High level stable expression of rat brain type IIA sodium channel α-subunit in CHO cells

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The neuronal sodium channels are responsible for the rising phase of action potential and are composed of three subunits, of which the α-subunit has been shown to be adequate for most of its functional properties. We have stably expressed the rat brain type IIA sodium channel α-subunit in CHO cell line using a CMV promoter-based vector. The expression was confirmed by detecting a 6.5 kb RNA corresponding to sodium channel α-subunit using Northern hybridization. The cells stably expressing the α-subunit, yield isolated sodium currents of amplitudes greater than 4 nA when studied in whole-cell configuration of the patch-clamp technique. The sodium currents are characterized by activation and inactivation properties similar to neuronal sodium channels, and are blocked by the voltage gated sodium channel blocker tetrodotoxin (TTX).

THE mammalian rat brain sodium channel is a heterotrimeric protein consisting of a 260 kDa α-subunit, a 36 kDa β_1-subunit and 33 kDa β_2-subunit. The channel mediates the transient inward sodium current in response to a depolarizing voltage pulse under voltage-clamp conditions and is responsible for the rising phase of the action potential. Different cDNA clones coding for different subtypes of α-subunit (I, II, IIA and III) have been isolated from rat brain[1]. Earlier work has shown that the α-subunit of rat brain type IIA sodium channel is adequate for its function when expressed in the mammalian CHO cells[2,3]. The expression and stable integration of cDNA encoding the rat brain type IIA sodium channel α-subunit in CHO cells was done initially using a SV-40 promoter based construct[2]. Subsequently a construct containing mouse metallothionein 1 (mMT1) inducible promoter was used to get higher expression levels[3].

The present study reports the use of a construct containing the CMV promoter to efficiently and stably express the rat brain type IIA sodium channel α-subunit in CHO cells. The advantage of using the CHO cell line over CHO-K1, is undetectable levels of endogenous sodium current and sodium channel specific mRNA in CHO cells[2–4]. On the other hand, CHO-K1 cells have endogenous sodium channels in their plasma membrane complicating the analysis of electrophysiological data. The level of highly stable and constitutive expression of sodium channel using CMV promoter reported here, is comparable to expression levels achieved using a construct containing the inducible mouse metallothionein 1 (mMT1) promoter in CHO-K1 cells. This supports the idea that CHO cells with their advantages can in fact be used for high level expression of channel proteins permitting detailed functional studies. Expression of sodium channels was functionally studied using the patch-clamp technique[5,6].

Plasmid pVA2580 carrying the coding portion for the rat brain type IIA sodium channel α-subunit[3,7–9] was a gift from Prof. Alan L. Goldin, University of California, Irvine, USA. The expression vector pCDM8 (ref. 10) used for the study was obtained from Invitrogen. Chinese Hamster Ovary (CHO) cells were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India and were grown in DMEM-F12 medium (Sigma) containing 7–10% fetal calf serum in 25 cm^2 culture flasks or 100 mm tissue culture dishes.

The expression plasmid pSN10 (Figure 1 *a*) was constructed as follows: The plasmid pVA2580 was first digested with *Cla*I, ends filled in with Klenow and subsequently digested with *Sal*I. This gave a 6.5 kb fragment carrying the coding region for rat brain type IIA sodium channel α-subunit, with one sticky end (*Sal*I), and another blunt end. Similarly, pCDM8 was digested with *Not*I, end filled in with Klenow followed by digestion with *Xho*I. This resulted in a 4 kb fragment with ends compatible with the insert. The recombinant (pSN10) obtained by ligating the above two fragments was checked by restriction mapping for proper orientation and integrity of the plasmid (Figure 1 *b*).

CHO cells were cotransfected with the plasmid pSN10 and pSV2neo (ref 11) at a molar ratio of 10 : 1, by calcium-phosphate DNA precipitation method as originally described by Graham and van der Eb[12] with some modifications[13]. After 48 h, the cells were tested for transient expression by recording isolated inward sodium currents using patch-clamp technique, and subsequently selected for stable expression in a medium containing 400 μg/ml (active) G418 (Sigma). The cells stably expressing sodium channel were maintained in the same medium containing 300 μg/ml G418. During later stages cells were maintained in the absence of G418 in the medium, without significant loss of expression levels.

