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Microtransplantation of ligand-gated receptor-channels from fresh or frozen nervous tissue into *Xenopus* oocytes: A potent tool for expanding functional information

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ABSTRACT

Despite huge improvements in neurobiological approaches for investigating the functional properties of neurotransmitter receptors and ion channels, many difficulties are still encountered when focusing on the human brain. Electrophysiological studies aimed at performing direct determinations on human nervous tissue are limited by neurosurgery and also by pathophysiological conditions prevailing before and after the resective operation. The electrophysiological study of receptors and channels becomes difficult also in animal models when the cells are not accessible and/or the experiments last many hours, during which the examined nervous tissue usually becomes unhealthy. To increase the possibility of doing optimal electrophysiological recordings, addressed to investigate the functional properties of receptors and channels, more than two decades ago, foreign mRNAs were injected into *Xenopus* oocytes to heterologously express the receptors; and about a decade ago cell membranes were injected into the oocytes to directly transplant the native receptors. While the first approach needs complex procedures for mRNA isolation, the membrane preparations are simpler to obtain and the embedded receptors are transplanted in their own membrane, with their own glycosylation and together with any ancillary proteins they may have. Using injections of membranes isolated from fresh nervous tissues several issues have already been addressed and many questions can be answered in the near future. Strikingly, with this approach it has been possible to "resuscitate" receptors and ion channels from tissues kept frozen for many years. This review focuses on recently obtained information and on some new lines of biological research using receptor microtransplantation into oocytes.

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Abbreviations: TLE, temporal lobe epilepsy; nAChRs, nicotinic acetylcholine receptors; GluRs, ionotropic glutamate receptors; $GABA_A$, γ-aminobutyric acid type A; CTLE, childhood temporal lobe epilepsy; CD, cortical dysplasia; BDNF, brain-derived neurotrophic factor; AD, Alzheimer's disease.

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1. Introduction

The experimental approach of injecting into *Xenopus* oocytes poly(A+) mRNA isolated from many tissues to express receptor proteins and channels was a revolutionary and powerful technique, first introduced by Miledi and colleagues (Metafora et al., 1976; Barnard et al., 1982), that made it possible to heterologously express, study and compare the properties of the major neurotransmitter receptors and transporters of many tissues and species. Despite the great wealth of information on receptor structure and function that has been obtained during the subsequent decades using mRNA injections into frog oocytes, there are some limitations concerning the complex procedures used to isolate fragile mRNA's with strong expressional potency and also the fact that one is losing the native protein processing as well as the lipids in which the original receptors were embedded. To overcome these problems Miledi and colleagues proceeded to inject oocytes with membranes isolated from the electric organ of *Torpedo*; and this led the oocytes to acquire *Torpedo* functional acetylcholine receptors and voltage-operated chloride channels, still embedded in their original lipid membrane (Marsal et al., 1995). Subsequently, nicotinic acetylcholine receptors (nAChRs) purified from the electric organ and reconstituted in a lipid matrix were used to see whether nAChRs embedded in heterologous lipids could be incorporated correctly in a host oocyte membrane. Proteoliposome injections into the oocytes led to the appearance of functional nAChRs, most of them with the correct orientation (Ivorra et al., 2002), ACh being substantially ineffective if applied inside the oocyte (Morales et al., 1995). The injection of proteoliposomes into *Xenopus* oocytes extends from ligand-gated receptors to transporters such as aquaporins (Le Cahérec et al., 1996; Bossi et al., 2007). We review here the use of the receptor microtransplantation technique focusing on ligand-gated receptor/channels and the results obtained to date, thus paving new roads for further development and applications.

2. Receptor microtransplantation by injecting membranes into *Xenopus* oocytes

2.1. The technical approach in brief

The heterologous expression of ligand-gated and voltage-gated ion channels in the oolemma of *Xenopus* oocytes is considered one of the most powerful tools for determining their function and structure. This experimental approach is particularly useful when the native cells are not easily amenable to extensive investigations, as for instance cells from the human brain. Two methods with comparable efficacies may be used to express receptors and channels in the oocyte membrane: (A) the cytoplasmic injection of *Xenopus* oocytes with poly(A+) mRNAs extracted from native tissues, or intranuclear injection of cDNAs encoding ion channels and (B) the cytoplasmic injections of membrane vesicles isolated from native tissues. The latter method shows some technical advantages compared to the first one:

- Easier preparation of the material to be injected;
- faster functional expression of neurotransmitter receptors compared to the classical mRNA injection;
- more stable preparation because degradation by RNAase is avoided;
- small amounts of tissue required and the same aliquots of membrane preparations can be used after thawing and freezing many times without many precautions.

A sketch of the"microtransplantation" method is shown in Fig. 1, while procedures routinely used are summarized in the following lines (and detailed in Miledi et al., 2002, 2006):

- 0.1–0.5 g of frozen tissue are homogenized in glycine buffer;
- the filtrate is centrifuged for 15 min at 9500 × *g*;
- the supernatant is centrifuged for 2 h at 100,000 × *g* with an ultra-centrifuge;
- the pellet is washed, re-suspended in assay buffer (glycine 5 mM) and used directly or aliquoted and kept at −80 °C for later usage;
- the preparation of *Xenopus laevis* oocytes and injection procedures are fully detailed in Miledi et al. (2002, 2006);
- oocytes are injected with membrane fractions (50–100 nl; 0.2–10 mg protein/ml) dissolved in 5 mM glycine, and maintained in modified Barth's solution plus antibiotics at 16 °C until the electrophysiological recordings are performed.

