Chapter 12

A Method for Sampling Rat Cerebrospinal Fluid with Minimal Blood Contamination: A Critical Tool for Biomarker Studies

Zhen He, John Panos, James Raymick, Tetyana Konak, Li Cui, Diane B. Miller, James P. O'Callaghan, Serguei Liachenko, Merle G. Paule, and Syed Z. Imam

Abstract

Sampling and analysis of cerebrospinal fluid (CSF) is a common clinical practice used in the diagnosis, treatment, and prevention of neurological diseases. A similar interest is the sampling of CSF from rats to bridge the gap between bench-to-bedside work and to foster the development of new CSF biomarkers for clinical use. Here, we describe an improved procedure with an instrument designed in-house, by which rat CSF was successfully collected with indiscernible blood contamination (via the naked eye/surgical microscope amplification). The sampled CSF amounts were over 100 μl regardless of the animal's body weight, hydration status, and symptoms of systemic damage including, but not limited to, seizure, delusion (such as repeated hemorrhagic self-biting), hematuria, and gastrointestinal bleeding. In adult Sprague-Dawley rats above 300 g, the sampled CSF amounts were reliably at 200 μl or above with this method. There were no deaths related to the CSF sampling procedure. In conclusion, the present method provides a reliable and reproducible approach for collecting 200 μl CSF in rats without blood contamination.

Key words Cerebrospinal fluid, In-house designed instrument, Rat

1 Introduction

The two major factors that define the quality of experimental CSF collections are the absence of blood contamination and the amount of sample volume collected. A CSF sample with no blood contamination is necessary because blood contamination can make biomarker analysis in CSF less reliable and the volume of the CSF sample can help in its utilization in exploring new assays, regardless of the limitation of a sample volume for a particular assay.

In the clinic, results of a normal conventional CSF collection and analysis include the criteria related to its appearance: clear, colorless and CSF cell count: 0–5 white blood cells (all mononuclear), and no red blood cells (https://www.nlm.nih.gov/medlineplus/

Michael Aschner and Lucio Costa (eds.), *Cell Culture Techniques*, Neuromethods, vol. 145,
https://doi.org/10.1007/978-1-4939-9228-7_12,

ency/article/003428.htm); in addition to the standards related to pressure, total protein amount, gamma globulin, glucose and chloride. We are not aware of the criteria for a normal CSF test in rats covering the same parameters as in humans. Currently, the one-time sampled CSF amount from a rat ranges between 50 and 180 μl [1–5], which could be a limitation for meeting all parameters tested as in human samples. There is always a dilemma in determining quality of a sampled rat CSF without compromising a part of CSF amount for the actual experimental analysis.

The total volume of rat CSF has been estimated to be between 400 and 500 μl [6, 7], and the cisterna magna and the subarachnoid cavity are the major areas containing CSF. As mentioned above, the current literature documents a one-time sampled amount of rat CSF from the cisterna magna to vary from 50 to 180 μl. Recently, an improved method with the ultrasound-guided approach led to a sample volume of 100–200 μl of CSF in male Sprague-Dawley rats (300–400 g), and the 200 μl CSF mark was reached in 2 of 40 rats [8]. In principle, CSF flows from the ventricles to the cisterns and/or subarachnoid space. It is believable that CSF from all surrounding areas will drain to the space of the lowest pressure/vacuum effect, indicating the potential to collect a higher CSF volume in rats.

In this study, we demonstrate a reliable and reproducible method to collect high-quality rat CSF in a sample volume of 200 μl and above via an approach from the cisterna magna. We also discuss the potential hurdles that lead to a smaller volume of CSF collection and blood contamination in relation to the replacement/orientation of the needle bevel into the cisterna magna for CSF collection.

