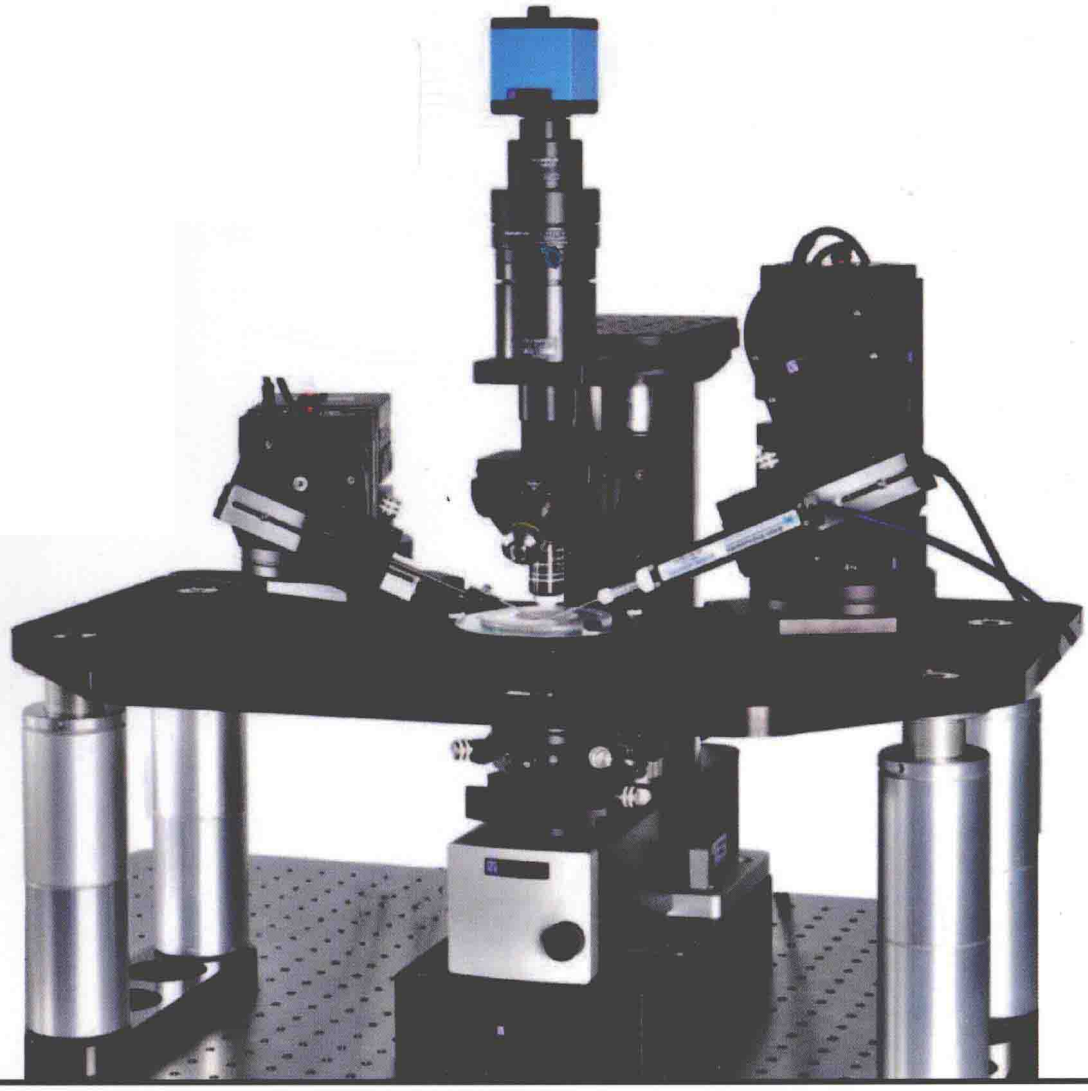

Patch Clamp Technique

Hank Loris

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Edited by **Hank Loris**

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Preface

This book is a leverage in understanding patch clamp technique. This book is a compilation of researches related to patch clamp technique accomplished by experts around the globe. It is basically an electrophysiological method for calculating the electric current created by a living cell. This innovative method was discovered by two professionals of Germany - Erwin Neher and Bert Sakmann. They were honored with the prestigious Nobel Prize in 1991 for their contribution to Medicine. It is even used for calculating the drug result against a sequence of diseases, and to find out the mechanism of disorders in animals and plants. It is also applied in getting the structure function actions of compounds and drugs; and major pharmaceutical firms use this method in clinical trials of their medicines. In simple words, this book deals with the theory of endogenous mechanisms of cells and its practical functions. It presents the fundamental principles and developing varieties, as well as deals with the most recent advancements in the established patch clamp method. This book intends to help students and experts in gaining more knowledge regarding this innovative technique.

All of the data presented henceforth, was collaborated in the wake of recent advancements in the field. The aim of this book is to present the diversified developments from across the globe in a comprehensible manner. The opinions expressed in each chapter belong solely to the contributing authors. Their interpretations of the topics are the integral part of this book, which I have carefully compiled for a better understanding of the readers.

At the end, I would like to thank all those who dedicated their time and efforts for the successful completion of this book. I also wish to convey my gratitude towards my friends and family who supported me at every step.

Editor

Contents

Part 1

Exploring Cellular Mechanisms Using Patch Clamp Technique

Patch Clamp Technique for Looking at Serotonin Receptors in B103 Cell Lines: A Black Box Test

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1. Introduction