Total RNA from the cells stably expressing sodium channel α-subunit (CNa18) and normal untransfected CHO cells were isolated by acid-guanidium-thiocyanate-phenol-chloroform extraction method[14]. Northern-hybridization was done as described in Sambrook *et al.*[15] Briefly 30 μg of total RNA was fractionated by 1% agarose–formaldehyde gel, transferred to Nylon membrane (Hybond N+, Amersham) and fixed by UV crosslinking. The blot was prehybridized in a prehybridization solution containing 5X SSC, 50% formamide, 50 mM Na-phosphate, 5X Denhardt's solution and 100 μg/ml of salmon sperm DNA at 42°C for 6 h.

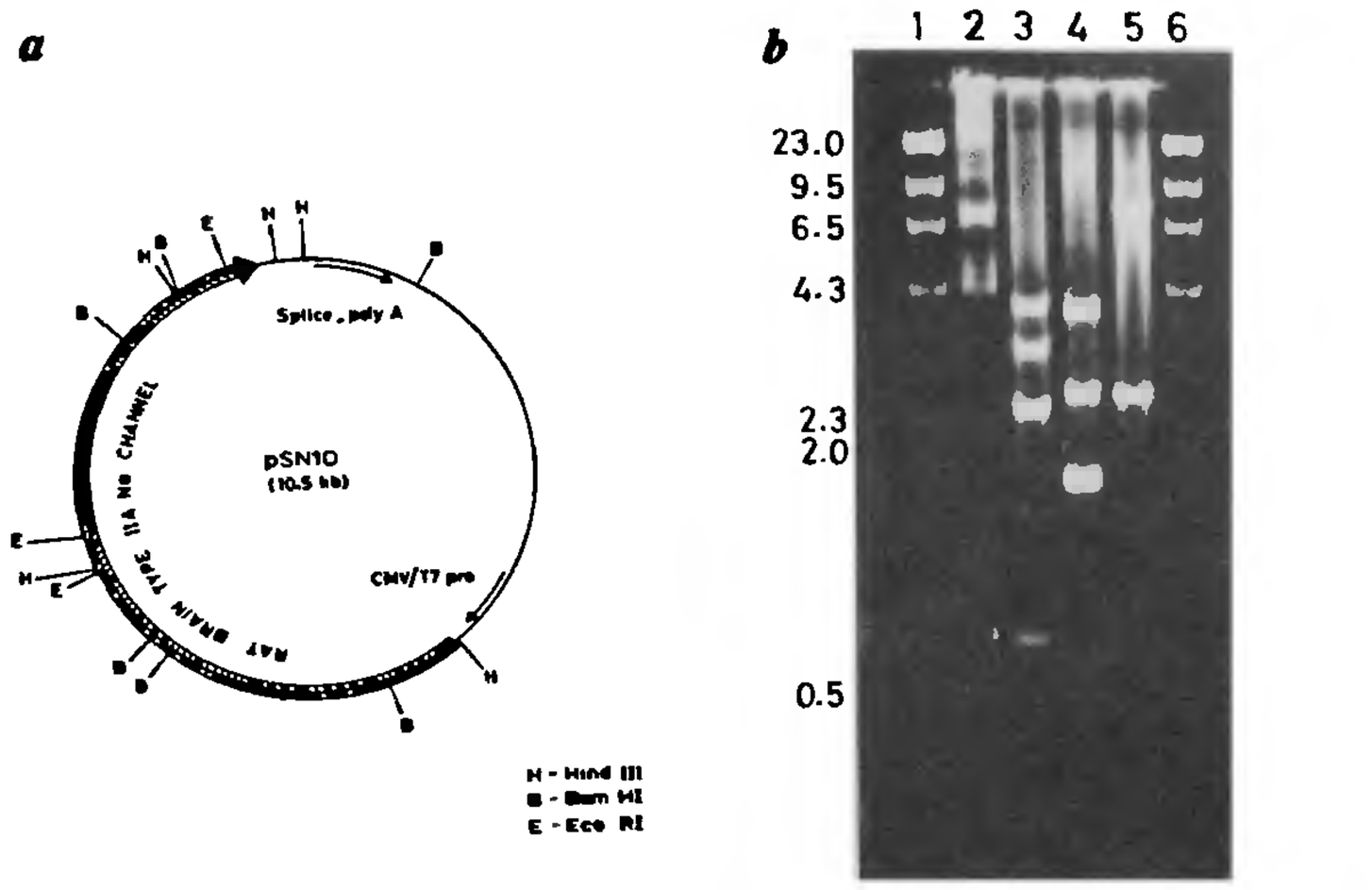

Figure 1. The construct pSN10 used to transfect CHO cells. *a*, The circular map of pSN10. The hatched region is the cDNA encoding the rat brain type IIA sodium channel α-subunit. The location of the CMV promoter, the splice signal and polyadenylation sites are shown by arrows. The direction of transcription is shown by arrowhead. Restriction sites are shown for three restriction enzymes as indicated. *b*, The restriction pattern as obtained from pSN10. Lanes 1 & 6: λ-*Hin*dIII markers, Lane 2: uncut plasmid, Lane 3: pSN10 digested with *Hin*dIII, Lane 4: pSN10 digested with *Bam*HI, Lane 5: pSN10 digested with *Eco*RI.

Radiolabelled probe was made by labelling the full length sodium channel cDNA (*Sal*I-*Sal*I fragment of pVA2580) with ^{32}P by random priming method and was added to the prehybridization mixture at a specific activity of 10^7 cpm/ml and hybridized for 12 h. The blot was then washed thrice at room temperature in 2X SSC, 0.1% SDS for 15 min, followed by three washes with 1X SSC, 0.1% SDS at 65°C for 20 min. Following a final rinse in 0.5X SSC, it was subjected to autoradiography.

Patch-clamp recordings were done at room temperature, on CNa18 cells grown to about 60% confluency in 35 mm tissue-culture dishes. Patch pipettes were pulled from thin walled omega-dot capillaries (1.5 mm, Intracel). The pipette solution composition was (in mM): 130 CsCl, 5 HEPES, 1 $CaCl_2$, 10 EGTA, pH 7.4; while the bath