Identification and Functional Expression of a Family of Nicotinic Acetylcholine Receptor Subunits in the Central Nervous System of the Mollusc Lymnaea stagnalis

We described a family of nicotinic acetylcholine receptor (nAChR) subunits underlying cholinergic transmission in the central nervous system (CNS) of the mollusc Lymnaea stagnalis. By using degenerate PCR cloning, we identified 12 subunits that display a high sequence similarity to nAChR subunits, of which 10 are of the α-type, 1 is of the β-type, and 1 was not classified because of insufficient sequence information. Heterologous expression of identified subunits confirms their capacity to form functional receptors responding to acetylcholine. The α-type subunits can be divided into groups that appear to underlie cation-conducting (excitatory) and anion-conducting (inhibitory) channels involved in synaptic cholinergic transmission. The expression of the Lymnaea nAChR subunits, assessed by real time quantitative PCR and in situ hybridization, indicates that it is localized to neurons and widespread in the CNS, with the number and localization of expressing neurons differing considerably between subunit types. At least 10% of the CNS neurons showed detectable nAChR subunit expression. In addition, cholinergic neurons, as indicated by the expression of the vesicular ACh transporter, comprise ~10% of the neurons in all ganglia. Together, our data suggested a prominent role for fast cholinergic transmission in the Lymnaea CNS by using a number of neuronal nAChR subtypes comparable with vertebrate species but with a functional complexity that may be much higher.

Nicotinic acetylcholine receptors (nAChRs) belong to the Cys loop family of pentameric ligand-gated ion channels (LGICs) together with the 5-HT3, GABA type A/C, and glycine receptors. nAChRs consist of an extracellular ligand-binding domain (LBD), a transmembrane ion channel, and an intracellular domain (1). In the mammalian central nervous system (CNS), eight α-type (α2–7 and α9–10) and three β-type (β2–4) nAChR subunits have been identified that selectively assemble into nAChR subtypes with different pharmacology, cation conductance, and cellular localization. In the mammalian CNS, nAChRs predominantly mediate presynaptic modulation of neurotransmitter release (for example see Ref. 2) and are to a limited extent involved in direct, fast synaptic transmission (for example see Refs. 3–7). In contrast, in the molluscan CNS, the mode of fast synaptic cholinergic transmission seems to prevail. The CNS of the freshwater snail Lymnaea stagnalis consists of ~20,000 large and identifiable neurons, of which many were shown to express functional nicotinic acetylcholine receptors (8, 9). In particular, in well described neuronal networks, various nAChR subtypes were shown to mediate synaptic transmission (10, 11). Uniquely, molluscs possess excitatory and inhibitory nAChR subtypes conducting cations and anions, respectively (9, 12–15). Also, different excitatory and inhibitory nAChR subtypes mediate cholinergic transmission at single identified synapses, the differential contribution of which is regulated by soluble extracellular factors (11, 16). Therefore, functionally identified neurons in the Lymnaea CNS are a valuable model to explore nAChR function and diversity related to signal transmission in the nervous system. The lack of molecular information on nAChRs in molluscs, however, prevents a comprehensive analysis of cholinergic transmission. To address this issue, we identified nAChR subunits in the Lymnaea CNS, and we assessed their cellular expression by in situ hybridization and quantitative PCR. Our data underscore the postulated widespread role for fast cholinergic excitatory and inhibitory transmission in the Lymnaea CNS, involving a number of nAChR subtypes that are comparable with vertebrate species but with a functional complexity that may be much higher.

MATERIALS AND METHODS

Animals—Adult L. stagnalis (shell length, 28–34 mm) bred under laboratory standard conditions (17) were used.

PCR Using Degenerate Oligonucleotides—Total RNA was isolated using Trizol® reagent (Invitrogen) from freshly dissected CNS or from pooled LePD (10×), RePD (10×), and VD (6×) neurons with axons attached and isolated by mechanical suction from Lymnaea brain ganglia. For CNS preparations, mRNA was isolated using (dT)25-coated magnetic beads (Dynal, Oslo, Norway). RNA was reverse-transcribed using hexanucleotide primers and Moloney murine leukemia virus-re-
verse transcriptase according to the manufacturer’s protocol (Promega, Madison, WI).

