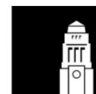


UNIVERSITY OF LEEDS

This is a repository copy of *A cytoplasmic Slo3 isoform is expressed in somatic tissues*.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/148447/

Version: Accepted Version

Article:

Chávez, JC, Vicens, A, Wrighton, DC et al. (5 more authors) (2019) A cytoplasmic Slo3 isoform is expressed in somatic tissues. Molecular Biology Reports, 46 (5). pp. 5561-5567. ISSN 0301-4851

https://doi.org/10.1007/s11033-019-04943-z

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

A cytoplasmic Slo3 isoform is expressed in somatic tissues

Julio C. Chávez[1*], Alberto Vicens[2*], David C. Wrighton[3], Karla Andrade-López[1], Carmen Beltrán[1], Rosa M. Gutiérrez[4], Jonathan D. Lippiat[3] and Claudia L. Treviño[1&]

[1]Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca Morelos, México.
[2]Department of Biochemistry, Genetics and Immunology, University of Vigo, 36310 Vigo, Spain.
[3]School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, United Kingdom.
[4]Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca Morelos, México

*These authors contributed equally

[&]**Corresponding author**
Departamento de Genética del Desarrollo y Fisiología Molecular
Instituto de Biotecnología, Universidad Nacional Autónoma de México,
Avenida Universidad 2001, Col. Chamilpa 62210
Cuernavaca Morelos, México.
E-mail: ctrevino@ibt.unam.mx
Phone number: +52 777 329 1777

Grant sponsors:
Dirección General de Asuntos de Personal Académico/Universidad Nacional Autónoma de México; Grant number: IN202519 to CT; IN215519 to CB; IA200419 to JCC. DCW was supported by a BBSRC PhD studentship.

Conflict of interests:
The co-authors of this manuscript declare that they have no conflict of interests

ABSTRACT

Slo3 is a pH-sensitive and weakly voltage-sensitive potassium channel that is essential for male fertility in mouse and whose expression is regarded as sperm-specific. These properties have proposed Slo3 as a candidate target for male contraceptive drugs. Nonetheless, the tissue distribution of *Slo3* expression has not been rigorously studied yet. Applying computational and RT-PCR approaches, we identified expression of two short *Slo3* isoforms in somatic mouse tissues such as brain, kidney and eye. These isoforms, which seem to result of transcription starting sites between exons 20 and 21, have an identical open reading frame, both encoding the terminal 381 amino acids of the cytosolic Slo3 domain. We corroborated the expression of these isoforms in mouse brain and testis by Western-blot. The complete isoform encoding the Slo3 ion channel was uniquely detected in testis, both at transcript and protein level. Although the functional role of the cytosolic Slo3 isoforms remains to be established, we propose that they may have a functional effect by modulating Slo channels trafficking and/or activity. This study confirms that expression of full-length Slo3 is sperm-specific but warns against developing contraceptive drugs targeting the C-terminal tail of Slo3 channels.

Abbreviations

cDNA: complementary DNA
EST: Expressed Sequence Tag
K^+: Potassium ion
RCK: regulator of K^+ conductance
Slo3-CT: Slo3 carboxyl terminal

Keywords

Potassium channels, Slo, alternative splicing, sperm.

INTRODUCTION

Potassium (K^+) channels of the Slo family exhibit functional properties that differ from those of classical voltage-gated K^+ (Kv) channels [1]. Slo channels possess a large C-terminal cytoplasmic domain containing two non-identical regulators of K^+ conductance (RCK) arranged in tandem, and which confer ligand-specific gating properties [1,2]. In vertebrates, the Slo family have four members, namely Slo1, two Slo2 paralogues, and Slo3. Slo1 channels, also referred to as BK, have been extensively investigated due to their wide expression profile and high unitary conductance (260 pS). Slo1 channels are activated both by depolarization and by an increase in intracellular Ca^{2+} [3,4]. The two Slo2 paralogues, Slo2.1 and Slo2.2, are structurally related channels but are modulated by different cytosolic factors, including ATP, Na^+ and Cl^- ions [5,6]. Slo3, on the other hand, is pH- and voltage-dependent [7,8]. Slo3 channel expression has been claimed to be restricted to mammalian testis, specifically in developing spermatocytes and mature spermatozoa [9], and is known to have a pivotal role in male fertility. Genetic ablation of the *Slo3* gene in mouse, results in infertile males.

Sperm cells from these knock out (KO) mouse exhibit several functional defects, such as absence of hyperactivated motility, lack of capacitation-associated membrane hyperpolarization (a maturational process needed for fertilization), and reduced acrosomal reaction, among others [10,11].

Recently, we reported that *Slo3* expression is in fact not restricted to mammals and is also present in testis from birds and reptiles, although its function in these species remains unknown [12]. Given its presumed testis-specific expression, the Slo3 channel has been proposed as an ideal target candidate to develop pharmacological agents to control fertility in humans. Before embarking in such an endeavor, it is essential to characterize the expression pattern of Slo3 in adult mammalian males. Expression of *Slo3* is presumed to be restricted to testis, a notion based on polymerase chain reaction (PCR) and northern blot analyses performed in a variety of mouse tissues, in which the *Slo3* transcript was detected solely in spermatozoa and testis [9]. However, as indicated by the authors of this study, their techniques could not rule out low *Slo3* transcription levels in the tested tissues. Moreover, its expression cannot be ruled out in tissues not included in their study.

