Eur Biophys J (1985) 12: 13–18

European Biophysics Journal

Interaction between sodium channels in mouse neuroblastoma cells

T. Kiss* and K. Nagy**

I. Physiologisches Institut der Universität des Saarlandes, D-6650 Homburg/Saar, Federal Republic of Germany

Received 8 May 1984/Accepted 16 January 1985

Abstract. Single sodium channels in mouse neuroblastoma cells (N1E 115) were studied in cell-attached patches. During a series of consecutive responses to depolarizing pulses, records with and without channel opening were seen to form clusters rather than appearing randomly. The probability of finding open channels on a record seemed to increase with increasing number of channel openings. The open times of channels became shorter with increasing closed time interval measured between consecutive channel openings. Overlapping openings showed a voltage-dependent open time, in contrast to single openings which had voltage-independent open time. On the basis of these observations interaction between neighbouring sodium channels is suggested.

Key words: Neuroblastoma, sodium channels, channel interaction

Introduction

The single channel recording technique (Neher et al. 1978) makes it possible to directly measure the current, i, through a single membrane channel. In this way, ensemble averages of many single channel currents (I) can be obtained and the probability, p, of a channel being open can be calculated according to the equation $I = Nip$, where N is the number of channels and i is the current through a single channel. However, this equation is only valid with the following conditions: (1) the channels have only one closed and one open state, (2) the population of channels is homogeneous, and (3) no interaction between neighbouring channels exists.

Interaction between channels has been studied in the node of Ranvier which has a high density of sodium channels. In noise measurements on the node of Ranvier, Sigworth (1980) found no significant changes in single channel conductance after reducing the channel number by a depolarizing prepulse or by adding tetrodotoxin. On the same preparation, Neumcke and Stämpfli (1983) observed negative cooperativity between sodium channels, presumably due to depletion of sodium ions outside the channel mouth, but no alteration of gating time constants which could be attributed to direct interaction between channels.

In the present work single channel measurements were done on mouse neuroblastoma cells in order to investigate possible channel-channel interaction between sodium channels. Some of the results have been reported in preliminary communications (Kiss and Nagy 1983; Nagy et al. 1984).

Materials and methods

All experiments were performed on cultured mouse neuroblastoma cells (N1E 115) which were grown and subcultured as described by Moolenaar and Spector (1978). The gigaohm-seal, patch-clamp technique was used to record Na currents from cell-attached membrane patches. Details of the procedure are described by Nagy et al. (1983). The bathing solution consisted of (in mM) 145 NaCl, 4 KCl, 1.8 $CaCl_2$, 0.4 $MgCl_2$, 5 HEPES, 5 glucose; pH was adjusted to 7.3. Solutions were filtered through a 0.2 μm filter before use.

Test pulses were generated by a microcomputer. The pulse duration was 80 ms, and the pulse interval was 1.0–1.5 s. Leakage and capacitative currents were compensated by analogue circuitry and digitally, by the computer. The cell membrane was hyperpolarized by 50–60 mV to achieve a holding potential of about −100 mV. Single channel currents were filtered at 1

Abbreviations: RP, resting potential; OT, channel open time

* Permanent address: Balaton Limnological Research Institute of the Hungarian Academy of Sciences, H-8237 Tihany, Hungary

** To whom offprint requests should be sent

kHz with a four-pole low-pass Bessel filter. Data were sampled at 200 μs intervals and were stored on floppy disks for off-line analysis.

