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A method for the evaluation of intracranial tetrodotoxin injections

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ABSTRACT

Tetrodotoxin is one of the most potent and oldest known neurotoxins. It acts by blocking the voltage-gated sodium channels in nerve cell membranes, leading to a transient silencing of neural activity. TTX is among the most widely used pharmacological agents for the temporary and selective blocking of neural structures. As such, an exact knowledge of the spatial and temporal diffusion gradient of TTX is important when planning pharmacological interventions. Here we report a method for the direct assessment of spatio-temporal TTX diffusion gradients using immunohistochemistry. TTX injections were performed in vivo via chronically implanted guiding cannulae, placed bilaterally in the dorsal entopallium of pigeons. To determine the temporal spread, animals were perfused at different time points after TTX injections. For visualization of the TTX affected area an immunohistochemical protocol was developed. The extension of staining was assessed 1 h after injection when TTX was diffused over 3 mm in all directions. TTX immunolabeling could be detected for up to 32 h; after 48 h no staining was found. Our findings provide a better understanding of the temporal decay and spread of intracranial TTX injections, thereby allowing a more reliable estimation of size and duration of TTX-effects.

1. Introduction

The toxicity of the puffer fish has been well known since 2700 BC. At the beginning of the 20th century, the poison of the puffer fish was named Tetrodotoxin (TTX) after the order of the puffer fish (*Tetraodontidae*) (Clark et al., 1999). In 1950, crystalline TTX was isolated by Yokoo (Evans, 1972). This was an important step in the understanding of TTX-poisoning. The action of TTX is straightforward. The TTX-molecule contains a guanidinium-group which binds to the external orifice of sodium-channels. Because the remainder of the molecule is too large to penetrate the channel it thereby blocks the orifice thus inhibiting the activation of voltage-gated sodium channels (Evans, 1972). As a result, the depolarization of cells is inhibited without affecting their resting-potentials thereby blocking neural signal transmission or muscle contractions.

The ability to transiently suppress neural signal transduction makes TTX an important tool for neurobiological research. It allows, for example, an in vivo reversible blocking of specific brain structures (Ambrogi Lorenzini et al., 1997). This technique has been used to investigate the contribution of specific brain structures to behavior (Cimadevilla and Arias, 2008; Kalenscher et al., 2003) and to development (Chapman et al., 1986; Prior et al., 2004; Sretavan et al., 1988).

For all experiments using TTX it is of high importance to know the exact size of the affected area, the effective period of action of TTX, as well as when TTX is fully cleared from the injection-site. To investigate the spread and effective period of TTX, Zhuravin and Bures (1991) examined the pupillary diameter in rats following TTX injections. With an injection volume of 1 μl and 10 ng TTX, they found diffusion in a spherical volume of 3 mm and an effective period of 2 h followed by decay over the subsequent 20 h. Although a good first attempt at understanding the spread and effectiveness of TTX, this method has certain shortcomings. It cannot be used with all other brain structures since it depends on a reliable physiological response. A general approach that can be used to define the spatio-temporal extend of TTX injections in any brain structure must be independent of such physiological manifestations since they are not always available. An additional limitation of such a functional evaluation is that we do not know if residual TTX is present even though the function of the area is restored. This information is of high importance to avoid potential accumulation of TTX over repeated injections. The aim of the present study is to use immunohistochemical methods described by Buckmaster (2004) to develop a method that allows controlling the temporal and spatial spread of TTX injected into neural tissue.

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2. Materials and methods

2.1. Subjects

Fourteen adult pigeons (*Columba livia*) of unknown sex were used in this study. The animals were obtained from local breeding stocks. All experiments were carried out according to the specifications of the German law for the prevention of cruelty to animals.

2.2. Surgery

For surgery animals were anaesthetized with 1 ml per kg of a 7:3 mixture of ketamine (100 mg/ml, Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and xylazine (20 mg/ml, Rompun, Bayer). The animals were placed in a stereotactic apparatus and the topical anaesthetic xylocaine (AstraZeneca, Wedel, Germany) was applied to the scalp, which was then cut and retracted to expose the skull. Two small holes were drilled above the left and right entopallium (anterior 9.5 and lateral ±6.0 according to the stereotaxic atlas of the pigeon brain (Karten and Hodos, 1967). Then two guiding cannulae of 6 mm length with dummy-cannulae of 6.5 mm length (Plastics one, Roanoke, USA) were placed in these holes and carefully lowered to a depth of 2.3 mm. The cannulae were fixed to the skull with dental acrylic (Austenal, Harrow, UK). The skin surrounding the implant was sutured and Tyrosur (Engelhard, Niederdorfelden, Germany) applied to the wound margin. After waking up, the animals were returned to their home cages and allowed to recover for 7 days.