3′- and 5′-directed degenerate oligonucleotides (Isogen Bioscience, Amsterdam, The Netherlands) were synthesized with an EcoRI or HindIII restriction site, respectively, at the 5′-ends. The 5′-directed oligonucleotides include the following: primer 1, 5′-gggagaaattcgaggcatcataagga-3′; primer 2, 5′-ggaacccaccaatacctaccaccag-3′; primer 5, 5′-ggaacccaccaatacctaccaccag-3′; primer 7a, 5′-ggaacccaccaatacctaccaccag-3′; primer 7b, 5′-ggaacccaccaatacctaccaccag-3′; primer 2, 5′-ggaacccaccaatacctaccaccag-3′; primer 4, 5′-ggaacccaccaatacctaccaccag-3′; primer 6, 5′-ggaacccaccaatacctaccaccag-3′; primer 8, 5′-ggaacccaccaatacctaccaccag-3′ (nomenclature according to IUPAC).

Primer combinations were used on a CNS-derived cDNA (≥1 CNS equivalent) or on cDNA templates of identified neurons (0.12 cell equivalent for VD4 and 0.20 cell equivalent for LPeD1/RPeD1) in an initial PCR (40 cycles: 94 °C for 30 s; 54 °C for 30 s; and 72 °C for 90 s) using a DNA thermal cycler (PerkinElmer Life Sciences). Nested primers were used in a subsequent PCR (40 cycles: 94 °C for 30 s; 54 °C for 30 s; and 72 °C for 90 s) using 1/50th of the initial PCR as template. Amplified cDNA products were separated on 2% agarose gels, and generated products of expected sizes were excised, digested with EcoRI and HindIII, and cloned and sequenced. For each independent PCR, several cloned products were sequenced by dideoxy chain termination sequencing.

**Full-length Cloning**—The cDNA of an amplified AZAPII (Stratagene, La Jolla, CA) cDNA library of the CNS of *L. stagnalis* was isolated by phenol extraction and ethanol precipitation. Nested oligonucleotide primer sets (Eurogentec, Seraing, Belgium) were designed based on partial PCR-derived nAChR subunit sequences. These primer sets were used together with nested AZAPII primer sets T33 (5′-ggaacccaccaatacctaccaccag-3′) and EV3 (5′-ggaacccaccaatacctaccaccag-3′) or T77 (5′-ggaacccaccaatacctaccaccag-3′) and EV2 (5′-ggaacccaccaatacctaccaccag-3′) in consecutive PCRs with identical parameters (40 cycles: 94 °C for 30 s; 58 °C for 30 s; and 72 °C for 210 s) using Advantage DNA polymerase (Clontech). DNA was separated on 1% agarose gels, and PCR products corresponding to 3′ (≥1000 bp) or 5′ (≥500 bp) parts were excised, cloned, and sequenced. A similar approach was used for SMART (18). Final sequences were obtained by sequencing three independently generated PCR products generated on dT-primed cDNA of pooled *Lymnaea* CNS. For Xenopus oocyte expression, cDNAs of open reading frames were cloned into pcDNA3 (Invitrogen). Sequences of cloned products were checked by dideoxy chain termination sequencing.

**Electrophysiology**—*Xenopus laevis* oocytes were prepared, injected, and recorded as described previously (23). Drugs were applied in the bath by rapid superfusion that was controlled by electromagnetic valves. Drugs and chemicals were purchased either from Sigma or Fluka. Current-voltage relationships (I-V curves) were determined either by holding the cell at a fixed potential and measuring the ACh-evoked current or by voltage ramps (from −100 to +80 mV in 2 s) applied during the ACh response. I-V plots were obtained by reporting the amplitude of the current as a function of the holding voltage after subtraction of the passive cell properties determined in absence of the agonist.

**Real Time Quantitative PCR**—RNA of the complete CNS, individual ganglia, and body tissues was isolated as described above followed by a DNase I (10 units; Roche Applied Science) treatment, according to the manufacturer’s instructions, and by phenol/chloroform extraction and ethanol precipitation. cDNA was made using hexanucleotides (300 pmol) and 200 units of Moloney murine leukemia virus H-reverse transcriptase (Promega, Madison, WI). RNA from pooled VD4 (6×) and LPeD1 (6×) neurons, cultured for 18 h, was isolated, and hexanucleotide-primed cDNA was transcribed according to van Minnen and van Kesteren (24).