Making use of the growing availability of genomic and expression data, we identified various mouse *Slo3* (m*Slo3*) expressed sequence tags (ESTs) in several non-reproductive tissues, particularly in neural tissue. This observation motivated us to confirm using PCR and Western blot (WB), whether such non-testicular ESTs correspond to non-canonical Slo3 transcripts and are in fact expressed in tissues other than testis.

MATERIALS AND METHODS

1.1. Tissue sources

CD1 male mouse (12-20 weeks old) were sacrificed by cervical dislocation, and tissues (testis, brain, skin, eye, heart, blood serum, kidney, lung, and liver) were dissected from fresh cadavers. All experimental procedures were approved by the Instituto de Biotecnología (Universidad Nacional Autónoma de México) Animal Care Committee and were performed in accordance with their Guiding Principles for the care and use of laboratory animals.

1.2. Rapid Amplification of cDNA Ends, PCR product cloning and cRNA synthesis

Marathon Rapid Amplification of cDNA Ends (RACE) PCR was performed using mouse brain Marathon-ready® cDNA (Clontech, St-Germain-en-Laye, France) to identify potential neuronal *Slo3* isoforms. Gene-specific primers were designed using NCBI primer blast design software (www.ncbi.nlm.nih.gov/tools/primer-blast/) utilizing the *Slo3*-related EST sequence identified in a cDNA library from mouse brain (GenBank entry: CV562866). First round PCR reactions were performed using Adaptor Primer 1 (AP1) with forward or reverse *Slo3*-specific primers. The products

of these reactions were used as templates for a second round of PCR reactions using nested primers. The RACE-PCR products were separated by agarose gel electrophoresis and bands were purified using the QIAEX II Gel Extraction Kit (Qiagen) before cloning using the StrataClone Blunt PCR Cloning Kit (Stratagene) according to the manufacurer's instructions, and sequencing (GATC Biotech AG).

1.3. RNA extraction and RT-PCR

Total RNA was purified from each mouse tissue sample using TRIzol® Reagent (Thermo Scientific) following manufacturer instructions. Reverse transcription was carried out using the RevertAid H Minus First Strand cDNA synthesis Kit (Thermo Scientific) with 1 μg of total RNA, following manufacturer protocol. The product of the first strand cDNA synthesis was used directly for RT-PCR using Taq DNA Polymerase (Thermo Scientific). PCRs were performed using Bio-Rad T100™ thermal cycler. PCR primers were designed using mouse *Slo3* transcript sequence (Ensembl ID ENSMUST00000098858). PCR products were sequenced by the Sanger method in an automatic sequencer provided by the Sequencing and Synthesis Unit of Instituto de Biotecnología (Universidad Nacional Autónoma de México).

1.4. Protein extraction, SDS-PAGE and western blot analysis

Testis and brain from CD1 were dissected and microsomal membranes (membrane fraction, MF) as well as their 200 000 x g supernatants (cytosolic fraction, CF) were obtained as reported by [13] and modified as follows. The tissues were mixed with 9 volumes of 0.32 M sucrose, 1mM EDTA, 1 mM 2-mercaptoethanol, cOmplete EDTA-free (Roche REF 05056489001), and 10 mM Tris-HCl, pH 7.4, and homogenized in a glass-Teflon Potter homogenizer. The suspensions were centrifuged at 12, 000 x g for 15 min at 4 °C and their supernatants at 200,000 x g for 45 min at 4 °C. The membrane fraction in the pellet, was resuspended in buffer containing 0.1 M sucrose, 1 mM EGTA, 0.2 mM 2-mercaptoethanol, cOmplete EDTA-free and 10 mM Tris-HC1, pH 7.4. Cytosolic fraction was concentrated in Centricon YM30 Amicon. The protein samples were resolved in a 10 % SDS-PAGE and electro-transferred (Semi-Dry Trans-Blot SD, Bio-Rad) to an Immobilon-P membrane. After blocking with 5% fat-free milk in TBS (50 mM Tris–HCl, 150 mM NaCl pH 7.6) containing 0.5% Twin-20 (TBS-T) at room temperature (1 hour), the Immobilon-P membranes were incubated at 4 °C, overnight with two anti-Slo3 antibodies: a mouse monoclonal produced against 1052-1121 amino acids of mSlo3 (NIH NeuroMab Facility, UC Davis, CA, USA) [10] (1:1000 in blocking buffer), and a chicken polyclonal produced against 878-892 amino acids of mSlo3 (New England Peptide LLC, Gardner, MA, USA) [14] (1:10000 in blocking buffer). Membranes were washed in TBS-T, and then incubated with secondary antibodies conjugated to horseradish peroxidase, either donkey anti-mouse

IgG (LI-COR Biosciences, Lincoln, NE, USA (1:5000) or goat anti-chicken (Zymed San Francisco, CA, USA) (1:2500), at room temperature/1 hour. Immobilon-P membranes were washed in TBS-T, TBS and revealed with the Chemiluminiscent substrate Super Signal West Pico (Thermo Fisher Scientific, Rockford, IL, USA, No.34080). After the WB, the Immobilon-P membranes were stained with amido black to visualize the total protein load.