However, when necessary, tissue samples 10–50 mg were used for membrane preparation.

2.2. Technical improvements of the method

Many attempts have been made to see whether protein concentration in the membrane preparation affects either the receptor expression delay or the density of functional receptors expressed, so far without very conclusive results. Preparations with different protein concentrations exhibit different time courses of receptor incorporation: the more concentrated samples generally incorporating more slowly. Moreover, a critical protein concentration for each preparation exists, above which the amplitude of the currents recorded does not increase further and the injection becomes deleterious to the oocytes. Since the injected membrane vesicles undergo a fusion process with the oolemma, it could be thought that cytosolic Ca^{2+} is involved in the mechanism of membrane fusion. Therefore, increasing the intracellular Ca^{2+} level could improve the incorporation of receptors after membrane injections. However, Morales and colleagues (Gal et al., 2000) showed that the incorporation of proteoliposomes into the *Xenopus* oocyte membrane is not triggered by an intracellular Ca^{2+} increase being a "constitutive mechanism" of oocytes, indicating that Ca^{2+} is not stringently required for the fusion process. The same authors suggested that the mechanism of membrane fusion could be accelerated by protein phosphorylation or by incorporation of fusion proteins with membranes before their injection (Gal et al., 2000). To date, these issues have not been addressed further. A complication of membrane microtransplantation from a nervous tissue to the

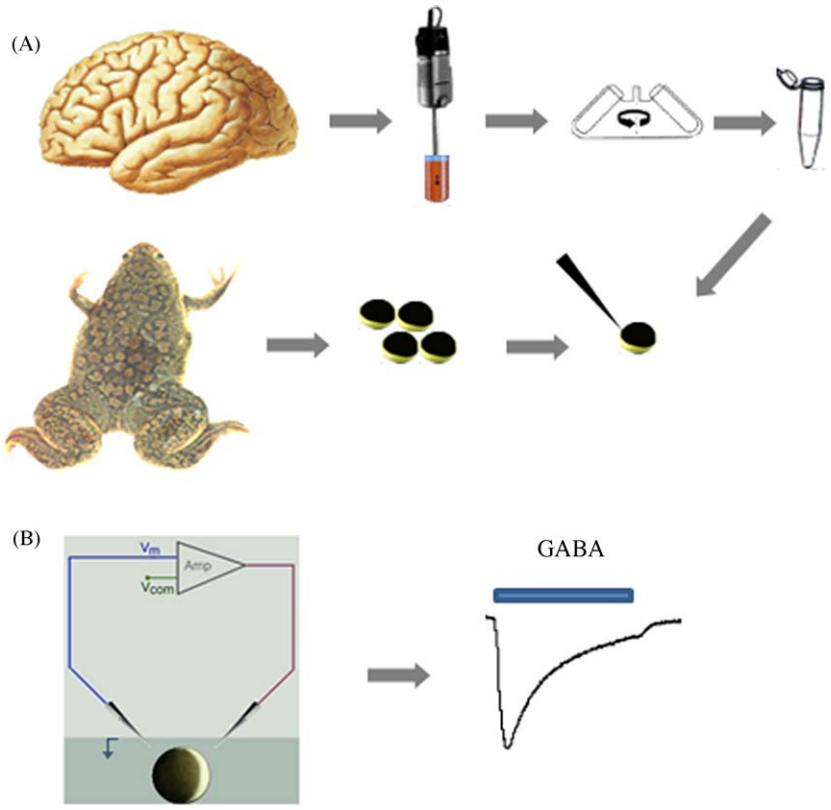

Fig. 1. Microtransplanting ligand-gated receptors from the brain to *Xenopus* oocytes (modified from Miledi et al., 2002, 2006). (A) Sketch of membrane extraction from human brain and oocyte injection and (B) sketch of voltage-clamp recordings of $GABA_A$ current from a membrane injected oocyte.

oocyte consists in the *in toto* preparation of the cell membranes; without discriminating their source; i.e. whether from neuronal or glial cells and from plasma or intracellular membranes. Sanna et al. (1996) tried to overcome this limitation by injecting rat cortical or nigral synaptosomes yielding the functional incorporation of $GABA_A$ receptors in *Xenopus* oocytes; and subsequently they used the same experimental procedures for transplanting cerebellar receptors in oocytes to express functional $GABA_A$ receptors (Sanna et al., 2003). A further improvement of the technique was attained by injecting into *Xenopus* oocytes a preparation of rat forebrain post-synaptic densities (PSDs) in order to study post-synaptic NMDA currents and their modulation by BDNF (Sandoval et al., 2007). In such a way they could study neuronal synaptic membranes.

Another limitation of the injection of membrane preparations from nervous tissue is the low level of expression of some types of ligand-gated receptors, such as neuronal nicotinic acetylcholine receptors. This low expression could be due to their inverse orientation in the oocyte membrane. Using radioactive labelling it has been calculated that, following injection of AChR reconstituted in lipids, 80% of the receptors are not properly assembled (Ivorra et al., 2002a,b). This problem can often be overcome by selecting tissues particularly rich in the appropriate receptors.

