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# Assembly of nicotinic α7 subunits in *Xenopus* oocytes is partially blocked at the tetramer level

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**Abstract The assembly of nicotinic α1β1γδ, α3β4, and α7 receptors and 5-hydroxytryptamine 3A ($5HT_3A$) receptors was comparatively evaluated in *Xenopus* oocytes by blue native PAGE analysis. While α1βγδ subunits, α3β4 subunits, and $5HT_3A$ subunits combined efficiently to pentamers, α7 subunits existed in various assembly states including trimers, tetramers, pentamers, and aggregates. Only α7 subunits that completed the assembly process to homopentamers acquired complex-type carbohydrates and appeared at the cell surface. We conclude that *Xenopus* oocytes have a limited capacity to guide the assembly of α7 subunits, but not $5HT_3A$ subunits to homopentamers. Accordingly, ER retention of imperfectly assembled α7 subunits rather than inefficient routing of fully assembled α7 receptors to the cell surface limits surface expression levels of α7 nicotinic acetylcholine receptors.**


*Keywords:* Blue native PAGE; α7 Nicotinic acetylcholine receptor subunit; 5-Hydroxytryptamine receptor subunit; Quaternary structure; Sucrose density centrifugation

## 1. Introduction

The cation-conductive nicotinic acetylcholine receptors (nAChRs) are members of the cys-loop superfamily of ligand-gated ion channels (LGICs) that includes the closely related 5-hydroxytryptamine type 3 ($5HT_3$) receptors as well as the anion-permeable inhibitory γ-aminobutyric acid receptors and glycine receptors (GlyRs). Neuromuscular junction nAChRs are pentamers composed of four homologous gene products with a stoichiometry of $(\alpha1)_2\beta1\gamma\delta$ (fetal) or $(\alpha1)_2\beta1\varepsilon\delta$ (adult). In the mammalian nervous system, an additional repertoire of at least 11 nAChR gene products exists comprising eight α (α2–α7, α9, α10) and three β subunits (β2–β4) [1–3].

All nAChRs are thought to share a pentameric structure, in which homologous subunits surround a central aqueous pore whose opening and closing is regulated by neurotransmitter binding. When reconstituted in expression systems, most α subunits (α2–α6) need to heteropolymerize with at least one of the β subunit isoforms to form functional nAChRs, yielding multiple heterogeneous receptor assemblies with distinct pharmacological and functional phenotypes [1–3]. α7 and α9 subunits differ from other mammalian nAChRs subunits in their capacity to form evidently homomeric acetylcholine-gated ion channels when expressed in *Xenopus* oocytes [4–6], and certain cells of neuronal origin such as SH-SY5Y cells [7], $GH_4C_1$ cells [8], and PC12 cells [9,10]. The existence of endogenous α7 homopentamers has been convincingly demonstrated in a PC12 cell line and rat brain [11,12]. Transient transfection of non-neuronal cell lines with α7 subunit cDNA, however, resulted in little or no production of functional nAChRs, although high levels of α7 mRNA and α7 protein were observed [10,13]. Also certain neuronal cell lines, including those PC12 cells which lack endogenous α7 nAChRs, did not produce functional nAChRs from transfected α7 cDNA [9]. Apparently, the formation of functional nAChRs from α7 subunits requires cellular factors aiding in receptor assembly, maturation and/or stabilization [9,13–16] that occur only in a subset of cells. Cell-specific receptor formation is not found with homooligomeric $5HT_3A$ or GlyR α1 receptors.

In this study, we used the blue native PAGE technique to compare the assembly behavior of α7 subunits and $5HT_3A$ subunits in *Xenopus* oocytes. Among the members of the nicotinic superfamily, the $5HT_3A$ subunit is most closely related with the α7 nAChR subunit [17], with which it shares 51% amino acid sequence homology (28% sequence identity). We find that a majority of α7 subunits forms homotetramers and aggregates in *Xenopus* oocytes that are retained in the ER, whereas $5HT_3A$ subunits assemble rapidly and completely to homopentamers. Also heteropentameric nicotinic receptors are formed productively from co-expressed muscle α1β1γδ or neuronal α3β4 subunits. Our results imply that oocytes do not have the capacity to guide the polymerization of α7 subunits with the same efficiency as of other LGIC subunits and that surface receptor expression level is limited by imperfect subunit assembly rather than by impaired routing of fully assembled α7 receptors to the cell surface.