solution contained (in mM) : 52 NaCl, 85 choline chloride, 5 HEPES, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, pH 7.4. A reduced concentration of sodium in the bath solution was used to decrease the current levels to minimize the access resistance problem[16]. Tetrodotoxin (TTX) (Sigma) was added to the bath solution from a concentrated stock. Patch pipettes filled with pipette solution had resistances of 1–2 MΩ. Currents were recorded by an EPC-7 Patch-Clamp Amplifier (List Electronics, Germany), digitized through a CED1401 A/D, D/A converter (Cambridge Electronics Design, UK) and analysed in a PC-AT 286 computer. The data were filtered at 3 kHz using the amplifier's built-in 3-pole bessel filter. Data acquisition, design of voltage protocols, leak subtraction and analysis were done using the WCP software (J. Dempster, University of Strathclyde, UK).

Following 48 h of transfection of CHO cells with the plasmids pSN10 and pSV2neo, the cells were tested for transient expression by recording isolated inward sodium currents using patch-clamp technique, and subsequently selected for stable expression in a medium containing 400 μg/ml G418. After screening 12 individual G418 resistant colonies for sodium channel expression by patch-clamp, 2 clones (CNa17 and CNa18) were found to exhibit large sodium currents. CNa18 was used for further study. During the course of experimentation we have found that the expression of sodium current is very stable without much loss of expression for more than 100 passages.

Total cellular RNA was isolated from CNa18 and CHO cell line and analysed by Northern blot. Figure 2 shows the result of Northern blot analysis of total RNA from the untransfected CHO cells and the CNa18 cells. The CNa18 cells express a 6.5 kb RNA corresponding to sodium channel α-subunit while the untransfected CHO cells do not. The full length cDNA was used as probe (see experimental section), and since the expression level was high, low molecular weight degraded sodium channel mRNA also lighted up giving a smear, but the smear starts from 6.5 kb position

