B'SYS GmbH

CHO $Na_V1.4$ Cell Line

Specification Sheet

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1. B'SYS $Na_V1.4$ CELL LINE

1.1 The voltage gated sodium channels $Na_V1.4$

SCN4A encodes $Na_V1.4$, a member of the sodium channel alpha subunit gene family. It is expressed in skeletal muscle and is responsible for the generation and propagation of action potentials that initiate muscle contraction. Mutations in this gene have been linked to several myotonia and periodic paralysis disorders. It is responsible for the generation and propagation of action potentials that initiate muscle contraction

1.2 Channelopathies related to muations in the SCN4A gene

Mutations in $Na_V1.4$ channels increase channel activity by impairing fast and/or slow inactivation causing hereditary sodium channelopathies of skeletal muscle such as: Hypokalaemic periodic paralysis, Paramyotonia congenital, Potassium-aggravated myotonia, Congenital myasthenic syndrome or Muscle stiffness.

VSGC blockers such as mexiletine, flecainide and other lidocaine analogues can reduce repetitive firing of action potential because of their use-depended properties, a mechanism that leads to a preferential action on channels with pathogenic gain-of-function mutations. These blockers reduce muscle stiffness in potassium-aggravated myotonia and paramyotoniacongenita by promoting the inactivated state of $Na_V1.4$ by

inducing a hyperpolarized shift in steady-state inactivation and by prolonging recovery time from inactivation.

1.3 B'SYS' CHO $Na_V1.4$ cells

B'SYS has designed a CHO $Na_V1.4$ cell line with constitutive coexpression of human $Na_V1.4$ sodium channels. The human $Na_V1.4$ cDNA (codon optimized) was cloned and transfected into CHO cells and then the functional properties of the $Na_V1.4$ channels validated by means of the patch-clamp technique. Results are outlined in section 3.

The cell line was tested with manual and automated (Q-Patch) patch-clamping.

2. PRODUCT SHIPMENT

2.1. Product Format

CHO cells stably transfected with recombinant human $Na_V1.4$ channel:

- 1 x 0.7 mL aliquots of frozen cells at 1-2 E+06 cells/mL
- Cells are frozen in complete medium with 10% DMSO

Cells are suitable for manual and automated patch clamping

2.2. Mycoplasma Certificate

B'SYS periodically tests cells for presence of mycoplasma by means of highly sensitive PCR based assays. All delivered cells are free of mycoplasma.

3. VALIDATION OF CHO $Na_V1.4$ CELLS

3.1. Biophysical characterization

$Na_V1.4$ currents were measured by means of manual and automated (Q-Patch) patch-clamp technique in the whole-cell configuration. The bath solution contained (in mM) NaCl 137 mM KCl 4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 10 mM, D-Glucose 10 mM, pH (NaOH) 7.4. The pipette solution consisted of (in mM) CsF 135 mM, NaCl 10 mM, HEPES 10 mM, EGTA 5 mM, pH (KOH) 7.3. After formation of a GΩ seal between the patch electrodes and individual $Na_V1.4$ stably transfected CHO cells, the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior. All solutions applied to cells were continuously perfused and maintained at room temperature. As soon as a stable seal could be established fast deactivating sodium currents were measured upon depolarization of the cell membrane from a holding potential of -100 mV to -130 mV up to +30 mV in 10 mV increments of 250 ms duration. Tail currents are elicited upon subsequent repolarization of the cell membrane to 0 mV for 10 ms. (Fig. 1 A, B). The voltage pulses were run at intervals of 5 s (n=11).

