Original Paper

Brain, Behavior and Evolution

Brain Behav Evol
DOI: 10.1159/000350196

Received: December 6, 2012
Accepted after revision: February 20, 2013
Published online: April 24, 2013

# Evolutionary Conservation of $K_v3.1$ in the Barn Owl *Tyto alba*

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**Key Words**
Barn owl · Voltage-gated potassium channel · Molecular adaptation · Cloning · Auditory system

**Abstract**
For prey capture in the dark, the barn owl *Tyto alba* has evolved into an auditory specialist with an exquisite capability of sound localization. Adaptations include asymmetrical ears, enlarged auditory processing centers, the utilization of minute interaural time differences, and phase locking along the entire hearing range up to 10 kHz. Adaptations on the molecular level have not yet been investigated. Here, we tested the hypothesis that divergence in the amino acid sequence of the voltage-gated $K^+$ channel $K_v3.1$ contributes to the accuracy and high firing rates of auditory neurons in the barn owl. We therefore cloned both splice variants of *Kcnc1*, the gene encoding $K_v3.1$. Both splice variants, *Kcnc1a* and *Kcnc1b*, encode amino acids identical to those of the chicken, an auditory generalist. Expression analyses confirmed neural-restricted expression of the channel. In summary, our data reveal strong evolutionary conservation of *Kcnc1* in the barn owl and point to other genes involved in auditory specializations of this animal. The data also demonstrate the feasibility to address neuroethological questions in organisms with no reference genome by molecular approaches. This will open new avenues for neuroethologists working in these organisms.


## Introduction

Sound localization requires fast, precise, and reliable neurotransmission of signals [Oertel, 1999; Trussell, 1999]. Auditory circuits involved in spatial hearing have therefore evolved multiple specializations to cope with this task. On the cellular level, these adaptations include unique synaptic structures such as the calyx of Held and the endbulb of Held, the two largest synapses in the brain [Trussell, 1997; Schneggenburger and Forsythe, 2006]. On the molecular level, specific sets of plasma membrane proteins are present that are involved in the generation of short-duration presynaptic and postsynaptic potentials in the synaptic region [Trussell, 1999]. Examples include an 'auditory' AMPA receptor, which is enriched in GluR-$D_{flop}$, and characterized by rapid channel gating [Mosbacher et al., 1994; Geiger et al., 1995] and rapid desensi-

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tization [Lawrence and Trussell, 2000] as well as a battery of voltage-gated potassium channels [Oertel, 2009; Johnston et al., 2010]. They help to achieve microsecond temporal precision, which is required for computation of interaural time and level differences, arising between the two ears [Trussell, 2002].

Further molecular specializations might be expected in auditory specialists such as the barn owl *Tyto alba*. This nocturnal hunter can locate prey in complete darkness using only the sense of hearing [Payne, 1971]. This necessitates an exquisite capability of sound localization. Indeed, the barn owl has evolved various unique specializations on the anatomical, cellular, and physiological levels. Examples include asymmetrically arranged ears, an extended basilar papilla, a hearing threshold close to the thermal noise, and an increased volume of auditory processing centers [Wagner et al., 2013]. Furthermore, the animal demonstrates very accurate processing in auditory circuits. Barn owls can utilize interaural time differences down to 10 μs to localize sound sources in azimuth [Moiseff and Konishi, 1981; Bala et al., 2003; Heffner, 2004]. Finally, the barn owl exceeds all other species investigated so far in the ability to phase lock, i.e. to fire preferentially at a certain phase of a stimulus. Electrophysiological analyses of the auditory nerve revealed that these cells can phase lock to the sound within the entire frequency range from about 200 to 10 kHz [Koppl, 1997]. This is about twice as high as observed in typical laboratory animals, where phase locking is limited to carrier frequencies below 5 kHz [Versteegh et al., 2011]. The quality of phase locking is such that at 5 kHz the temporal spread of the action potentials is around 35 μs [Koppl, 1997]. This time jitter decreases for even higher frequencies, although phase locking as expressed by vector strength decreases. This seemingly contradictory result is a consequence of phase locking being a relative measure that depends on frequency, while the time jitter is an absolute temporal measure.