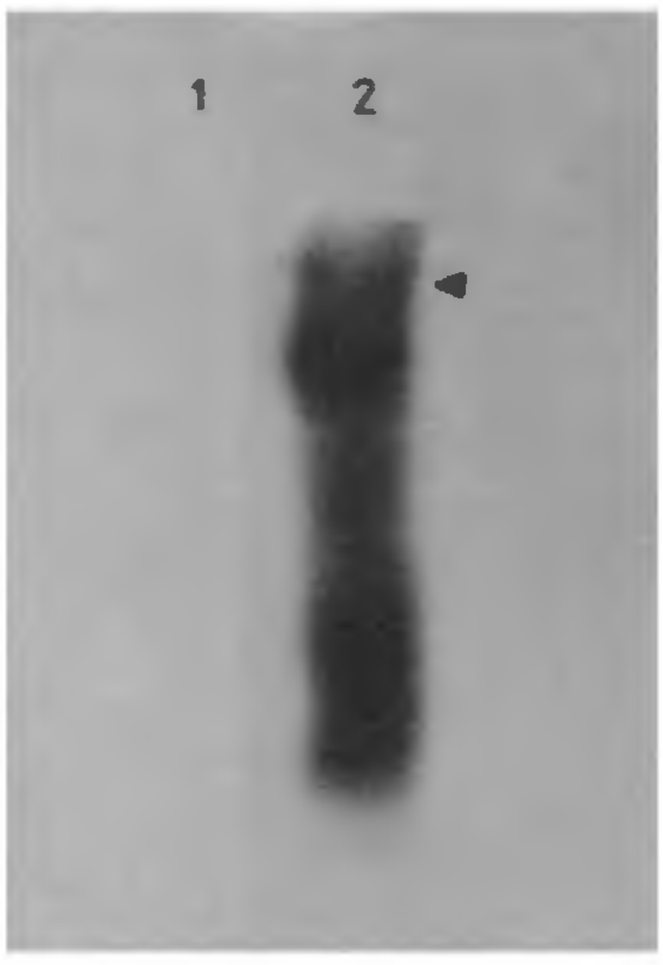

Figure 2. Northern hybridization of total cellular RNA from CHO and CNa18 cells, using 6.5 kb full-length sodium channel α-subunit cDNA as probe with 24 h exposure. Lane 1: RNA from CHO cells, Lane 2. RNA from CNa18 cells. Arrow head shows the position of 6.5 kb RNA

indicating intact sodium channel mRNAs[3]. No hybridization was detected in untransfected CHO cells even upon exposure for 100 h. Our observations of undetectable levels of sodium channel specific mRNA in the untransfected CHO cells therefore corroborate well with the earlier report of Scheuer *et al.*[2].

The CNa18 cells were voltage clamped using the whole-cell configuration of the patch-clamp technique. Figure 3 *a* shows large inward sodium currents elicited following depolarization of the membrane to potentials shown from a holding potential of −80 mV. The inward currents at membrane potentials positive to −35 mV were characterized by rapid activation following onset of the depolarization pulse followed by inactivation which was complete within 3 ms. The observations corroborate well with the earlier findings of West *et al.*[3]. Peak sodium current amplitudes analysed under similar voltage clamp conditions varied from 0.5 to 8.5 nA with a mean of 4.1 nA (SD 2.7 nA, n=12).

The sodium channels thus expressed, were found to be sensitive to the voltage-gated sodium channel blocker, tetrodotoxin (TTX). Figure 4 shows an example of TTX block of inward sodium current in the CNa18 cells