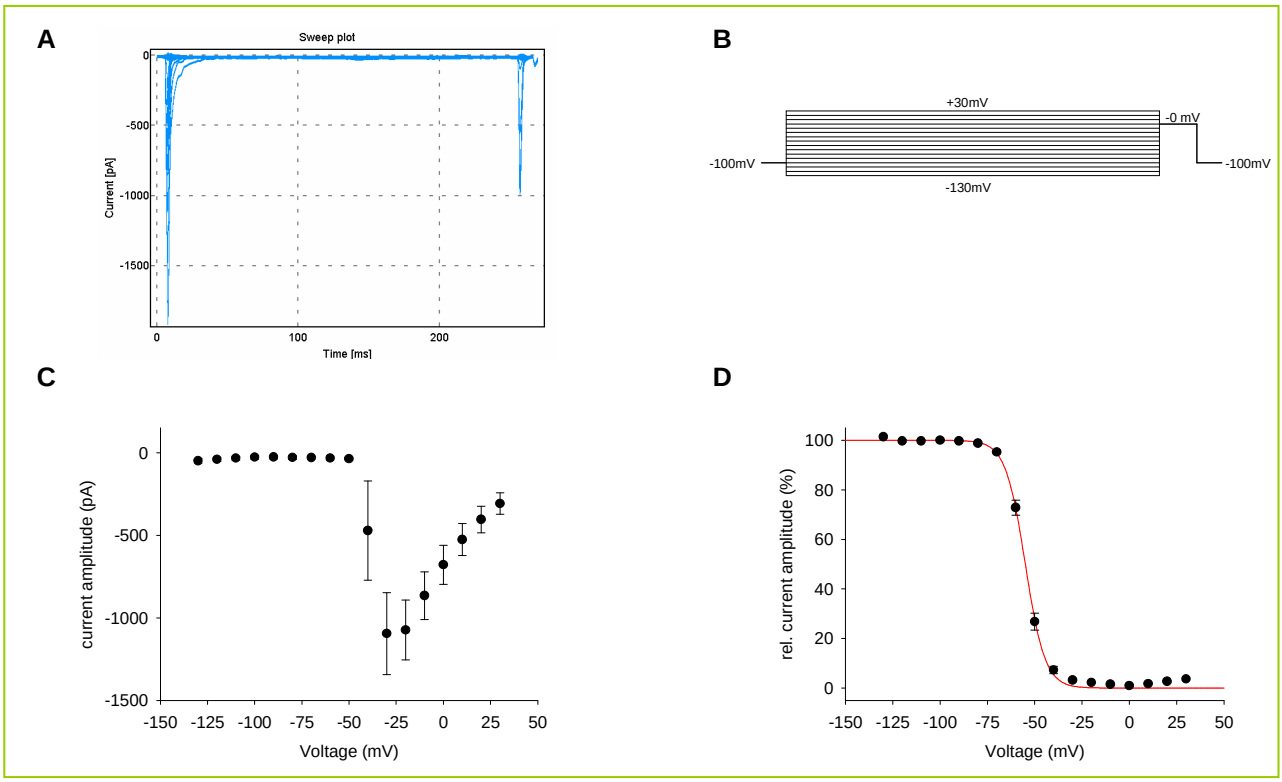

Fig. 1: Biophysical Characterization of stably transfected CHO $Na_V1.4$ cells. A) representative current recording. Cells were stimulated by the voltage protocol shown in b). C) IV curve if the $Na_V1.5$ channel. Peak current were measured at the beginning of the voltage pulse (n=11). D) Inactivation curve of the $Na_V1.4$ channel. The current amplitudes were measured at the beginning of the tail current. Data were fit by a Boltzmann equation. The $V_{0.5}$ value was -55.19 ± 0.83 mV (n=11). No currents were recorded in untransfected cells (data not shown).

For the IV curve the peak current amplitude of the pulses between -130 mV and +30 mV was plotted versus the applied voltage. This resulted in a bell shaped curve. The minimum of the IV curve was at -30 mV (Fig 1 C).

For the inactivation curve the peak currents of the tail current (pulse to 0 mV) was plotted versus the voltage of the prepulse. The resulting inactivation curve was fit with a Boltzmann equation. The $V_{0.5}$ value was -55.19 ± 0.83 mV (n=11), (Fig 1 D). This validation shows the same biophysical characteristics like described in the literature (Sheets at al. 2006).

