ESRF	**Experiment title:** X-ray scattering study of excitable membrane : correlation between membrane structure and nerve physiological states	**Experiment number**: SC 1965
Beamline: ID02	**Date of experiment**: from: December 15, 2005 to December 18, 2005	**Date of report**: August, 21, 2006
Shifts: 9	**Local contact(s)**: S. Finet & T. Narayanan	*Received at ESRF:*

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Report:

X-ray scattering experiments carried out at LURE on pike olfactory nerve (from 1998 to 2003) yielded valuable information on the structure of the axonal membrane and on its thermodynamic properties (Refs 2 to 4). The quality of the results – this was the first excitable membrane ever studied by X-ray scattering techniques – emboldened us to engage in the more ambitious project of exploring the structural phenomena that may underlie the propagation of electrical stimulation. Our hopes were founded on the technical facilities that had then become available at the ESRF.

After a few tests (November 2004) we were allocated 9 shifts in February 2005 and again 9 shifts in December 2005. The results of the November 2004 and the February 2005 experiments were reported in due time. We report here on the December 2005 experiments and on the present state of the analysis (which, we stress, is still underway).

In our August 2005 project we mentioned four questions to be addressed: i) – the kinetic correlation between membrane pairing and action potential; ii) –the pharmacological aspects of membrane pairing; iii) – the direct determination of membrane thickness dilation; iv) – to extend our observations to other nerves. The analysis of the February 2005 experiments, which we completed after writing the August 2005 report, led us to more sharply focussing the December 2005 experiments on the kinetic problems.

Technical aspects

We need not dwell on the trivial problems of animal handling, nerve dissection etc. which were satisfactorily solved with the skilled cooperation of the staff of the ESRF animal house.

The sample holder deserves a few comments. We made use of a home-made device designed to perform X-ray scattering experiments on a nerve kept under permanent electrophysiological control and to carry out flash X-ray scattering experiments triggered at specific times after the onset of electrical stimulation. In the design of the last experiment we greatly benefited from the sub-millisecond beam shutter available at ESRF.

It is also worthwhile to mention the problem of collimation, which took us some time to master. Indeed, the signals that we were seeking were not stronger than the intensity scattered by an air column a few millimetres long.

Disrupting effects of X-rays on the structure and the function of the nerve (radio-degradation)

Unexpected, and worrisome, observations showed that the signals which might hopefully be ascribed to electrical stimulation were at least one order of magnitude weaker than those due to radio-degradation. Our first worry was to disentangle the effects of X-ray exposure upon, on the one hand, the physiological state of the nerve and, on the other hand, the structure of both the membrane and the cytoskeleton of the axons. A series of experiments led us to the following conclusions: i) - the physiological state of the membrane is not too seriously upset by exposures times shorter than 1 second; ii) - the structure of the cytoskeleton is more sensitive to radio-degradation than that of the axonal membrane; iii) – in all cases the cumulative exposure times must be shorter than a few hundred milliseconds; iv) – the effects of radio-degradation must be taken into account and corrected for, even for the shortest exposure times (a few milliseconds).

Design of the kinetic experiments

After a number of trials we eventually adopted the following routine. Sequences of flash (5 ms) scattering experiments are performed, in which control spectra (recorded in the absence of electrical stimulation) are alternated with a small number (2-3) of spectra recorded at different times t_c after the onset of electrical simulation. Each sequence is repeated 6 times, so that the cumulative exposure is 30 ms at each t_c; 10-odd values of t_c were explored with each nerve. The analysis of the spectra involves the difference between the spectrum recorded at time t_c and the linearly interpolated spectrum of the pair of nearest control spectra.

Results

By way of example we present a few experiments performed on 15 nerves kept in their «native» state – namely immersed in the standard solution used in electrophysiological studies (Refs 1 and 2).

i – The spectra relevant to 3 of the 15 nerves had to be discarded.

ii – 5 ms spectra were recorded with each nerve at times t_c=25,30,..., 65,70,150 ms, with a control spectrum (in the absence of electrical stimulation) intercalated every third spectrum.

iii – Difference-spectra were computed between the spectrum recorded at time t_c and the linearly interpolated spectrum of the pair of nearest control spectra.

iv – The individual difference-spectra seem to have the same shape, some being of positive some of negative sign.

v - The strongest positive and negative difference-spectra of each nerve were selected and their average computed. The average of the positive difference-spectra is plotted in Figure 1 (the average of the negative difference-spectra is the negative of Figure 1).

These observations suggest the following **preliminary** conclusions:

1) – Although the transition from the intensity increment $\Delta I(s)$ to the electron density increment $\Delta\rho(r)$ necessarily involves some assumptions, we present in Figure 2 a function $\Delta\rho_{ax}(r)$ that is consistent with the data ($\Delta I(s)$) of Figure 1 and with the electron density profile $\rho_{ax}(r)$ of the unstimulated axonal membrane (Ref. 4).
2) – Since the structure of the axonal membrane is highly asymmetric, with its denser face exposed to the extra-axonal medium (Ref. 4), then the increment $\Delta\rho_{ax}(r)$ of Figure 2 corresponds to an increasing asymmetry.
3) – It thus appears that the propagation of action potential is accompanied by a change of structure equivalent to the asymmetry of the axonal membrane either increasing or decreasing.
4) – The apparently poor correlation of the structural effects with the electrophysiological state of the membrane may mirror the short duration (less than 5 ms) of the structural events and a loss of synchronization in fibres of different diameter (Ref. 2).

