Genetic Dissection of Active Electrogenesis in Paramecium aurelia

Neurophysiologists frequently use various drugs and toxins to interfere with specific membrane mechanisms. A genetic approach can achieve the same results. In taking this approach to the problem of excitation it is desirable to have an organism, like Paramecium, on which both formal genetic and orthodox electrophysiological studies can be made.

The behaviour of ciliated protozoa is under the direct control of the membrane. Thus, we have efficient methods of screening for membrane mutants which rely on their behavioural peculiarities. Wild-type paramecia show "avoiding reactions" to various stimuli. This is accomplished through a period of backward swimming in which the direction of ciliary beat is reversed. This behavioural reaction is correlated with membrane excitation. Mechanical, electrical and chemical stimuli can result in depolarisation of the membrane. Suprathreshold depolarisation activates a regenerative increase in Ca conductance. The increase in internal Ca concentration through the Ca influx apparently activates the reversal of ciliary beat. The system returns to normal through repolarisation and active ion transport.

Kung and more recently Chang and Kung have mutagenised populations of P. aurelia with N-methyl-N-nitro-N-nitrosoguanidine and, by screening, have obtained...
over 170 lines of mutants with various behavioural abnormalities indicating membrane malfunctions. Three unlinked genic mutants each representing a type were used here. These mutants were (1) "Pawns" (Stock d4-95, genotype pwB pwB), behaviourally characterised by the inability to reverse their ciliary beat even upon extreme stimulation. This behaviour deficiency was found to be correlated with the loss of proper membrane excitation\textsuperscript{10}, (2) "Fast-2" (Stock d4-91, genotype fna fna) having an accelerated forward movement and an insensitivity specifically to Na\textsuperscript{+} stimulation; and (3) "Paranoiac" (Stock d4-90, genotype Pa Pa) which show a form of violent over-reaction to Na\textsuperscript{+} but not to other stimuli. The wild type Stock 51s (non-kappa bearing) of synagen 4 of *P. aurelia* was used as control.

Much of the literature on the behaviour of *Paramecium* describes the immediate reactions when cells from one solution are put into a second solution of different ionic composition\textsuperscript{11,12}. The bulk of the electrophysiological work in *Paramecium* deals, however, with the evoked potential changes when the cells have been equilibrated for a few minutes to bath solutions of various ionic compositions\textsuperscript{13}. To study the physiological correlates to chemotactic responses against various cations, we continuously monitored the membrane potential through various changes of ionic solutions.

The cell was first adapted in a K solution of 4 mM KOH, 1 mM CaCl\textsubscript{2}, 1 mM citric acid and 1 mM Tris-HCl, pH 7.2. In this adaptation solution, membranes of wild type and all mutants were quiet and registered a resting potential of −20 to −30 mV.

To test the wild-type membrane reaction to Ba\textsuperscript{2+}, a Ba solution of 4 mM BaCl\textsubscript{2}, 1 mM CaCl\textsubscript{2} and 1 mM Tris-HCl, pH 7.2, was let into the bath to replace the K solution. The resting potential gradually diminished to −5 to 0 mV in 3 to 5 min. At this level the membrane spontaneously discharged. Such repetitive firings of all-or-none action potentials are known to be correlated with quick backward jerks observed in the free-swimming animals when they encounter the Ba solution.

In reaction to Ba solution, both Fast-2 and Paranoiac mutants generated the Ba spikes typical of the wild type (Fig. 1a, c and d). Considering the variations encountered within the same strain, the frequencies of these spikes observed in different strains are not meaningful. There was, however, a complete absence of action potentials in the case of Pawns treated identically with the other strains. Even under conditions known to increase the spike frequency and duration in wild type, such as a solution with Ca\textsuperscript{2+} concentration reduced to 0.3 mM, Pawn membrane never fired (Fig. 1b). This result agrees with the finding of Kung and Eckert\textsuperscript{7} that Pawn is not capable of proper electrogenesis due to an impairment of the voltage-sensitive Ca conductance changes. It also agrees with the behavioural observations that all strains discussed avoid Ba except Pawns\textsuperscript{8}.

We discovered a distinct pattern of active electrogenesis correlated with the chemotactic avoiding reactions against Na\textsuperscript{+} by the wild type. This pattern appeared in 15 to 25 s after an Na solution (4 mM NaCl, 1 mM CaCl\textsubscript{2} and 1 mM Tris-HCl, pH 7.2) began to flow into the bath in place of the K solution. No obvious change in the apparent resting level was observed, yet active depolarisations occurred. The rising phase of each episode began with a slow but sudden depolarisation rising from the resting level or rebounding from a slightly hyperpolarised level resulting from a previous episode. The rate of rise increased rapidly to that typical of the upstroke of action potentials. The spike peaked at about 20 mV from the resting level. The potential then oscillated for several hundred ms before returning to or slightly overshooting the resting level.

This gradually damped into patterns with lower spiking frequency, with spikes interspersed with spikeless depolarisations. Careful observation suggested that each spiked depolarisation episode was correlated with a sudden reversal of ciliary beat, and that in free swimming animals it most likely corresponded to the short avoiding reaction observed when the cell encountered Na\textsuperscript{+}. This behavioural correlate, and the fact that 10\textsuperscript{−7} to 10\textsuperscript{−5} g ml\textsuperscript{−1} of tetrodotoxin did not abolish the active electrogenesis, indicated that the action current was probably carried by Ca\textsuperscript{2+} although Na\textsuperscript{+} may play an important part in events previous to the spikes.