All cDNAs were ethanol-precipitated and dissolved in 60 μl of aquadest. Transcript-specific primers were designed using Primer Express (Applied Biosystems, Foster City, CA) to generate 70–120-bp amplicons as follows: LnAChR A forward, 5′-ggaacccaccaatacctaccaccag-3′; and LnAChR A reverse, 5′-gggaacccaccaatacctaccaccag-3′; LnAChR B forward, 5′-ggaacccaccaatacctaccaccag-3′; and LnAChR B reverse, 5′-gggaacccaccaatacctaccaccag-3′; LnAChR C forward, 5′-ggaacccaccaatacctaccaccag-3′; and LnAChR C reverse, 5′-ggaacccaccaatacctaccaccag-3′; and LnAChR D forward, 5′-ggaacccaccaatacctaccaccag-3′ and 5′-ggaacccaccaatacctaccaccag-3′.
FIGURE 1. Deduced protein sequences of LnAChR subunits. ClustalX alignment of deduced protein sequences of identified LnAChR A–L subunits. Residue numbering below the alignment is set to the mature LnAChR A sequence. Positions and names of primers used for amplification are indicated, as well as three regions of amplification delineated by the applied primer combinations (gray lines). Positions of identical and conserved amino acids are indicated (black and gray columns, respectively). The alignment shows putative signal peptides (italic) and transmembrane domains (labeled TM1, TM2, TM3, and TM4) as predicted by SMART analysis (18). Indicated are the conserved Cys pair that defines the Cys loop family of LGIC subunits, the conserved residues of the principal (loops A–C), and the complementary component (loop D) of the ligand-binding site (arrowheads). Also represented are sites in the LBDs of possible N-linked glycosylation (black boxes) and of predicted sites in the long intracellular loop of phosphorylation by cAMP and cGMP-dependent kinases (yellow shading), casein-dependent kinase (green shading), protein kinase C (orange shading), and tyrosine kinases (blue shading). Only phosphorylation sites as predicted by Prosite analysis with a significant score in NetPhos are represented. The alternatively spliced region in the long intracellular loop of LnAChR F is indicated (red box).
LnAChR D rev, 5'-cgacaagttagctgagcagtt-3'; LnAChR E fwd, 5'-cccgctgtaaaaccgcagttctt-3'; LnAChR F fwd, 5'-atccggtgctgctgctaa-3'; and LnAChR F rev, 5'-ccgctgtaaaaccgcagttctt-3'; LnAChR G fwd, 5'-ctgcagctgctgctaa-3'; LnAChR H fwd, 5'-tctgagctgtgtggttaaat-3'; LnAChR J rev, 5'-caatgctccttgggcctctttct-3'; LnAChR K fwd, 5'-ccatctcttgggcctctttct-3'; and LnAChR K rev, 5'-acgctgctgctgctaa-3'.

| Subunit | Accession Number |
|---------|------------------|
| A       | X15542           |
| B       | X15543           |
| C       | X15544           |
| D       | X15545           |

**Identification of nAChR Subunits from the CNS of L. stagnalis**—For the identification of nAChR subunits, we performed a nested PCR using degenerate primers on various CNS-derived cDNA templates (Table 1A). Sequencing of amplified DNA identified 13 different sequences that each showed similarity to a region of the LBD of nAChR subunits (see also Fig. 1). In addition, two sequences representing partial *Lymnaea* nAChR subunits were available from previous hybridization screening of a *A* phage CNS cDNA library for nAChR subunit-related sequences and from an ongoing *Lymnaea* CNS EST sequencing project (L. L. Moroz, University of Florida) (Table 1B). Full-length sequence information of the partial sequences was obtained by PCR cloning using a *Lymnaea* CNS cDNA library. In total, 12 individual transcripts were identified that were named *Lymnaea* nAChR subunits (LnAChR) A–L (Fig. 1). A full-length sequence for LnAChR L could not be obtained.

**RESULTS**

Identification of nAChR Subunits from the CNS of L. stagnalis—For the identification of nAChR subunits, we performed a nested PCR using degenerate primers on various CNS-derived cDNA templates (Table 1A). Sequencing of amplified DNA identified 13 different sequences that each showed similarity to a region of the LBD of nAChR subunits (see also Fig. 1). In addition, two sequences representing partial *Lymnaea* nAChR subunits were available from previous hybridization screening of a *A* phage CNS cDNA library for nAChR subunit-related sequences and from an ongoing *Lymnaea* CNS EST sequencing project (L. L. Moroz, University of Florida) (Table 1B). Full-length sequence information of the partial sequences was obtained by PCR cloning using a *Lymnaea* CNS cDNA library. In total, 12 individual transcripts were identified that were named *Lymnaea* nAChR subunits (LnAChR) A–L (Fig. 1). A full-length sequence for LnAChR L could not be obtained.