2.3. Morphological pattern of injected membranes

The protocol commonly used to obtain membrane preparations for microtransplantation (see Section 2.1) did not involve

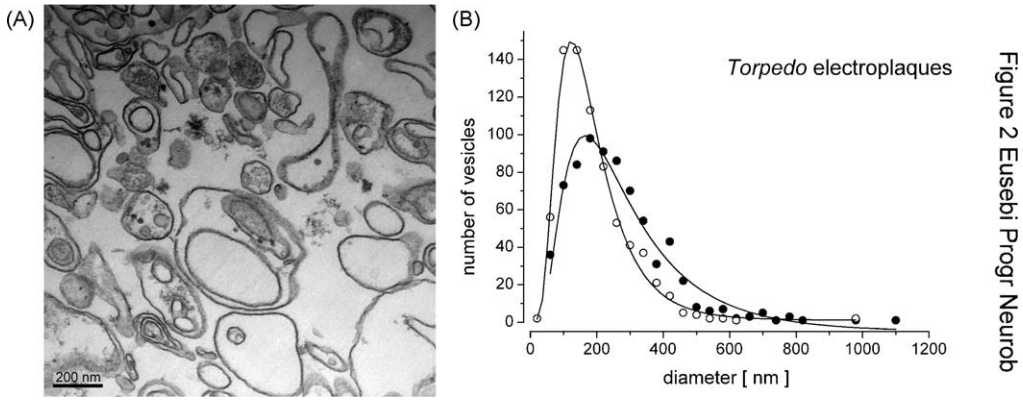

Fig. 2. (A) Representative electron microscope image of a *Torpedo* membrane preparation and (B) size distribution of the membrane vesicles with min (○) and max (●) diameters measured for each vesicle. Five TEM samples from three different membrane preparations ready for injections were analysed. Minimal and maximal diameters (i.e. short and long axis) were measured for a total of 726 vesicles, values were grouped in bins of 40 nm and plotted. Mean diameters ± S.E.M.: (○) 164 ± 3 nm; (●) 250 ± 10 nm.

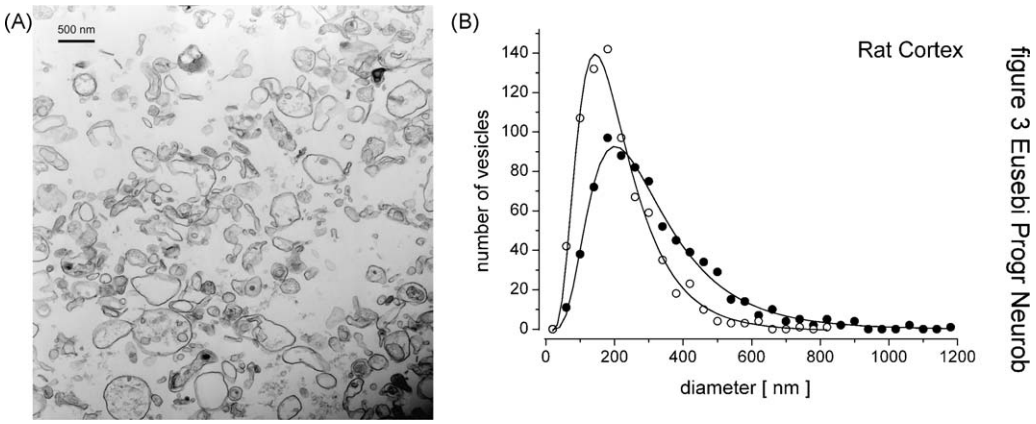

Fig. 3. (A) Representative electron microscope image of a rat cortex membrane preparation and (B) size distribution of the membrane vesicles with min (○) and max (●) diameters measured for each vesicle. Data values were obtained from three TEM samples from a single membrane preparation. Minimal and maximal diameters (i.e. short and long axis) were measured for a total of 736 vesicles, values were grouped in bins of 40 nm and plotted. Mean diameters ± S.E.M.: (○) 190 ± 4 nm; (●) 271 ± 4 nm.

purification procedures or the use of detergents to solubilise the membrane proteins. Accordingly, many different proteins are present in the membrane preparation, as can be seen by running gels of the samples. Some information on the morphology of the membrane preparations has been obtained by transmission electron microscopy. To evaluate the size distribution of the vesicles, several areas in different sections of the samples were inspected and the short and long axis (to which we refer as min and max diameter) were measured for each vesicle. Figs. 2A and 3Aillustrate respectively the electron microscopic appearance of membranes isolated from the electric organ of *Torpedo* and from the rat cerebral cortex. In both cases the membranes are seen mainly as heterogeneous uncoated vesicles of diameters ranging from 40 to 1200 nm, whose diameter distribution is illustrated in Figs. 2B and 3B. Such morphological inhomogeneity may contribute to the variability of receptor microtransplantation usually observed.

2.4. Time-course of receptor expression

Incorporation of receptors is detectable within a few hours following membrane injection (Marsal et al., 1995; Gal et al., 2000; Miledi et al., 2002; Sanna et al., 1996). This incorporation has been monitored by recording current-responses to the corresponding neurotransmitter over time after the oocyte injection of various types of membrane preparations: purified nAChRs from *Torpedo* reconstituted in asolectin lipid vesicles (Gal et al., 2000), rat cortical and nigral synaptosomes (Sanna et al., 1996), membranes isolated from human temporal neocortex (Miledi et al., 2002). Results reveal maximum responses c.a. 16–24 h after injection, with a plateau of c.a. 2–3 days duration (Marsal et al., 1995; Miledi et al., 2002). Fig. 4 shows the time-course of persistence of functional $GABA_A$ receptors over 2 weeks following the injection of membranes isolated from rat neocortex. It is likely that this decay mirrors the slowing incorporation of the receptors over time.