In this chapter, we would like to describe black box testing phenomenon of patch clamp technique while looking at the serotonin receptors in B103 cell lines.

In a black box test, the tester only knows the inputs and what the expected outcomes should be and but not the mechanisms of those outputs. Patch clamp method is a great method for quantifying the research on Pico or femto scales, but most of the time even very controlled experiments will not give us the expected results. We will begin our chapter by introducing serotonin receptors and B103 cell lines.

In mammals, serotonin or 5-hydroxytryptamine (5-HT) behaves primarily as an inhibitory neurotransmitter of the central nervous system (CNS), decreasing neuronal activity and facilitating behavioural relaxation, while peripherally it has an excitatory role, promoting inflammatory responses, pain, and muscle spasm (Kirk et al 1997). Centrally this neurotransmitter is produced nearly exclusively by a group of neurons found in the rostro-ventral brainstem comprising the raphé nuclei from which project two major serotonergic pathways (Dahlstrom & Fuxe, 1960).

There are more than seventeen types of serotonin receptors and almost all are associated with G-proteins except 5-HT_3R, which is a member of the ligand-gated ion channel superfamily. The 5-HT_3R was initially identified as a monovalent cation channel by studies indicating that extra-cellularly recorded depolarising responses were diminished by removal of Na^+ from extracellular solution (Wallis & Woodward, 1975). The native 5-HT_3R is a cation-specific ion channel, but is otherwise relatively non-selective (demonstrating poor cation discrimination) allowing the passage of even large molecules, such as Ca^{2+} and Mg^{2+} (Maricq et al., 1991).

Serotonin type 3 receptors have been identified in the enteric nervous system (Branchek, et al, 1984), on sympathetic, parasympathetic, and sensory nerve fibres in the CNS (Kilpatrick et al, 1987), and on several mouse neuroblastoma cell lines, including the NCB-20 (Lambert et al., 1989, Maricq et al., 1991), N1E-115 (Lambert et al., 1989), and NG 108-15 (Freschi & Shain, 1982). All of these lines exhibits a rapid membrane depolarisation accompanied by increased membrane conductance in response to exogenously applied 5-HT (Peters & Lambert, 1989).