2 Materials and Methods

The CSF collection method was developed for an ongoing project aimed at exploring fluidic biomarkers of central nervous system neurotoxicity in adult rat models of trimethyltin chloride (TMT) exposure [9]. The procedures and instruments were permanently set up at the beginning without any changes. However, during the progress of the project, the orientation of needle bevel (see details later) was modified to test the potential to maximize one-time CSF sampling amount without blood contamination.

Animals

All animal procedures were approved by the National Center for Toxicological Research (NCTR) Institutional Animal Care and Use Committee. For setting up the standard operation procedures and testing the effectiveness of this in-house designed instrument, six

male adult Sprague-Dawley rats were obtained from the NCTR breeding colony and used in a trial study. The trial ended in 2 days with encouraging outcomes: all animals survived the surgery with CSF sampled, and five of six animals displayed clear CSF, while one sampled CSF showed blood contamination. Accordingly, the formal experiment was started thereafter. In total, 103 adult male Sprague-Dawley rats (3 months old, Taconic, Inc.) were used in this study: 48 rats were exposed to a single dose of trimethyltin chloride (TMT) (7 mg/kg, ip), and 55 control rats received a similar volume of vehicle (saline) via ip injection [9]. Two, 6, 10, or 14 days posttreatment, CSF was collected.

Needle Bevel Orientations

In a separate project, we casted space of the cisterna magna in few rats (unpublished data) using colored silicone [10, 11]. We verified that the distance between the atlantooccipital membrane and the cisterna magna floor/the dorsal surface of the medulla oblongata was approximately 1 mm (image not shown) with normal physiological status/posture. Accordingly, during sampling, it was hypothesized that the bevel of a needle could be placed in three orientations: Type I, the bevel opening facing the cisterna magna floor; Type II, the bevel opening facing sideways (left or right); and Type III, the bevel opening facing the opposite of the cisterna magna floor (Fig. 1). Using same size of the bevel and the same angle in which a needle is inserted into, Type I was hypothetically favored because it would be less possible for the tip of needle to

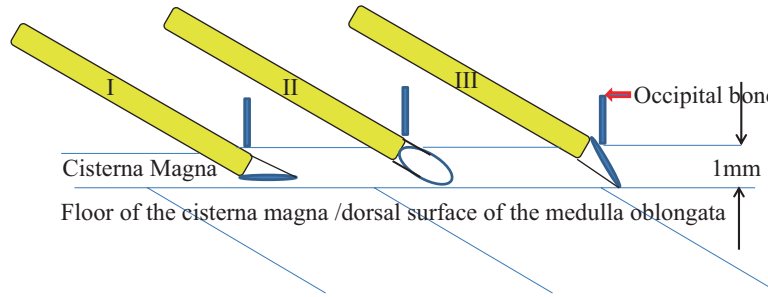

Fig. 1 Orientation of the bevel tip of the sampling needle. Orientation of the bevel tip of the sampling needle was designed based on our unpublished data that the distance between the atlantooccipital membrane and the cisterna magna floor/the dorsal surface of the medulla oblongata was about 1 mm when rats were in the physiological posture. Accordingly, Type I orientation was hypothetically ideal in attempting a complete placement of the needle bevel within the cisterna magna in such a limited space. In contrast, Type II or Type III would risk placing the bevel tail part outside of the cisterna magna without penetrating brain parenchyma in the same sized space. (I) The bevel tip of the sampling needle facing down; (II) the bevel facing sideways; (III) the bevel facing up

penetrate the cisterna magna floor (left panel of Fig. 1). Conversely, Type III would risk penetrating parenchyma of the medulla oblongata with the bevel tip, while the rear side of the bevel might remain the outside the cisterna magna (right panel of Fig. 1). Certainly, the entire bevel head must be placed into the cisterna magna so that the CSF could be effectively sampled by a vacuuming mechanism (see next paragraph). Nevertheless, all three bevel orientations were tested in the current study: Type I was conducted with 6 rats from the NCTR breeding colony in the trial study and 34 rats in the first portion of the TMT study, Type II was used in 34 rats in the middle portion, and Type III was conducted in 35 rats in the last portion of the TMT study.