**Neuronal nAChR Subunits of L. stagnalis**

With BLAST analysis each of the 12 deduced protein sequences share highest similarity with predicted as well as with functionally characterized nAChR subunits (data not shown). Sequence comparison with human LGIC subunits indicates the highest identity with nAChR subunits (units 2C). Previously described *Lymnaea* GABA receptor subunits (27, 28) do not show significant sequence identity with the identified protein sequences. All identified proteins possess four predicted transmembrane domains (TMs) with a relative spacing (three TMs at the center and one TM at the C terminus) that are similar to the sequence observed in nAChR subunits. Moreover, the subunits contain two cysteine residues separated by 13 amino acids (Fig. 1), which are features characteristic for proteins belonging to the so-named Cys loop family of LGICs.

Together, these results support the classification of the identified sequences as *Lymnaea* nAChR subunits. Because of the presence of the typical vicinal Cys residues in the loop C region of the principal component of ligand binding, LnAChR A–I and LnAChR K are classified as α-type nAChR subunits, and because of the absence of these Cys residues, LnAChR J is classified as β-type subunit. Most interestingly, in loop C the LnAChR H subunit shows an unusual His-X-Cys-X-X-X-Y motif rather than the conserved Tyr-X-Cys-X-X-X-Tyr motif.

Sequence Comparison of *Lymnaea* nAChR Subunits—Of all identified subunits, LnAChR F and I share the highest level of sequence identity (69% identity), whereas LnAChR H and K share the lowest level (25% identity) (Table 2A). For comparison, human nAChR subunits share sequence identity values of 35–84% (data not shown). LnAChR E shares the highest sequence identity with the α2 and α4 nAChR subunits (62%), whereas the lowest identity is found for LnAChR H and the human β2 subunit with only 24% identical residues (Table 2B).

Based on sequence relatedness (Table 2A), we defined three group of LnAChR subunits each with >60% inter-sequence identity, group B, I, and F, group C and E, and group D and G (Fig. 2A). None of the LnAChR subunits LBD domains displays a particular homology to AchBP, a molluscan protein secreted by glial cells homologous to the nAChR LBD (29, 30) (Fig. 2B). AchBP shares the highest sequence identity (27%) with the LnAChR K subunit (see Table 2A). The groups of subunits that include LnAChR H, the LnAChR B, F, I, and K, and the LnAChR D and G subunits branch off between human 5-HT3 receptors and nAChR subunits (Fig. 2C). Based on phylogeny, LnAChR H seems as distant from 5-HT3 subunits as it is from nAChR subunits. Most interestingly, LnAChR A displays a clear relation to the vertebrate α7 subunit that forms homopentameric receptors, whereas LnAChR J, C, and E are more related to subunits known to assemble into heteropentameric nAChRs.

**Functional Expression of LnAChRs in Xenopus Oocytes**—To determine the contribution of LnAChR subunits to functional receptors, we expressed LnAChR subunits in *X. laevis* oocytes. As a first step, LnAChR subunits were expressed individually allowing for only homopentameric nAChR subtypes to be formed. Expression of functional receptors was observed with LnAChR A or B subunits and with LnAChR I subunits (Fig. 3A). Oocytes that express the LnAChR A or B subunits responded to application of ACh but not to application of 5-HT (Fig. 3, B and C), glutamate, GABA, or glycine (data not shown). Receptors composed of LnAChR A or B subunits are sensitive to typical nicotinic agonists (i.e. nicotine and choline) and antagonists (i.e. meth-

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*E. Vreugdenhil and A. B. Smit, unpublished data.*
yllyaconitine, dihydro-β-erythroidine, α-bungarotoxin, and α-conotoxin-imI) (57). Although recorded currents are smaller, oocytes expressing the LnAChR I subunit respond equally to ACh or 5-HT applications (Fig. 3D). LnAChR I receptors are also insensitive to glutamate, GABA, and glycine (data not shown).

