu \text{m} \)) or on the vibratome (100 \( \mu \text{m} \)). If immunohistochemical localization of neurotransmitters was desired additionally, standard procedures with fluorescent secondary antibodies were applied. Sections were then dried quickly to avoid fluorescence fading and coverslipped with anti-fading medium (Serva, Fluoromount) or a glycerin-gelatin medium.

3. Results

3.1. Tracing techniques

The application of HRP demonstrated the well-known characteristics of this tracer: a dense spot of non-incorporated...
rated enzyme at the application site and intense retrograde as well as anterograde labeling of neuronal structures (Fig. 2A). The enzyme was taken up by the soma, by terminal structures, and by damaged fibers of passage; transport speed was approx. 0.5 mm/h. In contrast, the application of biocytin yielded intensely stained neurons at the application site without a diffuse background, indicating that most of the tracer had been taken up by the cells (Fig. 2B). The speed of transport was high (2 mm/h) and comparable to the situation in vivo (Luksch and Walkowiak, in preparation). The uptake of biocytin was not restricted to the soma, and resulted in anterograde as well as retrograde labeling. Anterograde labeling (Fig. 2C) was stronger compared to HRP, whereas retrograde labeling seemed to be less intense. However, even the retrograde label achieved with HRP did not stain entire neurons but comprised only primary and secondary dendrites. 10 kDa BDA labeling was comparable to that described for biocytin. Much faster axonal transport was observed, however, for 3 kDa BDA. With this fast dextran amine Golgi-like labeling of the entire neurons including secondary and tertiary dendrites was achieved (Fig. 2D).

Fig. 3 shows an experiment in *X. laevis* in which ascending projections to the torus semicircularis are demonstrated with 3 kDa BDA. Labeled cells are found in acoustic and vestibular cell areas, in lateral line related structures, in the dorsal column nucleus, in a lateral cervical nucleus and in the spinal cord. Comparable data were obtained from the other species studied.

The use of the dextran amine-coupled fluorescent dyes (FDA, RDA) yielded best results when applied in crystalline form, for both 3 and 10 kDa dextran amines, probably since the concentrations in the tissue achieved with the injection of aqueous solutions were too low to result in intense labeling of cells. The transport speed of the 10 kDa fluorescent dextran amines was slightly faster for the rhodamine-coupled derivative (3 mm/h) than for the fluorescein-coupled derivative (2 mm/h). Both tracers were transported retrogradely as well as anterogradely, and led to intense staining of somata and terminal structures up to a distance of 20 mm (Fig. 4A). Similarly to 3 kDa BDA, faster axonal transport was observed when using 3 kDa fluorescent dextran amines.

3.2. Intracellular recordings

The electrical stimulation at the auditory branchlets of the statoacoustic nerve led to various neuronal responses in

Fig. 3. (A) A representative experiment showing the application of 3 kD BDA to the torus semicircularis of *Xenopus laevis*; (B) example of labeling in the dorsal medullary nucleus; scale bar (in B) 100 μm.
several nuclei of the auditory brainstem (Fig. 5). Neurons in other areas of the brain, e.g., in the tectum mesencephali, did not show responses, indicating that the electrical stimulation selectively excited the auditory pathway. The quality of the nerve preparation had a strong influence on the stimulation current that was necessary to excite auditory nuclei; stimulation current was usually 2–10 μA in good preparations but had to be increased up to 50 μA if the nerve had been bruised or pulled. Intracellular recordings were possible for up to 4 days after CNS isolation without noticeable loss of activity, decrease in membrane potential in penetrated neurons, or any sign of tissue degeneration in stained structures compared to structures stained in vivo (Fig. 6). However, the stimulation current had to be increased probably due to the squeezing of the branchlets with the suction electrode. The longest recording time for one individual neuron was 4.5 h which greatly exceeded that in comparable in vivo recordings.