2.3. Injections

In this study, we used a TTX-concentration (10 ng in 1 μl saline) that is commonly used for the inactivation of selective neural structures and injected a standard volume of this solution (1 μl) (Ambrogi Lorenzini et al., 1997; Cimadevilla and Arias, 2008; Kalenscher et al., 2003; Rothfeld et al., 1986; Zhuravin and Bures, 1991). For the injections, one end of a connector assembly was connected to a syringe (5 μl syringe, Hamilton Company), the other end was connected to an injection cannula (Plastics One; 6.5 mm). The whole system was filled with distilled water and the syringe was fixed on a microinjection pump (Harvard Apparatus PHD 2000). Afterwards the filling was carried out; 1 μl of air and then about 7 μl TTX (Tocris; solved in citrate buffer and diluted to 10 ng/μl with Saline) were drawn up to the injection cannula. The dummy of the implanted guiding cannula was removed and the injection cannula was inserted to the guide. Via the microinjection pump, 1 μl TTX was injected with an injection rate of 0.2 μl/min. After 0.5, 1, 7, 15, 31 or 47 h the other hemisphere was injected in the same way.

2.4. Histology

Animals were perfused 1 h after TTX injection to the reference hemisphere. They received an injection of 200 units sodium heparin and were then deeply anaesthetized with an overdose of equithesin (0.45 ml per 100 g body weight). The transcardial perfusion was performed with 100 ml 0.9% sodium chloride and 800 ml ice-cold 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.4 (PBS). The brains were removed and stored for 2 h in 4% paraformaldehyde with supplement of 30% sucrose. For cryoprotection, the brains were put overnight in a solution of 30% sucrose in 0.12 PBS. Subsequently, they were cut in frontal plane at 40 μm on a freezing microtome and the slices were collected in PBS containing 0.1% sodium azide.

To visualise the TTX within the brain slices, the immuno-ABC-technique was used. The brain slices were first placed free-floating in 0.5% hydrogen peroxidase in distilled water for 30 min. After 1-h incubation in normal horse serum (Vectastain, Vector, Camon, Wiesbaden, Germany; 1% in 0.12 M PBS + 0.3% Triton-X-100 (PBS+)) and rinsing, sections were incubated overnight in the primary antibody (Hawaii Biotech 1/200 in PBS+) at 4 °C. After rinsing, the sections were incubated in the biotinylated secondary antibody (horse anti-mouse; Vectastain, Vector, Camon; 1/200 in PBS+). Another rinsing step followed and then sections were incubated in avidin–biotin-peroxidase solution (Vectastain ABC-Elite kit, Vector, Camon; 1/100 in PBS+). After rinsing peroxidase activity was detected using a heavy metal intensified 3′,3-diaminobenzidine reaction (DAB; Sigma), modified by the use of β-D-glucose/glucose oxidase (Sigma). The sections were mounted on gelatine-coated slides, dehydrated and coverslipped with Permount (Fisher Scientific, NJ, USA).

2.5. Data analysis

For quantification, slides were scanned as 8-bit greyscale image using a slide-scanner (Nikon Super Coolscan 5000 ED) with a resolution of 4000 ppi. Exposure was automatically adjusted for the entire slide; autofocus was applied to each brain slice. Image processing was performed using a custom Matlab-program (The Mathworks, Inc.) and Photoshop (Adobe Photoshop CS3, Extended Toolkit). The slides were masked in Photoshop and new layers were created, containing only pixels from the brain slice. The resulting area was used to calculate the overall mean and standard deviation of grey-values. For determination of the TTX-immunolabeled area we used a threshold of mean grey-value plus three standard deviations. Using this threshold a new layer was generated in Photoshop, any pixel above the threshold was depicted in black, the background in white. This representation was then visually inspected, and artifacts that were clearly not part of the stained area were removed.

To determine the spatial diffusion of TTX, all 1-h control injections were measured in Photoshop. In medio-lateral and dorso-ventral axis maximal extend of diffusion was measured, in anterior–posterior axis maximal diffusion was calculated by multiplying the number of stained slices with the thickness of (and distance between) the slices. Since the staining-intensity is rather variable depending on minute alterations in the staining-procedure, quality of perfusion etc. we calculated TTX-decay with reference to a control injection 1 h prior to perfusion. We did this by adding up the selected area across all sections in the critical hemisphere and in the control hemisphere of each brain. In the next step we divided the resulting total selected area of the critical hemisphere by the total selected area of the control hemisphere.

