Neuron-like cells in the chick spinal accessory lobe express neuronal-type voltage-gated sodium channels

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ABSTRACT
Ten pairs of protrusions, called accessory lobes (ALs), exist at the lateral sides of the avian lumbosacral spinal cord. Histological evidence indicates that neuron-like cells gather in the ALs, and behavioral evidence suggests that the ALs act as a sensory organ of equilibrium during bipedal walking. Recently, using an electrophysiological method, we reported that cells showing Na⁺ currents and action potentials exist among cells that were dissociated from the ALs. However, it was unclear which isoforms of the voltage-gated sodium channel (VGSC) are expressed in the ALs and whether cells having neuronal morphology in the ALs express VGSCs. To elucidate these points, RT-PCR and immunohistochemical experiments were performed. In RT-PCR analysis, PCR products for Nav 1.1–1.7 were detected in the ALs. The signal intensities of the Nav 1.1 and 1.6 isoforms were stronger than those of the other isoforms. We confirmed that an antibody raised against an epitope peptide of the rat VGSC had cross-reactivity to chick tissues by Western blotting, and we performed immunofluorescence staining using the antibody. The AL contained cells having neuron-like morphology and VGSC-like immunoreactivity at their cytoplasm and/or cell membranes. Filament-like structures showing GFAP-like immunoreactivity infilled intercellular spaces. The VGSC- and GFAP-like immunoreactivities did not overlap. These results indicate that the neuronal isoforms of the VGSC are mainly expressed in the AL and that the neuron-like cells in the ALs express VGSCs. Our findings indicate that AL neurons generate action potentials and send sensory information to the motor systems on the contralateral side of the spinal segment.

Many vertebrates walk on the ground by using their four limbs. In contrast, birds fly by using their forelimbs, which evolved into wings, and they walk by using their hindlimbs. It has long been suggested that birds require a special balance-sensing organ for bipedal walking on the ground because their hindlimbs are located behind the center of gravity, and some evidence supports this idea (1, 2, 4). The proposed location of such an organ is the lumbar region of the vertebral (14). In the avian spinal cord, ten pairs of protrusions, called accessory lobes (ALs), exist at the lateral sides of the lumbosacral spinal cord near the dentate ligaments (7). Histological evidence showed that neuron-like cells exist in the ALs and form the major marginal nuclei of Hofmann (6). The somata of the neuron-like cells are scattered in the pool of glycogen cells and show some morphological properties of mechanoreceptive neurons (16, 17). Morphological, histological and behavioral evidence suggests that ALs act as a sensory organ and have a role in maintaining body balance during walking on the ground (14, 15).

Although much experimental evidence suggests that ALs act as a sensory organ and that some neu-
rotransmitters exist in the ALs (9, 12, 13), there is little cellular evidence indicating that AL cells have neuronal functions. Previously, we reported that cells isolated from chick ALs showed voltage-gated Na⁺ and K⁺ currents under voltage-clamp conditions and generated action potentials under current-clamp conditions in whole-cell patch-clamp experiments (18). These results indicate that the voltage-gated Na⁺ channel (VGSC) is expressed and that functional neurons generating action potentials exist in ALs. In contrast to mammalian VGSCs, the identity and classification of the avian VGSC have not been well established. The VGSC is a multisubunit protein consisting of a pore-forming α subunit and accessory β₁ and β₂ subunits. In mammals, 9 subtypes of VGSC α subunits were identified and have been classified as Na⁺, 1.1–1.9. Na⁺, 1.1, 1.2 and 1.6 are expressed in both the central nervous system (CNS) and the peripheral nervous system (PNS), Na⁺, 1.3 is predominantly expressed in the CNS, and Na⁺, 1.7–1.9 are predominantly expressed in the PNS. On the other hand, Na⁺, 1.4 and 1.5 are predominantly expressed in skeletal muscles and cardiac muscles, respectively (3). In chicks, the base sequences of the cDNAs for Na⁺, 1.1–1.7 and 1.9 have been registered at the National Center for Biotechnology Information. Na⁺, 1.2 (5), Na⁺, 1.4 (20), Na⁺, 1.5 (20), Na⁺, 1.7 (5) and Na⁺, 1.9 (20) are registered as the base sequences of the identified cDNAs. On the other hand, the base sequences of the cDNAs for Na⁺, 1.1, Na⁺, 1.3 and Na⁺, 1.6 are registered as sequences predicted by the automated computational analysis of genomes by Gnomon. Although our previous study indicated that functional VGSCs are expressed in the ALs, it is unclear which VGSC isoforms are expressed in the AL and whether cells having neuronal morphology in section preparations of ALs express VGSCs. To clarify these points, we performed RT-PCR and immunohistochemical staining targeting VGSC α subunits in the present study.