The failure of many subunits to express as functional receptors as well as the small size of the currents mediated by receptors composed of the LnAChR I subunit suggests that additional subunits are required for functional expression. As a second step, subunit combinations were selected based on the inferred subunit classification and similarity of expression levels in the CNS (see Fig. 5). Co-expression of the β-type LnAChR I subunit with other subunits, including those resembling most the α-subunits found in mammalian neuronal nAChRs (LnAChR C or E), did not result in functional channels. Moreover, no currents

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**FIGURE 2.** Phylogenetic comparison of LnAChR subunits. A, phylogenetic tree of LnAChR subunits. Values of bootstrap analysis on 1000 data sets are indicated at branch points (black numbers). Sequence identity values are indicated for adjacent proteins (outlined numbers). Groups of LnAChR subunits with a ≥60% identity score (see Table 2) are highlighted (gray boxes). The partial LnAChR L sequence shows no particular relationship to any other subunit (data not shown). B, the procedure as under A was repeated, including AChBP now aligning residues belonging to regions corresponding to AChBP and the corresponding LBD of LnAChR subunits. The partially identified LnAChR L subunit was not included. C, phylogenetic tree of LnAChR subunits and all subunits that belong to the Cys loop family of LGICs identified in Aplysia, Lymnaea, Drosophila, and human. Values of bootstrap analysis on 1000 data sets are indicated at branch points. Related LnAChR subunits as defined under A are indicated (gray boxes). For human nAChR subunits, participation in the formation of neuronal or muscle nAChRs and of homo- or heteropentameric nAChRs are indicated. Black triangles indicate compressed sub-trees of closely related subunit types. Names indicate human subunits unless stated differently. Subunit sequences were received from the LGICdb (56).
could be observed with subunits combinations, including both LnAChR F and H that represent the most abundant subunits in the CNS (see below). Finally, responses of oocytes that co-expressed LnAChRI with LnAChRF or -J could not be distinguished from those of oocytes expressing LnAChR I alone, suggesting LnAChR I does not co-assemble into pentamers with these subunits (data not shown).

To assess the ionic selectivity of the receptors, I-V curves were determined (see “Materials and Methods”) in normal or low chloride concentration. As shown in Fig. 3E, reduction of the chloride concentration caused no major change of the reversal potential in LnAChR A (−13 to −20 mV). In opposition, a large shift of 49 ± 1.5 mV (n = 3) was observed when the same experiment was repeated with LnAChR B receptors, which suggests that the current flowing through these receptors is mainly carried by chloride ions (Fig. 3F). Receptors composed of LnAChR I display comparable characteristics of chloride-selective channels (data not shown). We therefore conclude that LnAChR A contributes to cation-selective receptors, whereas LnAChR B and I contribute to anion-selective receptors.

Expression of LnAChR Subunits in the Lymnaea CNS—The distribution and level of expression of LnAChR subunits was analyzed using ISH and real time qPCR, respectively.

qPCR analysis on cDNA templates derived from various tissues and organs showed that LnAChR subunits are expressed almost exclusively in the CNS (Fig. 4). In the CNS the transcripts for the identified

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**TABLE 2**

Protein sequence identity of Lymnaea and rat nAChR subunits

A, distance matrix of sequence identity (%) of aligned LnAChR subunits and Lymnaea AChBP (GenBank™ accession number P58154). B, distance matrix of identity scores (%) of aligned LnAChR and human nAChR subunits. The long intracellular loop was excluded from comparison. Dark and light boxes indicate the highest and lowest scores for each LnAChR subunit, respectively.

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* Data analyzed on partial sequence.
LnAChR subunits are exclusively found in neurons as shown by ISH (see Fig. 6). Expression was absent in non-neuronal cells in the CNS (glia and connective tissue) and in remnants of striated muscle included in the CNS tissue sections. We therefore concluded that all identified Lymnaea nAChR subunits are neuronal nAChR subunits.

In the CNS, LnAChR subunits are expressed at different relative expression levels (Fig. 5). LnAChR A, D, G, I, K, and L contribute to the total subunit expression in the CNS to a low degree. Most abundant are LnAChR F and H subunits that together represent approximately half of the total subunit expression. Only four subunits, LnAChR F and H together with the less abundant LnAChR C and E subunits, account for the majority of nAChR subunit expression in the CNS. Considerable differences are observed in the relative contribution of LnAChR subunits to expression at the level of individual ganglia. In most ganglia LnAChR F or H represent the most abundantly expressed subunits, in particular in the right pleural ganglion. However, some subunits display a particularly high contribution in only a few ganglia, such as LnAChR B in the buccal ganglia, LnAChR A in the left and right cerebral ganglia, and LnAChR E in the right pedal ganglion. LnAChR D and I subunits show very little contribution to expression in any of the ganglia.