RESULTS AND DISCUSSION

1.5. Identification of mSlo3 splicing isoforms from database mining

Genomic information about the *mSlo3* gene was explored using the Ensembl Genome Database (http://www.ensembl.org/index.html), and three putative *mSlo3* isoforms were found **(Fig. 1A)**. The isoform 1 (Ensembl transcript ID: ENSMUST00000098858) consists of 27 exons and is considered to be the canonical mSlo3 isoform, and corresponds to the mSlo3 transcript experimentally detected in mouse testis (AF039213) [9]. Several ESTs from testis libraries provide experimental support of this isoform's transcription **(Fig. 1A)**. The isoform 2 (ENSMUST000000120653) contains exons 21-27 of the full-length *mSlo3* isoform with an additional exon downstream the exon 20 **(Fig. 1A)**. This isoform is supported by a full-length cDNA (AK083174) as well as several ESTs from brain libraries **(Fig. 1A)**. The third *mSlo3* isoform (isoform 3, ENSMUST000000126226) is composed of exons 21-24, and exhibits an alternative splicing between canonical exons 20-21, resulting in two small 5' exons **(Fig. 1A)**. This isoform is supported by at least two short ESTs from brain (CV562866, CX213584), and its 3' is shown as truncated given that there is no supporting ESTs that includes this region. To determine the complete sequence of the isoform 3, we used Marathon RACE-PCR on mouse brain adaptor-ligated cDNA and used primers based on the EST clone CV562866 **(Supplementary Figure S1)**. The first round of RACE-PCR generated smears with faint bands at 1000-1500 bp. The products were used as templates for second round nested PCR. *Slo3* 3' RACE-PCR yielded products of ≈1.5 kb, whereas *Slo3* 5' RACE produced only low molecular weight bands, between 200-600 bp. If the cDNA transcript encoded a full length α-subunit, the 3' RACE-PCR would be expected to generate a band of ≈3.5 kb, thus the neural cDNA sample contains shorter sequences. This finding was further supported by performing PCR of a mouse brain cDNA library using pore region-specific primers, which failed to amplify any cDNA. We sequenced products from the Marathon RACE-PCR reactions and observed that this transcript has an identical 3' sequence to the second isoform **(Fig. 1A, Supplementary Figure S2).** Additionally, we identified two ESTs from eye libraries that map to exons in the terminal 3' sequence of *mSlo3* gene **(Fig. 1A)**.

We examined the putative transcription profile of *mSlo3* gene by exploring transcription factor binding sites (TFBS) and chromatin modification profiles based on mouse Chromatin immuno precipitation sequencing (ChIP-seq) data (ENCODE/LICR survey, 8-week adult), retrieved from the

UCSC genome browser (https://genome.ucsc.edu/). A strong signal of RNA polymerase II (Pol II) binding sites is observed in the 5' upstream sequence in testis cDNA **(Fig. 1B)**, supporting the strong promoter activity in this tissue. Interestingly, a potential binding site of Pol II was observed in cerebellum and cerebral cortex cDNA upstream the putative 5' end of shorter *Slo*3 isoforms. The histone methylation H3K4me1, which is a mark of active promoters, shows an enriched signal upstream to the 5' terminal sequence of *mSlo3* for testis cDNA. A remarkable signal was also observed upstream the putative 5' end sequence of the short isoforms for both testis and cortex cDNA libraries **(Fig. 1B)**.

Altogether, these data indicate that *mSlo3* gene would not solely encode the α-subunit of a sperm K^+ channel, but it also encodes shorter isoforms that can be expressed in non-reproductive tissues.

1.6. Expression of Slo3 isoforms in mouse tissues

To obtain experimental evidence of the expression of *mSlo3* isoforms, we carried out RT-PCR on cDNA obtained from several mouse tissues. To distinguish between the three *Slo3* isoforms, we used three different forward primers annealing to specific exons of each isoform, whereas a single reverse primer –mapping to exon 24– was utilized **(Fig. 2A, Supplementary Figure S2)**. RT-PCR experiments revealed expression of *mSlo3* isoforms in different mouse tissues **(Fig. 2B)**. The first isoform, coding for the α-subunit of Slo3 channel, was uniquely identified in testis. Isoform 2 was detected in a range of tissues such as testis, brain, eye and kidney. Isoform 3 transcripts were found in testis, kidney and brain. Identification of *mSlo3* transcripts in kidney was innovative as we had not evidence of *Slo3* expression in this tissue from genomic databases screening. None of the isoforms were amplified in liver, lung, heart and skin. Amplification of β actin was used as a positive control for all tissues. The PCR products obtained from testis and brain were purified and sequenced.

Since the *mSlo3* isoforms described in the present work lack the region spanning the S0-S8 hydrophobic segments, the two PCR primer pairs and the northern blot probe used by Schreiber et al. [9] would not anneal to these transcripts, which encode most of the S9-S10 segment. On the other hand, the divergence at the 5' end of the short *mSlo3* transcript variants, resulting from alternative splicing, would prevent binding of the PCR primer pairs they used to detect segment S9, as the target sequence is absent in them. This would explain why Schreiber et al. [9], were unable to detect cDNAs comprising the 3' end of the *mSlo3* sequence.