MATERIALS AND METHODS

Experimental animals and tissue preparation. All animal experiments were performed in accordance with the guidelines of Tottori University, and this study was approved by the Institutional Animal Care and Use Committee, Tottori University. Chick (Gallus gallus) embryos at embryological stages E16–E18 and male Wistar rats at 7–10 weeks of age were used. After the decapitation of a chick embryo, the spinal cord tissue containing the lumbar enlargement and the glycogen body (GB) was removed from the vertebra. Ten pairs of ALs were found at both lateral sides of the lumbosacral spinal cord. ALs were carefully dissected from the spinal cord with microscissors under a stereomicroscope. Dorsal root ganglia (DRG), cerebral cortex tissues, heart tissues, and skeletal muscle tissues were also dissected. All tissues were transferred to ice-cold phosphate-buffered saline (PBS) immediately after the dissection. Rats were deeply anesthetized by isoflurane and sacrificed by decapitation. Immediately, the cerebrum was removed and transferred to ice-cold PBS, and cerebral cortex tissues were dissected.

RT-PCR. The total RNAs used for the RT-PCR analysis were isolated from the ALs, DRGs, brain tissues (the cerebral cortex), heart tissues (the atrium) and skeletal muscle tissues of chick embryos using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Reverse transcription was performed with 2 μg of total RNAs and an oligo dT primer using SuperScript™ III Reverse Transcriptase (Life Technologies) according to the manufacturer’s instructions. PCR was performed with 1 μL of first-strand cDNAs, 8 pairs of primers that were designed for 8 subtypes of the VGSC (Na⁺, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7 and 1.9) as listed in Supplementary Table 1, and PrimeSTAR™ GXL (Takara Bio, Shiga, Japan). The PCR protocol involved 28 cycles of a series of incubations at 95°C for 10 s, 60°C for 15 s, and 68°C for 1 min. Amplified PCR products and a 100 bp DNA ladder (New England Ipswich, MA, USA) were electrophoresed with a Tris acetate EDTA buffer on a 1.5% agarose gel (Agarose S; Nippon Gene, Tokyo, Japan) containing 50 ppm Midori Green Advance DNA Stain (Nippon Genetics, Tokyo, Japan). The band signals on the gels were excited by UV light and photographed.

Western blotting. Chick ALs, chick cerebral cortex tissues and rat cerebral cortex tissues were homogenized by a Potter-Elvehjem homogenizer and an ultrasonic homogenizer (UD-201; TOMY, Japan) in ice-cold lysis buffer consisting of 150 mM NaCl, 1 mM EDTA, 1% Triton X 100, 5 mM HEPES, 1 μg/mL peptatin A (Sigma, USA), 1 μg/mL leupeptin (Sigma), 1 μg/mL aprotinin (Sigma), 0.2 mM Pefabloc SC (Sigma), 0.1 mg/mL benzamidine (Sigma), 8 μg/mL calpain inhibitors I (Sigma) and 8 μg/mL calpain inhibitor II (Sigma); the pH was adjusted to 7.4 with NaOH. The homogenate was centrifuged at 600 × g for 10 min at 4°C. Subsequently, the supernatant was centrifuged again at 8,000 × g for 10 min.
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Structure of chick spinal cord and ALs