The ability for high-frequency firing has been closely linked to the action of the $K_v3$ gene family of voltage-gated potassium channels [Rudy and Mcbain, 2001; Bean, 2007; Gu et al., 2012]. These channels belong to the family of high-threshold potassium channels, which activate rapidly at potentials positive to –10 mV. Among the four family members, $K_v3.1$, 3.2, 3.3, and 3.4, previous studies demonstrated that $K_v3.1$ is highly expressed in the auditory system [Luneau et al., 1991; Perney et al., 1992; Li et al., 2001; Ozaita et al., 2002] and plays an essential role for high-frequency firing of auditory neurons. Deletion of the channel prevents neurons of the medial nucleus of the trapezoid body from following high-frequency stimulation [Macica et al., 2003]. Furthermore, phosphorylation of $K_v3.1$ is regulated by the acoustic environment to adjust the intrinsic electrical properties to the demands of high firing rates [Song et al., 2005].

Previous comparison between two avian species revealed that the channel is expressed in the nucleus magnocellularis and the nucleus laminaris, two auditory nuclei of birds [Parameshwaran et al., 2001]. However, the expression pattern differed between the two birds [Parameshwaran et al., 2001]. In the barn owl, $K_v3.1$ immunoreactivity formed a gradient along the tonotopic axis of the nucleus magnocellularis with greater labeling observed in the region of high best frequency. This gradient was barely observed in the chicken, which might be due to the overall smaller frequency range in this bird. Furthermore, in the barn owl, the channel was concentrated in the axons of neurons of the nucleus laminaris and in their presynaptic terminals [Parameshwaran et al., 2001], whereas in the chicken, its axonal expression was restricted to the initial segment [Feng and Morest, 2006]. These data indicate cellular specializations for $K_v3.1$ in the barn owl and a role of this channel in reducing action potential duration within the terminal region of time encoding neurons. However, it remained unsolved whether this is accompanied by changes on the molecular level. $K_v3.1$ is encoded by the gene *Kcnc1*, and two alternative splice variants, *Kcnc1a* and *Kcnc1b*, exist [Luneau et al., 1991]. Both isoforms share a common N-terminus but differ in their C-terminus. The shorter $K_v3.1a$ isoform contains 10 additional amino acids, whereas the longer $K_v3.1b$ isoform has an extra 84 amino acids. Both splice variants differ in their expression during development. $K_v3.1a$ is found early in development, whereas $K_v3.1b$ is expressed later [Perney et al., 1992]. Here, we cloned both splice variants of *Kcnc1* from *T. alba* and analyzed the expression pattern by reverse transcription (RT)-PCR. Our data demonstrate the feasibility to approach evolutionary adaptations on the molecular level also in so-called non-model organisms such as the barn owl, which lack a reference genome.

## Materials and Methods

*Animals*

Three adult barn owls *(T. alba)* of both sexes were used in this research. All owls hatched in captivity and were raised in the breeding colony of the Institute for Biology II at the RWTH Aachen University. All procedures were in accordance with the National Institutes of Health guidelines for animal experimenta-

