

Functional Expression of Human $\alpha 9^*$ Nicotinic Acetylcholine Receptors in *X. laevis* Oocytes Is Dependent on the $\alpha 9$ Subunit 5' UTR

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Abstract

Nicotinic acetylcholine receptors (nAChRs) containing the $\alpha 9$ subunit are expressed in a wide variety of non-neuronal tissues ranging from immune cells to breast carcinomas. The $\alpha 9$ subunit is able to assemble into a functional homomeric nAChR and also co-assemble with the $\alpha 10$ subunit into functional heteromeric nAChRs. Despite the increasing awareness of the important roles of this subunit in vertebrates, the study of human $\alpha 9$ -containing nAChRs has been severely limited by difficulties in its expression in heterologous systems. In *Xenopus laevis* oocytes, functional expression of human $\alpha 9\alpha 10$ nAChRs is very low compared to that of rat $\alpha 9\alpha 10$ nAChRs. When oocytes were co-injected with cRNA of $\alpha 9$ and $\alpha 10$ subunits of human versus those of rat, oocytes with the rat $\alpha 9$ human $\alpha 10$ combination had an \sim fold higher level of acetylcholine-gated currents (I_{ACh}) than those with the human $\alpha 9$ rat $\alpha 10$ combination, suggesting difficulties with human $\alpha 9$ expression. When the ratio of injected human $\alpha 9$ cRNA to human $\alpha 10$ cRNA was increased from 1:1 to 5:1, I_{ACh} increased 36-fold (from 142 ± 23 nA to 5171 ± 748 nA). Functional expression of human $\alpha 9$ -containing receptors in oocytes was markedly improved by appending the 5'-untranslated region of alfalfa mosaic virus RNA4 to the 5'-leader sequence of the $\alpha 9$ subunit cRNA. This increased the functional expression of homomeric human $\alpha 9$ receptors by 70-fold (from 7 ± 1 nA to 475 ± 158 nA) and of human $\alpha 9\alpha 10$ heteromeric receptors by 80-fold (from 113 ± 62 nA to 9192 ± 1137 nA). These findings indicate the importance of the composition of the 5' untranslated leader sequence for expression of $\alpha 9$ -containing nAChRs.

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Introduction

Nicotinic acetylcholine receptors (nAChRs) are ACh-gated ion channels implicated in many physiological as well as pathophysiological processes. The role of nAChRs in mediating EPSPs at synapses in autonomic ganglia [1], [2] and at the skeletal neuromuscular junction is well established [3], [4]. In the CNS, nAChRs are involved in modulation of neurotransmitter release [5] and in attention and memory [6], [7]. The pathological conditions where involvement of nAChRs have been implicated include Alzheimer's and Parkinson's diseases [8], [9], nicotine addiction [10], [11] and schizophrenia [12], [13]. Seventeen vertebrate nAChR subunits have been cloned to date ($\alpha 1$ through $\alpha 10$, $\beta 1$ through $\beta 4$, γ , δ , and ϵ) [14]. The nAChR is formed from five subunits, either homomeric receptors ($\alpha 7$, $\alpha 9$) containing five identical subunits or heteromeric receptors (for example, $\alpha 4\beta 2$, $\alpha 3\alpha 5\beta 4$, $\alpha 6\alpha 4\beta 2\beta 3$, or $\alpha 9\alpha 10$).

$\alpha 9$ -containing nAChRs are unique among neuronal nAChRs in that they are found mainly outside of the CNS [15], [16], [17], [18], [19]. Also, unlike other nAChRs, they are inhibited by nicotine [15], [20], [21]. $\alpha 9$ -containing nAChRs play roles in pain [22], [23], [24], [25], [26], [27], inflammation, keratinocyte

adhesion [28], and in mediating synaptic transmission between the efferent olivocochlear fibers and cochlear hair cells [29], [30].