In the vertical sections of the lumbar enlargement of the chick spinal cord, a large GB at the dorsal side of the spinal cord and small ALs at the lateral sides of the spinal cord were found (Fig. 1A). The GB tissue was inserted into the spinal cord and reached the central canal. Cells showing typical neuronal cell morphology were found in the ventral horn (VH) region (arrow in Fig. 1B). The AL was connected to the VH regions and mainly consisted of neuron-like cells with large cell bodies (arrowheads in Fig. 1B) and cells resembling glycogen cells in the GB (Fig. 1B). Densely-packed glycogen of glycogen cells in the AL disappeared during a procedure to make section preparations. Peripherally concentrated nuclei and cell organelles, and plasma membranes remained. The immunoreactivity of the GB and ALs to the anti-GFAP antibody was somewhat different: filament-like structures infill intercellular spaces among neuron-like cells and glycogen cells in the GB (Fig. 1B). No tissues in GB showed GFAP-like immunoreactivity (Fig. 2), but no tissues in GB showed GFAP-like immunoreactivity.

RESULTS

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able. First, we selected a primary antibody that was expected to react with the chick VGSCs. We selected an antibody against Na\textsubscript{v} 1.1–1.7 because Na\textsubscript{v} 1.9 did not seem to be expressed in the ALs, as indicated by the RT-PCR analysis. The selected antibody (affinity purified rabbit anti-PAN VGSC, Chemicon) was raised against the purified peptide of the α subunit of rat Na\textsubscript{v} 1.1 (AA1500–1518, Accession# NM_030875). This amino acid sequence, “MTEEQ KKYYN AMKKL GSKK”, is positioned as an intracellular loop between the third and fourth repeats of the VGSC α subunit and is conserved in the predicted amino acid sequences of chick Na\textsubscript{v} 1.1–1.7 (Supplementary Table 2). Therefore, we expected that this antibody recognizes VGSC proteins in chick tissues and could be used in the present study.

**Cross-reactivity of the anti-VGSC antibody between rat and chick**

The rat brain (cerebral cortex) lysate at 5 µg protein/lane, the chick brain (cerebral cortex) lysate at 10 µg protein/lane and the chick AL lysate at 35 µg protein/lane were used for SDS/PAGE. The resolved proteins were electroblotted onto a PVDF membrane, and immunoreactive signals for the anti-VGSC antibody were detected (Fig. 4). In the lane of the rat brain, two different signals of approximately 260 kDa were detected. One was broader and positioned at the lower molecular size, and the other was sharper and positioned at the higher molecular size. These molecular sizes were consistent with the molecular sizes of the α subunits of the rat VGSC. No other signal was detected at different molecular sizes. In the chick brain, a dense signal at approximately 260 kDa and a weaker signal at approximately 130 kDa were detected. Similarly, in the chick ALs,
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The connecting region between the AL and the VH (arrowheads in Fig. 5A). Thin filament-like structures were stained by the Cy3-conjugated anti-GFAP antibody as a red signal in almost all regions of the preparation, except the GB region (Fig. 1C and Fig. 5B). Tissues located in the intercellular area among neuron-like cells and glycogen cells in the AL showed GFAP-like immunoreactivity. In the merged image (Fig. 5C), the green and red signals did not overlap each other except in red blood cells, which have strong autofluorescence (marked by asterisks). In negative control sections, the anti-VGSC antibody was preabsorbed by the control antigen (0.4 mg/mL, antigen:antibody = 1:1 in volume) for 1 h, or no primary antibody was applied; only a slight nonspecific signal was detected (data not shown).