In-House Designed Instrument for Sampling CSF in Rats

Due to the small physical size of the cisterna magna in rats and a possibility of needle penetration into the medulla oblongata, it could cause blood/tissue contamination to the sampled CSF. Therefore the accuracy, stability, and reliability in all procedures were essential to prevent sudden death/post-surgery paralysis. Accordingly, an in-house designed instrument (Fig. 2a) was developed integrating four parts: (1) a syringe infusion/suction pump set, (2) a 1058 mm length of PE50 tubing, (3) a needle head which was fixed onto (4) the final part, a micromanipulator; all were from commercially available resources. A needle head/the metal pipe part (BD 27G1-1/4) was detached from its tail part, inserted into one end of the PE50 tubing. The other end of the PE50 tubing was then mounted onto the needle of the syringe infusion/suction pump set. The needle head and tubing set was only used once for each animal, ensuring sterile status of the needle head and avoiding any cross-contamination. Noticeably, the tubing was marked with a 100 μl level, a 200 μl level, and a safety guard mark, which here served as a reminder that no CSF would pass across the mark so that the syringe installed on the syringe pump could be used repeatedly without contamination between samples. The needle head for each CSF sampling was mounted onto the micromanipulator which provided three-dimensional, screw-advanced, precise movements till the needle bevel completely penetrated the atlantooccipital membrane at the desired angle (see next paragraph). Finally, the syringe infusion/suction pump provided stable, controllable vacuum, and digitally controlled volume sampling. The in-house designed CSF sampling instrument worked via a volume-change-induced vacuum mechanism provided by the syringe infusion/suction pump.

CSF Sampling

After fur removal by shaving, the rat was placed into a stereotaxic frame (Fig. 1a) under anesthesia of 1–4% of isoflurane. A rhom-

Fig. 2 In-house designed instrument for CSF sampling in rats. The in-house designed instrument (Image **a**) integrates four parts: (I) a syringe installed in an infusion/suction pump and (II) a 1058 mm length of PE50 tubing, one end of which is mounted on the syringe needle and the other end installed on (III) a needle head (pointed by a red arrow head), which is fixed onto the final part, (IV) a micromanipulator, all of which can be purchased from commercial resources. Image (**b**) highlights the two black-ink markers: one close to syringe pump (red arrow) is for the warning sign indicating that the sampled CSF is far over 200 μl level and the sampled CSF may contaminate the syringe which is designed to be repeatedly used without any specific treatment; the other mark indicated by a yellow arrow is for labeling the 200 μl level (see text for detailed calculation per inner diameter of PE50 tubing and length of the tubing). Image (**c**) addresses the needle head-PE50 tubing connection (indicated by a red arrow head on Image (**a**); see text of how needle head was onsite made without compromising its sterile condition). Needle head and PE50 tubing set were used only once per animal ensuring its safety to the animal and preventing from cross-contamination

boid depressed area between the occipital bone and alar vertebrae was exposed under the surgical microscope and a midline incision of the skin, and the first layer of the muscle was followed by blunt separation of the muscles above the atlantooccipital membrane. The in-house designed instrument for CSF sampling was employed, and its needle bevel was advanced to target the atlantooccipital membrane at a 25° angle with the rat's mouth tilted down 5° (Fig. 3). Thereafter, the syringe pump was switched to withdraw 100–200 μl of CSF at a digitally controlled speed at 400 μl/min. The pump was switched on and off to control the vacuum accumulated within the system. After the predetermined CSF was sampled, the syringe pump was switched to infuse/inject the sampled CSF into a labeled 2 ml Monoject sterile blood collection tube (Tyco Healthcare Group).