Fewer but active depolarisations were observed when Pawns encountered the Na solution (Fig. 2b). Each depolarisation episode differed, however, from that of the wild type (Fig. 2a) by a clear lack of spikes. The plateau magnitude and duration of this depolarisation and the kinetics of repolarisation did not differ significantly from the wild-type pattern. This mutated form of active electrogenesis related to no observable behavioural response and apparently caused no ciliary reversal.

No sign of active electrogenesis was observed when Fast-2 first encountered the Na solution (Fig. 2c). There was not even the slow rise of resting level found in the other strains in reaction to the Na solution. After a long delay (more than 5 min), however, the membrane eventually drifted to −10 mV. At this time, trains of all-or-none action potentials were observed. This membrane also showed spike-like hyperpolarisations much more frequently than normal membrane.
of electrogenesis in the presence of Na⁺ could be viewed as modifications of the wild type pattern with single point blockages (Fig. 3).

The depolarisation pattern of Pawns can be viewed as the wild type pattern with a block on the upstroke of the action potential. Current injections of various strengths and durations up to $2 \times 10^{-4} \text{A}$ for 1 s (depolarising the membrane by 20 mV) during our experiments in Na solution evoked no spikes. The nature of the spikeless depolarisation pattern observed in Pawns in reaction to the appearance of Na⁺ is obscure. It is apparently triggered by Na⁺, never observed in the K-Ca or Ba-Ca solutions used and may relate to chemoreception of Na⁺.

![Fig. 3](image)

Fig. 3 A hypothesis showing that mutational blockages at various points of a normal depolarisation which occurs during Na chemotaxis can lead to different aberrant patterns. The three figures on the left are variations of the wild type patterns recorded in Fig. 2: the figures on the right are mutant patterns produced by blocks at different stages of a normal depolarisation. 

- **a.** The spiking component is blocked by the $\text{p}_{\text{w}}$ gene leading to the Pawn pattern.
- **b.** Gene $\text{fn}_2$ blocks the early depolarisation event and thus results in the Fast-2 pattern.
- **c.** Gene $\text{Pa}$ blocks the proper repolarisation process and thus sustains and prolongs the depolarisation. Calibration: 1 s and 10 mV.

The pattern of Fast-2 in reaction to Na can be viewed as the result of a mutational blockage in the normal depolarisation process, located before the Pawn blockage. This results in a total loss of depolarisation correlating with the specific loss of chemotactic reactions against Na⁺. Presence of Ba spikes, delayed action potentials in Na⁺ as well as electrically evoked action potentials indicates that the Fast-2 membrane is excitable. The mutational blockage, therefore, does not extend to the component blocked by the Pawn gene.

The intriguing patterns of Paranoiac in the Na solution may be viewed as the result of a genetic block of the normal process of repolarisation. Because of the lack of a proper shut-off mechanism related to Na⁺, the membrane potential stays near the peak of depolarisation for a greatly prolonged period and eventually drifts back to the resting level through a secondary repolarisation mechanism unaffected by this mutation.

As a description of the physiological abnormalities of various membrane mutants in *P. aurelia*, this study demonstrates the power of the genetic approach in systematically modifying a complicated biological phenomenon: the active electrogenesis during chemotactic reaction against Na⁺. This approach may aid in the search for a molecular mechanism for excitation by correlating the mutant phenotypic expression at various levels of investigation and by identifying the gene products. This rationale of applying genetics to neurobiology was first stated by Benzer.

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### Regulation of Spawning Behaviour in the Female Goldfish

**Although** the role of hormones in the control of sexual behaviour in female mammals is well established4, this is not the case with fish5. In spite of several reports that castration of female fish eliminates sexual behaviour2, only two investigations have demonstrated successful steroid replacement therapy3,4. We report here, however, the induction of sexual behaviour in female goldfish (Carassius auratus) by injection of ovulated eggs into the ovarian lumen of individuals with vitellogenic ovaries, and also by administration of both eggs and oestrogen to individuals with regressed ovaries.

In goldfish, ovulation can be induced in several days by a change in water temperature from 12° to 20° C. At this 'summer' temperature, females ovulate once or several times within a month, after which the ovaries regress. At ovulation, the eggs are released into an ovarian cavity which is continuous with the oviduct. Ovoposition (release of eggs through the ovidopore) occurs during a series of spawning acts. The female approaches and enters floating vegetation in a head-up position, the male following from below. Both turn on their sides and, as they break the water surface, the male presses against the female and appears to push her out of the way. Eggs and sperm are released this time and fertilisation occurs. This procedure may be repeated up to several hundred times during 1–3 h. Spawning behaviour of the female is seen only in the presence of male goldfish and green plants5.

Encouraged by Yamazaki's5 findings that (1) spawning behaviour was seen only in goldfish which had ovulated, and (2) hand-stripping of ovulated fish terminated this behaviour, we investigated the role of ovulated eggs. We found that when ovoposition is prevented by a plastic and glass plug in the ovidopore of an ovulated female, spawning behaviour is extended. Whereas spawning usually lasts less than 2 h, 'plugged' females perform the spawning act (without oviposition) throughout the day of ovulation and sometimes continue the following day.

To determine whether placement of eggs in the ovarian lumen would induce, rather than prolong, spawning, ovulated females were allowed to spawn with a male for 20 min to obtain individual normal spawning rates. The fish were then