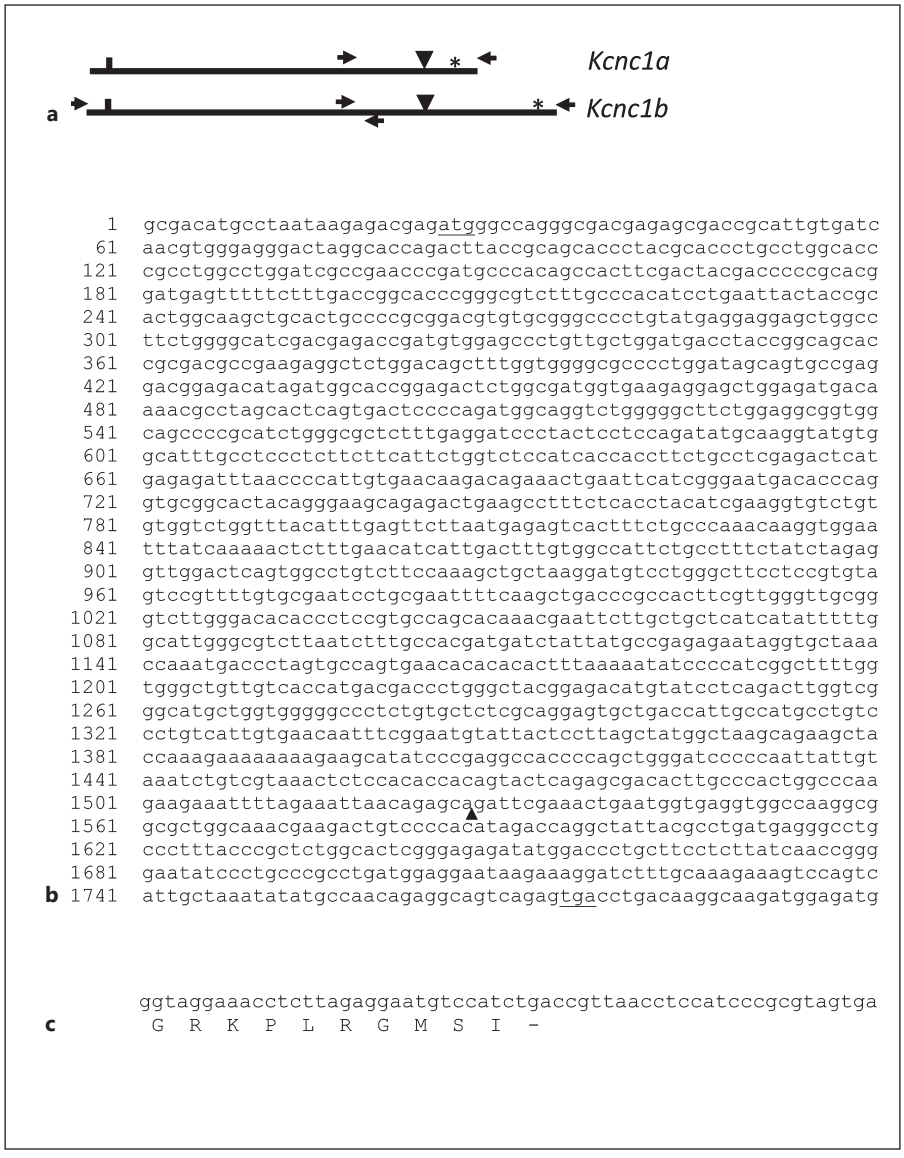


**Fig. 1.** Nucleotide sequence of *Kcnc1* from *T. alba*. **a** RT-PCR strategy employed to amplify both splice variants of *Kcnc1*. The vertical bars indicate the start codon, the asterisks denote the stop codon, the filled arrowheads indicate the splice site, and the arrows indicate positions of primers used for amplification. The nucleotide sequences of the long splice variant *Kcnc1b* (**b**) and the short splice variant *Kcnc1a* (**c**) are shown. For *Kcnc1a,* only the sequence after the splice site is depicted, together with the deduced amino acid residues. The splice site is indicated by a filled arrowhead, and the start and stop codons in *Kcnc1b* are underlined. Note that the sequences of the outermost primers are not part of the final DNA sequences.

tion and approved by the Landespräsidium für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Recklinghausen, Germany).

*Tissue RNA Isolation and RT-PCR*

Animals were deeply anesthetized with an overdose of a barbiturate (Nembutal) and decapitated shortly before the heart stopped beating. Total RNA was isolated from various organs and tissues of the barn owls by the guanidine thiocyanate method [Chomczynski and Sacchi, 1987]. The quality and quantity of RNA samples were assessed by gel electrophoresis and optical density measurements. RT of total RNA (20 µg) was performed by using standard protocols with a mixture of random hexanucleotide and polyT primers and Revert Aid™ M-MuLV reverse transcriptase (Thermo Scientific, St. Leon-Rot, Germany) as enzyme [Schroer et al., 1999].

*Kcnc1*-specific PCR was performed for 30 cycles in a total volume of 50 µl. Primers were as follows: 5′ part of *Kcnc1b*: Kcnc1b5′-for 5′-TTGGATGAGKCYRCGACATG-3′ and Kcnc1b5′-rev 5′-GAAAATTCGCAGGATTCGCAC-3′, 3′ part of *Kcnc1b*: Kcnc1b3′-for 5′-TCTATCTAGAGGTTGGACTC-3′ and Kcnc1b3′-rev 5′-GTCCAAATGAGAAGGTCTTC-3′, and 3′ part of *Kcnc1a*: Kcnc1a-for 5′-GACTCAGTGGCCTGTCTTCC-3′ and Kcnc1a-rev 5′-CTAAGAAAGCAGTCTGGCTG-3′. The quality of the RT reactions was assessed in a PCR reaction using *γ-actin*-derived primers (for 5′-ACAATGGCTCCGGCATGTGC-3′ and rev 5′-CCACATCTGCTGGAAGGTGG-3′). Denaturing was at 94°C for 30 s, annealing temperature was 56°C for 30 s, and elongation was at 72°C for 90 s. 5 µl of each reaction were loaded onto a 1.5% agarose gel containing GelRED (GeneON, Ludwigshafen, Germany). PCR products were purified using a commercial PCR product isolation kit (Roche Applied Science, Mannheim, Germa-

