NeuroToxicology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

NeuroToxicology

Full length article

Evaluation of microtransplantation of rat brain neurolemma into *Xenopus laevis* oocytes as a technique to study the effect of neurotoxicants on endogenous voltage-sensitive ion channels

Edwin Murenzi[a,c], Abigail C. Toltin[b], Steven B. Symington[b], Molly M. Morgan[c], John M. Clark[a,c,*]

[a] Molecular and Cellular Biology Program, University of Massachusetts, Amherst, MA, United States
[b] Department of Biology and Biomedical Science, Salve Regina University, Newport, RI, United States
[c] Department of Veterinary and Animal Science, University of Massachusetts, Amherst, MA, United States

ARTICLE INFO

Article history:
Received 11 December 2015
Received in revised form 31 March 2016
Accepted 4 April 2016
Available online xxx

Keywords:
1,1,1-trichloro-2,2-di(4-chlorophenyl) ethane
1,1-bis-(4-chlorophenyl)-2,2-dichloroethene
Tetrodotoxin
Xenopus laevis
Neurolemma
Microtransplantation
Voltage-sensitive sodium channel

ABSTRACT

Microtransplantation of mammalian brain neurolemma into the plasma membrane of *Xenopus* oocytes is used to study ion channels in their native form as they appear in the central nervous system. Use of microtransplanted neurolemma is advantageous for various reasons: tissue can be obtained from various sources and at different developmental stages; ion channels and receptors are present in their native configuration in their proper lipid environment along with appropriate auxiliary subunits; allowing the evaluation of numerous channelpathies caused by neurotoxicants in an *ex vivo* state. Here we show that *Xenopus* oocytes injected with post-natal day 90 (PND90) rat brain neurolemma fragments successfully express functional ion channels. Using a high throughput two electrode voltage clamp (TEVC) electrophysiological system, currents that were sensitive to tetrodotoxin, ω-conotoxin MVIIC, and tetraethylammonium were detected, indicating the presence of multiple voltage-sensitive ion channels (voltage-sensitive sodium (VSSC), calcium and potassium channels, respectively). The protein expression pattern for nine different VSSC isoforms ($Na_v1.1$–$Na_v1.9$) was determined in neurolemma using automated western blotting, with the predominant isoforms expressed being $Na_v1.2$ and $Na_v1.6$. VSSC were also successfully detected in the plasma membrane of *Xenopus* oocytes microtransplanted with neurolemma. Using this approach, a "proof-of-principle" experiment was conducted where a well-established structure-activity relationship between the neurotoxicant, 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT) and its non-neurotoxic metabolite, 1,1-bis-(4-chlorophenyl)-2,2-dichloroethene (DDE) was examined. A differential sensitivity of DDT and DDE on neurolemma-injected oocytes was determined where DDT elicited a concentration-dependent increase in TTX-sensitive inward sodium current upon pulse-depolarization whereas DDE resulted in no significant effect. Additionally, DDT resulted in a slowing of sodium channel inactivation kinetics whereas DDE was without effect. These results are consistent with the findings obtained using heterologous expression of single isoforms of rat brain VSSCs in *Xenopus* oocytes and with many other electrophysiological approaches, validating the use of the microtransplantation procedure as a toxicologically-relevant *ex vivo* assay. Once fully characterized, it is likely that this approach could be expanded to study the role of environmental toxicants and contaminants on various target tissues (e.g. neural, reproductive, developmental) from many species.