Intracellular application of biocytin yielded intensely stained neurons (Fig. 7). The tracer was distributed homogeneously in soma, dendrites and axonal structures; no gradient was observable so that the recording site in the neuron could not be detected. Transport of the tracer within the cell was rapid (approx. 2 mm/h) and comparable to that found after extracellular biocytin application. The quality of cell staining depended not only on the duration of the iontophoreses and the current applied but also on the electrode characteristics; in some cases, neurons seemed to be completely stained after application of 1 nA for only 1 min. Occasionally, we observed ensembles of stained neurons (2–3) lying closely together. Such simultaneously stained neuronal ensembles were not likely to be artifacts due to accidental extracellular application of biocytin, since they were also observed when the resting membrane potential of the injected neuron was still high after the iontophoreses, indicating an intracellular position of the electrode. These simultaneously stained neurons usually showed similar dendritic and axonal patterns.

3.3. Immunohistochemical procedures

The survival time of the preparation, which was defined as the time between the dissection of the animal and the fixation of the isolated CNS, ranged between 16 h for the 3 kDa dextran amine tracing and 4 days for intracellular biocytin studies. Even after the longest survival times, no sign of tissue degeneration was observed. Labeled neuronal structures had an inconspicuous appearance and could be traced over long distances (several centimeters) without any sign of inhomogeneous tracer distribution or membrane disruption indicating that the fixation by immersion was sufficiently fast to avoid tissue damage. In general, every result obtained in the in vitro preparation was comparable to those collected with an in vivo approach. In those experiments where immunohistochemistry for neuromodulator localization followed the transport phase of the tracer, the distribution of the immunolabeled terminals (e.g., leucine-enkephalin) was indistinguishable from the pattern found in in vivo brains that had been fixed by perfusion of an anesthetized animal (compare Walkowiak and Luksch, 1994).

In the experiments in which fluorescent dextran amine tracers and immunohistochemical techniques for neuromodulator localization were combined, the protocols did not interfere with each other significantly. Fig. 4B shows an example of retrogradely labeled neurons of the nucleus laminaris (torus semicircularis) traced with rhodamine-coupled dextran amine (red) and terminal structures containing the neuromodulator leucine-enkephalin detected with a secondary antibody coupled to FITC (green).

Each of the various procedures tested for the localization of HRP, biocytin or BDA gave comparable staining of labeled structures. However, since the diffusion of the streptavidin- or avidin-biotin-coupled HRP complexes is limited, the thickness of the sections should not exceed 70 μm when processed free-floating or 20 μm when processed already mounted onto slides. Staining of erythrocytes that sometimes remained after the perfusion disappeared completely after the blocking of endogenous peroxidases with H2O2; this step was only possible for biocytin and BDA.

4. Discussion

4.1. Methodological considerations

Various physiological studies using in vitro brain approaches in amphibians have been published during the last decades, comprising superfused CNS preparations of X. laevis embryos (e.g., Kahn and Roberts, 1982; Roberts and Clarke, 1982; Roberts et al., 1986) brain slices (Holohean et al., 1990), brainstem preparations (Schmidt, 1976; Schaffer, 1982; Cochran et al., 1987; Straka and Dieringer, 1993; Atzori and Nistri, 1994; Dicke and Roth, 1994; McLean et al., 1995) and combined spinal cord-muscula-
ture preparations (Sagawa et al., 1987; Wheatley and Stein, 1992). To our knowledge, the use of the complete isolated CNS for tracing experiments has only been reported for

Fig. 5. Intracellular recordings from auditory neurons in the torus semicircularis of *Discoglossus pictus*. The responses were elicited by electric stimulation of the auditory branchlets of the eighth nerve. The stimulus was a 200 μs lasting positive DC pulse. The vertical line at the beginning of the trace represents the stimulus artifact. (A) Action potential followed by long lasting depolarisations; (B) excitatory postsynaptic potential; (C) inhibitory postsynaptic potential. The resting potentials were: −72 mV (A), −45 mV (B) and −53 mV (C).

Fig. 6. Average resting potentials from day 1 to 4 after brain isolation do not differ significantly (day 1, n = 7; day 2, n = 15; day 3, n = 14; day 4, n = 9).

HRP (McCormick and Braford, 1984; González and Muñoz, 1987; Straka and Dieringer, 1991).