ny) and sequenced by LGC Genomics (Berlin, Germany). At least two independent PCR products were sequenced. Sequences were deposited in GenBank and are accessible with the following GenBank accession No.: KC197026 *(Kcnc1a)* and KC197027 *(Kcnc1b)*.

*Bioinformatics*

Multiple alignments were performed using Clustal Omega [Sievers et al., 2011] using default settings. *Kcnc1* sequences from various species were obtained from GenBank with the following accession No.: *Anolis carolinensis* (green anoles) XM_003214808, *Gallus gallus* (chicken) XM_003641325, *Homo sapiens* (human) NM_001112741, *Meleagris gallopavo* (wild turkey) XM_003206255, *Mus musculus* (mouse) NM_001112739, *Rattus norvegicus* (rat) NM_012856, and *Taeniopygia guttata* (zebra finch) XM_002197833. The genomic sequence of *Kcnc1* was retrieved from the following GenBank accession No.: NC_006092.3 *(G. gallus)*, NC_011469.1 *(T. guttata)*, NC_015015.1 *(M. gallopavo)*, and NC_014776.1 *(A. carolinensis)*, respectively.

## Results

To clone *Kcnc1* from *T. alba*, we used a PCR-based strategy to amplify first the long splice variant *Kcnc1b* (fig. 1a). We extracted *Kcnc1b*-related cDNA and genomic sequences from birds and the green anoles and performed multiple alignments to identify evolutionarily conserved sequence areas in Sauropsida (reptiles and birds). These conserved nucleotide sequences were then used to design primer pairs to amplify two overlapping fragments corresponding to the 5′ and 3′ fragments of *Kcnca1b*. The two outermost primes were located in the 5′- and 3′-untranslated regions of the chicken, respectively (fig. 1a). RT-PCR of brainstem RNA of *T. alba* yielded a single fragment of the expected size for each of the two primer pairs. Sequence analyses demonstrated that both fragments together contained the entire open reading frame (ORF) of *Kcnc1b* (NCBI: KC197027). The ORF has a length of 1,758 nucleotides (fig. 1b) and encodes a protein of 585 amino acids with a calculated relative molecular mass of 65,900 (fig. 2). The nucleotide sequence of the ORF revealed 98% identity (1,722/1,758 nucleotides) with that of the chicken and the zebra finch. On the protein level, the amino acid sequence was completely conserved between the barn owl and the chicken, and only few amino acid exchanges were observed compared to other species such as the green anoles (fig. 2). Between the barn owl and the zebrafish, the sequence identity was still 82%.

To clone the shorter splice variant *Kcnc1a*, a similar strategy was performed. This time, a primer pair was designed with one primer binding to the *Kcnc1* sequence upstream of the splice site and one primer located downstream of the putative stop codon of *Kcnc1a* (fig. 1a). Again, a single fragment was amplified by RT-PCR. Sequence analyses revealed that the encoded amino acid residues of the splice variant were identical to those in chicken, zebra finch, green anoles, mouse, and man. In total, the ORF has a length of 1,536 nucleotides and encodes a protein of 512 amino acids with a calculated relative molecular mass of 57,900 (NCBI: KC197026).

Taken together, our data reveal identical amino acid sequence of both isoforms between the barn owl, an auditory specialist, and the chicken, an auditory generalist.

*Expression Analysis of Kcnc1*

Expression of the mammalian *Kcnc1* is restricted to the nervous system [Luneau et al., 1991]. To analyze its expression in the barn owl, we performed RT-PCR on various adult tissues of *T. alba*. We used the primer pair for the 5′ end which is common to both splice variants (fig. 1a). PCR products were only obtained from neural tissue, i.e. brainstem, forebrain, and cerebellum (fig. 3). Previous analysis in the chicken had revealed expression both in neurons and astrocytes [Feng and Morest, 2006]. Since neural subtype-specific cell lines are not yet available from the barn owl, we could not address whether neuronal and glial expression of *Kcnc1* is also true for the barn owl. Immunohistochemical data point mainly to neuronal expression in this bird [Parameshwaran et al., 2001; Parameshwaran-Iyer et al., 2003]. The other organs tested, heart, kidney, lung, stomach, and muscle, were negative (fig. 3). These data demonstrate that *Kcnc1* expression in the barn owl is restricted to the nervous system, similar to the expression in mammals.