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*Abbreviations:* LGIC, ligand-gated ion channel; GlyR, glycine receptor; $5HT_3$, 5-hydroxytryptamine; nAChR, nicotinic acetylcholine receptor

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## 2. Materials and methods

### 2.1. cDNA constructs

Mutations were inserted using the QuikChange™ site-directed mutagenesis kit (Stratagene). Plasmids containing cDNAs encoding the rat nAChR subunits α3, α7, and β4 were kindly provided by Dr. J. Patrick [6,18,19]. The complete coding regions of these cDNAs were subcloned into vector pNKS2 [20]. A plasmid (5HT$_3$A-pSGEM) containing the cDNA for the mouse 5HT$_3$A subunit [21] was kindly provided by Dr. M. Garcia-Guzman (Max Planck Institute for Experimental Medicine, Göttingen, Germany). Double stranded oligonucleotides encoding a C terminal His tag were inserted without changing any other amino acid into restriction sites generated just before the stop codons to generate nAChR α3-His, nAChR α7-His, nAChR β4-His, and 5HT$_3$A-His. All constructs were confirmed by sequencing. Plasmids encoding the muscle type rat nAChR α1-His subunit with a C-terminal hexahistidyl tag (His) as well as the non-tagged β1, γ and δ subunits were available from previous studies [22,23].

### 2.2. Expression of LGIC subunits in Xenopus laevis oocytes

Completely defolliculated oocytes were obtained by collagenase treatment [24] and maintained in sterile frog Ringer's solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4) supplemented with 50 μg/ml of gentamycin. Two-electrode voltage-clamp recordings were performed as described previously [23,25]. nAChRs and 5HT$_3$A receptors were activated by 200 μM (−)nicotine (hydrogen tartrate salt) or 10 μM serotonin (both from Sigma–Aldrich, Taufkirchen, Germany), respectively, in nominally $Ca^{2+}$-free ORi solution (designated Mg-ORi) to avoid $Ca^{2+}$ activation of endogenous Cl-channels. The holding potential was −60 mV in all experiments.

For radiolabeling, cRNA-injected oocytes and non-injected control oocytes were incubated overnight with L-[$^{35}$S]methionine (>40 TBq/mmol, Amersham Biosciences) at ~25 MBq/ml in ORi (~0.1 MBq/oocyte). For selective labeling of plasma membrane bound proteins, cRNA-injected oocytes were kept for 3 days at 19 °C and then labeled with freshly radioiodinated ($Na^{125}I$, Amersham Biosciences) sulfo-succinimidyl-3-(4-hydroxyphenyl)proprionate ($^{125}$I-sulfo-SHPP) [26].

### 2.3. $Ni^{2+}$–NTA affinity chromatography, blue native PAGE and SDS–PAGE

Proteins were purified under non-denaturing conditions from digitonin or dodecylmaltoside extracts of radiolabeled oocytes in the presence of iodoacetamide and resolved by blue native PAGE as described previously [24,27]. For SDS–PAGE, proteins were denatured for 10 min at 37 °C with SDS sample buffer containing 20 mM dithiothreitol (DTT), and then electrophoresed in parallel with [$^{14}$C]-labeled molecular mass markers (Rainbow, Amersham Biosciences) on SDS–polyacrylamide gels. To investigate the glycosylation status, samples were treated for 1–2 h with either endoglycosidase H (Endo H) or PNGase F (New England Biolabs) in the presence of reducing SDS sample buffer supplemented with 1% (w/v) NP-40 to counteract SDS inactivation of PNGase F. Experiments were repeated at least three times with consistent results except of radioiodinations, which were reproduced only once.

### 2.4. Velocity gradient centrifugation

Metabolically labeled oocytes expressing α7-His or 5HT$_3$A-His LGICs were extracted with 1% digitonin as above. Cleared detergent extract (0.2 ml) was loaded atop of 11 ml of a 10–30% (w/v) linear sucrose gradient containing 150 mM NaCl, 50 mM Tris/HCl, pH 7.4, and 0.05% digitonin, and centrifuged at 40 000 rpm (~200 000*g*) for 16 h in a SW 41 Ti rotor (Beckman). 0.5-ml Gradient fractions were collected from the bottom of the tube and subjected to $Ni^{2+}$–NTA agarose affinity chromatography as above. Bound proteins were released by incubation with 250 mM imidazole/HCl, pH 7.4, and 1% digitonin, treated with the appropriate PAGE sample buffer, and resolved on both blue native PAGE gels and SDS–PAGE gels.

## 3. Results

### 3.1. Unlike 5HT$_3$A subunits, α7 subunits assemble inefficiently to homopentamers

The functional expression of the muscle type (αl-His)β1γδ nAChR, neuronal (α3-His)β4 and α7-His nAChRs, and 5HT$_3$A-His receptors in *Xenopus* oocytes was confirmed by two-electrode voltage-clamp electrophysiology. The introduction of a C terminal hexahistidine tag into the α7 subunit did not affect the shape of the current trace (Fig. 1A), but resulted in a ~25% reduction of the average current amplitude (Fig. 1B). Absolute amplitudes of the homomeric α7-His nAChR-mediated current of <0.6 μA were small when compared to >25 μA mediated by homomeric 5HT$_3$A-His receptors under equivalent conditions.