A crucial question for the evaluation of data collected in such a preparation is whether they are comparable to findings in vivo. We believe that the transferability is

Fig. 7. Intracellularly labeled neuron in the magnocellular nucleus of the torus semicircularis of *Discoglossus pictus*. Section thickness 20 μ.m; scale bar 100 μ.m.
supported by several arguments. First, no cellular degeneration of the brains was observed even after several days in the Ringer solution; intracellularly stained neurons as well as anterogradely and retrogradely labeled structures showed no differences to neuronal structures stained in vivo (Straka and Dieringer, 1991; Walkowiak and Luksch, 1994; Muñoz et al., 1995). The pattern of labeling in experiments such as the one shown in Fig. 3 is comparable to that obtained in vivo experiments (e.g., Wilczynski, 1981; Will et al., 1985; Feng and Lin, 1991). Second, intracellularly recorded neurons had resting potentials of up to $-90$ mV even after 3 days in the Ringer solution, indicating a good physiological state of the brain. Third, our intracellular recordings showed that the electrical stimulation of the auditory nerve elicited reactions in all nuclei of the auditory pathway including the dorsal medullary nucleus, the superior olive, various structures in the tegmentum mesencephali, and the torus semicircularis, but not in other brain areas. Since afferents to the midbrain include at least two synapses (up to four), the essential neuronal circuits seem to be intact. Additionally, we did not find general differences when recording in vitro and in vivo (Luksch and Walkowiak, 1993). Fourth, the immunohistochemical data on neuromodulator localization showed no differences when compared with results yielded in another study (Luksch and Walkowiak, 1992) where the brains were fixated by perfusion of the anesthetized animal. This finding indicates that even after several days in vitro cellular synthesis and transport systems are functioning properly. Taken together, we think that in amphibians, the in vitro approach maintains a physiological status of the brain, allowing anatomic as well as physiological studies.

Of the tracing substances applied extracellularly, HRP has been used for several decades and is well characterized (Mesulam, 1982). In combination with a heavy metal intensification (Adams, 1981) and the tissue preserving glucose-oxidase modification (Shu et al., 1988), anterogradely and retrogradely labeled structures are intensely stained. However, even if the retrograde transport exceeds the anterograde one, retrograde labeling of neurons comprises only the soma and the main dendrites. Biocytin has been introduced as an extracellular neuronal tracer only recently (King et al., 1989). The uptake of biocytin seems to rely on a specific, sodium- and ATP-dependent mechanism at the soma (King et al., 1989), and biocytin was therefore characterized as an anterograde tracer. The amount of retrograde transport is described contradictory in the literature (King et al., 1989; Diamond et al., 1991; Izzo, 1991; Kenan-Vaknin et al., 1992) and might depend on the density of terminal structures at the application site (Lapper and Bolam, 1991). In our experiments, retrogradely labeled structures were only weakly stained but comprised all known afferents. The main advantages of biocytin are the fast and strong anterograde transport, a comparatively weak background at the application site which allows the precise identification of the neurons labeled, the possibility of blocking endogenous peroxidases and the sensitivity and variability of the detection system which allows DAB precipitation as well as fluorescence labeling. Biotinylated dextran amines have similar advantages. These tracers are transported retrogradely as well as anterogradely. The retrogradely labeled neurons have an excellent dendritic filling, whereas the tracer can be identified at long distances from the injection site. Particularly the rapid 3 kDa BDA is extremely useful in the in vitro preparation.

Fluorescent dyes coupled to dextran amines have been introduced as neuronal tracers several years ago (Glover et al., 1986; Fritzsch and Wilm, 1990; Nance and Burns, 1990). Small dextran amines with a molecular mass of 3 kDa diffuse faster than the larger 10 kDa ones (Popov and Poo, 1992; Tao and Nicholson, 1992; Fritzsch, 1993). In the in vitro approach, the substances have characteristics comparable to the in vivo situation, i.e. they are transported anterogradely and retrogradely with 2–3 mm/h and are taken up by intact as well as damaged cells (Glover et al., 1986). Similar findings have been reported in a mammalian brain slice preparation (Boulton et al., 1992). The main advantages of the fluorescent dextran amines are their fast and bidirectional transport, the uptake by all cellular compartments, the possibility of applying two or three differently coupled substances for easy double and triple labeling and the ease of localization. Moreover, double-labeled structures can be analyzed with confocal laser scanning microscopy in great detail. One disadvantage is the instability of some fluorescent dyes (e.g., FITC) to ethanol treatment, thus allowing only very rapid dehydration or coverslipping with water-soluble media. Another problem arises if the combination of fluorescent tracers with immunohistochemistry is desired since glutaraldehyde, which may be necessary for the binding of the antibody to its epitope, cannot be used because it causes autofluorescence.