## Discussion

The rapid kinetics of $K_v3.1$ enable auditory neurons to rapidly repolarize without compromising a closely following second action potential. This channel therefore presented a strong functional candidate for the ability of the barn owl to enable phase locking up to 10 kHz. We therefore cloned both splice variants from *T. alba*. However, our sequence analyses rebutted the conjecture that molecular divergence of the protein contributes to the extraordinary performance of this auditory specialist. Therefore, species adaptation on the molecular level has to involve other proteins. Indeed, many membrane proteins were already shown to participate in shaping action potentials and postsynaptic potentials [Bean, 2007; Johnston et al., 2010; Golding, 2012]. Important proteins

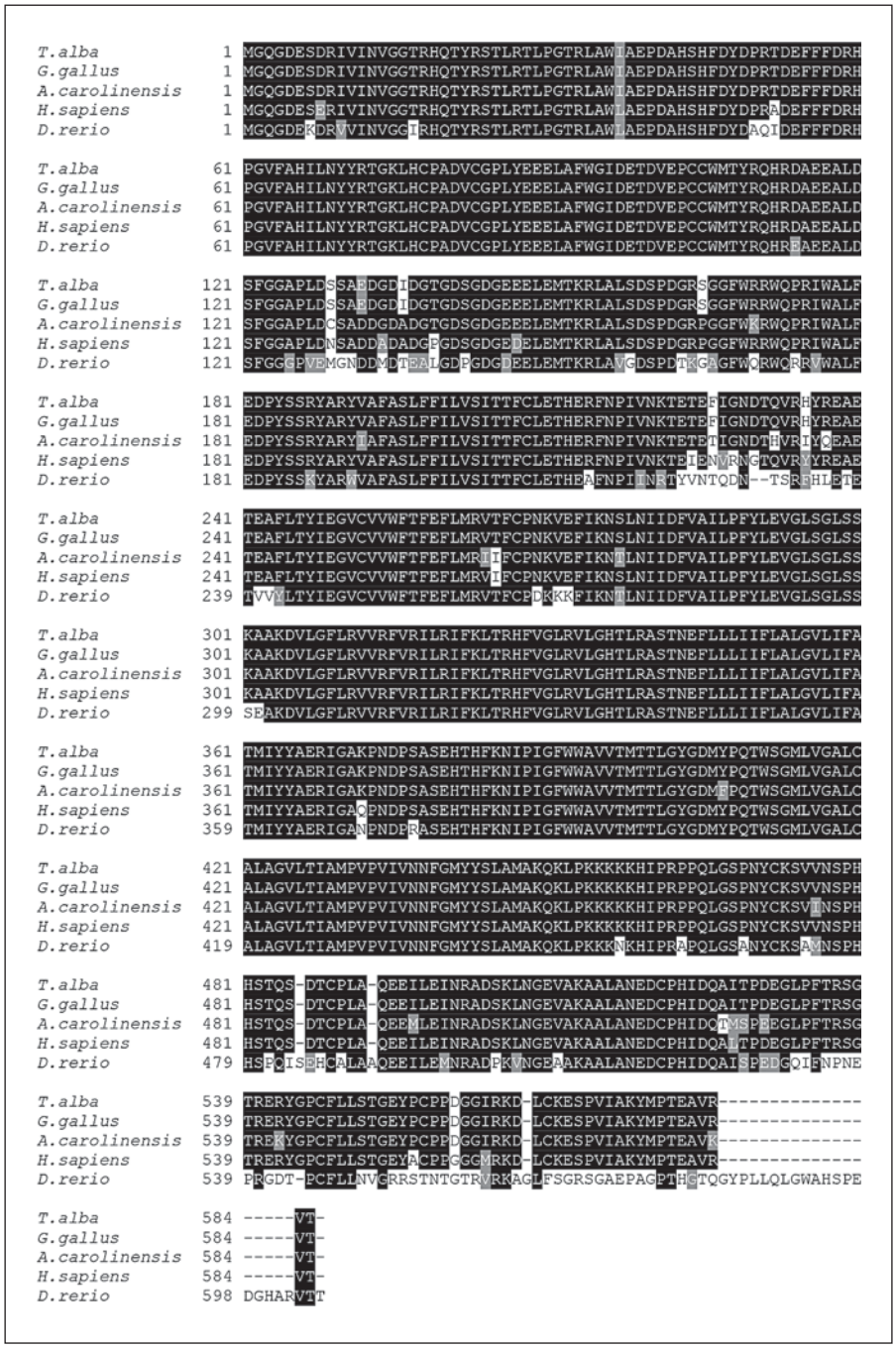


**Fig. 2.** Multiple sequence alignment of $K_v$3.1b. Amino acid alignment of the long splice variant $K_v$3.1b from *T. alba*, *G. gallus, A. carolinensis*, *H. sapiens*, and *Danio rerio.* Black-shaded amino acids are highly conserved during evolution, whereas grey-shaded boxes indicate substitutions by chemically similar amino acids.

include other high-threshold voltage-gated potassium channels such as $K_v$3.3 [Grigg et al., 2000; Chang et al., 2007] or low-threshold voltage-gated potassium channels such as $K_v$1.1 and $K_v$1.2. These channels are also abundant in auditory neurons [Grigg et al., 2000; Chang et al., 2007; Hirtz et al., 2011; Satheesh et al., 2012] and their expression is required for temporal precision [Kopp-Scheinpflug et al., 2003; Gittelman and Tempel, 2006]. Further candidates are passive leak channels and hyperpolarization-activated cation channels, which both contribute to short membrane time constants [Golding, 2012], and AMPA receptors for fast synaptic excitation [Trussell, 1999].

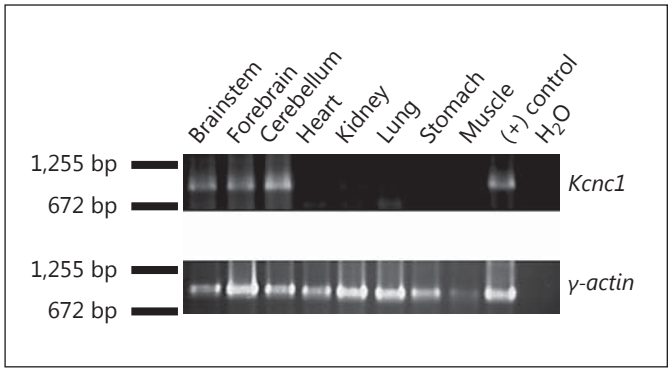