The results obtained by qPCR are in good agreement with findings made using ISH (Fig. 6). Expression of LnAChR subunit transcripts can be observed in all ganglia of the Lymnaea CNS. The LnAChR F and H subunits are abundantly expressed throughout the CNS as indicated by numerous labeled neurons that represent ∼10% of the neurons in all ganglia.

Expression of the Vesicular Ach Transporter in the Lymnaea CNS—To establish the extent of acetylcholinergic transmission in the Lymnaea CNS, we also mapped the expression of the Ach-synthesizing cells by identifying the cellular expression of the Lymnaea vesicular Ach transporter (LVACHT). The vesicular Ach transporter has been used previously as a marker of cholinergic neurons (25, 31). ISH shows strong labeling of an estimated 10% of the neurons throughout the CNS (Fig. 6). The highest relative LVACHT expression level is observed for the left pedal ganglion. LVACHT in other ganglia is lower, with the cerebral ganglia, the parietal ganglia, and the visceral ganglion representing the lowest expression (i.e., <25% of LVACHT expression in the left pedal ganglion).

Expression of LnAChR Subunits in Identified Neuronal Populations—The Lymnaea CNS contains various identified clusters of neurosecretory cells, which synthesize neuropeptides and have been shown to be implicated in various aspects of physiology, such as animal growth, copulation behavior, and egg laying. To investigate whether these neurons might be controlled by cholinergic transmission or whether they synthesize Ach as transmitter, the expression of LnAChR subunits and of LVACHT, respectively, was determined. Five types of neurosecretory cells were investigated, i.e. caudodorsal cells (CDCs, egg laying), light green cells (LGCs, growth), light yellow cells (LYCs, function unknown),...
anterior lobe (AL, copulation behavior) neurons, and PeIB cluster neurons (copulation behavior).

qPCR profiling detected expression of all identified LnAChR subunits in each of these neuronal cell populations (Fig. 7). However, differences in expression levels were observed and generally were more pronounced compared with the expression detected in the complete ganglia. In view of the high sensitivity of the qPCR technique, this observation indicates that the neuronal clusters express only a selective subset of LnAChR subunits rather than the full complement. All neuronal clusters display the highest relative expression of the LnAChR F subunit, with exception of the left and right cerebral AL clusters, in which LnAChR H is most abundant.

The expression of these abundant transcripts was confirmed by in situ hybridization that showed a moderate labeling of LnAChR F in the CDCs and a strong labeling in the LGCs. A low level labeling is observed for other subunits, besides LnAChR F and H, such as the LnAChR I in the CDCs and LnAChR D in the LGCs. Expression of LnAChR E in the AL and PeIB neurons as indicated by qPCR was not observed with ISH. Finally,ISH reveals heterogeneity in the expression of LnAChR subunits within the neuronal populations, such as LnAChR F expressed by the lateral LGCs, but not by the medial group of LGCs. Most interestingly, CDCs display a heavy labeling when probed for LVAChT expression, suggesting the neuroendocrine CDC egg-laying hormone cells are all producing ACh, in addition to the egg-laying hormone peptides. No LVAChT labeling of the LGC, LYC, AL, and PeIB cell types was observed.

Relative LnAChR Subunit Expression Levels Determined in the Identified VD4 and LPeD1 Neurons—In principle, the newly obtained sequence information of nAChRs subunits could be used to analyze nAChR subtype expression by the identified neurons described in the Lymnaea CNS. As proof of principle, we determined LnAChR subunit expression in cultured LPeD1 and VD4 neurons that have been shown to express different types of functional nAChRs by means of electrophysiology (11). Although Lymnaea β-tubulin expression is readily detectable, expression of LnAChR subunits is close to the detection limit of the technique (Ct values 35–40). qPCR is unable to detect expression of a number of subunit types, in particular in VD4 preparations (Fig. 8). For other subunit types, i.e. LnAChR B, C, D, F, G, I, J, and K in LPeD1 and LnAChR C, D, F, and K in VD4, expression can be detected, but not in all preparations. Finally, expression of the LnAChR E subunit in LPeD1 could be detected in each sample. Compared with LPeD1, relative expression levels in VD4 are ~10-fold lower. The LnAChR E and F subunits that are measured most reliably in LPeD1 and VD4, respectively, display the largest mean level of expression accompanied by a large variation.