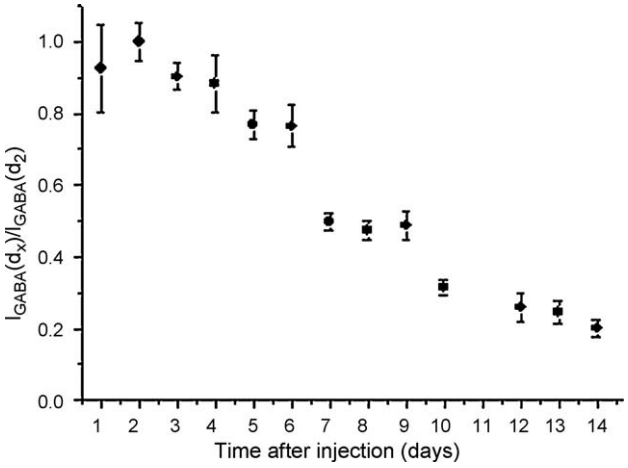

Fig. 4. Time-course of the "expression" of $GABA_A$ receptors. Expression is shown as the ratio between the GABA current determined over time (days post-injection d_i) and that at day two, when the expression was maximal. *Rat cortex membrane preparation*: experiments were performed at room temperature (19–21 °C), oocytes were continuously perfused with Ringer's solution (flow rate 10 ml/min) and GABA 1 mM was bath applied. Mean $I_{GABA}(d_2) = 98 \pm 7$ nA (78 oocytes; 4 donors).

2.5. Site of injection and receptor sprouting

It is known that the native muscarinic receptors present in the oocyte membrane are located mainly in the animal hemisphere (Kusano et al., 1982) and that the GABAρ1 and α7 nicotinic receptors expressed by their respective cRNAs are also concentrated mainly in the animal hemisphere plasma membrane (Martinez-Torres and Miledi, 2001; Palma et al., 2002a,b; Limon et al., 2007). Because of this polarization it was interesting to find out the amounts and sites of insertion of the membranes carrying the transplanted receptors. For that purpose Amici and Miledi injected *Torpedo* electric-plaque membranes at the animal pole, equator or vegetal pole and measured the ACh-currents generated by the oocytes, finding that, while the neurotransmitter sensitivity is rather uniform through all the vegetal pole and the equator, it is quite variable when membranes have been injected into animal pole. Thus, the best procedures suggested by these findings are to inject membranes in the vegetal pole or the equator. Using α-bungarotoxin conjugated with a fluorescent probe (Alexa Fluor-488) to visualize the distribution of *Torpedo* nAChRs and imaging oocytes with a confocal laser scanning microscope, it is possible to monitor the nAChRs distribution in oocytes injected with *Torpedo* membranes. Fig. 5 shows that nAChRs are distributed around the site of membrane injection in the oocyte.

Fig. 5C shows a certain clustering of receptors in the vegetal and animal pole sides when membranes from electric organ of *Torpedo* are injected into the equator.

By contrast, in oocytes injected with αβγδ-subunit cRNAs coding for *Torpedo* AChR, a wide spread fluorescence signal was visualized (e.g. Fig. 5D) rather than patchy distribution obtained with injected membranes.

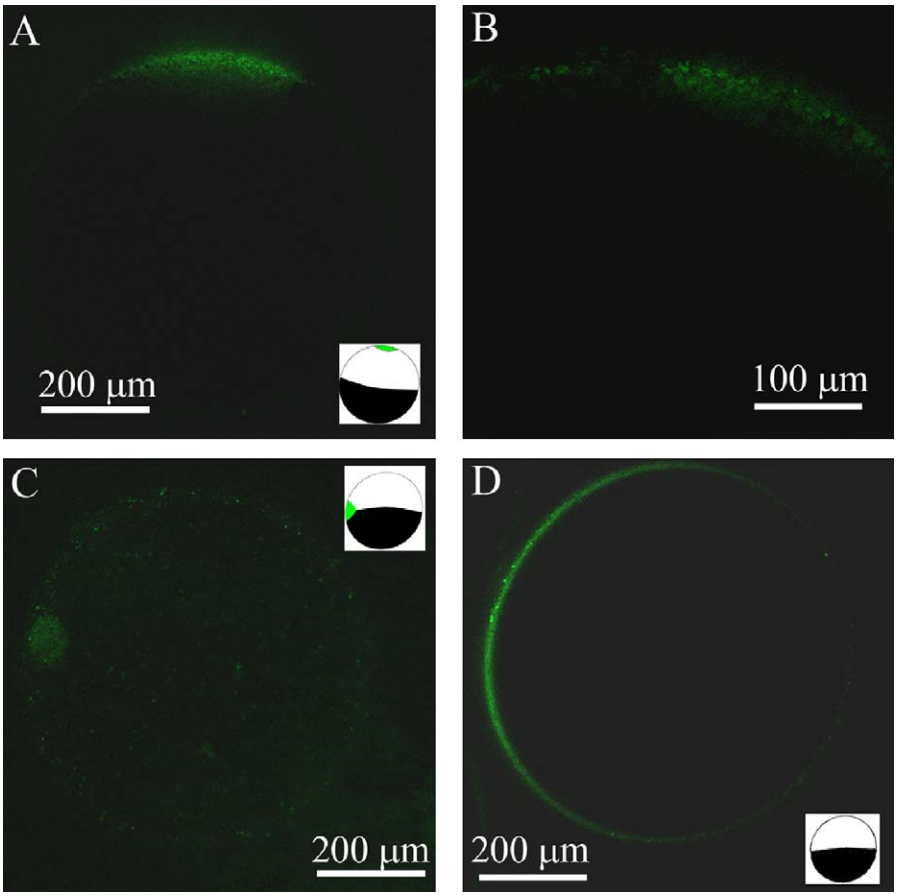

Fig. 5. Confocal images of oocytes expressing nAChRs labelled with α-bungarotoxin conjugated with Alexa Fluor-488, images were taken 2–3 days after membrane injection and 6 days after cRNA injection. (A) Oocyte injected with *Torpedo* membranes, injection site: vegetal pole; in (B) the same oocyte after 1 h exposure to Ringer's solution without α-bungarotoxin. (C) Oocyte injected with *Torpedo* membranes, injection site: equator. (D) Oocyte injected with αβγδ-AChR subunit *Torpedo* cRNAs.