**DISCUSSION**

Previously, we found that enzymatically isolated cells from ALs of chick embryos showed depolarization-activated Na⁺ current in the electrophysiological recording (18). In addition, it was reported that an intracellular concentration of Cl⁻ in neuron-like cells isolated from chick embryonic ALs was as low as that of neurons of adult chicks (19), suggesting that a developing stage of neurons of chick embryos at E16–18 is similar to that of adult animals. To confirm whether neuron-like cells of chick embryo,
correct. The signal intensities of the Na, 1.1 and 1.6 isoforms of the ALs were as strong as those of the DRG and brain. Since Na\textsubscript{v} 1.1 and 1.6 are specifically expressed in the CNS and PNS in mammals, this result further supports the hypothesis that ALs contain neurons. In addition, the AL differed from the DRG in that ALs do not express Na\textsubscript{v} 1.9, which is known to be specifically expressed in sensory neurons in mammals (3). ALs may have different functional properties from neurons in the PNS because the expression pattern of the VGSC isoforms in the ALs resembles that of the brain rather than the DRG.

Previously, we reported evidence showing that enzymatically isolated chick AL cells showed TTX-sensitive VGSC currents and generated action potentials in whole-cell voltage-clamp and current-clamp recordings, respectively (18). Although these results clearly indicated the expression of VGSCs in AL cells, it was unclear which cells observed in the section preparation of chick ALs express VGSCs. In the present study, we used the primary antibody for the rat VGSC α subunit raised against the epitope peptide that is completely conserved in all predicted amino acid sequences of the TTX-sensitive VGSC α subunits of the chick. In Western blotting analysis, immunoreactive signals with a similar size were detected in the lanes for the rat and chick brains, suggesting that the antibody used in this study has cross-reactivity to chick VGSCs and is useful for detecting VGSC proteins in chick tissues. Since both rats and chicks express multiple subtypes of the VGSC α subunit and the immunogenic amino acid sequence for this antibody is conserved regardless of the subtype and the species, it was expected which we had examined in the previous studies, express mRNA and proteins of VGSCs, we used chick embryo in this study. The AL region contained neuron-like cells with large cell bodies and glycogen cells and both types of cells did not show the GFAP-like immunoreactivity. GFAP is the major component of astrocytic intermediate filaments. It was reported that astroglial cells in the spinal cord and AL of pigeons were stained by the anti-GFAP antibody (13, 17). Although it is considered that the glyogen cells in the GB and ALs are derived from astroglial cells (8), glycogen cells in neither the GB nor ALs express GFAP in the pigeon (13, 17). The same situation occurred in the chick: the cells in the glycogen body were not stained by the anti-GFAP antibody. Filament-like structures in the intercellular regions among neuron-like cells and glycogen cells showed GFAP-like immunoreactivity in the ALs, indicating that astrocytes exist in the chick AL similarly to the pigeon. Glycogen cells are reported to have short processes (17). Thus, it is also possible that filament-like processes extending from cell bodies of glycogen cells showed GFAP-like immunoreactivity, because glycogen cells were considered to be derived from glial cells. To confirm this possibility, further detailed analysis is necessary.

Using RT-PCR analysis, we confirmed that ALs express Na, 1.1–1.7 mRNAs. This result is consistent with the previous report that TTX-sensitive VGSCs are expressed in isolated AL cells (18). The cDNAs for Na, 1.1, 1.3 and 1.6 that are designated predicted sequences in the database were detected in isolated cDNA preparations, suggesting that mRNAs corresponding to the predicted cDNA are expressed in native tissues and that their base sequences are