Results

Figure 1 shows single channel currents through Na^+ channels measured in the cell-attached configuration. A 50 mV pipette potential was added to the resting potential (RP) in order to obtain a holding potential between −80 and −100 mV. 681 consecutive depolarizations were applied using 70 mV pulses, i.e. the pulse potential was +20 mV relative to the resting potential. Inspection of Fig. 1 gives the impression that records with or without openings have a tendency to be clustered. To prove this subjective impression we

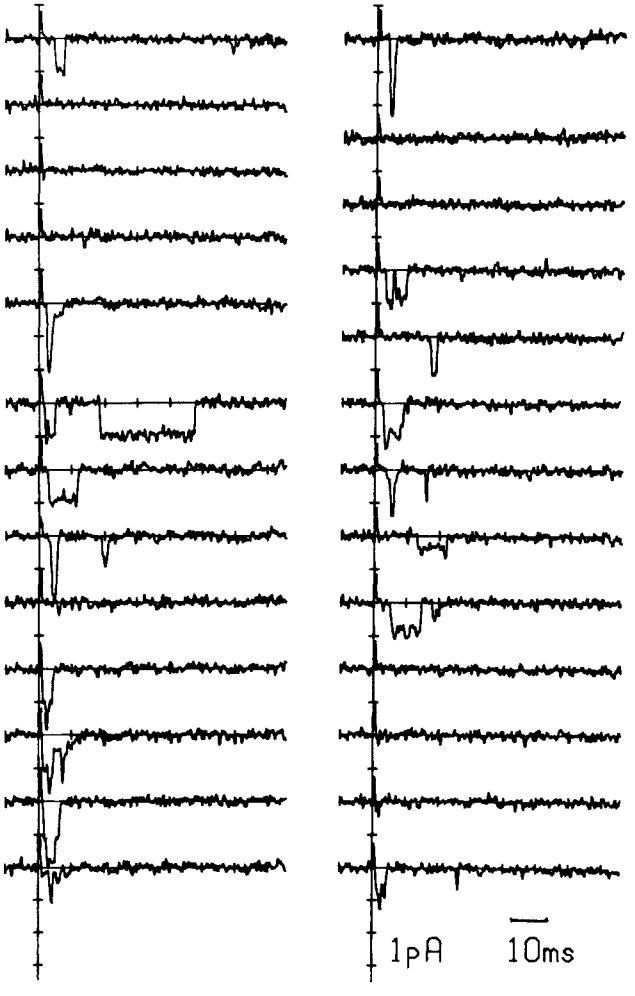

Fig. 1. Clustering of records with and without openings from a cell-attached patch. 681 depolarizing pulses were delivered with a frequency of 1/s. From top to bottom, starting on the left column, records with serial number between 76 and 101 are shown. The 76th record is the last one from a cluster of 12 records with openings. Holding potential = RP −50 mV. Test pulse to +20 mV relative to the RP. Temperature 10°C. The patch contained 6 channels

applied "runs analysis" to our records. Analysis of runs is an effective statistical method for testing randomness in sequences of two types of elements (Wald and Wolfowitz 1940; Mood 1940; Swed and Eisenhart 1943). A "run" is a sequence of like elements, i.e. in our case, records with and without openings. According to the runs test a sequence of elements forming clusters has a smaller number of runs than expected for a random sequence. In Fig. 1 the number of runs is 9. (Remember, the first record was preceded by 11 records having openings.) For a random process 9 or fewer runs should be observed with a probability of 0.013, according to the equations of Swed and Eisenhart (1943). The most likely number of runs for Fig. 1 is 14 and 14 or fewer runs are observable with a probability of 0.43. Therefore, the most likely number of runs is 33 times more probable than the 9 runs in our example.

The distribution of the number of runs is approximated by a standardized random variable, Z, which is defined by the equation

$$Z = -\frac{R - 2np\,(1-p)}{2\sqrt{np}\,(1-p)}\,,$$

(Mood 1940), where R is the number of runs, n is the total number of trials and p, in our case, is the probability of finding at least one open channel during a trial. Z is normally distributed with zero mean and unit variance. Positive values for Z indicate clustering of records with openings. At a certain significance level the one-tailed test of the standard normal distribution can be used for disregarding the null hypothesis. At 5% significance level, Z is 1.64. For the whole experiment measured with 1 s interpulse interval, which is partly presented in Fig. 1, $Z = 4.24$ obtained with $R = 221$, $p = 0.74$ and $n = 681$ at +20 mV. Thus, records with and without openings show a significant tendency for clustering.