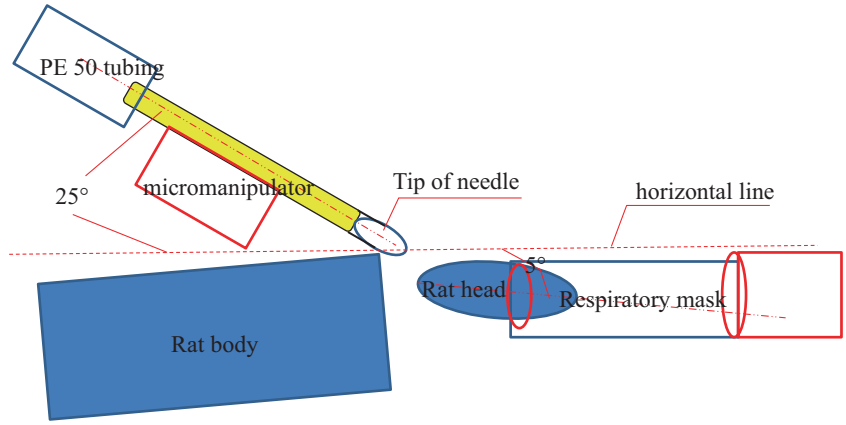

Fig. 3 Illustration of side view of the CSF sampling system. The illustration addresses how the head of a rat is fixed to access a respiratory anesthetic setting and at what angle the CSF-sampling needle is set to approach the cisterna magna

CSF Quality and Quantity Assessment

Quality of the sampled CSF (i.e., visual appearance) was verified by the naked eye and/or with the aid of 10–25× amplification of the surgical microscope to determine whether there was visible blood. The sampled CSF quantity was first estimated by a formula calculating inner volume of the commercially purchased PE50 tubing: 200 μl (desired CSF amount) = $r^2\pi \times L$. The standard inner diameter of the PE50 tubing is 0.58 mm, and accordingly r is 0.29 mm. The supposed PE50 tubing length (L) for 200 μl volume of a CSF sample then is 757 mm per calculation with the formula. To ensure the sampled CSF is 200 μl or above, the eventual 200 μl mark was arbitrarily enforced at the length of 870 mm of each CSF-sampling PE50 tubing, a ~13% increase in addition to a volume in the needle head (~30 mm in length) (see Discussion for reasons of intended increase of length of the sample collection tubing). The 100 μl mark was enacted at the half of the 870 mm tubing length, and the safety guard mark was imposed at the 1058 mm length of the tubing. Theoretically, 123 μl of CSF was sampled at the 100 μl mark of the PE50 tubing, and 238 μl of CSF was sampled at the 200 μl mark of the PE50 tubing per calculation of the formula: volume (100 μl mark)=$(0.29\text{ mm})^2 \times 3.14159 \times$ (435 mm of tubing length+30 mm of needle head length) and volume (200 μl mark) = $(0.29\text{ mm})^2 \times 3.14159 \times$ (870 mm of tubing length + 30 mm of needle head length), respectively. To verify the sampled CSF at 100 μl or more and 200 μl or more, a pipetting method was further applied: typically, all the declaimed 200 μl of CSF samples were redistributed into four collection tubes, each with 50 μl or above.

3 Results

Body weights were 409.9 ± 35.7 g in the vehicle control group and 411.1 ± 32.5 g in TMT treatment group at day 0 with no significant difference between groups (Fig. 4). During paired feeding, animals in vehicle control group slightly gained body weight, ranging between 370 and 546 g, while the animals in TMT treatment group displayed progressive body weight loss, varied between 309 and 492 g. Body weight in TMT treatment group was significantly lower than that in vehicle control group at day 12 (443.1 ± 44.5 g vs. 345.9.1 ± 33.8 g). Totally, 103 rats were subjected to the CSF-sampling surgery. The overall success rate (no visible blood contamination) was 89%. If calculating the success rate by examining the sample in the collection tubes, the rate increased to 91% because the visible contaminated blood portion in the sampling tubing in two samples were immediately separated by cutting down the tubing ~20 mm length from the visible blood portion: the bleeding/visible blood occurred after clear CSF was sampled for >200 μl in one rat and >100 μl in another (so called delayed blood contamination). Those samples were marked with questionable quality while awaiting further analysis.