Fig. 5 VGSC- and GFAP-like immunoreactivity at AL regions. (A) VGSC-like immunoreactivity visualized by the Alexa 488-conjugated secondary antibody. (B) GFAP-like immunoreactivity visualized by the Cy3-conjugated primary antibody. (C) Merged image of A and B. The inset shows an expanded image of the cell with a yellow arrow. White arrows, positively stained neuron-like cell; red arrows, positively stained glycogen cells; arrowheads, positively stained cells at the connecting region between the AL and the ventral horn; asterisks, red blood cells. Scale bar, 50 μm.
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that multiple bands would be detected at approximately 260 kDa. In the rat brain lane, a thin band at the larger molecular size position and a broad band at the smaller molecular size position were detected at approximately 260 kDa. It was reported that Na, 1.1, 1.2 and 1.3 are preferentially expressed in the CNS (3). These two signals with different molecular sizes may reflect the expression of different subtypes of the VGSC α subunit. Similar to the results for the rat brain, multiple bands were detected in the chick brain at approximately 260 kDa. This result in combination with the RT-PCR results suggests that multiple subtypes of the VGSC α subunit are also expressed in the chick cerebral cortex. A broad and weak band at approximately 130 kDa was detected in the chick brain and AL lane, but not in the rat brain lane. It is unknown what caused the 130 kDa signal. Nuclei of both neuron-like cells and glycogen cells in ALs showed immunoreactivity to the anti-VGSC antibody, but such signals in nuclei were not detected in rat brain sections (data not shown). Immunoreactivity in the nuclei may not be caused by VGSC proteins, because VGSCs are expressed on plasma membranes and function as ion channels. Therefore, an unknown protein in nuclei of chick tissues may cause a 130 kDa signal in the Western blotting. It is also possible that endogenous proteases digested VGSC α subunit proteins and that the resulting fragments containing the immunogenic region showed another immunoreactivity at this smaller molecular size. No other nonspecific signals were detected at different molecular size positions, even the lowest molecular size position. These results suggest that the anti-VGSC antibody used in the present study recognizes VGSC α subunit proteins in tissue sections of chicks also. Since the density of neuron-like cells was lower in the ALs than in the brain, weaker signals may be detected although a much larger amount of the protein was used for SDS/PAGE. However, since signals similar to those detected in the chick brain and no other nonspecific signals were observed, the selected anti-VGSC antibody is expected to specifically stain VGSC proteins in AL regions.

We developed a method to immunostain chick VGSC α subunits using an anti-rat VGSC antibody, making immunohistochemical analysis of the avian VGSC α subunit expression possible. In the AL region, cytoplasm and nuclei of neuron-like cells showed clear VGSC-like immunoreactivity and no GFAP-like immunoreactivity, indicating that neuron-like cells express VGSC proteins. In glycogen cell, peripherally delimited small region where cyto-

plasmic organelles and nuclei were concentrated also showed VGSC-like immunoreactivity and no GFAP-like immunoreactivity, suggesting that glycogen cells also express VGSC proteins. We reported that cells that were dissociated from ALs and had abundant intracellular structures showed VGSC currents in the patch-clamp recording (18). The present results in combination with the previous studies leastwise indicate that cells having neuronal morphology in the AL express the neuronal subtypes of the VGSC and show neuronal functions. On the other hand, although glycogen cells are considered to be derived from glial cells (17), the present results suggest that glycogen cells have neuron-like immunoreactivity. However, we did not observe electrophysiological responses of isolated glycogen cells, because glycogen cells punctured by touching with a tip of glass pipettes for the patch clamp recording. To clarify whether glycogen cells express functional VGSC on their membranes, further analysis is required.

As equilibrium sensors, the ALs are expected to have a role in maintaining body balance during bipedal working on the ground (11, 14, 15). Neurons in the ALs projected to the motoneurons in the contralateral side of the spinal cord (10). Therefore, it is necessary for AL neurons to generate action potentials that are propagated to the neurons located in the other side of the same spinal segment. The present and previous results provide evidence that AL cells generate action potentials and support the hypothesis that AL cells send sensory information to the motor systems. However, it is still unclear how AL neurons sense the body balance of birds. It is possible that the AL neuron itself has a role as a mechanosensor or that other cells that have a mechanosensory function provide sensory information about equilibrium to AL neurons. To clarify the underlying mechanisms, further histological and functional analysis is required.