Both shorter isoforms (2 and 3) contain an identical open reading frame (ORF) whose translation would yield a protein of 381 amino acids (**Supplementary Figure S2)**. This protein would span a portion of the cytosolic RCK-2 domain and the C-terminal tail of the Slo3 channel, lacking the transmembrane region. In addition, this protein also would lack the RCK-1 domain, which contains

the assembly interface necessary to adopt the tetrameric gating ring structure of Slo channels [14]. These observations suggest that shorter Slo3 isoforms will be expressed as a soluble and monomeric proteins.

To examine the expression of *mSlo3* isoforms at the protein level, testis and brain protein extracts were separated by SDS-PAGE and analyzed by WB using a mouse monoclonal anti-Slo3 antibody (from Neuromab) that recognizes an epitope contained in all mSlo3 isoforms **(Supplementary Figure S2)**. We observed bands (around 50 kDa), the predicted molecular size of cytosolic isoforms (about 47 kDa, UniProt ref: D3Z5P2), in extractions of both testis and brain (not shown). However, the high molecular size protein (~130 kDa) corresponding to the full-length Slo3 channel was not detected in the membrane fraction from testis, albeit this protein has been previously identified using this antibody [10]. Additionally, this antibody also recognizes bands around 50 kDa in samples for Slo3 KO testis, according to the data sheet of the manufacturer. To overcome these technical problems, we used a custom-made polyclonal chicken anti-Slo3 that also targets epitopes contained in all mSlo3 isoforms **(Supplementary Figure S2)**. This antibody was validated in a previous paper form our group using the antigenic peptide and the pre-immune serum [15]. Unfortunately, when we tested this antibody in testis from Slo3 KO mice we also detected non-specific bands in the range of 25-70 kDa (not shown). However, using this antibody, we did visualize the ~130 kDa band of Slo3 channel in the membrane fraction from testis but not from brain (**Figure 3**). A band of ~80 kDa was detected in the membrane fraction from brain. Several bands (50-70 kDa) were detected in the cytosolic and membrane fraction of brain and testis. Since this antibody yields nonspecific bands at these molecular weights samples, we were unable to demonstrate unambiguously the expression of the shorter Slo3 protein by immunodetection procedures in brain and testis.

Unlike Slo3 channels, which are specifically expressed in testis and restricted to spermatocytes and mature spermatozoa, the soluble *Slo3* isoforms are expressed in several tissues. These cytosolic proteins appear to share similar tissue expression with Slo1 channels, expressed in many different organs [1]. Interestingly, expression of *Slo3* C-terminus was primarily detected in brain, where Slo1 channels have a key role controlling the excitability of neurons [16]. A recent study using a proteomic approach identified Slo3 as a possible interacting protein of Slo1 in mouse brain [17]. Slo1 gene products include a large number of alternative isoforms that vary in their gating properties, susceptibility to modulation, and trafficking to the plasma membrane and through intracellular organelles [18–21]. Regarding the evolutionary interpretation of this interaction, it is possible that the expression of soluble Slo3 isoforms in somatic tissues may be favored due to their positive effects on Slo1 trafficking. The presence of cytosolic Slo3 isoforms in testis could increase the surface expression of Slo1 in pre-meiotic germ cells [22].

Although our findings initially challenge the widely-accepted view about the existence of a unique Slo3 protein specifically expressed in testis/spermatozoa, the full-length *Slo3* transcript encoding a functional K^+ channel remains exclusively detected in testis. Our findings thus would not run against the consideration of the Slo3 channel as a candidate target for male contraceptive drugs but call for caution for using drugs targeting the C-terminal tail of Slo3 channels.

ACKNOWLEDGMENTS

The authors thank Jose Luis De la Vega, Paulina Torres-Rodríguez, Yoloxóchitl Sánchez, Gabriela Cabeza, Sergio González, Timothy Munsey, and Elizabeth Mata for technical support. We also thank Juan Manuel Hurtado, Roberto Rodríguez, David Castañeda, Arturo Ocádiz and Shirley Ainsworth for computer and library services.

REFERENCES

[1] Salkoff L, Butler A, Ferreira G, Santi C, Wei A. High-conductance potassium channels of the SLO family. Nat Rev Neurosci 2006;7:921–31. doi:10.1038/nrn1992.

[2] Xia X, Zhang X, Lingle CJ. Ligand-Dependent Activation of Slo Family Channels Is Defined by Interchangeable Cytosolic Domains. J Neurosci 2004;24:5585–91. doi:10.1523/JNEUROSCI.1296-04.2004.

[3] Barret JN, Magleby KL, Pallotta B. Properties of Single Calcium-activated Potassium Channels in Cultured Rat Muscle. J Physiol 1982;331:211–30.

[4] Magleby KL. Gating mechanism of BK (Slo1) channels: so near, yet so far. J Gen Physiol 2003;121:81–96. doi:10.1085/jgp.20028721.

[5] Yuan A, Santi CM, Wei A, Wang ZW, Pollak K, Nonet M, et al. The sodium-activated potassium channel is encoded by a member of the Slo gene family. Neuron 2003;37:765–73. doi:10.1016/S0896-6273(03)00096-5.

[6] Bhattacharjee A, Joiner WJ, Wu M, Yang Y, Sigworth FJ, Kaczmarek LK. Slick (Slo2.1), a rapidly-gating sodium-activated potassium channel inhibited by ATP. J Neurosci 2003;23:11681–91. doi:23/37/11681 [pii].