We are using B103 cell lines to study this fast acting receptor channel. The B103 rat neuroblastoma cell line was produced via transplacental exposure to nitroethylurea (Druckrey et al., 1967) and literature (Tyndale et al., 1994; Kasckow, et al., 1992) indicated the possibility that this line could be derived from cells of the raphé nuclei, and so might be representative of cells from the serotonergic pathway. The B103-line has been used as a model in a number of studies looking at GABA function, including GABA uptake (Schubert, 1975), and binding (Napias, et al., 1980). Studies looking at the functionality of $GABA_AR$s in a number of the lines initially generated (Schubert et al.,1974) via the patch-clamp technique indicated that while all lines were suitable for patch-clamp studies, none showed appreciable $GABA_A$-induced chloride conductance. Although the B103-line was not used in this study, it was reasonable to assume that it might exhibit similar characteristics and be suitable for electrophysiological studies (Hales & Tyndale, 1994). This was supported by the findings of (Kasckow et al., 1992) where patch clamping detected no functional $GABA_A$ chloride channels in the B103-line. Other studies involving the B103-line have centred around exploring the characteristics of Alzheimer's disease (specifically neuritic plaques) with particular focus paid to the β-amyloid peptide (Mook-Jung, 1997), and β/A4 protein precursor (Ninomiya et al, 1994).

Membrane excitability of the line was initially confirmed using anode-break stimulus, while ^{125}I-α-neurotoxin binding indicated the presence of AChRs. B103 cells were shown to contain the neurotransmitter GABA, and both choline acetyl transferase and glutamic acid decarboxylase activities - enzymes acting in ACh and glutamate anabolism (Schubert et al., 1974). This cell line has also been used for looking at the effects of extracellular Ca2+ influx on endothelin-1-induced mitogenesis, as B103 neuroblastoma cells predominantly express endothelin ETB receptors (Yoshifumi et al, 2001)

It has been shown previously that metastatic cells express high levels of voltage-gated Na+ channels (VGSCs) in prostate cancer (Laniado et al., 1997), breast cancer (Fraser et al., 2002; Roger, et al., 2003) and melanoma (Alien, et al, 1997).

Although, the cell line has previously proven suitable for patch clamp study, no work had yet been conducted about the presence of serotonin type 3 receptor channels and their relationship with the types of VGSCs for these cells.

The patch clamp technique has been applied to the B103 cell line in this experimental series in order to explore the native voltage-gated channels (VGCs) and serotonin sensitivity to type 3 receptors present in these cells. This project is aimed to explore whether these cells presented active/functional serotonin type 3 receptors ($5\text{-}HT_3R$) and voltage-gated sodium channels (VGSCs) and the link between each other.

2. Experimental procedures and methods

2.1 Cell culture

The B103 cells were donated by Dr Phil Rob (Cell Signalling Unit, Westmead). Stock aliquots were stored at -80°C and active stocks used for 20-25 passages before a new aliquot was revived - passage limitation decreased the incidence of cellular mutation (Figure 1).

Twice a week confluent active stocks were split and new flasks seeded in neuronal growth medium (NGM) (DMEM (TRACE), 10% foetal calf serum (FCS), 2% of 7.5% sodium

bicarbonate, 200 mM L-glutamine, 2% 1 M HEPES). Five minute incubation in trypsin at 37°C, 5% CO_2, 90% humidity (Forma Scientific incubator) degraded the extra cellular matrix of the culture, releasing cells from flask adhesion (effective dislodging turned the trypsin cloudy).

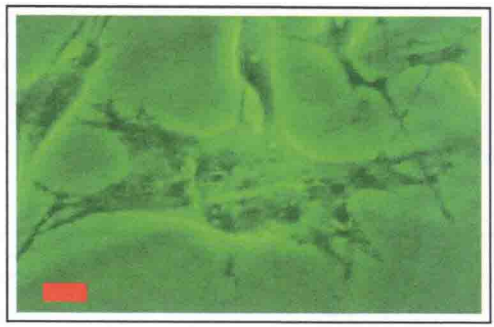

Fig. 1. A Sample Mutated Cell from the B103 Clonal-Line. Taken with an Olympus inverted microscope at 30× magnification showing dramatically altered morphology. These cells were typically seen to engulfing neighbouring cells.

Trypsin was inactivated by adding NGM, preventing continued digestion, which would have resulted in cell lysis. The suspension was spun at 400 rpm for 8-10 minutes in a megafuge (Heraeus Instruments). Supernatant was discarded and cell pellet gently resuspended in 10 ml NGM.

Later on cells were replated (Figure 2) and cover slips were prepared for patch clamp experiments.