3.2. Pharmacological characterization

For the pharmacological characterization of the $Na_V1.4$ channel Mexiteline was tested at concentrations of 0.1, 1.0, 10, 100 and 1000 µM and TTX at concentrations of 0.1, 1.0, 10, 100 and 1000 nM (n≥3 cells). The stimulation frequency was 0.2 Hz. Both test items were tested at a holding potential of -100 mV and -70 mV. The dose response curves were generated (see Fig. 2) and the IC_{50} values calculated.

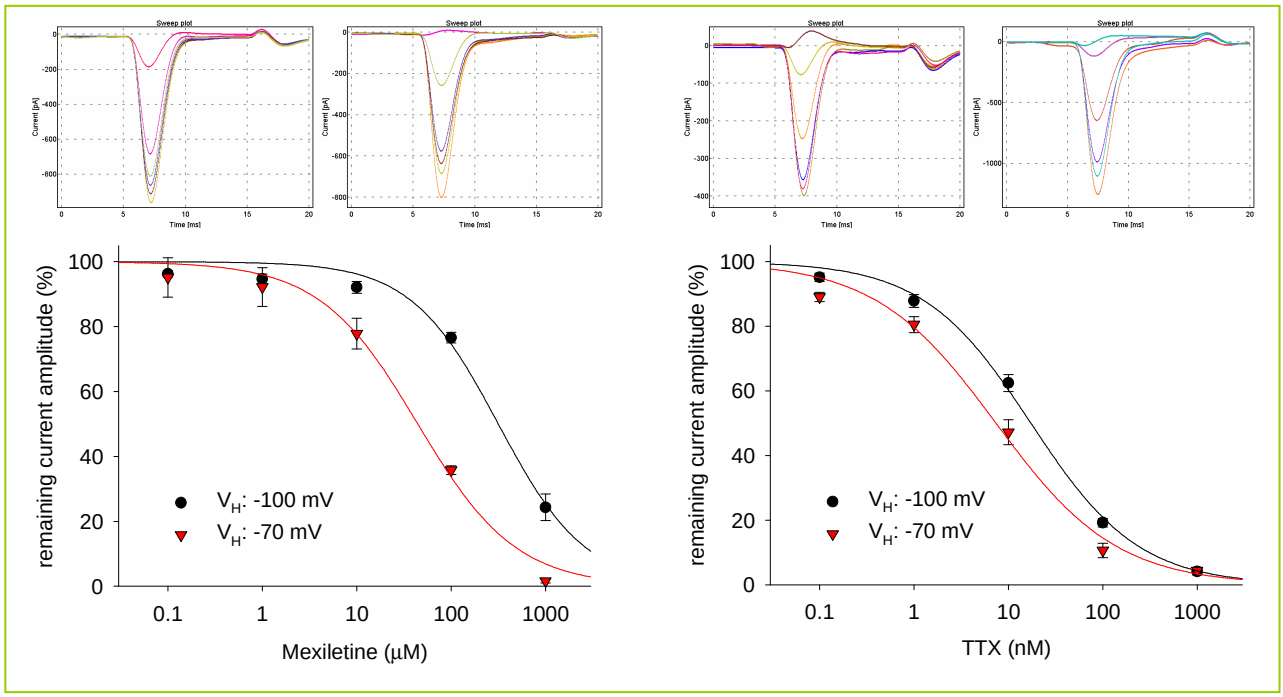

Fig. 2: Top: Representative current traces recorded in the steady state in the presence of Mexiletine (left) and TTX (right). The traces were recorded at a holding potential of -100 mVare shown left, the right traces were recorded at -70 mV. Bottom: Dose response curves for Mexiletine and TTX at an holding potential of -100 mV and -70 mV.

The results can be summarized as follows.

Application / stimulation frequency	**IC_{50} value (µM)**	**Hill coefficient**
Mexiletine -100 mV	315.79	0.94
Mexiletine -70 mV	44.33	0.84
TTX -100 mV	17.31	0.76
TTX -70 mV	7.39	0.68

The observed inhibition of $Na_V1.4$ inward peak currents by Mexiletine and TTX are in line with its known pharmacological profile. The effect of Mexiletine is strongly voltage dependent, while the effect of TTX is only slightly changed by the holding potential. Theses results indicate, that Mexiletine shows an increased affinity to inactivated $Na_V1.4$ channels.