FIGURE 4. Relative levels of expression of LnAChR subunit transcripts in Lymnaea organs. Relative LnAChR subunit transcript levels were determined by real time qPCR on cDNA preparations of various Lymnaea organs and tissues pooled from 5 to 10 animals (n = 1). LnAChR transcript levels were normalized to the levels of Lymnaea elongation factor α. For each subunit, individual tissue measurements are expressed as a fraction of the combined expression of the subunit in all organs.

FIGURE 5. Relative transcript expression levels of LnAChR subunits in the central ring ganglia of L. stagnalis. Expression of LnAChR transcripts was measured on the cDNA preparations of independent pools of the complete CNS (n = 17), the left and right buccal (n = 3), the left cerebral (n = 5), the right cerebral (n = 5), the left and right pleural (n = 5), the left pedal (n = 4), the right pedal (n = 3), the left parietal (n = 4), the right parietal (n = 3), and the visceral (n = 5) ganglia and were normalized to the expression of Lymnaea β-tubulin. The pie charts reflect the mean expression level of LnAChR subunits relative to the total LnAChR subunit expression in the cDNA preparations. Note that data and analysis of pleural ganglia, and of buccal ganglia, are based on RNA pooled from the left and right ganglia.
FIGURE 6. Expression of LnAChR subunits and LVAcHT in the Lymnaea CNS. The expression of LnAChR subunits and of the LVAcHT in the central ring ganglia of the Lymnaea CNS was investigated using qPCR and ISH. Each graph (A–LVAcHT) indicates the relative expression levels in the different ganglia as determined by qPCR under Fig. 5. The drawings provide an overview of the location and the intensity of labeled neurons observed with ISH; please note the number of spheres does not correspond to the exact number of neurons. The photographs show examples of labeled neurons (black outlined arrowheads) obtained with ISH on serial sections of the Lymnaea CNS. With the LVAcHT, the identified RPed1 neuron (white outlined arrowhead) was found unlabeled. Scale bars represent 100 μm. The abbreviations used are as follows: L/R Buc, left and right buccal ganglia; L/Cer, left cerebral ganglion; R/Cer, right cerebral ganglion; L/R Pleu, left and right pleural ganglia; L/Ped, left pedal ganglion; R/Ped, right pedal ganglion; L/Par, left parietal ganglion; R/Par, right parietal ganglion; Visc, visceral ganglion.
DISCUSSION

From the moment of the identification of ACh as a neurotransmitter, the receptors of ACh have been investigated in the CNS of mollusks by means of electrophysiology (8, 9, 12, 13, 32, 33). Molluscan CNS studies, as opposed to those in mammalian species, demonstrated that fast ACh-mediated synaptic transmission is predominant in the CNS and is mediated by multiple and unique nAChR subtypes (10, 11, 34–36). In this paper, for the first time, a comprehensive analysis of the molecular complexity of subunits potentially involved in fast cholinergic transmission in a molluscan species is presented.

**LnAChR Subunit Diversity in the Lymnaea CNS**—In total, we identified 10 α-type and 1 β-type full-length Lymnaea nAChR subunits, as well as one presumed partial nAChR subunit. Whether this describes the full complement of nAChR subunits in the Lymnaea CNS is unknown; however, the number of Lymnaea subunits characterized matches that found in other species. For instance, in the human genome 8 α-type and 3 β-type neuronal nAChR subunits exist, and insects possess 7 α-type and 2 β-type nAChR subunits, which all represent neuronal nAChR subunit types (37, 38). As in mammals and Drosophila, the observed nAChR subunit diversity in Lymnaea does not resemble the exceptionally high number of Caenorhabditis elegans (at least 20 α-type and 7 β-type) (39) or of the pufferfish Fugu rubripes (16 α-type and 12 β-type) (40).