3. Functional properties of microtransplanted receptors

The method, developed to microtransplant already assembled neurotransmitter receptors from brains and other tissues to the plasma membrane of *Xenopus* oocytes, raised two main questions.

The first question was whether the findings obtained with the original approach of injecting heterogenic mRNAs isolated from tissues (Gundersen et al., 1984; Wagner et al., 2000; Bossi et al., 2007) are equivalent to those obtained by injecting membranes isolated from the same tissues. The initial microtransplantation study (Marsal et al., 1995) already showed that electroplaque acetylcholine receptors and chloride channels, expressed by mRNAs or microtransplanted, had similar properties. An extensive study on the physiological and pharmacological properties of human $GABA_A$ and glutamate receptors (GluRs) was performed by isolating mRNAs from samples of human epileptic brains and injecting them into *Xenopus* oocytes (Palma et al., 2002). The properties of the receptors expressed were compared with those of the receptors embedded on the tissue membranes isolated from the same human brain tissues and injected into oocytes, obtaining equivalent results (Miledi et al., 2002). Thus, *the answer to the first question* was that the ligand-gated receptors studied share functional properties independent of their heterologous origin following injections of mRNAs or membranes into *Xenopus* oocytes.

The *second question* raised by microtransplantating receptors through membrane injection was whether the oolemma incorporates the membranes and acquires neurotransmitter receptors with the same properties as those of the receptors while they were still in the "donor" cells. Three types of neurotransmitter receptors, the homomeric glutamate GluR1 receptor and the heteromeric neuronal nicotinic α4β2 receptor, both expressed in HEK cells, and the homomeric neuronal nicotinic receptor α7, expressed in GH(4)C1 cells, were microtransplanted to oocytes (Palma et al., 2003), and the receptors behaved as those present in the native cells. Namely, *I–V* relationships, dose–response curves, and modulator or antagonist sensitivity were perfectly maintained after transplantation. Therefore, *the answer to the second question* was that, in general, the properties of the receptors in their native membrane are retained after transplantation to the oocytes, indicating that microtransplantation is a useful method to study proteins present in the membranes of cultured cells.

It is noteworthy that one advantage of the microtransplantation method is to "normalize" the functional properties of the receptors rendering them independent of the many types of native host cells where the receptors are originally expressed, thus permitting the analysis of their gating and kinetics under the same environmental conditions keeping in mind, however, that these conditions including intra- and extra-membrane signaling pathways and cytosolic and extracellular ambients are far from the original ones. Another limitation of this method is represented by the oocyte's dimension, altering the kinetics of macroscopic currents generated by the transmitter. These are generally much slower than the currents generated in smaller cells, with a few exceptions as, for instance, the current decay of GluR1 receptors, which is faster in the oocyte compared to HEK cells (Palma et al., 2003). Patch-clamp single channel recordings can be used to overcome this limitation, as after the injection of poly(A+) mRNAs or cRNAs (Miledi et al.,

1983; Leonard et al., 1987; Mileo et al., 1995). All these findings confirm that, even with some drawbacks, the microtransplantation of receptors to oocytes is a very powerful method to conveniently and extensively investigate the properties of ligand-gated receptors, and also to compare *ex vivo* their functional properties.

4. Physiological and pharmacological issues addressed

At its beginning, the microtransplantation method was developed injecting already assembled AChRs and Cl^- channels from the electric organ of *Torpedo* into *Xenopus* oocytes. Namely, membrane vesicles from fresh *Torpedo* electroplaque tissue were injected into the oocytes and, within a few hours, the oocyte membrane acquired AChRs and Cl^- channels (Marsal et al., 1995). The mechanism of incorporation of those AChRs receptors and Cl^- channels was very different from that following the injection of mRNA into the oocytes, since the appearance of receptors after membrane injection does not require *de novo* protein synthesis, N-glycosylation or other post-translational modifications. That work, the first, indicated that the foreign receptor-bearing membranes fuse with the oocyte plasma membrane and cause the appearance of functional receptors and channels in the oocyte membrane. A parallel line of research consisted in injecting oocytes with purified *Torpedo* AChRs incorporated in an artificial lipid matrix. This led to the appearance of clusters of AChRs that retained their native properties (Morales et al., 1995). This latter line of research yielded a number of interesting results on the functional incorporation of exogenous proteins into the oolemma. One notable conclusion was that the fusion process is independent of intracellular calcium increase (Gal et al., 2000) contributing towards a better understanding of the fusion of lipid vesicles, including cortical granules, with the egg membrane. Further results were obtained by injecting purified AChRs and investigating the action of quaternary ammonium cholinesterase inhibitors on AChR function (Olivera et al., 2005; Olivera-Bravo et al., 2005, 2006, 2007) demonstrating that this method of inserting exogenous ligand-gated receptors in oocytes is very useful also for the screening and assay of drugs.