To assess the oligomeric state of the 5HT$_3$A-His and α7-His subunits, we exploited the blue native PAGE technique, which has been demonstrated to have the capacity to display the pentameric nature of other members of the nicotinic receptor superfamily such as the inhibitory $(\alpha_1)_5$ glycine receptor [28,29] and the muscle type $(\alpha1)_2\beta1\gamma\delta$ nAChR [23,24] (see also Fig. 2A, lanes 7–8). Also, recombinant 5HT$_3$A-His subunits isolated under non-denaturing conditions from *Xenopus* oocytes migrated predominantly as a single defined oligomer (Fig. 2A, lane 4). To display the number of 5HT$_3$A-His subunits incorporated in this oligomer, we exposed it to 8 M urea or 0.1% SDS to weaken non-covalent subunit interactions, thus inducing a dissociation into lower order intermediates. Consistent with the remarkable stability of LGIC complexes, these treatments did not lead to a complete dissociation into monomers, but generated a ladder of five bands, which

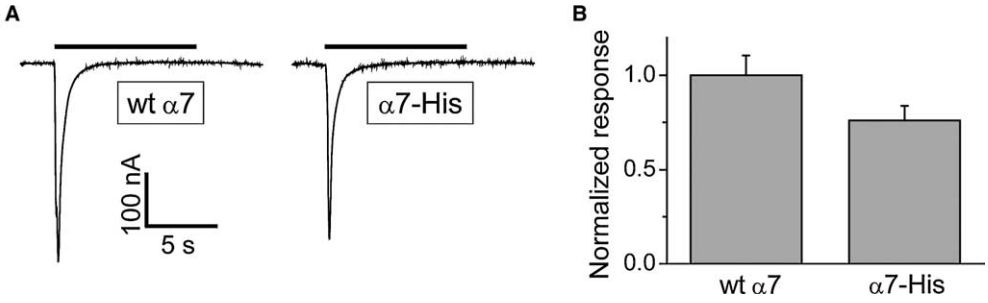
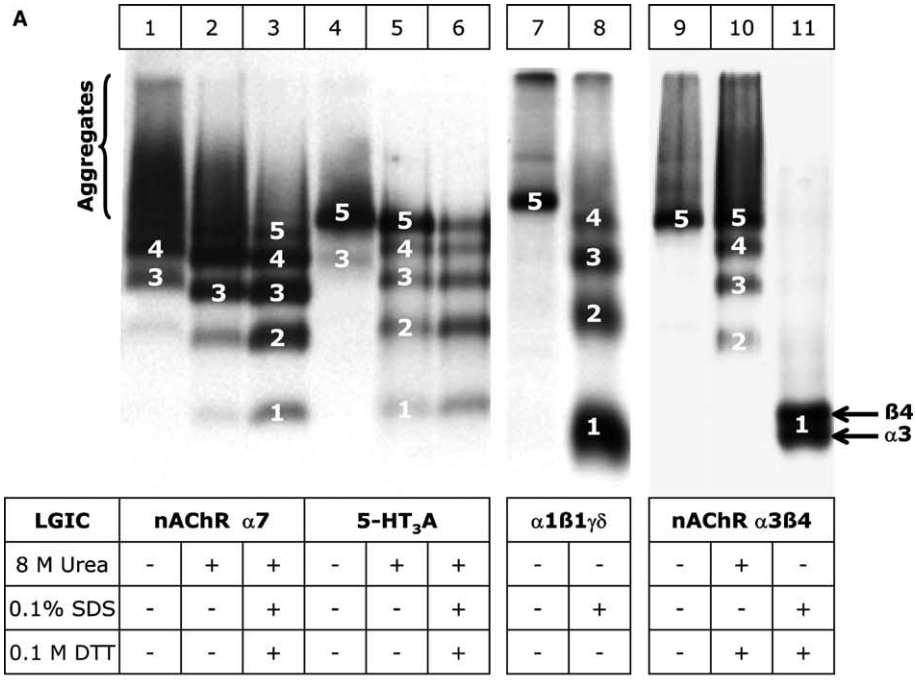



Fig. 1. Functional characterization of His-tagged α7 nAChR expressed in *Xenopus* oocytes. (A) Shown are typical current traces elicited at a holding potential of −60 mV by saturating test pulses of 200 μM nicotine (horizontal lines) in Mg-ORi two days after injection of the indicated cRNAs. (B) Bars are means ± S.E.M. of normalized current responses from two independent experiments, each with five oocytes per α7 type (unity corresponds to 340 ± 53 nA).

consisted of one, two, three, four and a maximum of five copies of the $5HT_3A$ subunit (Fig. 2A, lanes 5–6). Accordingly, the bulk amount of the non-treated $5HT_3A$ oligomer (lane 4) must be a pentamer, in agreement with previous studies [30,31]. The sole additional protein band resolved by blue native PAGE in the non-dissociated sample represents a minor




| LGIC | nAChR α7 | | | 5-HT$_3$A | | | α1ß1γδ | | nAChR α3ß4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 M Urea | - | + | + | - | + | + | - | - | - | + | - |
| 0.1% SDS | - | - | + | - | - | + | - | + | - | - | + |
| 0.1 M DTT | - | - | + | - | - | + | - | - | - | + | + |