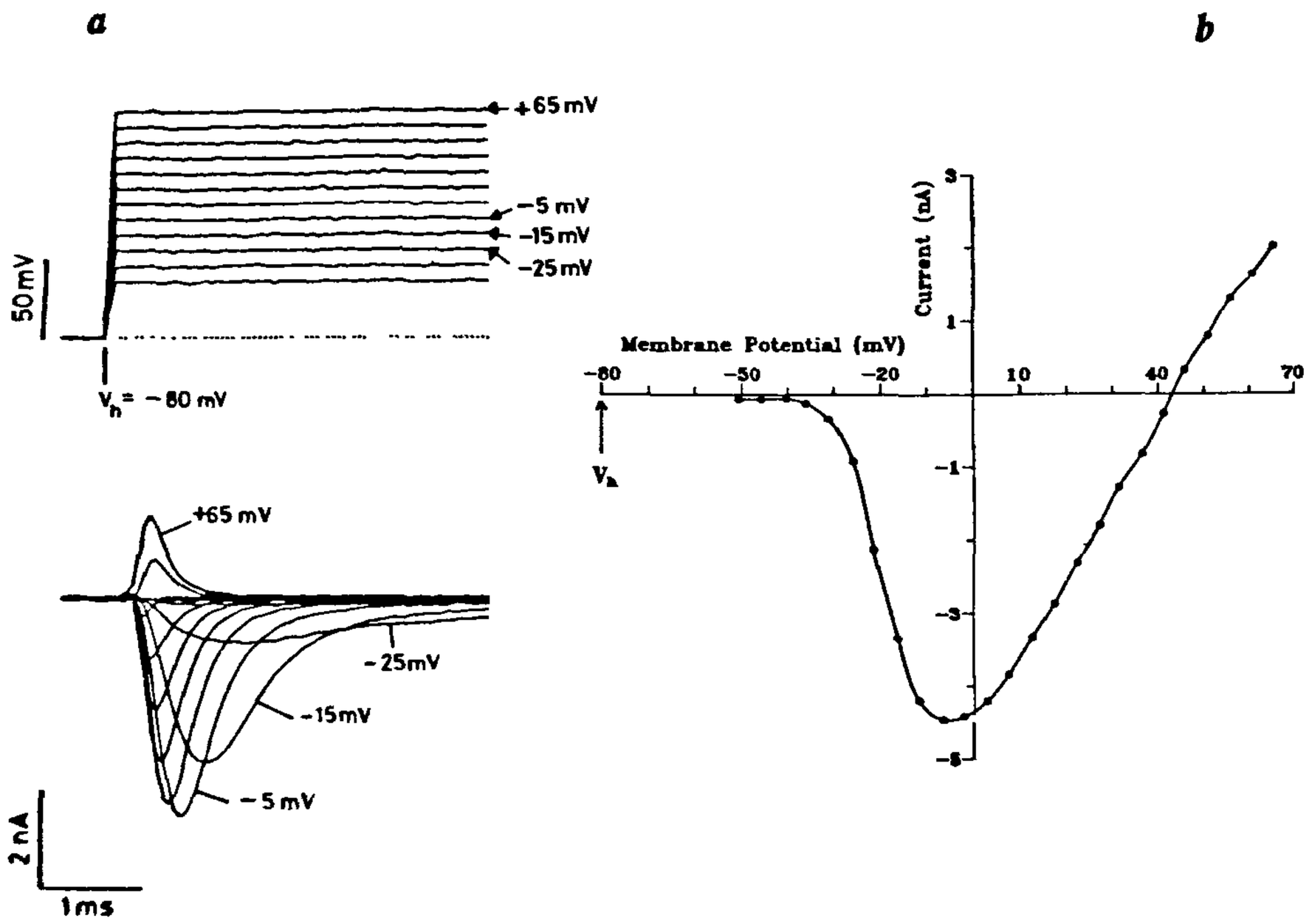

Figure 3. Whole cell sodium current from a CNa18 cell *a*, Whole cell sodium currents (bottom traces) elicited by step depolarizations (upper traces) from a holding potential of −80 mV to −45, −35, −25, −15, −5, 5, 15, 25, 35, 45, 55, 65 mV. Four current traces are marked to indicate the membrane potentials at which they were elicited *b*, Current voltage relationship of sodium current The peak sodium currents (I) elicited by 5 mV step depolarizations to −50 to 65 mV from a holding potential of −80 mV (V_h), were plotted against the membrane potential (V). Each trace in (*a*) and point in (*b*) is an average of five records. The s.e.m bars about the mean in (*b*) were smaller than the symbol size. (*a*) and (*b*) were recordings from the same cell

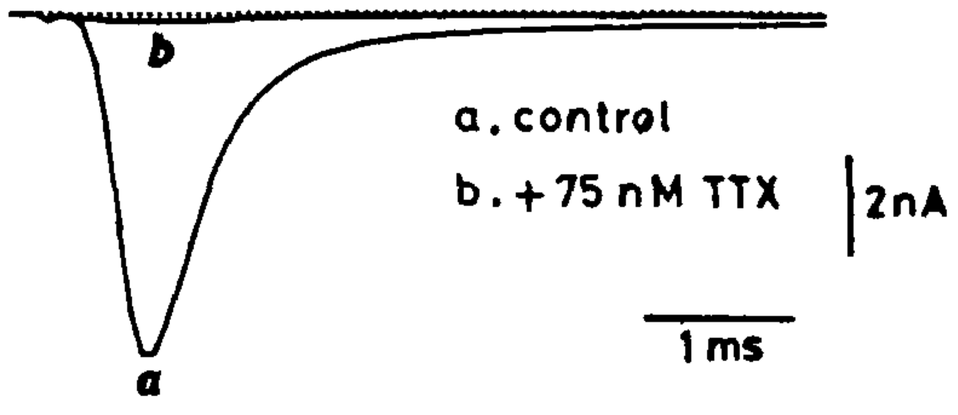

Figure 4. Tetrodotoxin (TTX) block of sodium currents from CNa18 cells. Averaged sodium current (n = 5) elicited by 70 mV depolarization step from a holding potential of –80 mV (trace *a*). Same sodium current after 5 min exposure to 75 nM TTX (trace *b*).