Z changed with the magnitude of the depolarization and especially with the number of channels in the patch, which explains the very different values obtained for different patches. However, a satisfactory normalization for the channel number could not be found. Variation of Z with the membrane potential for different patches is shown in Fig. 2A. Here, filled circles indicate Z for patches having three or fewer channels (estimated from the highest current level). These values do not indicate clustering of records with openings as $Z < 1.64$, the 5% significance level. For patches with more than three channels (filled triangles in Fig. 2A) the non-random order of channel openings is not evident at $V_m < 10$ mV ($Z < 1.64$, the value of 5% significance), while at $V_m \geq 10$ mV the clustering parameter is above the 5% significance level. At V_m 30 mV the number of records without openings is

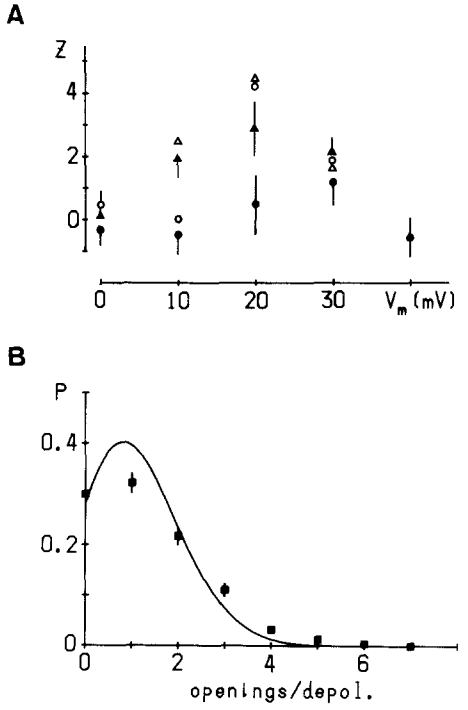

Fig. 2. A Change of the clustering parameter, Z, with membrane potential. Filled circles and filled triangles are the mean values of Z for patches having three or fewer channels and for patches having 4 to 6 channels, respectively. Mean values and standard deviations were calculated from 3–6 experiments at 10–16°C. Open circles show the parameter Z for the same patch as in Fig. 1. Open triangles were obtained from a single patch with 6 channels at 22°C. Each Z value was calculated from at least 100 depolarizations. **B** The probability, P, of finding a certain number of open channels on a record. Dots and standard deviations are obtained from the same experiment as in Fig. 1. The continuous curve is the binomial distribution fitted to the experimental points with $n = 6$ channels in the patch

strongly reduced or is zero in patches with four or more channels, therefore the calculation of Z is impossible here.

Horn et al. (1984) reported clustering of channel openings in GH_3 cells, when the interpulse interval was short (0.8 s). In our measurements, values of Z showed no significant dependence on the interpulse interval in the range of 1.0–1.5 s.

Horn et al. (1984) concluded that the clustering is due to a non-inactivatable, hibernating state of the sodium channels. We suppose that the clustering is induced by the interaction between channels. If this is the case, the clustering must be connected with the probability of channel openings on a record. This view is supported by Fig. 2B, which shows the probability of finding a certain number of channel openings per depolarization. The plot is from the same patch as in Fig. 1. The probability, P, of finding x channels opened independently on a record is given by the binomial distribution

$$P(x) = \binom{n}{x} p_0^x (1 - p_0)^{n-x},$$

where n is the number of channels in the patch, p_0 is the time independent closed-open transition probability. The continuous line in Fig. 2B is the binomial distribution obtained with $p_0 = 0.19$ and $n = 6$ channels in the patch. The experimental values (dots in Fig. 2B) show that the probability of finding one opening on a record is considerably smaller, but the probability of finding 3, 4, etc. openings/record is larger than the theoretical value. The deficiency (theoretical – measured value) at one opening/record equals the sum of the excesses at 3, 4, etc. openings/record. In other words, if a channel opens the opening of the next channel is more probable than it would be expected from independent statistical estimations. We suppose that the non-random behaviour of openings and the deviation of the distribution of the openings/record from that predicted by the binomial distribution is the result of cooperative interaction between sodium channels.