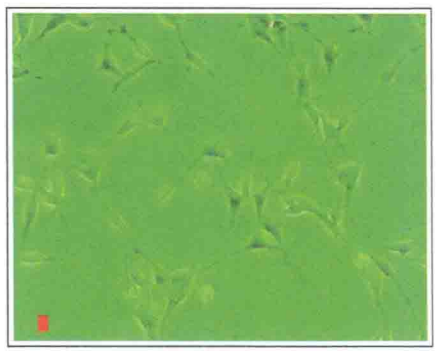

Fig. 2. A Typical B103 Cell Culture. Image at 10× magnification after 48 hours of incubation, showing a cellular concentration of 4.0×10^5. Note the extensive branching network generated.

2.1.1 Cell counting

Cells were counted from the outer four segments of a **hemocytometer** (Improve Neubave Weber) under **10×** magnification (using an Olympus CK2 microscope) and a total mean

value was calculated. This value was used to determine the concentration of cells per millilitre in the diluted cell suspension by employing the formula:

mean cell count × 100 000 (gave a per ml value) = cells/ml

After cell concentration was calculated, the cell suspension was diluted to **1.0 × 10^5 cells/ml** and the cells plated at varying concentrations onto sterilised collagen-coated coverslips (see heading Collagen-Coating the Coverslips) in **35 × 10 mm tissue culture dishes** (Corning). The cellular concentration required for later work was **4.0 × 10^5** and because cells roughly **doubled** every **24 hours**, plates were seeded with four different cellular concentrations (Table#1).

Day of Use	Plating Cell Concentrations	Cell Culturing	
		FCS Media (ml)	Cell Suspension (ml)
Day 1	seeding performed	-	-
Day 2	2.0×10^5*	0	2
Day 3	1.0×10^5*	1	1
Day 4	5.0×10^4*	1.5	0.5
Day 5	2.5×10^4*	1.75	0.25

* Because of the doubling rate of neuronal cells, plates reached a concentration of 4.0×10^5 on their respective days of use.

Table 1. Cell Culturing Schedule

2.1.2 Collagen-coating the coverslips

Collagen provided a matrix for B103 cell adhesion when plated. Coverslips and culture dishes were coated with sterile **10 µg/ml** rat tail **collagen solution** (Roche) diluted in **phosphate buffered saline** (PBS), and incubated at **37°C** for **2 hours**. The collagen solution was removed and dishes washed with PBS to ensure complete removal of residual collagen.

2.2 Solutions

Cells were patched under two different sets of bath and pipette solutions. Initial results were obtained from physiologically normal solutions (normal pipette solution: 120 mM KCl, 3.7 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM TEACl, 10 mM HEPES, 11 mM EGTA (pH 7.4); normal bath solution: 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, 10 mM D-glucose (pH 7.4)) which were designed to mimic normal cellular conditions. Later recordings utilised solutions with symmetrical cation concentrations (normal pipette solution: 140 mM NaF, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA (pH 7.4); experimental bath solution: 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10mM

D-glucose (pH 7.4)). To promote long-term cell viability bath solution osmolarity was kept between 300-320 mOsm. A difference of 20 mOsm rendered cells non-viable for electrophysiological study (adversely affecting plasma membrane structure and function) either resulting in cell swelling (<300 mOsm) or shrinking (>320 mOsm) leading to premature cell death. The bath perfusion system was used to elute the cell cultures and was comprised of a solution reservoir connected to the bath via plastic tubing. A regulator was attached to the tubing allowing for control of solution flow - unrestricted flow was 0.38 ± 0.009 ml/sec.

2.2.1 Bath solution perfusion

Solution was removed from the bath and emptied into a waste reservoir via a system of tubing connected to a **miniport motor** (Neuberger). Between the waste reservoir and the motor was a second reservoir containing **silica gel crystals** which prevented moisture from reaching the motor.

The bath perfusion system was particularly prone to contamination, especially with bacteria which fed on the solution glucose. To prevent contamination the system was rinsed with distilled water after every use to remove any trace glucose. However when the inevitable contamination did occur **antibacterial solution** (Milton hospital-grade disinfectant) was used to flush the lines.