when used at nanomolar concentrations (75 nM).

Scheuer *et al.*[2] previously reported expression of rat brain type IIA sodium channel α-subunit in CHO cells using the pECE vector containing SV40 promoter resulting in sodium currents whose characteristics mimicked mammalian neuronal sodium channels. However, the current amplitudes were low precluding detailed functional studies. Subsequently, by using another vector containing the mMT1 promoter, a 30-fold increase in expression levels was observed using CHO-K1 cells[3]. The present findings reveal the use of a different expression system using pCDM8 vector containing the CMV promoter for efficient and high level stable expression of type IIA sodium channel α-subunit, comparable to the levels of expression achieved by West *et al.*[3].

Analysis of isolated whole-cell currents revealed the peak current amplitude occurring at –10 to –5 mV (n=12). The occurrence of complete inactivation within 3 ms, and pharmacological sensitivity to TTX suggests similarity in physiological and pharmacological properties of the expressed channels with the neuronal sodium channels. Such properties indicate that the channel proteins are synthesized, processed completely to mature size and inserted appropriately in the membrane to confer functional activity in the absence of β_1 and β_2 subunits, and are reminiscent of native neuronal voltage-gated sodium channels.

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Light intensity and the seasonal testicular cycle in a male wild bird, Spotted munia

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Different light intensity levels (0.11, 0.48, 0.96 and 1.86 mW/cm^2) of constant white light did not affect annual gonadal cyclicity of a male wild bird, Spotted munia; under high intensity (0.96 and 1.86 mW/cm^2) gonads were not fully developed indicating their inhibitory nature. But the low light intensity (0.11 mW/cm^2) accelerated the testicular function.

Of all the environmental factors regulating avian reproduction, light is known to be the most important one. Although a good deal of literature is available on the effects of light in the reproduction of birds[1–4], almost all are based on effects of duration of light exposure. Whatever little information is available on the influence of light intensity is restricted to poultry birds[5–8]. There has not been any systematic study on wild birds so far. The present investigation was therefore undertaken to reveal the importance of light intensity, if any, in the seasonal reproduction of a wild bird, Spotted munia, *Lonchura punctulata*.

In the first week of March 1992, adult Spotted munia were procured from a local bird supplier and were acclimatized to the laboratory conditions for 14 days.

The birds were then sexed by laparotomy and only healthy males were used in the experiment. Five groups of nine each were established in separate wirenet cages (20 × 16 × 14 inches). Birds of the groups 1, 2, 3 and 4 were exposed to continuous illumination of white incandescent light at 0.11, 0.48, 0.96 and 1.86 mW/cm^2 levels respectively. The light intensity for each group was measured by a radiometer (Int. Light Inc., USA). The birds of Group 5 received normal daylight (NDL) and served as controls. All these birds were exposed to ambient humidity and temperature. Food and water were provided *ad libitum*. The study was terminated in January 1993, once it covered the entire reproductive cycle. Every month laparotomy was performed and the left testis of each bird was measured *in situ*. Finally the gonadal volume was recorded (calculated from the size of the long and short axes of the testis).

Data were subjected to analysis of variance (ANOVA) and where appropriate Student's *t* test was applied for further comparison of the means.

Annual gonadal cycle was observed in all four experimental groups irrespective of their treatments (Figure 1). ANOVA analysis, however, indicated significant differences between the different groups in November and December 1992. While an apparent increase in testicular volume was observed in the birds treated with lowest (0.11 mW/cm^2) intensity of light, in most of the months, it decreased both in 0.96 and 1.86 mW/cm^2 treated groups, significantly in November 1992 ($P < 0.01$ for both 0.96 and 1.86 mW/cm^2 groups compared to the NDL control value). Compared to the lowest intensity group also, in these birds testicular volume was significantly less ($P < 0.05$ both for 0.96 and 1.86 mW/cm^2 groups). However, no significant difference was noted between the values of NDL group and 0.48 mW/cm^2 intensity group in any of the months.