If there is an interaction between successive openings, a plot of channel open time (OT) against the closed time intervals between openings should reveal a correlation. Figure 3A presents such a plot. The open time of overlapping openings can be calculated in two ways: (a) supposing that a channel opened first closes first, and (b) supposing that a channel opened first closes last. The calculation shown in Fig. 3A was made on the basis of the first supposition. This supposition is not a strong restriction because overlapping openings generally appear at the onset of the depolarization only and the first closed time is measured from the last closing of these openings. For the data of Fig. 3A, linear regression resulted in a slope of -0.12 for the first 10 ms of the closed time. At closed times longer than 10 ms no correlation could be found. However, as the relation between the channel open time and the preceding closed time is not known it is better to characterize that relation with a general, rank correlation. For the 0–10 ms closed time interval, Spearman's correlation coefficient, r_s (Colquhoun 1971) was -0.45 with a significance level of $u = -2.98$ resulting in a definite correlation for the two-tailed test at $\alpha = 0.01$ (Graf et al. 1966). At closed times longer than 10 ms no correlation could be found because here the number of events is strongly reduced, as shown by Fig. 3B.

One can argue that the decrease of the open time with increasing closed time is due to the time-dependence of the channel open time. As the first channel opens soon after the onset of the depolarization the first closed time approximates the delay time of the second opening. We have studied this possibility and the results are shown in Figs. 3C and D. Here, records having no overlapping events were selected, because

the sequence of openings could then be determined unambigously. Figure 3C shows the open time plotted against the preceding closed time, while in Fig. 3D the open time of the same events is plotted as a function of their delay times measured from the onset of the depolarization. The slope of the line is −0.14 in Fig. 3C and +0.07 in Fig. 3D, obtained by applying linear regression to the points between 0 and 10 ms. Spearman's correlation coefficients were $r_s = -0.53$ ($u = -3.24$) and $r_s = 0.16$ ($u = 0.97$) for Fig. 3C and D, respetively. Thus, the correlation of the open time with increasing closed time is significant for the two-tailed test at $\alpha = 0.01$, while the correlation for the plot of open time against delay time is significant only at $\alpha = 0.40$ (Graf et al. 1966), i.e. no correlation is observable. Therefore, we may state that the decrease of the open time is exclusively influenced by the closed time and not by the time-dependence of the channels. This observation can be interpreted as an interaction between the channels.

The conclusion from Fig. 3 is that channels which open closely after each other have the tendency to stay open longer. Extrapolating to infinitely short closed time (i.e. openings not separated by closed intervals) the case of overlapping openings is reached. Thus, we might expect that overlapping openings would have longer mean open time than the single ones. It is known that the open time of channels is voltage-dependent (Fenwick et al. 1982; Nagy et al. 1983) and that the probability of findings overlapping openings also depends on voltage. Therefore we analyzed the voltage-dependence of the OT on records which had overlapping events separately from those which had only single events. The maximum likelihood estimates of channel open time (Fenwick et al. 1982; Horn and Lang 1983; Horn and Standen 1983) as a function of the membrane voltage are shown in Fig. 4. The OT of single openings (open symbols in Fig. 4) have no voltage-dependence over a voltage range of +10 to +40 mV (relative to the RP). Overlapping events, however, show voltage-dependence of the OT in the same range. With increasing depolarization there is an increasing probability of observing overlapping events, and therefore more possibility for interaction; therefore, the interaction of channels might contribute to the voltage-dependence of the channel open time.