3. Results

We developed an immunohistochemical staining protocol to reveal the region affected by TTX injection and the temporal decay of injected TTX. Areas affected by TTX were indicated by a diffusely black to brown stained area around the injection canal comprising some individually labeled cells or fibers (Fig. 1A). The size of the labeled area depended on the time point after TTX injections indicating that the immunohistochemical labeling pattern represents diffusion and decay of the injected TTX (Fig. 1C).

To verify reliability of the immunohistochemical signal we compared the dimension of labeled areas 1 h after injection. At this time point, the spatial spread of the TTX-labeling averaged to 3.0 mm (±0.7 mm) in dorsal ventral, 2.7 mm (±0.8 mm) in medial lateral and 3.1 mm (±1.0 mm) in anterior posterior direction (Fig. 1B).

In addition to the injections 1 h prior to perfusion, all animals received a second injection into the contralateral hemisphere. For

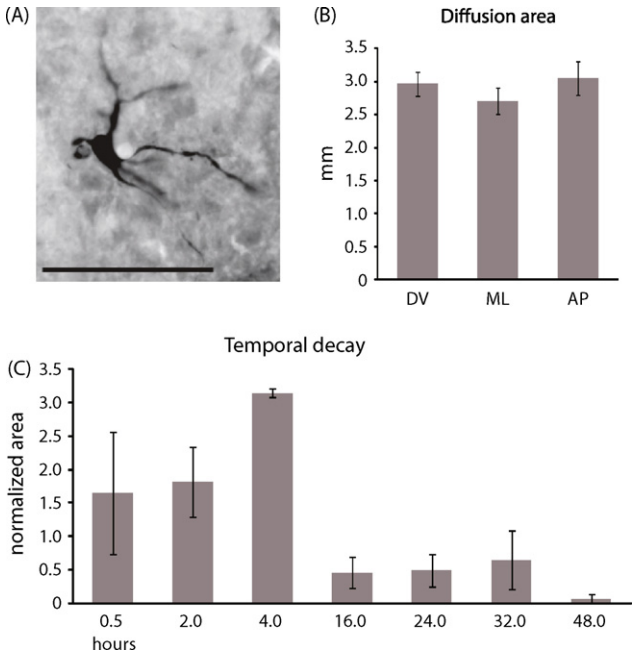

Fig. 1. Results. (A) A TTX-positive neuron. Scale bar represents 100 μm. (B) The spatial diffusion of TTX, 1 h after injection. DV: Dorsal–ventral, ML: medial–lateral, AP: anterior–posterior. (C) TTX-staining at different time points after injection. The stained area is divided by the area of a 1-h control injection to the contralateral hemisphere.

each time point (0.5, 2, 4, 16, 24, 32 and 48 h) two animals were used. These injections were performed at different time points to allow comparison of the stained area with the 1-h control staining. This approach revealed that within the first 2 h after injection the size of the labeled area was nearly constant (Fig. 1C). Four hours after injection, the maximal amount of staining was reached. At this time, the affected area was about three times as large as 1 h after injection. This peak subsided after 16 h post injection, when the amount of staining was reduced to half. The size of the affected area remains constant at 24 and 32 h. Staining disappeared 48 h after injection (Fig. 2).

4. Discussion

The aim of the present study was to develop a reliable technique for the evaluation of the spread and temporal decay of TTX injections. Here we introduce a novel protocol for the immunohistochemical detection of TTX in brain slices. This technique can be used to verify intracerebral TTX injections and requires only standard laboratory equipment and a commercially available antibody. Importantly, this method is universal and can be applied to any species or experiment.

We applied this technique to assess the diffusion and temporal decay of TTX injections into the entopallium of pigeons. One hour after injection, we found that the diffusion of 1 μl of a 10 ng/μl TTX solution averages to about 3 mm in all directions. This result is in line with the functional study by Zhuravin and Bures (1991), who report the same spread for intracranial injections of TTX to the Edinger-Westphal nucleus of rats. It is important to note, however, that the dimensions of the affected area may depend on several parameters. Traversing fiber tracts may for example act as diffusion barriers, lamination and proximity to ventricles or to the surface of