1. Introduction

Electrophysiological approaches used to study the effect of neurotoxic insecticides, such as DDT and pyrethroids, on various ion channels include; external cell recordings, whole cell patch clamp and heterologous expression of cloned channels, such as

* Corresponding author at: Department of Veterinary and Animal Science, University of Massachusetts, Amherst, MA, United States.
E-mail addresses: emurenzi@vasci.umass.edu (E. Murenzi), atoltin@salve.edu (A.C. Toltin), steven.symington@salve.edu (S.B. Symington), mmemorgan5@gmail.com (M.M. Morgan), jclark@vasci.umass.edu (J.M. Clark).

http://dx.doi.org/10.1016/j.neuro.2016.04.004
0161-813X/

cRNAs injected into *Xenopus* oocytes (Goldin and Sumikawa, 1992). The speed of electronic data collection matches that of the gating processes of ion channels and allows for a direct assessment of the effect of neurotoxicants on these toxicologically-germane channel kinetic events. Nevertheless, many of the preparations used to examine native channel activity are either non-neural (Yoshii et al., 1985) or non-mammalian (Davies et al., 2007; Clark et al., 1995). Additional problems are presented using heterologously expressed channels. A variety of voltage-sensitive ion channels, including sodium, calcium and chloride, are assembled in mammals by using multiple subunit isoforms, alternative splicing and posttranslational modifications. The outcome of such a scenario is that there is no such thing as "the sodium", "the calcium" or "the chloride" channels but that there is a diversity of different channels with unique kinetic and pharmacological properties. In most cases, we do not know what suites of channel isoforms are actually expressed in a tissue nor do we know how their expression varies with time. Thus, the assessment of the action of neurotoxicants, such as DDT on individual channels, one by one using heterologous expression, becomes a daunting if not impossible task.

The majority of biochemical data collected concerning the neurotoxic action of DDT and the commonly-acting pyrethroids has been by using *in situ* preparations, such as tissue slices and isolated presynaptic nerve terminals (Bloomquist and Soderlund, 1988; Komulainen and Bondy, 1987; Symington et al., 2008). The benefits of these preparations are that they are easily obtained from the mammalian CNS and retain a variety of functional attributes for study, such as ion flux, membrane depolarization and neurotransmitter release. The disadvantages are that non-physiological means are employed to evoke depolarizing conditions and data collections occur over time intervals in excess of those occurring in the intact nervous system (Shafer et al., 2005).

There does exist, however, an alternative approach that brings together the strengths of both electrophysiological and biochemical methodologies and eliminates many of the problems mentioned above. In a series of papers from several authors (Aleu et al., 2002; Canals et al., 1996; Limon et al., 2008; Marsal et al., 1995; Miledi et al., 2002; Miledi et al., 2004; Miledi et al., 2006; Eusebi et al., 2009), a system has been reported that uses *Xenopus laevis* oocytes microinjected with plasma membranes fragments from nervous tissue (neurolemma) of a variety of organisms that allows the direct, real-time, recording of ion currents using standard two electrode voltage clamp (TEVC) electrophysiological protocols. The actions of neurotoxic insecticides, such as DDT, have not been assessed and additional ion currents besides those elicited by calcium ion and GABA and glutamate receptors have not been yet characterized (Conti et al., 2013).

One of the major limitations of neurolemma injections into *Xenopus* oocytes, however, is the lack of control of incorporation. Results published on the incorporation of acetylcholine receptors from *Torpedo marmorata* electric organs showed variability in the amplitude of responses on an oocyte-to-oocyte basis as well the magnitude of the effect in the oocytes expressing the necessary proteins (Morales et al., 1995; Marsal et al., 1995).

In this research, we evaluated the utility of using *X. laevis* oocytes, injected with rat brain neurolemma, to characterize the various ion currents that are present and the direct and comparative action of the neurotoxic insecticide DDT and its non-neurotoxic metabolite, DDE, on pharmacologically-isolated, native, TTX-sensitive voltage-sensitive sodium channels (VSSCs), a major target site of DDT and commonly-acting pyrethroid insecticides.