The use of biocytin for the intracellular staining of neurons has several advantages: Very short application time is required for complete staining of a neuron, the substance is transported rapidly and distributes homogeneously in the cell and the axon, the tip diameter of the electrodes can be small since the molecule is small and does not tend to clog, and the detection system is very sensitive. The finding of simultaneously stained neuronal ensembles is probably due to coupling of these cells via gap junctions (Simpson et al., 1977) rather than unspecific application of biocytin. Similar effects have been reported by other groups working with biocytin (Kawaguchi et al., 1989; Kita and Armstrong, 1991; Wiggers and Roth, 1994; Schulte-Mattler and Luhmann, 1995). This hypothesis is supported by the fact that simultaneously stained neurons usually had comparable dendritic and axonal organization. However, since a comparable percentage of neuronal ensembles is found in vivo, this finding can not be attributed to the isolated brain preparation.
4.2. General considerations

In our experiments with the isolated brains of amphibians, we did not encounter any technical limitations concerning the application of tracing substances. We have not tested the application of tracers via iontophoretic injections into deep tissue areas. However, since neuronal circuits remain intact and can be activated across several synapses, the exact localization of nuclear boundaries by multi-unit recordings and the subsequent stereotactic tracer injection (e.g., HRP and biocytin) as described elsewhere (Luksch and Walkowiak, in preparation) should be possible. Moreover, we have not tested the combination of biocytin application and immunohistochemistry with different DAB protocols as described by Norgren and Lehmann (1990) or Veenman et al. (1992) to yield different light-stable reaction products, which should be possible as well. In general, we consider that every tracing technique developed in vivo may be applied in an isolated brain preparation as well.

The analysis of physiological parameters in an isolated brain has some obvious limitations, e.g., stimulation of sensory systems with physiological stimuli is excluded. On the other hand, the stimulation of sensory systems with electrical stimuli leads to excitation of complete sensory pathways and complex reaction patterns in individual neurons comparable to the findings in intact animals. We therefore think that the basic physiology of neurons can be investigated well in vitro. In some cases, the complete deafferentiation of the brain may even be an advantage of this preparation. In vivo, many physiological parameters of the animal and the environment cannot be controlled entirely, e.g., variations due to experimental conditions such as immobilization stress, changing oxygen supply, or the influence of other sensory modalities. The 'constancy' of these parameters is guaranteed in the isolated brain, offering the possibility of analyzing the response of single neurons and networks to a reliably constant stimulation and of manipulating the network via the application of neuropharmacological agents or via the stimulation of different afferents.

Besides the limitations for physiological work mentioned above, the use of an isolated CNS has many advantages. First, virtually all areas are readily accessible at the same time without having the problem of blood vessels that hinder access. Second, large lesions and massive tracer applications are possible without survival problems of the animal, and tracers will not be translocated by blood circulation. Third, as pulsations caused by the pressure changes of blood circulation do not occur, intracellular recordings are comparatively easy and stable. Additionally, since the meninges can be completely removed, the penetration of the electrode is facilitated and no limitations to the electrode shape exist. Fourth, this approach offers the possibility of working on the same brain for several days by storing the tissue in a refrigerator overnight at low temperatures, thus allowing extensive utilization of a single preparation and a reduction in the number of experimental animals required. Taken together, we think that the isolated frog CNS is well suited for a variety of neuroanatomical standard procedures and, furthermore, may bridge the gap between isolated cellular physiology and the analysis of complex brain functions.

The present study shows that an isolated anuran CNS preparation has many advantages. Adequate physiological integrity can be maintained without perfusing the vascular system as is necessary in isolated mammalian brains (e.g., Llinás et al., 1981; Mühlethaler et al., 1993). Therefore, no elaborate support strategy is necessary to reduce the effects due to absence of a blood supply. Isolated CNS preparations of anuran brains can be used with equal success and ease as the lamprey (e.g., Brodin and Grillner, 1990) and turtle (e.g., Hounsgaard and Nicholson, 1990; Keifer et al., 1992) preparations.

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