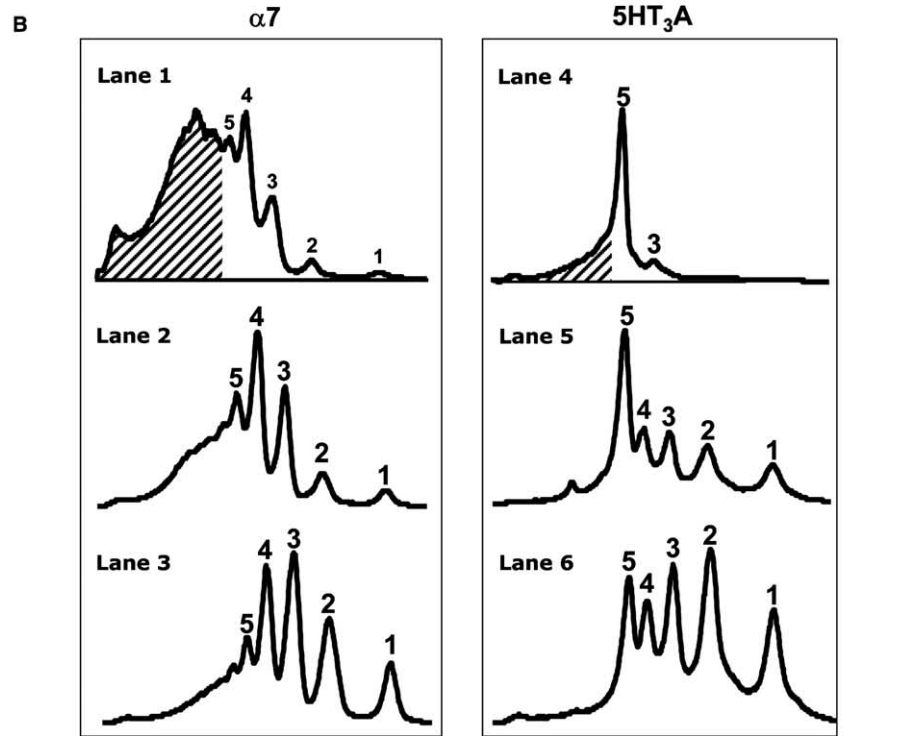



Fig. 2. Assembly state of homomeric nAChR α7-His and $5HT_3A$-His subunits and heteromeric nAChR subunit combinations shortly after their synthesis. (A) *Xenopus* oocytes were injected with cRNAs for His-tagged α subunits and non-tagged accessory subunits as indicated. After [$^{35}$S]methionine labeling, LGICs were natively purified from digitonin or dodecylmaltoside extracts of these cells. LGICs were immediately resolved by blue native PAGE either without further treatment or after a 1 h incubation at 37 °C in the presence of 8 M urea, 0.1 M DTT or 0.1% SDS as indicated. (B) A quantitative profile of the radioactivity incorporated in the various homooligomers was established by PhosphorImager analysis of individual lanes of the blue native PAGE gel shown in (A). The origin of the abscissa corresponds to the top of the blue native PAGE gel. Numbers indicate the oligomeric state of the corresponding protein bands. Hatched areas indicate aggregated protein. Lane numbers are the same as in (A). Experiments were repeated $\geqslant 3$ times with virtually identical results.

fraction of homotrimers (cf. Fig. 2B, right panel, lane 4). The absence of significant amount of intermediate assembly states indicates that the 5HT3A receptor attained a pentameric state during or shortly after synthesis while still in the ER.

In contrast, the recombinant α7-His protein isolated directly after a 4 h [$^{35}$S]methionine pulse existed in several oligomeric states and also in an aggregated form as indicated by the high molecular mass proteins that migrated at a broad range of masses above that of the pentameric receptor (Fig. 2A, lane 1). By comparison with the pattern of bands produced by dissociating treatment of the α7-His protein with urea or SDS (lanes 2–3), the discrete protein bands in lane 1 could be assigned to homotrimers and homotetramers of the α7-His subunit. Quantitative scanning of the protein bands with a PhosphorImager revealed the presence of monomers, dimers, and pentamers besides the dominant homotetramer (Fig. 2B, left panel, lane 1). From the quantitative profile, it is also apparent that another significant portion of α7-His subunits is contained in aggregates larger than the homopentamer. The relative amount of oligomers and aggregates did not change significantly during a subsequent 36 h chase interval (results not shown). Quantitative scanning established further that $5HT_3A$-His subunits are largely incorporated in a homopentameric complex directly after a 4 h pulse (Fig. 2B). Primarily, properly assembled pentameric complexes were also formed from heteromeric nAChR combinations such as muscle type α1β1γδ subunits (Fig. 2A, lanes 7 and 8) and neuronal α3β4 subunits (lanes 9–11).