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Supplementary Table 1  Sequences of RT-PCR primers for the VGSC isoforms of the chick (Gallus gallus)

| Target | Sequences | Product size (bp) | Accession # |
|--------|-----------|------------------|-------------|
| Na, 1.1 | Forward: 5'-gtagttcatagcctgcaaagg-3' | 495 | XM_003641586 |
| Reverse: 5'-tctgecatagtaggaacacctg-3' | | |
| Na, 1.2 | Forward: 5'-ccacaattaagtgtttgtaaag-3' | 529 | NM_001293281 |
| Reverse: 5'-ecctccctttctttctttgctt-3' | | |
| Na, 1.3 | Forward: 5'-agttgcgcaactgcgagcagg-3' | 535 | XM_015289740 |
| Reverse: 5'-egagacccagagcacttcac-3' | | |
| Na, 1.4 | Forward: 5'-gagaaccctggcagctcaa-3' | 603 | NM_001318445 |
| Reverse: 5'-cttgctgtgccggtacat-3' | | |
| Na, 1.5 | Forward: 5'-ccatattgcgacccaaacctc-3' | 642 | NM_001318446 |
| Reverse: 5'-ctcgaggatgctgccgtcaaa-3' | | |
| Na, 1.6 | Forward: 5'-tctctgtgctctgactctctc-3' | 709 | XM_424477 |
| Reverse: 5'-ctctccacgctgctcccaattc-3' | | |
| Na, 1.7 | Forward: 5'-gqgdif  MTEEQ KKYYNAMKKL GSKK pqkpip 1481–1510 XM_003641586 | 665 | NM_001293282 |
| Reverse: 5'-gqgdif  MTEEQ KKYYNAMKKL GSKK pqkpip 1481–1510 XM_003641586 | | |
| Chick Na, 1.9 | Forward: 5'-gqgdif  MTEEQ KKYYNAMKKL GSKK pqkpip 1481–1510 XM_003641586 | 725 | NM_030875 |
| Reverse: 5'-gqgdif  MTEEQ KKYYNAMKKL GSKK pqkpip 1481–1510 XM_003641586 | | |

The sequences of the PCR primers against the cDNA for the Na, 1.1–1.7 and 1.9 isoforms of the chick VGSCs and expected sizes of the PCR products are shown.

Supplementary Table 2  Comparison of the amino acid sequences of VGSCs around the immunogenic region

| Molecular Identity | Amino acid sequence | AA Position | Accession # |
|--------------------|---------------------|-------------|-------------|
| Rat Na, 1.1        | gqdif MTEEQ KKYYNAMKKL GSKK pqkpip | 1500–1518 | NM_030875 |
| Mouse Na, 1.1      | gqdif MTEEQ KKYYNAMKKL GSKK pqkpip | 1500–1518 | NM_001313997 |
| Chick Na, 1.1      | gqdif MTEEQ KKYYNAMKKL GSKK pqkpip | 1481–1510 | XM_003641586 |
| Chick Na, 1.2      | gqdif MTEEQ KKYYNAMKKL GSKK pqkpip | 1491–1509 | NM_001293281 |
| Chick Na, 1.3      | MTEQQI MMKKL GSKK pqkpip | 1497–1515 | XM_015289740 |
| Chick Na, 1.4      | gkdif MTEEQ KKYYNAMKKL GSKK pqkpip | 1304–1322 | NM_001318445 |
| Chick Na, 1.5      | gqdif MTEEQ KKYYNAMKKL GSKK pqkpip | 1510–1528 | NM_001318446 |
| Chick Na, 1.6      | MTEEQ KKYYNAMKKL GSKK pqkpip | 1471–1497 | XM_424477 |
| Chick Na, 1.7      | MTEQQI MMKKL GSKK pqkpip | 1480–1498 | NM_001293282 |

The boxed sequences indicate the epitope region used to develop the primary antibody for rat Na, 1.1. The same amino acid sequences were found in all seven amino acid sequences of chick (Gallus gallus) Na, 1.1–1.7.