All rats, control or TMT treated, survived the CSF sampling surgery/anesthesia although NCTR's attending veterinarian judged some animals experiencing an unscheduled emergent surgery as "terminal status." Noticeably, three rats with manifestations including but not limited to "signs of recumbency, non-responsiveness, labored respiration, seizures, diarrhea, and significant weight loss" endured the CSF sampling surgery and displayed relatively stable living signs including the rhythmic respiration after withdrawing CSF. One of these three animals experienced temporary respiratory and circulatory arrest during

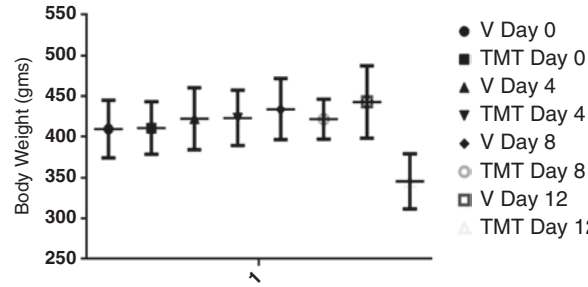

Fig. 4 Time course of changes in body weight following trimethyltin chloride (TMT) exposure. Using software GraphPad Prism6, *t*-test for day 12 shows a significant difference between vehicle (V) group and TMT group, $p < 0.05$. Repeated measures ANOVA cannot be done due to loss of subjects. ANOVA test shows $p < 0.001$

anesthesia induction and recovered after conducting resuscitation (rhythmic chest squeezing) before the CSF sampling surgery. Every drop of CSF was harvested from these three animals as observed by an empty gap displayed in the CSF-sampling tubing after fulfillment of the CSF sampling. The sampled CSF passed the 200 μl mark in two of these three animals, and in the remaining one, ~180 μl CSF was sampled.

4 Discussion

The CSF sampling method described here advances in two aspects compared with the available literature: (1) the instrument established in this study provides a confidence in sampling high-quality CSF with no visible blood contamination and 86–95% success rate, and (2) the present approach ensures a predictable CSF amount sampled: ≥200 μl in normal adult male Sprague-Dawley rats. Noticeably, an average of ~200 μl CSF was sampled even in the "terminal" animals with a significant reduction in body weight (as low as 267 g).

As demonstrated in Fig. 1, there are three bevel orientations of the sampling needle head. It appeared that Type II had a highest successful rate with no visible blood contamination in sampling 100 μl, while no significant difference was detectable between the other two bevel orientations. Type III approach led to sample the highest CSF amount (≥200 μl in 33 of 35 rats) although animals in Type I and Type II groups were targeted at the level of 100 μl. Actually, two CSF samples from Type III group displayed a delayed blood contamination, one after the sampling amount was over the 100 μl mark/123 μl (the calculation amount by tubing size and length plus needle head-hold volume) and another after sampling over 200 μl mark/238 μl per that calculation, indicating that Type II approach might risk similar odds if the sampling amount had been targeted at the level of 200 μl. It is worth mentioning that the operator knew the bleeding reason in one of the two CSF samples that displayed blood in the first drop of CSF in Type III group: miscalculation in selecting the needle penetrating place led a portion of the sloped rear bevel of the needle head to scratch the edge of the occipital bone during needle advance. Such experience throughout the project played a major role in the enhanced success rate in Type II and III groups, which were executed in the later portions of the project. In some cases, minor adjustments of needle penetrations were made to ensure such hemorrhage did not occur. Those adjustments are as follows: (1) the needle advance was halted after entering the atlantooccipital membrane with a length of one and half of its bevel if no CSF was sampled with the syringe pump while advancing the sampling needle head after its bevel passed the membrane; (2) the needle was removed (screwed back), and the