To assess the oligomeric state of α7-His subunits also by a method more commonly used in this respect, digitonin extracts of LGIC-expressing oocytes were subjected to sucrose density centrifugation. His-tagged LGICs were subsequently purified by native $Ni^{2+}$–NTA chromatography from each sucrose fraction. Blue native PAGE analysis (Fig. 3A) resolved the $5HT_3A$ receptor protein as a uniform homopentamer of identical mass in all the sucrose fractions where it was present. In contrast, the oligomeric state of the α7-His protein displayed by blue native PAGE increased with the sucrose density of the fraction from which the protein was purified. SDS–PAGE analysis of the same samples (Fig. 3A) followed by PhosphorImager quantification (Fig. 3B) showed a much broader distribution across the sucrose gradient for the α7-His protein than for the $5HT_3A$-His protein. A similar broad sedimentation profile was previously observed for Triton X-100-solubilized recombinant α7 nAChRs isolated from HEK293 cells [10]. The similar results obtained by either method demonstrates that blue native PAGE represents a convenient alternative for sucrose density centrifugation.

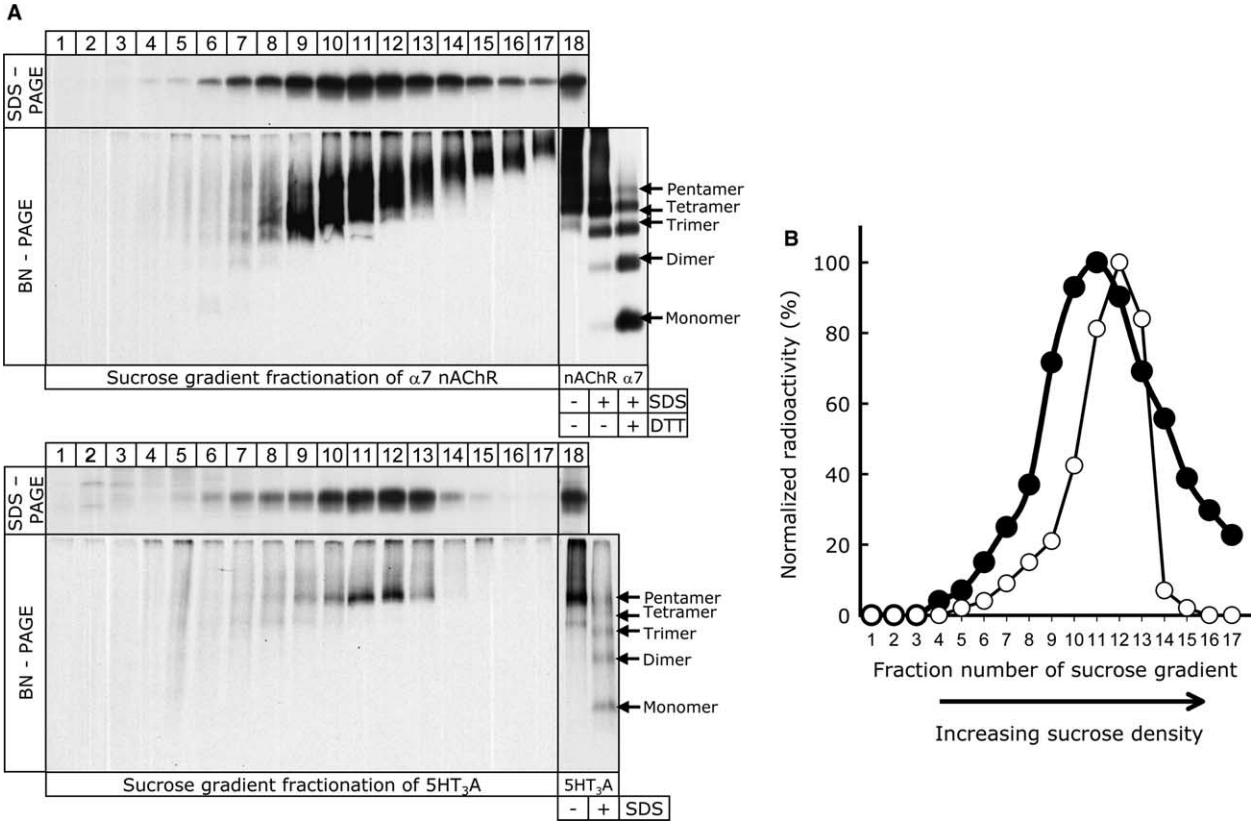



Fig. 3. Blue native PAGE and SDS–PAGE analysis of α7 nAChRs and $5HT_3A$ receptors purified from fractions derived from sucrose density centrifugation. (A) Autoradiographies of the indicated PAGE gels. LGICs purified directly from digitonin extracts of oocytes are also shown (lane 18). Samples analyzed by SDS–PAGE were denatured by reducing SDS–PAGE sample buffer before loading. (B) Radioactivity of the $5HT_3A$-His polypeptide (○) and the α7-His polypeptide (●) as resolved by SDS–PAGE and shown in (A) was quantified by PhosphorImager analysis and normalized to the highest signal of the appropriate gel.