Although gonadal cyclicity was not affected in any of the experimental groups and none of the light intensity levels could prevent testicular regression, in higher intensity the testes were not fully developed as seen in NDL birds. While in 0.48 mW/cm^2, no significant difference was found between the testicular volume of different groups of birds in any of the months studied, in 0.11 mW/cm^2, it was rather more in most of the months compared to the NDL values. Similar observations have also been made in a poultry bird, turkey hen[8], where high intensity was of no advantage on its reproductive performance.

In Spotted munia, gonadal development starts with the onset of monsoon in Indore (22.4° N, 78.54° E). However, our recent finding rules out the involvement of humidity as a proximate factor in the testicular function of this bird[9]. The effects of different photoperiods on its reproduction have also been studied earlier[10, 11]. These reports indicated no significant change in testicular cycle following exposure to different light hours. However, the importance of light intensity was not studied, despite the fact that in Spotted munia maximum development of testes occurs in November (winter time in Indore), when natural light intensity is relatively low. Our present finding indicates that low light intensity is favourable for the testicular function of Spotted munia.

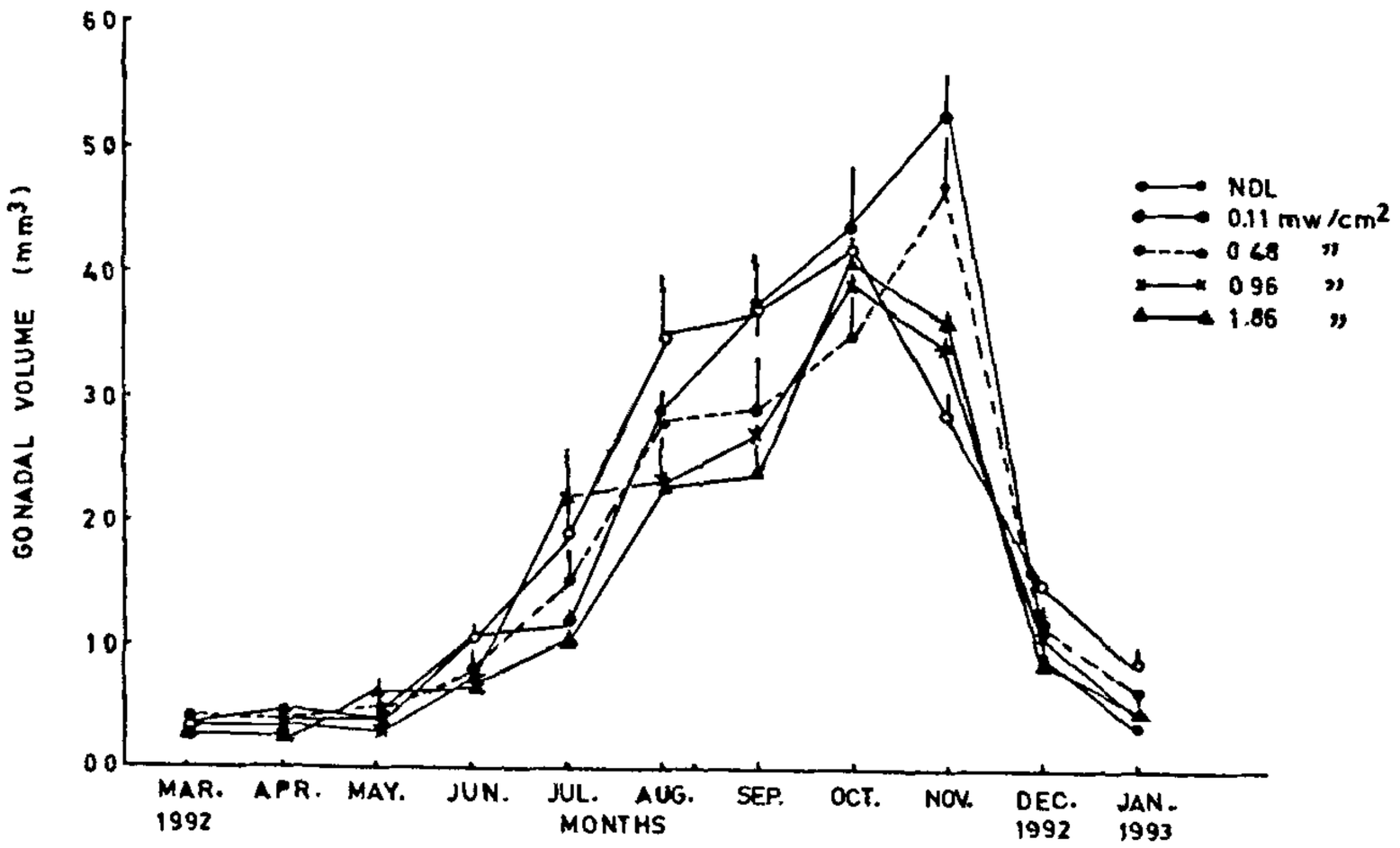

Figure 1. Testicular cycle of Spotted munia, exposed to continuous white incandescent light of 0 11, 0 48, 0 96 and 1 86 mW/cm^2 intensity or to natural day light (NDL). Vertical lines indicate the standard errors of the means ($n = 9$).

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Concentration of proteins in the midgut epithelium of sixth instar larvae, pupae and adult of *Spodoptera mauritia* Boisduval (Lepidoptera: Noctuidae)

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The protein content increased in the sixth instar larvae from 24 h to 120 h (prepupa), this was the highest level observed which later declined to a minimum in the adult. The midgut tissue as a storage site for proteins has been reported.

PROTEINS stored in tissues like haemolymph and fat body are subsequently utilized for adult growth and development[1,2]. Midgut is the synthetic site for haemolymph proteins in insects[3]. Changes in protein level of gut tissue during development have been studied as this has not been attempted so far.