Another relevant result obtained by the approach of protein purification and injection of proteoliposomes into oocytes was the discovery and characterization of a novel member of the aquaporin family, the P25 polypeptide from the MIP family found in digestive tract of a sap sucking insect *Cicadella viridis* (Le Cahérec et al., 1996). That work demonstrates that the insertion of functional foreign membrane proteins into the oocyte membrane is not restricted to receptors and ionic channels but extends to other transmembrane proteins.

The alpha4beta2 nicotinic ACh receptors have been microtransplanted from HEK cells into oocytes and a number of relevant findings, including the effects of long-term exposure to nicotine, have been obtained (Moroni et al., 2006). NMDA-receptors have been microtransplanted from rat forebrain post-synaptic densities into oocytes with the notable demonstration of antagonistic effects of TrkB and $p75^{NTR}$ on NMDA currents. Specifically it has been reported that $p75^{NTR}$ is inhibitory for NMDA currents and antagonizes TrkB-mediated NMDA-R potentiation, a mechanism likely playing an important role on synaptic plasticity (Sandoval et al., 2007). Expression of native $GABA_A$ receptors by the injection of rat brain synaptosomes has been obtained giving evidence that $GABA_A$ receptors retain the main pharmacological properties of native tissues (Sanna et al., 1996). All together, these reports indicate that the membrane microtransplantation approach is a strong and simple method for studying receptor pharmacology, and is useful also for transplanting transduction pathway elements together with ligand-gated receptors. However, perhaps the most important results to be obtained, using the membrane microtransplantation approach, are achieved when addressing pathophysiological issues of the human brain, as shown below.

5. Pathophysiological issues addressed

5.1. Intractable epilepsies

Epilepsy is a large group of heterogeneous neurological disorders characterized by recurrent seizures, with several types of currents activated simultaneously in many neurons. Specifically, in focal epilepsy the seizures develop in localized areas of a cerebral hemisphere. Usually, patients undergo substantial benefit following treatments with antiepileptic drugs. However, patients with medically intractable epilepsy are seizure-free only after resective surgery, where some brain tissues are removed. Several experimental approaches, including molecular biology, immunohistochemistry, and genetics have been used extensively trying to decipher the pathogenesis of epilepsies. These have yielded the general view that an imbalance between excitatory and inhibitory pathways in the brain may be mainly responsible for the epilepsies. Taking advantage of the nervous tissue resection in patients afflicted with intractable epilepsies, a large body of work has been done on the GABA-ergic system in epilepsy and relevant results have been obtained with the experimental approach of receptor microtransplantation into *Xenopus* oocyte.

5.1.1. Human adult temporal lobe epilepsy (TLE)

The human adult temporal lobe epilepsy is a focal neurological disorder accounting for 30–35% of all epilepsies, and the almost unique therapy with favourable outcomes is resective neurosurgery. This epileptic syndrome, specifically in mesial TLE, is categorized as cryptogenic since the cause of the disorder is hidden. Experimental models indicate that excitability is altered by changes in voltage-operated or ligand-gated channels and in ion or neurotransmitter transporters; and neuromodulators or second messengers have also been implicated in seizure expression. Nevertheless, despite large efforts to understand its aetiopathology, a convincing picture of the events underlying TLE has not been disclosed and beneficial treatments have not yet been established. Besides many experimental procedures, one of the most promising approaches to disclose possible changes in the properties of the receptors of the epileptic brain is actually the microtransplantation of ligand-gated receptors from the human epileptic brain to *Xenopus* oocytes.

It is well known that *Xenopus* oocytes injected with brain mRNAs of many species are able to express many types of functional neurotransmitter receptors, due to translation of the exogenous mRNA by the oocyte's own protein synthesizing machinery (Miledi et al., 1989; Matute et al., 1992). Evidence has been provided that *Xenopus* oocytes, injected with poly(A)$^+$ RNA extracted from the human epileptic temporal lobe and hippocampus, express functional ionotropic glutamate receptors as well as γ-aminobutyric acid type A ($GABA_A$) receptor, with peculiar properties that have been reported in a series of papers that opened previously unexplored avenues towards the diagnosis and treatment of epileptic patients (Palma et al., 2002). In most of those papers (Palma et al., 2005a,b, 2006, 2007a,b; Ragozzino et al., 2005) the cytoplasmic membrane injection technique, rather than poly(A+) mRNA injections, was used to gain information on the receptors as isolated from the epileptic brain. An important relevant result, which may have a role in TLE, was that the stability of the "epileptic" $GABA_A$ receptors was much less than that of non-TLE $GABA_A$ receptors. Specifically, the receptor instability, monitored by the use-dependent run-down of GABA receptors type A, is an amplitude decrease of the ionic current generated by the repetitive activation of $GABA_A$ receptors. That decrease is due