penetrating hole was cleaned for removing any fluid/blood; (3) the needle was then advanced again after adjusting/increasing the angle between the animal's trunk and the animal's head. An explanation for these cases is that there is anatomic variation that some animals may have such as an epidural and/or a subdural space before the sampling needle head penetrated the subarachnoid membrane and reached into the cisterna magna. Our unpublished data indicated existence of this cavity when making a model of subarachnoid hemorrhage in rats: a few of the rats failed to display blood deposit on the cranial base (the injected blood went "nowhere"/place not confirmed) after injecting 0.4 ml of fresh blood via penetrating the atlantooccipital membrane.

Techniques to Avoid Blood Contamination

CSF can be contaminated with blood from the injured tissue where blood vessels exist ubiquitously. In contrast to the method via a puncture through skin with or without instrumental guidance such as an ultrasound imaging [8], the present method exposes the atlantooccipital membrane and thus avoids the possibility that the sampling needle will penetrate a vessel between the subcutaneous tissue and the overlaying muscular tissues of the atlantooccipital membrane. Furthermore, vasculature existing within the atlantooccipital membrane can be viewed clearly with the surgical microscope and thus can be circumvented when advancing the needle with the micromanipulator. Nevertheless, the atlantooccipital membrane should be verified free from blood from all the surrounding tissues in the surgical incision/field before advancing the sampling needle through the atlantooccipital membrane. In general, the blood contamination from the surgical field and the injured atlantooccipital membrane shows a reddish color in the first drops of the sampled CSF within the needle head tubing set. In the present method, the operator was "blind" after the needle tip pierced the atlantooccipital membrane. A beginning blood contamination of the sampled CSF might originate from the brain structures that form the cisterna magna. For example, a hemorrhage might be caused by the injury of the floor of the cisterna magna/the dorsal surface of the medulla oblongata due to an over advanced sample needle (referring to Fig. 1). A reddish color could be displayed in the middle or final drops of the sampled CSF in the needle head tubing set: it occurred three times in the present study when the sampled CSF passed the 100 or the 200 μl mark. We surmised that a high vacuum pressure had accumulated within the tubing set and would account for the bleeding.

Obstacles Affecting CSF Sampling Amounts

(1) Dehydration. The present study demonstrated that there were three animals that appeared to have limited CSF volume (maximum at ~200–238 μl) via the cisterna magna approach in the TMT

treated group. These animals displayed "terminal" signs with significant body weight loss, indicating dehydration as the potential explanation. (2) CSF leakage. The operator did see that CSF leakage occurred around the sampling needle head when advancing the needle across the atlantooccipital membrane. An increased pressure in the cisterna magna or an uneven cutting along the bevel sides (which caused leakage) had been surmised as the potential culprit preventing CSF sampling at the maximum. On the other hand, there were several pragmatic guesses that might lead to a decreased sample volume. First, the vacuuming force was critical. A much higher vacuuming force (a rather "high"-speed vacuum) appeared to lead to a delayed hemorrhage, which interrupted the CSF sampling process. Theoretically, a reduced vacuuming force would also be accountable. The sampled CSF was frequently (not counted but estimated at ~10% of total surgeries) seen with air bubbles in the sampling tubing. As mentioned, an epidural/subdural cavity might exist between the atlantooccipital membrane and the arachnoid membrane as the tent/roof of cisterna magna in some animals. It was hypothesized that a sample needle might be placed with its bevel opening part in the cavity and another part in the cisterna magna, resulting in the bubble-sampled CSF. Also, the bubble-sampled CSF might be the result of an unsealed spot between the sampling needle head and the surrounding tissues (the atlantooccipital membrane). Screening the sampling needle quality before beginning the sample may thus partially avoid the occurrence of bubble-sampled CSF.