Several observations suggest that we did not identify the full complement of LnAChR subunits. Although the partially characterized LnAChR L subunit might represent a β-type nAChR subunit (see below), we were unable to design degenerate primers that confidently target β-type subunits, which explains why the only β-type subunit was obtained from another source. Most likely, additional β-type nAChR subunits remain to be characterized. In addition, we did not observe labeling of glial cells using ISH, which suggests that the subunit(s) belonging to an α-bungarotoxin-sensitive nAChR shown in previous work to be present on AChBP-expressing glial cells (29) remain(s) to be identified.

The functional expression of the subunit types A and B reveals their sensitivity for ACh and indicates that the related subunits are also involved in the formation of nAChRs. The absence, however, of efficient functional expression of the other subunits indicates that for their functional expression additional (β) subunits are probably necessary. It is important to recall that functional expression of insect receptors was obtained only by the combination of an insect α-like subunit with a vertebrate β-type subunit (41, 42). Also, our attempts to express various α-type subunits together with the cloned and predicted β-type subunit did not result in a functional channel in Xenopus oocytes. Either the appropriate combination of subunits or, alternatively, accessory proteins comparable with RIC-3 (43) might be missing. Further work will be required to clarify this issue.

**Evolution of Lymnaea nAChR Subunits**—Sequence homology analysis shows that LnAChR A, C, E, I, and possibly L subunits are sequence-related to subunits from human and Drosophila. Based on this similarity, we might infer possible functional properties of these subunits. Indeed, LnAChR A that is related to the homo-oligomeric human α7 nAChR and Drosophila Da6 nAChR (44, 45) also has the capability to form functional homopentameric receptors when expressed in Xenopus oocytes. Similarly, LnAChR I (β-type) and LnAChR C and E are related to the heteropentameric group of human nAChR subunits and can be predicted to participate in heteromeric receptors. Although insignificant with phylogenetic analysis, the partial LnAChR L subunit might be related to Lymnaea (LnAChR J), Aplysia, and Drosophila (ARD) β-type subunits (Fig. 2C), agreeing with LnAChR L most closely resembling the β-type nAChR subunits from various insects when compared with GenBank™ sequences (data not shown).

Although clearly belonging to the nAChR subunits, the LnAChR B, F, I, and K and the LnAChR D and G groups display no particular similarity to individual mammalian or insect nAChR subunits. Possibly, these subunits are typical products of molluscan evolution. In line with this, LnAChR B and LnAChR I, and most likely LnAChR F and K, seem to represent constituents of the long known chloride-conducting nAChR subunits known so far to only exist in molluscs (9, 12–15). The results of BLAST analysis and the presence of specific residues in loop C of ligand binding conserved in α-type nAChR subunits to a large extent support classification of LnAChR H as an α-type nAChR subunit. Phylogenetic analysis, however, suggests that LnAChR H has diverged considerably from nAChR subunits appearing almost as distant from human nAChR subunits as from 5-HT3 receptor subunits. In addition, the histidine residue in loop C of ligand binding of LnAChR H is difficult to reconcile with an interaction with the quaternary ammonium of ACh, an interaction suggested for tyrosines at corresponding positions conserved in...
other α-type nAChR subunits (46). In conclusion, the contribution of LnAChR H to ligand binding of LGICs remains to be determined and might involve sensitivity to neurotransmitters other than ACh. The widespread presence of LnAChR H in the Lymnaea CNS suggests that this particular subunit mediates a general physiological function, which also pledges further functional characterization.

Acetylcholine-mediated Transmission in the Lymnaea CNS—The data presented in this paper for the first time provide a CNS-wide description of the expression of molecular components involved in cellular reception and vesicular release of acetylcholine in L. stagnalis. Based on our study, nAChRs are detectably expressed by at least 10% of the neurons in the CNS. That this number might actually be higher comes from reports that suggest that virtually all neurons in the Lymnaea CNS respond to application of ACh through presumed nicotinic receptors that are mainly depolarizing and excitatory in nature (8, 9). Our data reveal large differences in expression levels and distribution patterns of LnAChR subunit types and suggest the presence of nAChR subtypes with ganglion specificity (LnAChR A or B), cell type specificity (LnAChR D), and of subunits involved in more generic tasks (LnAChR F and H).

The expression of LVACHT reveals large numbers of acetylcholine-synthesizing neurons present in all ganglia of the Lymnaea CNS. Although cholinergic interneurons have been identified, the molluscan CNS is known to contain numerous motor neurons possibly accounting for many of the cholinergic neurons. For example, Giller and Schwartz (47) have functionally identified most cholinergic neurons in the Aply-
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