2.2.2 Technical difficulties

The technique employed for electrophysiological study of the B103 cell-line was not conducted under aseptic conditions therefore the cells were particularly prone to **bacterial infection**. Bacteria tended to attack the cellular **cytoplasm** forming small **vacuoles** (Figure#3) and rendering the cells unfit for study. Once an infection had been noted, in order to prevent further contamination (particularly of the surrounding equipment) the patch-clamp system had to be immediately decontaminated using **70% ethanol** and/or **antibacterial solution**. The coverslip had to be immediately discarded and the stage and bath had to be thoroughly disinfected to prevent contamination of subsequent coverslips.

2.3 Pharmacological agents

The following pharmacological agents were used: Serotonin, Ondansetron, Tetrodotoxin (TTX), Phenytoin, and d- Tubocurarine. All these were purchased from Sigma, except TTX (Alomone).

2.4 Patch clamp experiments

Cells were visualised with an Olympus IX70 inverted microscope and images recorded with a KOBI digital colour camera and the ASUS Live 3D Multimedia software. Electrophysiological manipulation and recordings were undertaken with a HEKA EPC9 amplifier and HEKA *Pulse* software package which supersedes older amplifier models by having a fully interactive, PC-compatible data retrieval and storage facility. The *PULSE* program allowed for automatic electronic noise adjustments such as fast and slow capacitative transients' nullifications.

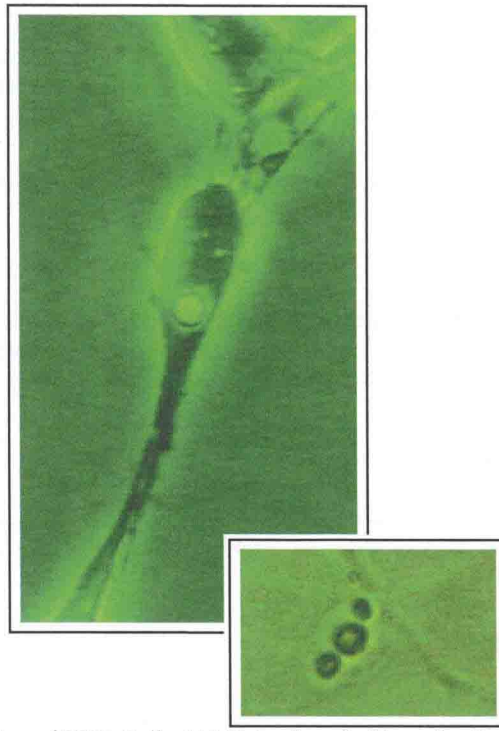

Fig. 3. Bacterial Infection of B103 Cells. **(A) Cytoplasmic Vacuole.** Bacteria entered the cells by generating holes in the cell membranes where they formed vacuoles in the cytoplasm. Image generated under phase-contrast filtering at 30× magnification. **(B) Bacterial Aggregate.** Image generated at magnification under bright-phase filtering at 60× magnification.

Thin walled borosilicate glass capillaries (1.5 mm O.D. × 1.17 mm I.D) were used to produce patch pipettes with a 3 MΩ resistance. Pipettes were half-filled using both the front- and back-filling techniques. Solution-filled glass pipettes were attached to an Ag/AgCl recording electrode and manipulated using a PCS-5000 series patch clamp micromanipulator (Burleigh Instruments). Cellular patching was performed according to the protocol outlined by (Hamil et al., 1981) Figure 4.

An appropriate B103 cell was chosen for patching on the basis of its general morphology: approximately 25 μm in diameter, well-defined clean cell membrane, and relatively isolated from contact with other cells. Morphological cellular standardisation was a critical component of the protocol. All cells were tested for their viability in the physiological saline before changing into symmetrical solutions (sodium on both side of the cell membrane) for measuring voltage activated sodium currents. 5-HT3 receptor channel currents were observed in B103 cells, when they were exposed to serotonin (endogenous currents of B103 cells were completely abolished by using TTX or Phenytoin).