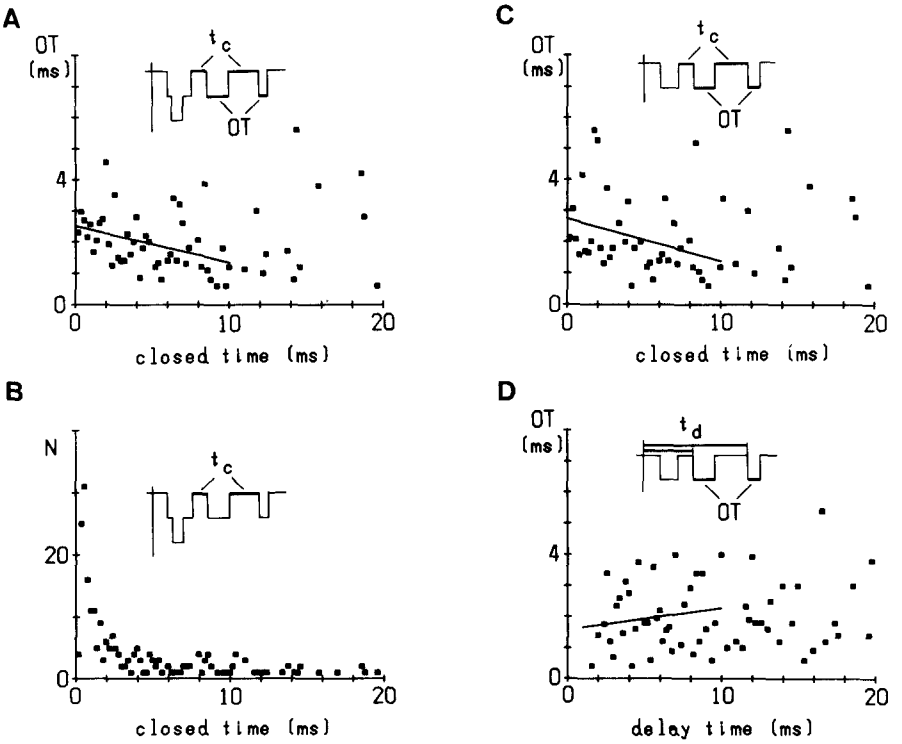

Fig. 3. Open times of successive events as function of the closed time interval between openings and of delay time. Cell-attached patch, V_H = RP −50 mV, V_m = 20 mV, relative to RP, T = 10°C. **A** Open times of channels plotted against the preceding closed time. All records were used for calculation. Open times of overlapping events were determined using the assumption that the channel which opened first also closes first. The closed time intervals were measured from the opening of a channel back to the closing of the preceding open channel or channel group (see *inset*). Therefore this plot does not contain the OT of first events. The line is a linear regression line with a slope of −0.12. **B** The number of events as a function of the closed time interval; same experimental data as in **A**. **C** Open time of channels as function of the preceding closed time for records having no overlapping openings. The slope of the line is −0.14. **D** The OT of the same events as in **C** but plotted against their absolute delay time measured from the onset of the depolarization (see *inset*). The slope of the line is +0.07

It is important to note that for records having single events the number of events per depolarization increases only slightly with increasing membrane potential, while for records also having overlapping events it increases greatly. For example, in the patch indicated by circles in Fig. 4 the mean number of openings per depolarization for single events was 1.52 and 1.82 at 10 and 30 mV, respectively, and for records having overlapping events it was 0.42 and 3.48 at the same potentials. This effect is probably related to the clustering behaviour and also suggests interaction between sodium channels.

It is necessary to mention that the selection of records for the plot in Fig. 4 results in longer OT for the overlapping and shorter OT for the non-overlapping openings than the maximum likelihood estimate. Namely, if overlapping events are excluded from the calculation the mean open time becomes considerably shorter (Horn and Standen 1983). However, we selected records with overlapping events and generally these records have single openings too. This fact somewhat balances the one-sided selection, especially when the number of openings per record (e.g. at +30 mV) is high. Furthermore, the conclusion that the overlapping openings have a prolonged open time has been reached from the open time of single events, where no selection was applied.