### 3.2. Solely complex-glycosylated α7 pentamers arrive at the cell surface

To allow for a direct comparison of the oligomeric state of the total pool of α7-His subunits with plasma membrane-bound α7-His subunits, α7-His protein was purified under non-denaturing conditions in parallel from surface-radioiodinated and metabolically labeled subgroups of cRNA-injected oocytes and resolved on the same blue native PAGE gel. While the metabolically labeled oocytes contained α7 subunits of different assembly states (Fig. 4A, lane 4), the plasma membrane contained exclusively homopentameric α7 nAChRs, albeit in comparably low amounts (lane 3). No intermediate bands corresponding to tetramers or lower order complexes were observed to exist at the cell surface. The muscle type $(\alpha 1)_2\beta 1\gamma\delta$ analyzed as a positive control appeared in significantly larger amounts at the cell surface (Fig. 4A, lane 2) than α7 nAChRs.

As an additional indicator for the subcellular localization of membrane proteins, we monitored the glycosylation status of the α7-His subunits. The [$^{35}$S]methionine-labeled α7 subunit migrated at around 63 kDa (Fig. 4B, lane 5) as compared to a protein core of 54 kDa calculated from the protein sequence. Deglycosylation with both Endo H or PNGase F resulted in a 8–9 kDa mass shift to about 55 kDa (Fig. 4B, lanes 6 and 7) consistent with the release of three high-mannose type *N*-glycans at $N^{24}$, $N^{68}$, and $N^{111}$ of the mature α7-His polypeptide [32]. In contrast, the plasma membrane-bound α7 subunits migrated at 66 kDa and turned out to be entirely Endo H resistant (Fig. 4B, lane 5), indicating that all the α7-His subunits capable of reaching the plasma membrane acquired complex-type carbohydrates during transit of the Golgi apparatus. Accordingly, the apparent lack of Endo H-resistant complex-type α7-His subunits in metabolically labeled oocytes is consistent with the ER retention of the vast majority of α7-His subunits. As the fraction of complex-glycosylated α7-His subunits is low, their particular glycosylation status can be visualized only by selective labeling of the plasma membrane-bound pool of α7-His nAChRs.

## 4. Discussion

### 4.1. Incomplete assembly of α7 subunits in Xenopus oocytes

Many mammalian cell lines do not express functional nAChRs when transiently transfected with α7 subunits [10,33], even though α7 subunits are synthesized in substantial amounts. *Xenopus* oocytes are considered as an established exception of the rule that functional α7 nAChRs are produced by neuron-derived cells only. Accordingly, oocytes serve frequently in the analysis of electrophysiological and pharmacological properties of the homomeric α7 nAChR. We show here that α7 subunit assembly in *Xenopus* oocytes is also an only partially productive process that leads to fully assembled homopentamers, but also a large portion of homotetramers and aggregates. Both blue native PAGE analysis and sensitivity to Endo H of the oocyte expressed α7 subunits add to the view that only a limited fraction of the totally synthesized α7 subunits is able to polymerize to homopentamers capable of leaving the ER. En route to the plasma membrane, this small amount of homopentameric α7 nAChRs acquires complex-type carbohydrates in the Golgi apparatus. A majority of α7 polypeptides, however, remains trapped in the ER in the high-mannose form, apparently as a result of incomplete subunit assembly.

Incomplete homopolymerization in *Xenopus* oocytes is neither found with homooligomeric $5HT_3A$ subunits (present study) nor GlyR α1 subunits [28,29]. The small amount of $5HT_3A$ homotrimers observable directly after the [$^{35}$S]methionine pulse may represent a transient assembly intermediate equivalent to the αβγ trimer, which constitutes the earliest identifiable intermediate during assembly of muscle type nAChRs [23,34]. Consistent with this viewpoint, the $5HT_3A$ homotrimer disappeared completely during a subsequent chase period (unpublished results). In contrast, the relative amounts of α7 tetramers and aggregates, persisted during the chase, suggesting that a significant percentage of the α7 subunits is permanently misfolded and misassembled.