[7] Zhang X, Zeng X, Xia X, Lingle CJ. pH-regulated Slo3 K^+ Channels : Properties of Unitary Currents. Currents 2006;128:301–15. doi:10.1085/jgp.200609551.

[8] Zhang X, Zeng X, Lingle CJ. Slo3 K + Channels : Voltage and pH Dependence of Macroscopic Currents 2006;128:317–36. doi:10.1085/jgp.200609552.

[9] Schreiber M, Wei A, Yuan A, Gaut J, Saito M, Salkoff L. Slo3 , a Novel pH-sensitive K^+

Channel from Mammalian Spermatocytes. J Biol Chem 1998;273:3509–16.

[10] Santi CM, Martínez-lópez P, Luis J, Vega-beltrán D, Butler A, Alisio A, et al. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. FEBS Lett 2010;584:1041–6. doi:10.1016/j.febslet.2010.02.005.

[11] Zeng X-H, Yang C, Kim ST, Lingle CJ, Xia X-M. Deletion of the Slo3 gene abolishes alkalization-activated K^+ current in mouse spermatozoa. Proc Natl Acad Sci U S A 2011;108:5879–84. doi:10.1073/pnas.1100240108.

[12] Vicens A, Andrade K, Cortez D, Gutiérrez RM, Treviño C. Pre-mammalian origin of the sperm-specific Slo3 channel. FEBS Open Bio 2016;7:382–90. doi:10.1002/2211-5463.12186.

[13] Maeda N, Kawasaki T, Nakade S, Yokota N, Taguchi T, Kasai M, et al. Structural and functional characterization of inositol 1,4,5-trisphosphate receptor channel from mouse cerebellum. J Biol Chem 1991;266:1109–16.

[14] Yuan P, Leonetti MD, Pico AR, Hsiung Y, MacKinnon R. Structure of the human BK channel Ca^{2+}-activation apparatus at 3.0 A resolution. Science 2010;329:182–6. doi:10.1126/science.1190414.

[15] López-González I, Torres-Rodríguez P, Sánchez-Carranza O, Solís-López A, Santi CM, Darszon A, et al. Membrane hyperpolarization during human sperm capacitation. Mol Hum Reprod 2014;20:619–29. doi:10.1093/molehr/gau029.

[16] Swensen AM, Bean BP. Ionic mechanisms of burst firing in dissociated Purkinje neurons. J Neurosci 2003;23:9650–63. doi:23/29/9650 [pii].

[17] Singh H, Li M, Hall L, Chen S, Sukur S, Lu R, et al. MaxiK channel interactome reveals its interaction with GABA transporter 3 and heat shock protein 60 in the mammalian brain. Neuroscience 2016;317:76–107. doi:10.1016/j.neuroscience.2015.12.058.

[18] Chen L, Tian L, MacDonald SHF, McClafferty H, Hammond MSL, Huibant JM, et al. Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) α-subunits generated from a single site of splicing. J Biol Chem 2005;280:33599–609. doi:10.1074/jbc.M505383200.

[19] Zarei MM, Eghbali M, Alioua a, Song M, Knaus H-G, Stefani E, et al. An endoplasmic reticulum trafficking signal prevents surface expression of a voltage- and Ca^{2+}-activated K^+ channel splice variant. Proc Natl Acad Sci U S A 2004;101:10072–7. doi:10.1073/pnas.0302919101.

[20] Wang SX, Ikeda M, Guggino WB. The cytoplasmic tail of large conductance, voltage- and Ca^{2+}-activated K^+ (MaxiK) channel is necessary for its cell surface expression. J Biol Chem 2003;278:2713–22. doi:10.1074/jbc.M208411200.

[21] Kwon S-H, Guggino WB. Multiple sequences in the C terminus of MaxiK channels are involved in expression, movement to the cell surface, and apical localization. Proc Natl Acad Sci U S A 2004;101:15237–42. doi:10.1073/pnas.0404877101.

[22] Gong XD, Li JCH, Leung GPH, Cheung KH, Wong PYD. A BKCa to Kv Switch During Spermatogenesis in the Rat Seminiferous Tubules. Biol Reprod 2002;67:46–54. doi:10.1095/biolreprod67.1.46.

FIGURE CAPTIONS

Figure 1. Transcript isoforms and expressed sequence tags (EST) of mouse Slo3. A, Schematic alignment of mSlo3 isoforms (top) with associated ESTs (bottom). First, second and third isoform are colored in red, blue and green, respectively. Exons are numbered according to the first isoform, indicating spliced exons as blank boxes. Arrowed region in isoform 3 represent the portion of sequence experimentally obtained by RACE-PCR. EST accessions are shown on left arranged by tissue source. Red, blue and green schemes represent EST unambiguously assigned to first, second and third Slo3 isoforms respectively. Black schemes represent ESTs unable to be unambiguously associated to a specific Slo3 isoform. **B,** Cis-regulatory elements in *mSlo3* gene. Transcripts are displayed as blue schemes on top. Tracks of histone modification H3K4m3 and RNA Polymerase II (PoI2) binding sites are shown as signal intensity bars. Information retrieved from UCSC Genome Browser (https://genome.ucsc.edu/).