Figure 1

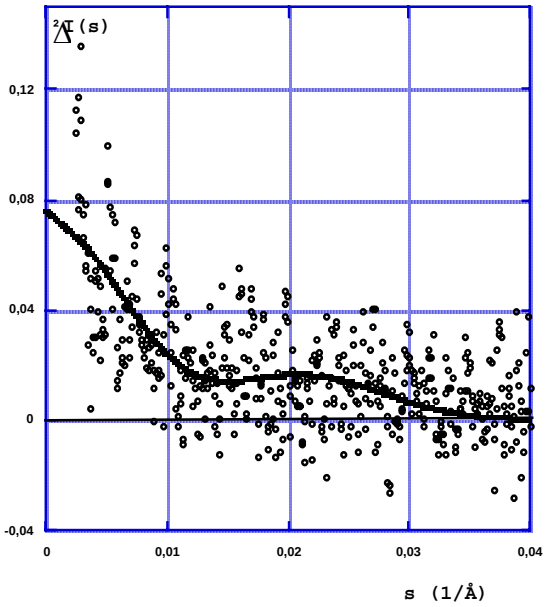

Figure 1. Difference-spectra were computed for each nerve and for each time t_c after the onset of action potential. The average of the maximal positive difference-spectra observed were averaged and plotted (**dots**). The **full line** is the least-square fit to the data and corresponds to the electron density profile [$\rho_{ax}(s)+\Delta\rho(s)$] (Figure 2).

Figure 2

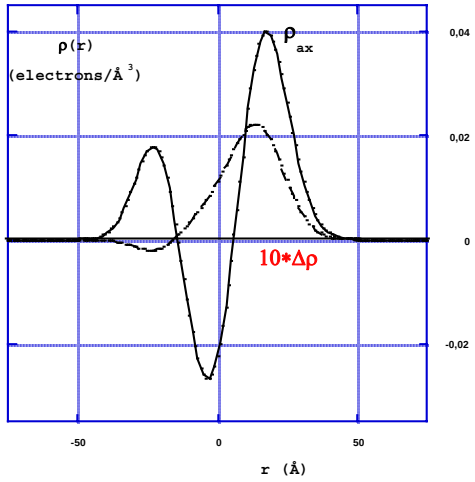

Figure 2. Electron density profile of the axonal membrane (Ref. 3) (solid line) and the increment corresponding to the intensity increment ΔI(s) of Figure 1 (10*Δρ, dashed line).

Pharmacological aspects of membrane pairing

We corroborated the previous observation that the replacement of Na^+ by K^+ in the external medium leads to the pairing of a large fraction of the axonal membranes by the apposition of their **extra-axonal** faces (Refs 2 and 4). Moreover we made the interesting observation, that may have physiological implications, that tetradotoxin (TTX), a specific blocker of the Na^+ channel, has no effect on the K^+-induced pairing whereas no pairing takes place in the presence of tetraethylammonium chloride (TEA), a molecule known to block the K^+ channels.

We also studied the effects of Lqh III, a drug known to increase the duration of action potential (Ref. 5). We observed a dramatic alteration of the spectra which seems to mirror an interaction of the axonal membranes *via* the apposition of their **intra-axonal** faces. This observation, moreover, prompted us to inspect more closely the spectra previously recorded in the presence of other neurotropic drugs (Ref. 4) and thus to observe traces of the same phenomenon that had escaped our attention.

Other nerves

We performed a few experiments on trout olfactory nerves. We identified signals typical of the axonal membrane and of the cytoskeleton, although almost 10 times weaker than those recorded with pike olfactory nerves. The structure of the axonal membrane thus seems to be similar in the olfactory nerves of two animals. The replacement of Na^+ by K^+ in the outer medium seems to induce a fraction of the membranes to pair, much like in the pike nerve, but the pairing seems to be less regular.

References

1 – Benoit, E., Charpentier, G. Mateu, L., Luzzati, V. & Kado, R. (2000). Electrophysiology of the olfactory nerve of pike *Esox lucius*: a pilot study on optimal experimental conditions. *Cybium Rev. Eur. Ictyol*., **24**, 241-248.

2 – Luzzati, V., Mateu, L., Vachette, P., Benoit, E., Charpentier, G. & Kado, R. (2000). Physical structure of the excitable membrane of unmyelinated axons : X-ray scattering study and electrophysiological properties of pike olfactory nerve. *J. Mol. Biol.*, **304**, 69-80.

3 – Luzzati, V., Vachette, P., Benoit, E. & Charpentier, G. (2004). X-ray scattering study of pike olfactory nerve, **I** -intensity of the axonal membrane, solution of the phase problem end electron density profile. *J. Mol. Biol.*, **343**, 187-197.

4 – Luzzati, V., Benoit, E., Charpentier G. & Vachette, P. (2004). X-ray scattering study of pike olfactory nerve. **II** – Elastic, thermodynamic and physiological properties of the axonal membrane. *J. Mol. Biol.*, **343**, 199-212.

5 – Benoit, E. & Gordon, D. (2001). The scorpion alpha-like toxin Lqh III specifically alters sodium channel inactivation in frog myelinated axons. *Neuroscience*, **104**, 551-559.