**Fig. 3.** Expression of *Kcnc1b* in adult tissues from *T. alba*. RT-PCR was performed on 8 different tissues. *Kcnc1* mRNA was only present in neural tissue (brainstem, forebrain, and cerebellum), whereas the other organs tested (heart, kidney, lung, stomach, and muscle) were negative. The positive control represented the total brainstem cDNA, from which *Kcnc1* was originally amplified. Water as template served as a negative control. RT-PCR using *γ-actin* served as a control for successful RT of the tissue samples. One of two experiments with identical results is shown.

Our data failed to detect differences in the amino acid sequence of *Kcnc1* between an auditory specialist and a generalist. This confirms the high evolutionary conservation of potassium channels [Jan and Jan, 2012]. This lack of molecular adaption on the genetic level might be rooted in the fact that the function of $K_v3.1$ can be strongly modulated on the posttranslational level. Phosphorylation by protein kinase C results in a significant reduction of $K_v3.1b$-mediated current [Macica et al., 2003], and this phosphorylation is controlled by the acoustic environment [Song et al., 2005]. In future studies, it will therefore be interesting to compare the phosphorylation pattern of $K_v3.1$ in the barn owl with that in other species.

Although our initial hypothesis was rejected, our results clearly demonstrate that the time has come to address neuroethological questions on the molecular level. Our cloning strategy revealed that available and ongoing genome projects provide a solid basis to extend molecular biology tools to species with poor sequence coverage so far. Furthermore, the advent of next-generation sequencing techniques, which produce millions of sequence reads in a single run, in combination with sophisticated bioinformatic tools [Metzker, 2010; Ozsolak and Milos, 2011] will provide affordable access to '-omics' techniques. This will open exciting new avenues in the field of neuroethology and auditory research alike.


## Acknowledgements

We thank J. Schröder for excellent technical support, Sandra Brill for help with tissue preparation, and C. Köppl for providing tissue.


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