Fig. 2. Examination of mSlo3 transcripts. **A,** Mapping of primers used for RT amplification of mSlo3 transcripts. Green and red arrows indicate forward and reverse primers, respectively. Expected size of amplicons is indicated. **B,** RT-PCR of mSlo3 transcript isoforms in indicated mouse tissues. Products were separated on ethidium bromide agarose gels. DNA size markers are shown on the left side. Is1: isoform 1; Is3: isoform 3; Is2: isoform 2; βac: β-actin control (554 bp). (n = 3).

Fig. 3. Immunodetection of Slo3. Immunoblotting of Slo3 in mouse brain **(A, left)** and testis **(A, right)** extracts using polyclonal chicken anti-Slo3. Protein extracts were isolated in membrane and cytosolic fractions. Proteins were separated by SDS-PAGE on 10% acrylamide gels. Molecular size ladders are indicated on the left side. (Data are representative of three independent gels). Loading controls (membranes stained with Amido Black 2X) are shown in **B**.

SUPPLEMENTARY MATERIAL

Figure S1. Marathon RACE PCR of CV562866. PCR products were separated on ethidium bromide agarose gels. In first round of Marathon RACE-PCR were used two CV specific primers (CvS;CvE) against the outer adaptor primer (AP1). In the second round of RACE-PCR EST specific internal primers (F*; R*) against the inner AP2 were applied. DNA size ladders are indicated in each gel (n=3).

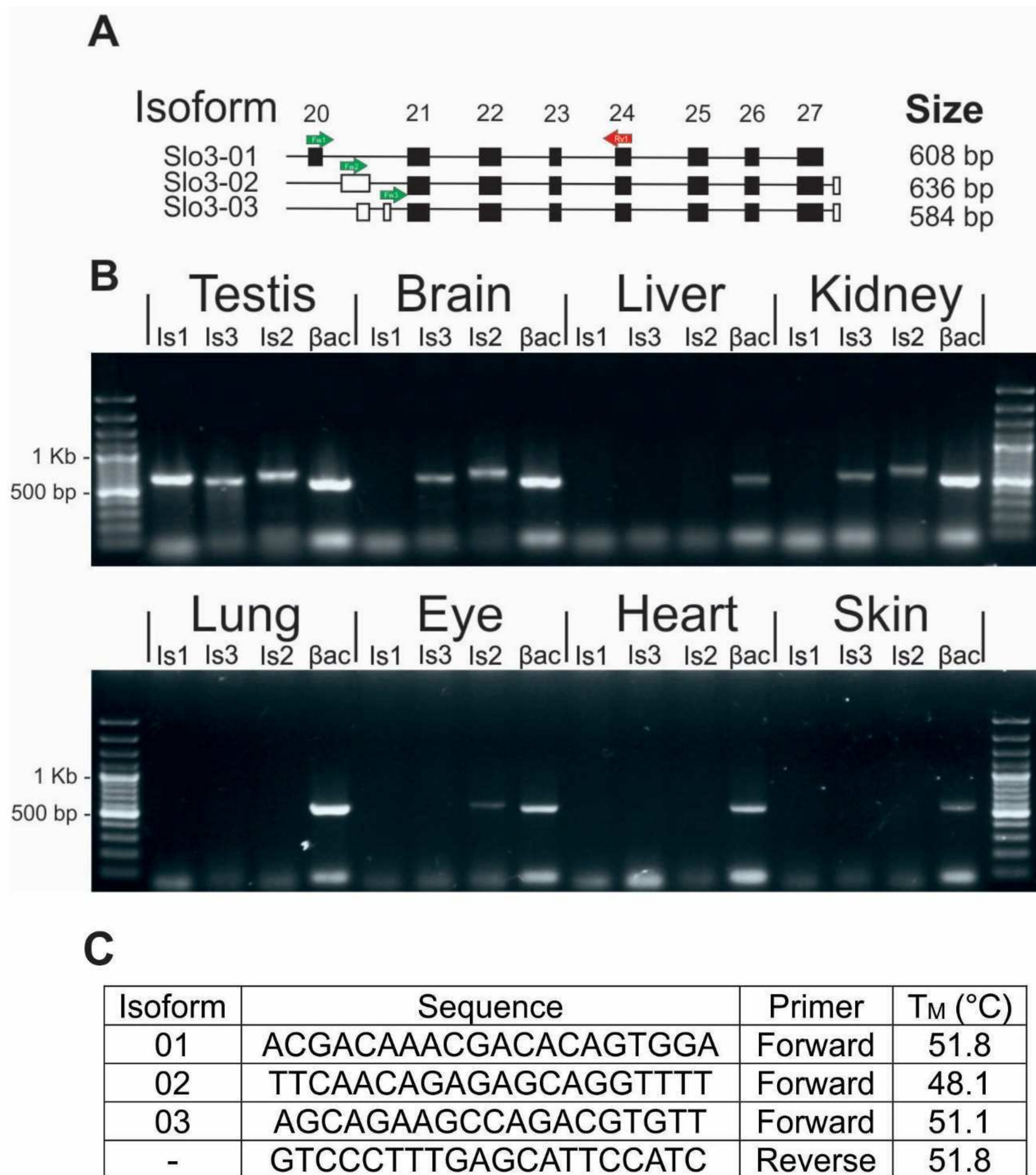
Figure S2. Alignment of mouse Slo3 isoforms. Translation frames are shown under each sequence. Transmembrane segments (S0-S6) and regulator of K^+ conductance (RCK) domains are indicated above alignment. The epitope of the antibody α-Slo3 (chicken α-Slo3) used for Western-Blot is indicated.