The sixth instar larvae were separated immediately after moulting from the colony reared in the laboratory. The midgut was dissected out in an ice-cold ringer and the gut contents were removed. Protein in the tissue homogenate was precipitated with 80% aqueous ethanol. The precipitate was then successively extracted with ethanol–chloroform, ethanol ether and ether at room temperature and with 0.5 N perchloric acid at 90°C for 15 min. The final residue left on hot acid extraction was dissolved in 0.5 N sodium hydroxide and estimated for protein[4].

The results of estimation for proteins in the different stages are given in Table 1. The concentration of proteins in the midgut epithelium increased from 24 h to 120 h larva. But it declined sharply at the time of transformation of pupa to adult. Changes observed in the protein content of midgut tissue during the period of development of 6th instar larva of *S. mauritia* indicate that midgut tissue acts as a storage organ for proteins during its late larval and early pupal stages.

Table 1. Protein content of midgut

Insect stages	µg/mg fresh tissue	µg/gut
24 h larva	8 96 ± 0 17	229.57 ± 12 87
48 h larva	12 57 ± 0 12	295.00 ± 12 71
72 h larva	13 33 ± 0 14	340 27 ± 31 82
120 h larva (pre-pupa)	53 68 ± 3 02	521 8 ± 22 63
Pupa (0 day)	41 11 ± 1 69	411 09 ± 16.86
Pharate adult	17.27 ± 0 15	73 88 ± 4.34
Adult	8 06 ± 0 22	46.29 ± 10 00

Five samples each were used in the estimations and the results are the mean of the five determinations with standard deviations.

The midgut forms the major part of the gut which is a prominent organ in the larva of lepidopterous insects. It undergoes considerable reduction in size during larval–pupal transformation. In silkworm, when the larva stops eating before cocoon spinning, silk synthesis is maintained at the cost of other tissues, mainly the gut and the integument[5]. Protein from the gut of *Neodiprion sertifer* provides necessary materials for the development of its adult tissues[6]. In *Malacosoma* and *Rothschildia*, fat body is not the only site for protein uptake. The transfer of blood proteins into midgut, heart muscle, accessory gland, ovary and testis occurs at various stages[7].

In *S. mauritia* the level of proteins in the midgut wall gradually shoots up during larval period when the larva is feeding actively. It has therefore been suggested that the protein synthesized during the larval period is stored in the midgut tissue in the prepupal stage. The fall in the level of proteins in the gut of late pupa and adults of *S. mauritia* points to the utilization of these stored proteins during metamorphosis.

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