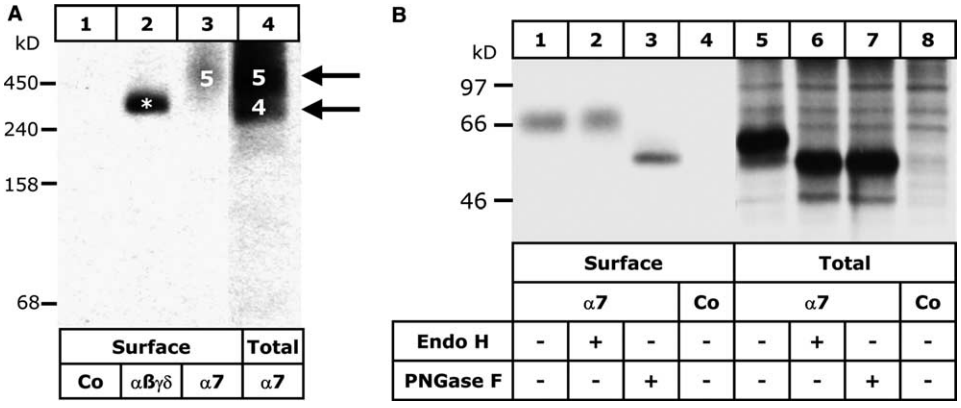



Fig. 4. Plasma membrane bound α7 nAChR is a complex-glycosylated homopentamer. (A) Three days after injection of the indicated cRNAs, oocytes were surface labeled with $^{125}$I-sulfo-SHPP and extracted with digitonin. Proteins were purified under non-denaturing conditions and resolved by blue native PAGE (4–13% acrylamide). For direct comparison, α7-His protein isolated after an overnight [$^{35}$S]methionine pulse is also shown (lane 4). The asterisk indicates the pentameric surface-expressed muscle type nAChR, which was analyzed in parallel. (B) Glycosylation status of $^{125}$I-labeled (surface) and [$^{35}$S]methionine-labeled (total) α7 subunits. The same samples as in (A) were denatured with reducing SDS sample buffer and then incubated for 2 h with Endo H or PNGase F as indicated. Note that the $^{125}$I-labeled plasma membrane-bound α7 polypeptide is entirely Endo H resistant, whereas the [$^{35}$S]methionine-labeled α7 subunit is in an Endo H sensitive form. Co, non-injected control oocytes.

### 4.2. N-Glycan status of α7 nAChRs in Xenopus oocytes

Unexpectedly, the plasma membrane-bound α7 subunits turned out to harbor exclusively Endo H resistant, complex-type carbohydrates. In previous studies, α7 subunits were found to carry solely high-mannose type carbohydrates irrespective of whether they were isolated from native tissue, i.e., brain, or from host cells such as *Xenopus* oocytes or COS cells [32]. In our experiments, the detection of complex-type carbohydrates required the selective visualization of the plasma membrane-bound α7 nAChR by surface radioiodination. We were unable to detect complex-type α7 subunits among metabolically labeled α7 subunits, apparently because complex-glycosylated α7 subunits constitute only a minor fraction of the total α7 subunit pool. Hence, it is possible that a small amount of complex-glycosylated α7 subunits remained undetectable also in previous studies, in which the plasma membrane-bound nAChRs were not selectively labeled.

### 4.3. Incompletely assembled α7 nAChRs are retained by the quality control system in Xenopus oocytes

Surprisingly, the mechanisms that account for the paucity of functional α7 nAChRs at the cell surface of transiently transfected cells are cell type specific. In tsA201 cells of a human kidney epithelial cell line, α7 subunits arrive in substantial numbers as multimers at the cell surface, but neither function nor bind α-bungarotoxin [35]. COS cells, on the other hand, produce properly folded and assembled α7 nAChRs, yet these nAChRs remain located in an intracellular pool because they lack trafficking motifs needed for distribution to the plasma membrane [36]. In contrast, our results in *Xenopus* oocytes can be readily reconciled with selective retention of incompletely assembled α7 subunits by the ER quality control system [37]. A similar mechanism appears to be responsible for the low surface expression of functional α7 nAChRs in HEK293 and fibroblast QT6 cells. These cells also produce the α7 protein, but largely fail to assemble it properly, in contrast to neurons, which converted virtually all α7 subunits into nAChRs [13]. Both the need for structural subunits such as a homologous subunits, splice variants or folding isomers or specific helper proteins could explain the limited capacity of α7 subunits to polymerize. One such helper protein, RIC-3, has recently been identified to be involved in functional maturation of nAChRs in *Caenorhabditis elegans* [15].

### 4.4. Possible assembly pathway of α7 subunits in Xenopus oocytes

In *Xenopus* oocytes a major obstacle appears the addition of a fifth α7 subunit to the emerging receptor complex, leading to assembly block at the homotetramer level (Fig. 5 pathways A and C). Since α7 subunits must adopt two distinct conformations to assemble into functional nAChRs [35], homopentameric α7 receptors may be regarded as multimers composed of two folding isomers. In analogy to the "heterodimer" model of the assembly of the αβγδ subunits of the muscle type nAChRs [38,39], newly synthesized α7 subunits may first fold and combine to dimers of two isomers, which polymerize further to pentamers by enclosure of an unassembled α7 subunit (Fig. 5, $B_1$–$B_3$). *Xenopus* oocytes may belong to those cells in which α7 subunits fold mainly into only one conformation, and thus low levels of the second α7 folding isomer limit the formation of isomer dimers and eventually functional nAChRs. An "enclosed" tetramer may be formed preferentially and thus assembly interfaces needed for incorporation of the fifth subunit may become permanently inaccessible (Fig. 5, $A_1$–$A_3$). The incompletely assembled α7 oligomers may polymerize further giving rise to higher order oligomers that appear as undefined aggregates.