Figures Slo3 fragments

Figure 1

Figure 1. Transcript isoforms and expressed sequence tags (EST) of mouse Slo3. A, Schematic alignment of mSlo3 isoforms (top) with associated ESTs (bottom). First, second and third isoform are colored in red, blue and green, respectively. Exons are numbered according to the first isoform, indicating spliced exons as blank boxes. Arrowed region in isoform 3 represent the portion of sequence experimentally obtained by RACE-PCR. EST accessions are shown on left arranged by tissue source. Red, blue and green schemes represent EST unambiguously assigned to first, second and third Slo3 isoforms respectively. Black schemes represent ESTs unable to be unambiguously associated to a specific Slo3 isoform. **B,** Cis-regulatory elements in *mSlo3* gene. Transcripts are displayed as blue schemes on top. Tracks of histone modification H3K4m3 and RNA Polymerase II (PoI2) binding sites are shown as signal intensity bars. Information retrieved from UCSC Genome Browser (https://genome.ucsc.edu/).

Figure 2



Isoform	Sequence	Primer	T_M (°C)
01	ACGACAAACGACACAGTGGA	Forward	51.8
02	TTCAACAGAGAGCAGGTTTT	Forward	48.1
03	AGCAGAAGCCAGACGTGTT	Forward	51.1
-	GTCCCTTTGAGCATTCCATC	Reverse	51.8

Fig. 2. Examination of mSlo3 transcripts. **A,** Mapping of primers used for RT amplification of mSlo3 transcripts. Green and red arrows indicate forward and reverse oligonucleotides, respectively. Expected size of amplicons is indicated. **B,** RT-PCR of mSlo3 transcript isoforms in indicated mouse tissues. Products were separated on ethidium bromide agarose gels. DNA size markers are shown on the left side. (C) Oligonucleotides sequence refered in A in green and red arrows, in addition to the melting temperatura for each of them. Is1: isoform 1; Is3: isoform 3; Is2: isoform 2; βac: β-actin control (554 bp). (n = 3).

Figure 3

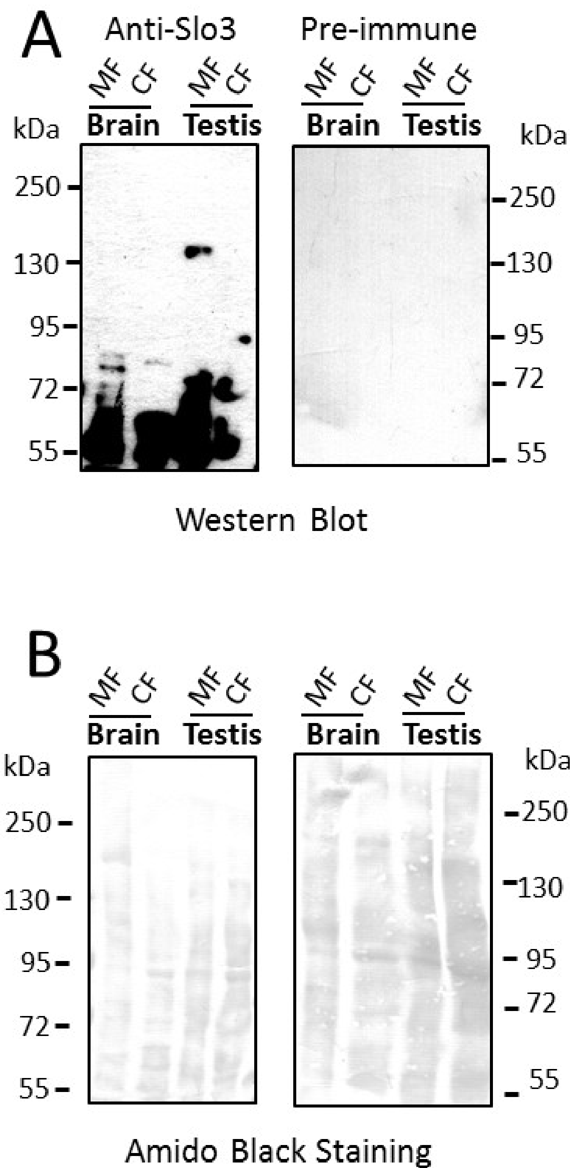


Fig. 3. Immunodetection of Slo3. Immunoblotting of Slo3 in mouse brain **(A, left)** and testis **(A, right)** extracts using polyclonal chicken anti-Slo3. Protein extracts were isolated in membrane (MF) and cytosolic fractions (CF). Proteins were separated by SDS-PAGE on 10% acrylamide gels. Molecular size ladders are indicated on the left side. (Data are representative of three independent gels). Loading controls (membranes stained with Amido Black 2X) are shown in **B**.