mostly to receptor desensitization, involving a shift of channels from the open to a desensitized state (Hille, 2002). The run-down is a process that occurs both in reconstituted systems and in native neurons. It is noteworthy that, in refractory TLE, the $GABA_A$ run-down is greater, and the recovery of the GABA current is slower, compared to non-TLE tissues (Palma et al., 2004), presumably due to the participation of additional mechanisms, such as receptor endocytosis (Goodkin et al., 2007). In the brain, the $GABA_A$ receptor run-down reduces the efficacy of the GABA-ergic signal and is expected to facilitate and reinforce seizures. Noteworthy, another mechanism contributing to $GABA_A$ action run-down and facilitating seizures could be the endocannabinoid-mediated reduction of GABA release (Wilson and Nicoll, 2001): injection of synaptosomes extracted from hippocampal GABA-ergic terminals into oocytes may provide a strong experimental approach to give another clue to mechanisms of seizures. Moreover, this picture of the inhibitory GABA-ergic system in intractable epilepsy becomes more complicated in the TLE hippocampal subiculum, a region considered to be responsible for the interictal discharges likely due to perturbed chloride homeostasis (Huberfeld et al., 2007), where the inhibitory neurotransmitter GABA switches to excitatory in ~20% of TLE pyramidal neurons (Cohen et al., 2002). It is noteworthy that, with the membrane injection approach, the shift of equilibrium potential underlying the excitatory nature of GABA in the epileptic subiculum, disclosed with whole-cell patch-clamp recordings in native neurons, was clearly detected also in oocytes expressing exogenous TLE $GABA_A$ receptors (Palma et al., 2005, 2006). This last observation indicates that Cl^--transporters together with ionotropic receptors, both possibly altered by the disease as shown by quantitative RT-PCR in TLE patients (Palma et al., 2006), were microtransplanted to the oocytes. Therefore, the membrane injection approach is also a potent tool for functional investigation of transporter systems (see also Bossi et al., 2007).

Given that $GABA_A$ receptor stability may be relevant for epileptogenesis, a critical objective is to discover neuromodulators of $GABA_A$ receptor function that may help to develop new antiepileptic treatments for refractory epilepsy. Using the membrane injection approach a list of $GABA_A$ receptor run-down modulators of the human TLE brain has been obtained, including brain-derived neurotrophic factor, phosphatase blockers, Zn^{2+} and levetiracetam, the potent antiepileptic drug largely employed in clinical practice (Palma et al., 2004, 2005b, 2007a,b). All these drugs, to various extents, reduce the GABA current run-down, rendering more stable the "epileptic" $GABA_A$ receptors.

In conclusion, microtransplanting ligand-gated receptors from human TLE to *Xenopus* oocytes led to the discovery of a peculiar feature of epileptic $GABA_A$ receptors, its larger instability compared to non-epileptic peri-tumoral tissues (taken as control), a feature that may play an important role in seizure development. Thus, the microtransplantation approach is a potent tool for testing new drugs, or endogenous substances such as neurotrophic factors or neuromediators, against epilepsy.

5.1.2. Lesional epilepsies

Lesions of various types are identified in patients with intractable temporal and extratemporal epilepsy. Lesions may be neoplastic, vascular, dysgenetic, traumatic or ischemic. $GABA_A$ receptors microtransplanted from patients afflicted with lesional epilepsies, specifically traumatic, dysgenetic and neoplastic exhibited larger stability compared to TLE, indicating that the $GABA_A$ receptors instability is a feature specific for cryptogenic TLE (Ragozzino et al., 2005). This result was obtained through the membrane injection approach and confirmed by whole-cell patch-clamp recordings from neurons in human brain slices (Ragozzino et al., 2005). Furthermore, injecting into oocytes membranes isolated from epileptic patients with subcortical lesional epilepsy, such as hypothalamic hamartomas (HH), led to the appearance of $GABA_A$ receptors with normal functional and pharmacological properties (Wu et al., 2007), thus supporting the view that, with the notably exception of the $GABA_A$ receptor increased instability in TLE, the dysfunction of the epileptic GABA-ergic system should be related to other network characteristics. Interestingly, HH tissue could be intrinsically epileptogenic since some neurons exhibit immature features with Cl^- equilibrium potential favouring excitation by GABA (Wu et al., 2008).

5.1.3. Pilocarpine-rat TLE

Adult "epileptic" rats exhibit recurrent seizures, caused by pilocarpine-induced *Status epilepticus*, and represent one of the best animal models of human TLE. $GABA_A$ receptors microtransplanted from the "pilocarpine" rat cortical neurons to oocytes show a greater run-down than oocytes injected with rat brain control tissue, an event also occurring in patch-clamped rat pyramidal neurons (Palma et al., 2007). The run-down of epileptic rat receptors resembles that of human TLE receptors, being enhanced by Zn^{2+} and sensitive to the antiepileptic agent levetiracetam, the neurotrophin brain-derived neurotrophic factor, and the phosphatase blocker okadaic acid. These findings point to the run-down of $GABA_A$ receptors as a hallmark of TLE, discovered using the microtransplantation technique and subsequently confirmed by patch clamping of the native neurons. These results provide additional confirmation that the GABA-ergic system of the pilocarpine treated rat is an excellent model of TLE. Furthermore, since long-lasting exposure of the nerve cells to antiepileptic drugs could change receptor properties (Macdonald et al., 1992; Raol et al., 2005), the fact that results obtained with drug-naive rat tissue are similar to those obtained from human epileptic tissue indicates that the peculiar run-down properties of the $GABA_A$ receptors are epilepsy-related rather than drug-induced.