3.3. Patch-clamp Success Rates

The patch-clamp properties of the CHO $Na_v1.4$ cell line were elucidated at typical working passage numbers (passage 5-17). A total of 32 cells were analyzed. Success for establishment of on-cell configuration was defined as follows: > 1 GΩ. The whole-cell configuration was not accepted if the membrane resistance was below 500 MΩ. A successful recording had to be free of rundown effects and variations in series resistance.

- On-cell successful: **100%** (n=32)
- Whole-cell successful: **97%** (n=32)
- Recording (15 min) successful: **94%** (n=32)

4. CELL CULTURE CONDITIONS

4.1. General

CHO $Na_V1.4$ cells are incubated at 37°C in a humidified atmosphere with 5% CO_2 (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing F12 (HAM) medium supplemented with 9% fetal bovine serum, 0.9% Penicillin/Streptomycin solution and 100 µg/mL Hygromycin. The CHO $Na_V1.4$ cells are passaged at a confluence of about 80%. For manual electrophysiological measurements the cells are seeded onto e.g. 35 mm sterile culture dishes containing complete medium. For automated patch-clamping, cells from confluent T25 flasks are used.

- All solutions and equipment coming in contact with the cells must be sterile.
- Use proper sterile technique and work in a laminar flow hood.
- Be sure to have frozen cell stocks at hand before starting experiments.
- Cells should be split every 2-3 days at 70% - 80% confluency at 1:3 to 1:5 ratio.

4.2. Recommended Complete Medium

- F12 (HAM) with GlutaMAX I or Glutamate
- 9% FBS
- 0.9% Penicillin/Streptomycin

4.3. Antibiotics

- CHO $Na_V1.4$ clones were selected under 500 µg/mL Hygromycin antibiotic pressure.
- To cultivate CHO $Na_V1.4$ cells, also a reduced antibiotic pressure (100 µg/mL) can be used.
- To separate CHO $Na_V1.4$ cells from untransfected cells, use 500 µg/mL Hygromycin.

Remark: The permanent application of high antibiotic pressure has no effect on current density.

4.4. Thawing Cells

- Remove vial of cells from liquid nitrogen and thaw quickly at 37°C.
- Decontaminate outside of vial with 70% ethanol.
- Transfer cells to a T-75 culture flask containing 10 mL complete medium.
- Incubate cells at 37°C for 4-6 h to allow the cells to attach to the bottom of flask.
- Aspirate off the medium and replace with 10 mL complete medium & antibiotics.
- Antibiotics: 100 µg/mL Hygromycin.
- Incubate cells and check them daily until 70% - 80% confluency is reached.

4.5. Splitting Cells

- When cells are 70% - 80% confluent remove medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Passage cells into new flask with complete medium and antibiotics at 1:3 to 1:5 ratio.
- Use remaining suspension for counting the cells.

4.6. Freezing Medium

- Mix 0.9 mL fresh complete medium and 0.1 mL DMSO for every 1 mL freezing medium.
- Sterilize freezing medium by means of appropriate micro filter (0.1 µm – 0.2 µm).

4.7. Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluency prior to freezing.
- Remove the complete medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Pellet cells with centrifuge and carefully aspirate off medium.
- Resuspend cells at a density of approximately 2.0 E+06 cells per mL with fresh freezing medium.
- Aliquot 0.5 mL of cell suspension into each cryovial.
- Overnight incubate cells in a styropor box at –80°C.
- The next morning transfer cryovial in liquid nitrogen tank for long-term storage.