The single channel conductance should reflect the type of interaction described by Neumcke and Stämpfli (1983). To study this we measured the distribution of single channel amplitudes. Again, we plotted amplitude histograms from single and overlapping events separately. The amplitude histogram of single events was symmetrical, a Gaussian curve, but that of the superimposed events was asymmetrical. The maximum of the latter histogram was shifted to smaller current values by about 10% compared to the histogram of single openings. This observation is in accordance with the results of Neumcke and Stämpfli (1983), however the effect is so small that we cannot regard it as proof.

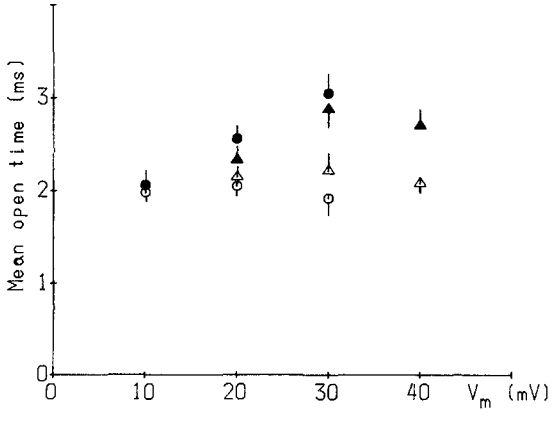

Fig. 4. Potential dependence of mean open time. Open symbols represent non-overlapping, closed symbols overlapping events. Data are from two cell-attached patches. V_H = RP −50 mV, 10°C. Bars indicate SEM. Values were calculated from 200–500 events

Discussion

The results indicate an interaction between sodium channels in mouse neuroblastoma cells. This conclusion is based on the following observations: (a) In a sequence of depolarizations, records with and without openings are not randomly distributed, but create clusters. This peculiar behaviour of sodium channels was recently observed in rat pituitary cells and interpreted as a transition into a „hibernating" state (Horn et al. 1984). We suggest that the clustering may be due to channel-channel interaction, since the theory of runs is applied in physics for the study of cooperative phenomena (Feller 1968). The interaction of channels may cause the change of the closed-open transition probability, when the number of openings increases during a depolarization (Fig. 2). (b) The open time of a channel is influenced by the "neighbouring" channels. It is longer for channels which open soon after a previously opened channel and becomes shorter with prolonged closed time (Fig. 3). A significant voltage-dependence of the open time is observed for overlapping events but not for isolated openings.

In our earlier work (Nagy et al. 1983) we measured the voltage-dependence of channel open time for single and overlapping events in outside-out patches generally having more than 4 channels. Those data and the open time of the overlapping openings plotted in Fig. 4 show a voltage-dependence similar to that reported by Vandenberg and Horn (1984). In contrast to our observation, Aldrich et al. (1983) found voltage-dependent channel open time only at small depolarizations. We suppose that the discrepancy is due to (a) the smaller channel number in their experiments, (b) the higher temperature in their experiments (16–21.8°C) which makes it more difficult to observe small changes in the channel open time, (c) the uncertainty of membrane potential in cell-attached patches or (d) the different method of calculating the channel open time in the case of overlapping openings.

Our observations argue against independent gating of sodium channels and suggest that neighbouring channels interact to a certain degree. However, interaction can only occur between channels which are close to each other. According to our estimations (from the whole-cell-clamp measurements) the sodium channel density in cultured neuroblastoma cells is of the order of $10/\mu m^2$, which is somewhat lower than the range of $30–100/\mu m^2$ reported by Catterall (1980). In any case, the channel density is considerably smaller than that in the node of Ranvier

(Conti et al. 1976). To explain the interaction in our preparation we must assume that, in mouse neuroblastoma cells, sodium channels are arranged in pairs or in groups. Likewise, Almers and Stirling (1984) suggested the occurrence of sodium channels as oligomers; Aldrich et al. (1983) reported that the number of sodium channels in a patch is usually even.