5.1.4. Perspectives: childhood epilepsy

Out of all newly diagnosed cases of childhood temporal lobe epilepsy (CTLE), about 10% remain intractable to medical therapy and are referred for presurgical evaluation. Cortical dysplasia (CD) is frequently associated with pediatric epilepsy surgery patients, with unknown ontogenetic mechanisms. At a molecular level, pediatric epilepsy patients exhibit $GABA_A$ receptors with a large heterogeneity of subunit expression. Since GABA is the main excitatory neurotransmitter in early cortical development, and since it is generally believed that GABA networks contribute to childhood epileptogenesis, the first line drugs used to treat pediatric epilepsy, which enhance GABA-ergic neurotransmission, seem to be not very appropriate. Therefore, insights into the mechanisms by which GABA-ergic neurotransmission is modulated could be very useful to better treat seizures in pediatric epilepsy. As already mentioned, a feature of some $GABA_A$ receptors is their instability upon repetitive activation, resulting in the GABA-activated current run-down. Therefore, impairment of the run-down should be considered an epileptogenic event in the adult brain, where GABA is the main inhibitory neurotransmitter, but an antiepileptogenic in the brain of pediatric patients where in many neurons GABA may be excitatory (Cepeda et al., 2007). Accordingly, a goal that can be clearly reached using the microtransplantation technique is to determine the properties of $GABA_A$ receptors in cortical nervous tissue after pediatric resective surgery. This would pave the road towards the development of new and more rational treatments through a better knowledge of $GABA_A$ receptor modulation. Specifically, it would be important to (A) investigate whether neuromodulators of the $GABA_A$ rundown, namely BDNF, Zn^{2+} and phosphatase inhibitors, are effective also on the rundown of CTLE $GABA_A$ receptors and (B) to discover new modulators of

CTLE $GABA_A$ receptor rundown. A very recent paper began to fill these deficiencies (Jansen et al., 2008).

5.2. Alzheimer's disease

Since the original description of the dementia of Auguste by Alzheimer in 1906, many different hypotheses have been proposed to explain Alzheimer's disease (AD), including amyloid β-peptide deposition and synaptic dysfunction (Yankner and Lu, 2008). It is well known that neurotransmitter receptors are key players in the process of synaptic transmission. Nevertheless, almost nothing was known about the functional characteristics of the neurotransmitter receptors of the AD brain before the microtransplantation of cell membranes from the AD-brain to the membrane of frog oocytes. With this approach, it was found that cell membranes from the postmortem brains of humans that had died of Alzheimer's disease can be microtransplanted to the plasma membrane of *Xenopus* oocytes. Those membranes carried neurotransmitter receptors and voltage-operated channels that were still functional, even after they had "hybernated" for many years (Miledi et al., 2004). Specifically, $GABA_A$ and GluRs from postmortem brains kept frozen for >10 years were still functional and behaved like the native receptors (Bernareggi et al., 2007). The numbers of receptors and channels transplanted by membranes from AD-brains were smaller than those transplanted from non-AD membranes. Much work is still needed to determine in detail the electrophysiological and pharmacological properties, as well as the molecular structure, of these $GABA_A$ and GluRs, which, remarkably, still function so many years after the death of the patient

5.3. Autism

Autism is a neurodevelopmental disorder characterized by the early presentation of deficits in social abilities and repetitive behaviours or obsessions (Costa e Silva, 2008). Most suspected etiologies or associations of autistic spectrum disorders implicate inhibitory and excitatory neurotransmission pathways in the brain. While at a macroscopic level MRI and EEG techniques are widely used to study the autistic brain function, at the cellular level the only technique used to provide a direct and powerful insight into the process of neurotransmission in the autistic brain is the microtransplantation of neurotransmitter receptors from the postmortem autistic brain to *Xenopus* oocytes. With this technique Limon et al. (2008) microtransplanted functional receptors to GABA and glutamate, and calcium channels from the temporal cortex and cerebellum, both receptors thought to be the target of genetic factors associated with autism. So far, the properties of the "autistic" receptors appear similar to those of control receptors. We are only at the start of this new scientific road, but it already seems clear that the microtransplantation method will provide very important insights into the etiology of Autistic Spectrum Disorders as well as ways of treating them.

6. Conclusions and future developments

Molecular and physiological studies reveal a wide diversity of receptor and channel subtypes, which have peculiar pharmacological and physiological properties that are mechanistically related to the pathophysiological conditions of the host cells. One of the most powerful approaches to study cell membrane receptors and channels is their microtransplantation to *Xenopus* oocytes (Miledi et al., 2002, 2006). In this way receptors and channels retain their original functional properties and also their regulation by native associated elements. Neurotransmitter receptors and channels are subjected to dynamic regulation by specific receptor associated proteins or by receptor phosphorylation. Therefore, it is clear that the strategy of expressing native receptors in classical heterologous expression system such as transfected cell lines or RNA-injected oocytes suffers important limitations for investigating receptor and channel behaviors. The microtransplantation approach can overcome this problem, because the microtransplanted receptors retain fully the characteristics they had while still being embedded in their native surrounding environment. Using this experimental approach, studies are being done on intractable epilepsies, Alzheimer's disease and autism, adding important molecular information that will help clarify their pathogenesis.

The method of microtransplanting foreign membrane proteins to *Xenopus* oocytes has already been applied successfully to study proteins from various animal tissues and yeast; and it is clear that further developments will make it possible to carry out similar highly revealing investigations on non-nervous tissues such as endothelia, blood, derma, and gland cells, as well as plant cells. Furthermore, for patients where surgery is performed, their frozen tissues may be used for "*ad personam*" assays to develop or optimize their treatments.

Another important feature of the microtransplantation method derives from the use of postmortem tissues generously donated by patients to brain banks, which store them frozen. It is fascinating that neurotransmitter receptors have been "resuscitated" even after postmortem intervals of a few hours (AD) to more than 1 day (autistic). Therefore, donated specimens should be graciously accepted even if they have postmortem intervals of more than 1 day. Some of the tissues we have used had been kept frozen for more than 10 years. We look forward to the day when a prehistoric animal is found frozen and a sample of the brain is studied. We feel confident that his receptors will be resuscitated and we wonder what he will tell us.

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