The alternative "sequential" assembly model takes into account that αβγ trimers represent the earliest assembly product of muscle type nAChRs [23,39], and could hence explain the existence of homotrimers in *Xenopus* oocytes. Trimer formation in the assembly pathway of the muscle type nAChR is

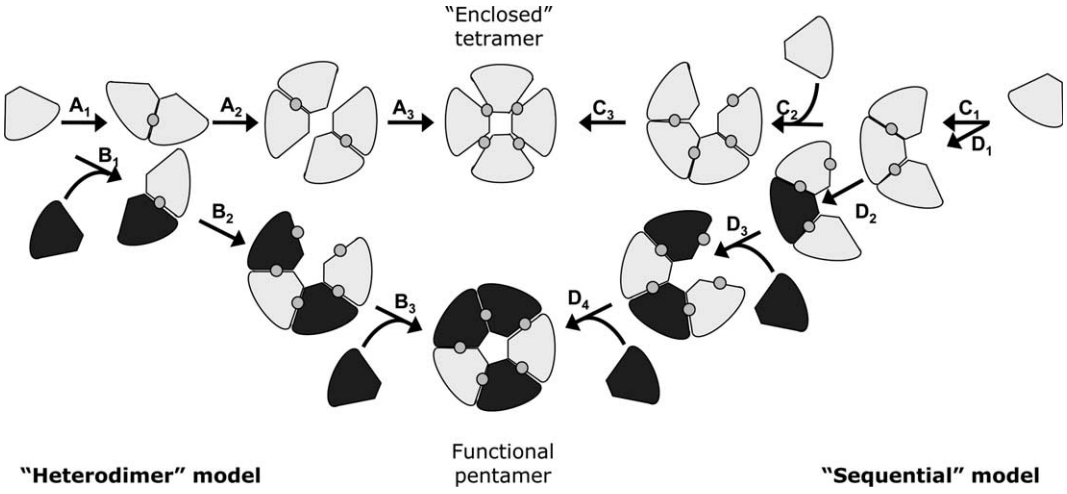



Fig. 5. Possible ER assembly pathway for α7 subunits in *Xenopus* oocytes. In analogy to the "heterodimer" model of the assembly of muscle type nAChR (for review, see [39]), functional α7 nAChRs may be formed by dimerization of two α7 dimers ($B_1$), each composed of two α7 folding isomers [35]. Limited availability of one of the folding isomers enables only a small portion of α7 subunits to assemble properly ($B_1$–$B_3$). The assembly of the remainder becomes blocked at the tetramer level ($A_1$–$A_3$). Alternatively, in analogy to the "sequential" model, a homotrimer may first be formed ($C_1$, $D_1$) as a prerequisite for subsequent folding steps ($D_2$) that generate recognition sites for the addition of the fourth and eventually the fifth α7 subunit ($D_3$–$D_4$). Like in the heterodimer model, lack of properly folded α7 subunits is proposed to block assembly at the level of the homotrimer and homotetramer ($C_1$–$C_3$). α7 folding isomers are shown in black and white; gray circles indicate subunit recognition sites.

considered as a prerequisite for subsequent slow posttranslational folding reactions of the α subunit (within the αβγ trimer) as part of a process that exposes appropriate amino acids for contact with the next incoming subunit (Fig. 5, $D_1$–$D_4$). Like in the adapted "isomer heterodimer" model above, the lack of appropriate folding isomers could explain the failure to complete the assembly process (Fig. 5, $C_1$–$C_3$). Interestingly, also the assembly of the muscle nAChR is blocked at the (αβγδ) tetramer level if a β subunit mutant is co-expressed that cannot form the cystine loop [40]. Although our data do not allow us to discriminate between the two assembly models, they provide additional support of the view that the α7 subunit itself contains insufficient information to warrant proper folding without support of cell specific helper proteins [13].

In summary, our results show that nicotinic α7 subunits are efficiently synthesized in *Xenopus* oocytes, but homopolymerize less faithfully than expected. Incomplete subunit assembly rather than a disturbed intracellular trafficking of fully assembled α7 receptors provides an explanation for why only a limited number of functional α7 nAChRs arrive at the oocyte surface. Efficient α7 nAChR formation apparently needs co-assembly with other nAChR subunit isoforms such as β2 [41] and β3 subunits [42] or support from cell type-specific chaperones that guide the assembly process.

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