5. $Na_V1.4$ SEQUENCE

5.1. Human $Na_V1.4$ Accession Number NP_000325.4

The human sequence of the $Na_V1.4$ channel gene was codon optimized. The resulting cDNA encodes for NP_000325.4.:

MARPSLCTLVPLGPECLRPFTRESLAAIEQRAVEEEARLQRNKQMEIEEPERKPRSDLEAGKNLPMIYGDPPPEVIGIPL
EDLDPYYSNKKTFIVLNKGKAIFRFSATPALYLLSPFSVVRRGAIKVLIHALFSMFIMITILTNCVFMTMSDPPPWSKNV
EYTFTGIYTFESLIKILARGFCVDDFTFLRDPWNWLDFSVIMMAYLTEFVDLGNISALRTFRVLRALKTITVIPGLKTIV
GALIQSVKKLSDVMILTVFCLSVFALVGLQLFMGNLRQKCVRWPPPFNDTNTTWYSNDTWYGNDTWYGNEMWYGNDSWYA
NDTWNSHASWATNDTFDWDAYISDEGNFYFLEGSNDALLCGNSSDAGHCPEGYECIKTGRNPNYGYTSYDTFSWAFLALF
RLMTQDYWENLFQLTLRAAGKTYMIFFVVIIFLGSFYLINLILAVVAMAYAEQNEATLAEDKEKEEEFQQMLEKFKKHQE
ELEKAKAAQALEGGEADGDPAHGKDCNGSLDTSQGEKGAPRQSSSGDSGISDAMEELEEAHQKCPPWWYKCAHKVLIWNC
CAPWLKFKNIIHLIVMDPFVDLGITICIVLNTLFMAMEHYPMTEHFDNVLTVGNLVFTGIFTAEMVLKLIAMDPYEYFQQ
GWNIFDSIIVTLSLVELGLANVQGLSVLRSFRLLRVFKLAKSWPTLNMLIKIIGNSVGALGNLTLVLAIIVFIFAVVGMQ
LFGKSYKECVCKIALDCNLPRWHMHDFFHSFLIVFRILCGEWIETMWDCMEVAGQAMCLTVFLMVMVIGNLVVLNLFLAL
LLSSFSADSLAASDEDGEMNNLQIAIGRIKLGIGFAKAFLLGLLHGKILSPKDIMLSLGEADGAGEAGEAGETAPEDEKK
EPPEEDLKKDNHILNHMGLADGPPSSLELDHLNFINNPYLTIQVPIASEESDLEMPTEEETDTFSEPEDSKKPPQPLYDG
NSSVCSTADYKPPEEDPEEQAEENPEGEQPEECFTEACVQRWPCLYVDISQGRGKKWWTLRRACFKIVEHNWFETFIVFM
ILLSSGALAFEDIYIEQRRVIRTILEYADKVFTYIFIMEMLLKWVAYGFKVYFTNAWCWLDFLIVDVSIISLVANWLGYS
ELGPIKSLRTLRALRPLRALSRFEGMRVVVNALLGAIPSIMNVLLVCLIFWLIFSIMGVNLFAGKFYYCINTTTSERFDI
SEVNNKSECESLMHTGQVRWLNVKVNYDNVGLGYLSLLQVATFKGWMDIMYAAVDSREKEEQPQYEVNLYMYLYFVIFII
FGSFFTLNLFIGVIIDNFNQQKKKLGGKDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPQNKIQGMVYDLVTKQAFDITIM
ILICLNMVTMMVETDNQSQLKVDILYNINMIFIIIFTGECVLKMLALRQYYFTVGWNIFDFVVVILSIVGLALSDLIQKY
FVSPTLFRVIRLARIGRVLRLIRGAKGIRTLLFALMMSLPALFNIGLLLFLVMFIYSIFGMSNFAYVKKESGIDDMFNFE
TFGNSIICLFEITTSAGWDGLLNPILNSGPPDCDPNLENPGTSVKGDCGNPSIGICFFCSYIIISFLIVVNMYIAIILEN
FNVATEESSEPLGEDDFEMFYETWEKFDPDATQFIAYSRLSDFVDTLQEPLRIAKPNKIKLITLDLPMVPGDKIHCLDIL
FALTKEVLGDSGEMDALKQTMEEKFMAANPSKVSYEPITTTLKRKHEEVCAIKIQRAYRRHLLQRSMKQASYMYRHSHDG
SGDDAPEKEGLLANTMSKMYGHENGNSSSPSPEEKGEAGDAGPTMGLMPISPSDTAWPPAPPPGQTVRPGVKESLV*

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