2. Materials and methods

2.1. DDT, DDE and other chemicals

Technical grade (98% pure, Lot#: 341-50A) p,p′-dichlorodiphenyltrichloroethane (DDT) and technical grade p,p′-dichlorodiphenyldichloroethylene (DDE) (98% pure, Lot#: 341-48A) were provided by Chem Service Inc. (West Chester, PA). Pesticide stock solutions were prepared in dimethylsulfoxide (DMSO 99.9% pure, Lot#: 0613C024, AMRESCO, LLC. Solon, OH) as a solvent vehicle and diluted as required. At no time did the concentration of DMSO exceed 1.0% w/w in any experimental assay. Chlorotoxin (CTX, 98% pure, Lot#: SLBC8745 V), ω-conotoxin MVIIC (MVIIC, 95% pure, Lot#: 110M4798 V), tetraethylammonium chloride (TEA, 98% pure, Lot#: 1427167 V), KB-R7943 (KB-R, 98% pure Lot#: 021M4743 V), niflumic acid (NFA, 98% pure, Lot#: BCBB6165), DL-dithiothreitol (DTT, 99% pure, Lot#: SLBG9110 V) were purchased from Sigma-Aldrich, Corp. (St. Louis, MO). Tetrodotoxin with citrate (TTX, 98% pure, Lot#: 9T0202-92190) was purchased from Biotium, Inc. (Hayward, CA). All automated western blotting reagents and standards were purchased from ProteinSimple (San Jose, CA). All antibodies were purchased from either Alomone Labs (Jerusalem, Israel) or ABcam (Cambridge, MA). Deglycosylation reagents were purchased from New England Biolabs (Ipswich, MA). Rhodamine reagents were purchased from Life Technology (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich, Corp. (St. Louis, MO) at the highest purity available.

2.2. Animal rearing

Post-natal day 90 (PND90) CD® IGS Sprague-Dawley female rats, were purchased from Charles River Laboratories (Wilmington, MA) and maintained at the University of Massachusetts Central Animal Facility (Morrill Science Center, Amherst, MA). Female *X. laevis* frogs were purchased from Nasco (Fort Atkinson, WI) and maintained on a 12:12 day:night light cycle at 19 °C in a vivarium (X-Hab Systems, Aquatic Eco-Systems INC, Integrated Sciences Building, Amherst, MA). All animal procedures were conducted in accordance with IACUC guidelines (Protocol ID # 2013-0026 and 2013-0007).

2.3. Preparation of neurolemma tissue fragments

Neurolemma tissue fragments (neurolemma) were prepared from whole rat brain according to Dunkley et al. (1986) with minor modifications. Rats were sacrificed by decapitation, whole brains including brain stem removed, and placed into ice-cold sucrose buffer (0.32 M sucrose, 1 mM EDTA, and 0.25 mM DTT, pH 7.4). The dissected brain was homogenized using a Teflon-glass homogenizer (30 ml Tissue Grinder, Potter-ELV, Ctd, WHEATON®, Millville, NJ) equipped with a motor-driven pestle (Model K43, Tri-R Instruments, Rockville Centre, NY) for 3–4 cycles at 500 rpm (~2–5 min) and the homogenate centrifuged at 900*g* for 10 min at 4 °C. The supernatant was decanted, transferred to a new tube and re-centrifuged at 15,000*g* for 30 min at 4 °C. The resulting crude mitochondrial pellet, containing the neurolemma, was gently re-suspended in glycine/HEPES buffer (50 mM glycine, 20 mM HEPES, pH 7.0) using a hand-operated Teflon-glass homogenizer (5 ml Tissue Grinder, Potter-ELV, Ctd, WHEATON®, Millville, NJ) to a final protein concentration of 2 mg/ml as reported (Marsal et al., 1995; Miledi et al., 2002). Neurolemma aliquots (1 ml) were transferred to cryogenic vials and pelleted by centrifugation at 1500*g* for 5 min. The supernatant was removed, the vial capped and snap frozen with liquid nitrogen and stored at −80 °C until use. Under these conditions, neurolemma fragments remain viable for ~1 year as described by Canals et al. (1996).

Please cite this article in press as: E. Murenzi, et al., Evaluation of microtransplantation of rat brain neurolemma into *Xenopus laevis* oocytes as a technique to study the effect of neurotoxicants on endogenous voltage-sensitive ion channels, Neurotoxicology (2016), http://dx.doi.org/10.1016/j.neuro.2016.04.004