In summary, we recommend the following to be able to sample ≥200 μl of CSF without blood contamination:

1. Detailed training in surgical skill.
2. Selecting Type II or III bevel orientation of the sampling needle head. It is possible that a locally increased vacuum/negative pressure may play a role in increased bleeding cases in Type I bevel orientation since the bevel faces the floor of the cisterna magna. On the other hand, Type II or Type III bevel orientation dodges this possibility.
3. Awareness of a possible epidural and/or subdural cavity.
4. Slow suctioning of CSF (arbitrarily ~2 min for sampling 200 μl).

Acknowledgments

This work was supported by the National Center for Toxicological Research/Food and Drug Administration [Protocol # E0758001 to M.G.P. and S.Z.I]. The authors are grateful for the technical expertise provided by the animal care staff of the Priority One Corporation and technical support provided by Susan Lantz and Bonnie Robinson.

Disclaimer

This document has been reviewed in accordance with the US Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect the position or opinions of the FDA nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the FDA.

References

1. Bouman HJ, Van Wimersma Greidanus TB (1979) A rapid and simple cannulation technique for repeated sampling of cerebrospinal fluid in freely moving rats. Brain Res Bull 4:575–577
2. Consiglio AR, Lucion AB (2000) Technique for collecting cerebrospinal fluid in the cisterna magna of non-anesthetized rats. Brain Res Brain Res Protoc 5:109–114
3. Pegg CC, He C, Stroink AR et al (2010) Technique for collection of cerebrospinal fluid from the cisterna magna in rat. J Neurosci Methods 187:8–12
4. Mahat MY, Fakrudeen Ali Ahamed N, Chandrasekaran S et al (2012) An improved method of transcutaneous cisterna magna puncture for cerebrospinal fluid sampling in rats. J Neurosci Methods 211:272–279
5. Li Y, Zhang B, Liu XW et al (2016) An applicable method of drawing cerebrospinal fluid in rats. J Chem Neuroanat 74:18–20
6. Lai YL, Smith PM, Lamm WJ et al (1983) Sampling and analysis of cerebrospinal fluid for chronic studies in awake rats. J Appl Physiol 54:1754–1757
7. Frankmann SP (1986) A technique for repeated sampling of CSF from the anesthetized rat. Physiol Behav 37:489–493
8. Lu YG, Wei W, Wang L et al (2013) Ultrasound-guided cerebrospinal fluid collection from rats. J Neurosci Methods 215:218–223
9. Imam SZ, He Z, Cuevas E et al (2018) Changes in the metabolome and microRNA levels in biological fluids might represent biomarkers of neurotoxicity: a trimethyltin study. Exp Biol Med (Maywood) 243:228–236
10. He Z, Yamawaki T, Yang SH et al (1999) An experimental model of small deep infarcts involving the hypothalamus in rats: changes in body temperature and postural reflex. Stroke 30:2743–2751
11. He Z, Yang S, Naritomi H et al (2000) Definition of the anterior choroidal artery territory in rats using intraluminal occluding technique. J Neurol Sci 182:16–28

Neuromethods 145

Springer Protocols

Michael Aschner
Lucio Costa *Editors*

Cell Culture Techniques

Second Edition

Humana Press

Cell Culture Techniques

Second Edition

Edited by

Michael Aschner

Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, USA

Lucio Costa

Department of Environment/STE 100, University of Washington, Seattle, WA, USA

Humana Press

Editors
Michael Aschner
Department of Molecular Pharmacology
Albert Einstein College of Medicine
Bronx, NY, USA

Lucio Costa
Department of Environment/STE 100
University of Washington
Seattle, WA, USA

ISSN 0893-2336 ISSN 1940-6045 (electronic)
Neuromethods
ISBN 978-1-4939-9227-0 ISBN 978-1-4939-9228-7 (eBook)
https://doi.org/10.1007/978-1-4939-9228-7

This Humana Press imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.