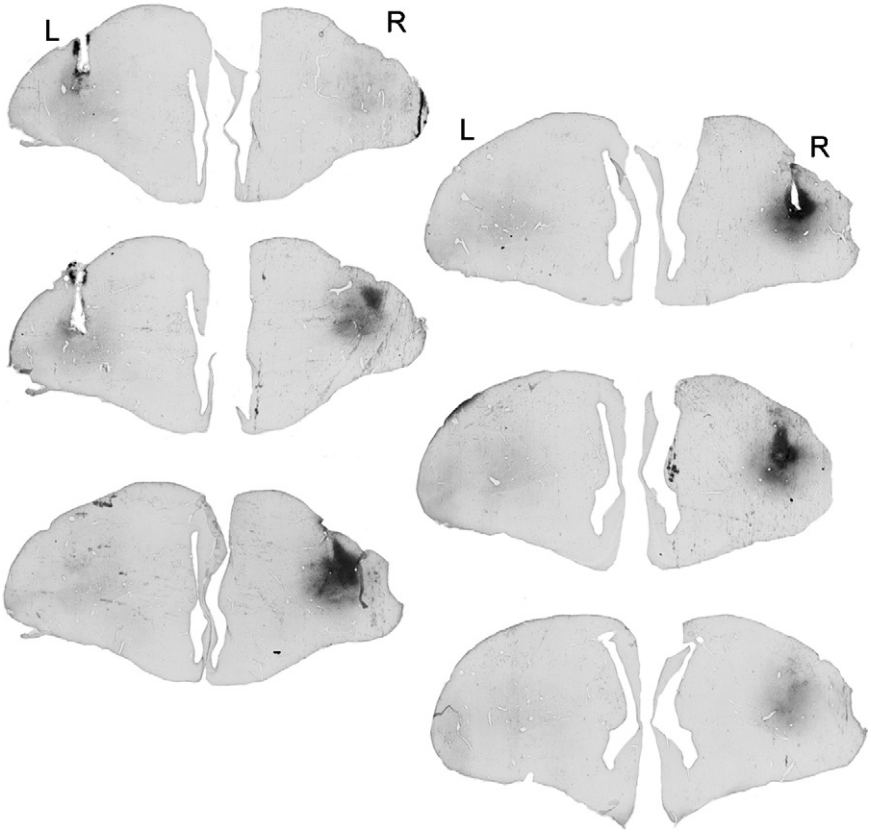

Fig. 2. A stained series. The right hemisphere (R) received a control injection, 1 h prior to the perfusion and shows clear staining. The left hemisphere (L) was injected 48 h before perfusion and shows virtually no TTX-staining.

the brain can also affect the diffusion. Furthermore, the diameter and length of the cannula and the details of the injection-procedure may play an important role for the diffusion of TTX. Therefore we recommend adjusting all injection-parameters in a preparatory study before using TTX to block a brain structure. With the introduced method to immunohistochemically stain injected TTX it is easy to verify the affected area at a given time point and to adjust the amount of injected TTX for the kind of tissue, injection-procedure and cannula. Furthermore the method is useful to verify the efficiency of the injections after an experiment. Animals can be perfused after the last test session and immunohistochemical staining will show the affected region. It can be verified if the whole region of interest was blocked, if the region was blocked only partially or if surrounding regions have been affected.

Apart from the spatial distribution, the temporal evaluation of TTX injections is a second critical factor for the planning of experiments. In the present study we found a larger stained area 0.5 h after injection compared to 1 h after injection. This might show that the spread of TTX decreases after injection, however, we find a considerable rise at the consecutive time points. Considering the large standard error at 0.5 h, the value at this time point might also be explained by technical problems in one animal. The size of the stained area expands continuously between 1 and 4 h after injection. We believe this indicates a continuous spread of TTX from the time of injection lasting at least 4 h. At 16 h we find staining of about half the area compared to 1 h, this area remains roughly constant 24 and 32 h after injection. With the method presented here, it was 48 h after injection that TTX could no longer be detected at the injection site. This result does not imply that TTX remains functional for that long. Zhuravin and Bures (1991) observed that TTX affects the functionality of the pupillary reflex for 2 h and that the effect decays over 20 h. The fact that we could detect TTX quite longer could mean that TTX still remained in the tissue but no longer affected the function of the target area. But it could also be that the duration of the TTX effects depends on the functionality of the blocked region. In contrast to the results by Zhuravin and Bures (1991), Rothfeld et al. (1986) reported that full recovery of the lordosis reflex was not observed before 2 days after TTX injection. Furthermore, electrophysiological data showed that neural activity in the inferior colliculus recovered between several hours and maximally 2 days after TTX injection (Nwabueze-Ogbo et al., 2002). These observations fit with our immunohistochemical data.

Note, however, that the method we introduce here does not allow an interpretation of the functional role of the stained TTX, only the presence or absence of TTX in a certain region can be assessed with this method. Independent of an exact knowledge about the functionality, we recommend waiting for at least 48 h after TTX injections before continuing with the experiment. This recovery period will ensure that functionality is restored and most importantly prevents the potential accumulation of TTX. The immunohistochemical protocol introduced in this study makes the blocking of specific brain regions with TTX more reliable because it allows to easily assess the spatial extend of the injections and the duration until TTX is fully cleared from the brain.

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