SUPPLEMENTARY MATERIAL

Supplementary figure 1

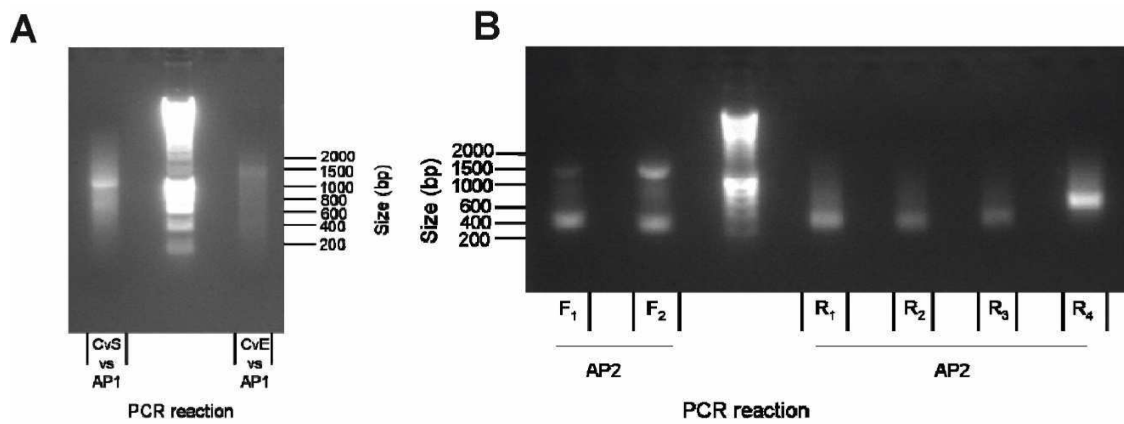


Figure S1. Marathon RACE PCR of CV562866. PCR products were separated on ethidium bromide agarose gels. In first round of Marathon RACE-PCR were used two CV specific primers (CvS;CvE) against the outer adaptor primer (AP1). In the second round of RACE-PCR EST specific internal primers (F*; R*) against the inner AP2 were applied. DNA size ladders are indicated in each gel (n=3).

Supplementary figure 2

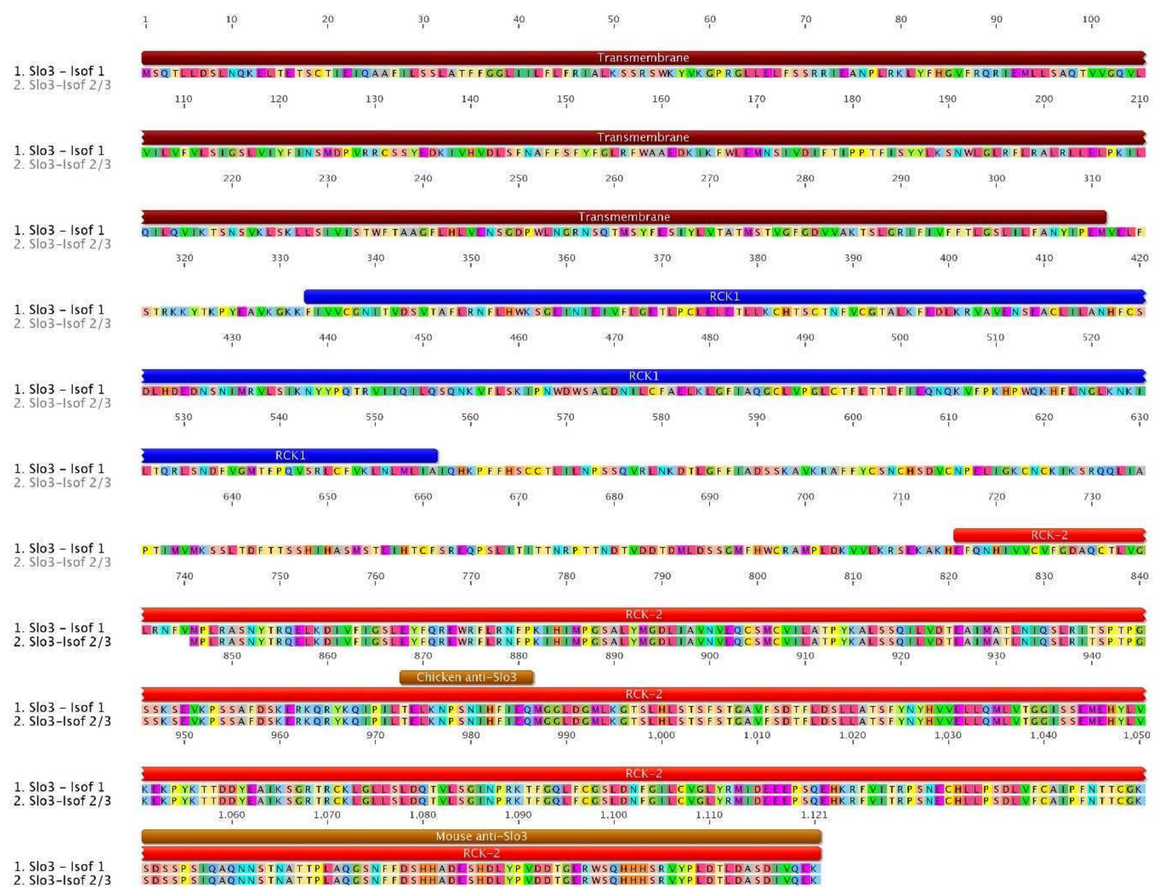


Figure S2. Alignment of mouse Slo3 isoforms. Translation frames are shown under each sequence. Transmembrane segments (S0-S6) and regulator of K^+ conductance (RCK) domains are indicated above alignment. The epitope of the antibody α-Slo3 (chicken α-Slo3) used for Western-Blot is indicated.