Acknowledgements. We are grateful to Professor H. Meves for his kind support during the course of the work, to Professor B. Neumcke and Prof. R. Horn for helpful comments on the manuscript and to Dr. D. Hof for writing computer programs. We thank Professor B. Hamprecht for supplying the cell line, Dr. T. D. Plant for reading and Mrs. R. Stolz for typing the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 38). T. Kiss is a fellow of the Alexander von Humboldt-Stiftung.

References

Aldrich RW, Corey DP, Stevens CF (1983) A reinterpretation of mammalian sodium channel gating based on single channel recording. Nature 306:436–441

Almers W, Stirling C (1984) Distribution of transport proteins over animal cell membranes. J Membr Biol 77:169–186

Catterall WA (1980) Neurotoxins that act on voltage-sensitive channels in excitable membranes. Annu Rev Pharmacol Toxicol 20:15–43

Colquhoun D (1971) Lectures on biostatistics. Clarendon Press, Oxford

Conti F, Hille B, Neumcke B, Nonner W, Stämpfli R (1976) Conductance of the sodium channel in myelinated nerve fibres with modified sodium inactivation. J Physiol 262:729–742

Feller W (1968) An introduction to probability theory and its applications, vol 1. John Wiley & Sons, New York

Fenwick EM, Marty A, Neher E (1982) Sodium and calcium channels in bovine chromaffin cells. J Physiol 331:599–635

Graf U, Henning H-J, Stange K (1966) Formeln und Tabellen der mathematischen Statistik. Springer, Berlin Heidelberg New York

Horn R, Lange K (1983) Estimating kinetic constants from single channel data. Biophys J 43:207–223

Horn R, Standen NB (1983) Counting kinetic states: the single channel approach. In: The physiology of excitable cells. AR Liss, New York, pp 181–189

Horn R, Vandenberg CA, Lange K (1984) Statistical analysis of single sodium channels. Effects of *N*-bromoacetamide. Biophys J 45:323–335

Kiss T, Nagy K (1983) Characterisation of two open states of Na channels in neuroblastoma cells. Neurosci Lett (Suppl) 14:198

Moolenaar WH, Spector I (1978) Ionic currents in cultured mouse neuroblastoma cells under voltage-clamp conditions. J Physiol 278:265–286

Mood AM (1940) The distribution theory of runs. Ann Math Statistics 11:367–392

Nagy K, Kiss T, Hof D (1983) Single Na channels in mouse neuroblastoma cell membrane. Indications for two open states. Pflügers Arch 399:302–308

Nagy K, Kiss T, Hof D (1984) Kinetic behaviour of single sodium channels in neuroblastoma cells (N1E 115). Pflügers Arch (Suppl) 400:45

Neher E, Sakmann B, Steinbach JH (1978) The extracellular patch clamp: A method for resolving currents through individual open channels in biological membranes. Pflügers Arch 375:219–228

Neumcke B, Stämpfli R (1983) Alteration of the conductance of Na^+ channels in the nodal membrane of frog nerve by holding potential and tetrodotoxin. Biochim Biophys Acta 727:177–184

Sigworth FJ (1980) The conductance of sodium channels under conditions of reduced current at the node of Ranvier. J Physiol 307:131–142

Swed FS, Eisenhart C (1943) Tables for randomness of grouping in a sequence of alternatives. Ann Math Statistics 14:66–87

Vandenberg CA, Horn R (1984) Inactivation viewed through single sodium channels. J Gen Physiol 84:535–564

Wald, A, Wolfowitz J (1940) On a test whether two samples are from the same population. Ann Math Statistics 2:147–162