With advances in molecular biology, it became possible to isolate and sequence the genes encoding nAChRs. $\alpha 9$ and $\alpha 10$ subunits were among the last nicotinic receptor subunits to be isolated and characterized. The clone encoding the $\alpha 9$ subunit was originally obtained from a rat olfactory epithelium cDNA library [15]. *X. laevis* oocytes injected solely with rat $\alpha 9$ cRNA yielded homomeric receptors that responded to 100 μ M ACh with currents that ranged from 20 to 500 nA [15]. The clone encoding the rat $\alpha 10$ subunit was isolated from an adult rat cochlea cDNA library [31]. The coinjection of rat $\alpha 9$ and rat $\alpha 10$ cRNAs into oocytes resulted in oocytes with \sim 100-fold larger ACh-gated currents (I_{ACh}) than oocytes injected solely with $\alpha 9$ cRNA. Subsequently, the sequences of human $\alpha 9$ and $\alpha 10$ subunits were determined from keratinocytes [28] and inner-ear neuroepithelium [32], respectively.

To study the pharmacological properties of nAChRs, heterologous expression systems are often used. Mammalian cell lines such as HEK293 and SH-EP1 cells are frequently used to characterize nAChRs [33], [34]. Besides mammalian cells, oocytes of *Xenopus laevis*, the African clawed frog, have been frequently

used for heterologous expression. These oocytes provide several advantages for the study of receptors. They are large and thus easy to handle and to inject with RNA, have long life-times (several days) and can be maintained under relatively simple culture conditions. Oocytes are largely free of endogenous receptors that could interfere with the signals of exogenously expressed channels/receptors. Thus, oocytes have been extensively used to characterize the biophysical and pharmacological properties of nAChRs. They have also been used to study the stoichiometry of receptor subunits, the contribution of different subunits to the properties of receptors, and the structure-function relationships with various ligands. For most nAChRs, oocytes have worked extremely well as an expression host [35], [36], [37]. However, in some instances cRNA-injected oocytes have failed to yield readily detectable I_{ACh} . For instance, human $\alpha 9$ cRNA-injected oocytes have only small I_{ACh} compared to oocytes injected with its rat counterpart [38], [39]. There is no report to date of successful functional expression of human $\alpha 9$ -containing receptors in mammalian cell lines and few reports of successful transfection of rat $\alpha 9$ -containing receptors [40], [41].

The translational efficiency of nAChRs in oocytes is influenced by the structure of the injected cRNA [42], [43] including the Kozak sequence [44], the secondary structure [45], [46] and composition of untranslated regions [47], [48]. The 5' leader sequence preceding the coding region plays an important role in the binding of cap-binding proteins and in facilitation of translation initiation [49]. One approach to improve the translation in oocytes is to flank the gene-encoding sequence with the untranslated regions of highly translatable proteins of *X. laevis*, such as β -globin [50], [51]. When 5' and 3' untranslated regions (UTRs) of human interferon- β mRNA are replaced by those of *X. laevis* β -globin mRNA, the translation is increased as much as 20- and 300- fold in reticulocyte lysates and in *X. laevis* oocytes, respectively [52]. The *X. laevis* β -globin leader sequence exerts its facilitatory effect presumably by increasing translation initiation, and not by increasing the binding of limiting factors [50]. However, for human $\alpha 9$, the addition of the *X. laevis* β -globin sequence to the 5' and 3' UTRs is not sufficient to produce high expression levels.

In this report, we show that the human $\alpha 9$ subunit is the limiting factor in the expression of human $\alpha 9\alpha 10$ nAChRs in *X. laevis* oocytes. Furthermore, we found that this expression can be substantially improved by the insertion of the 5' leader sequence of alfalfa mosaic virus RNA4 (AMV) to the human $\alpha 9$ 5' UTR.

Materials and Methods

Ethics Statement

Isolation of oocytes from *X. laevis* frogs was performed in accordance with and under approval of the Institutional Animal Care and Use Committee of the University of Utah.

cDNA constructs

cDNAs encoding $\alpha 9$ and $\alpha 10$ nAChR subunits from rat were provided by A. B. Elgoyhen (University of Buenos Aires, Argentina). The rat $\alpha 9$ cDNA was in a pGEMHE [51] vector between SmaI and EcoRI restriction sites, and the rat $\alpha 10$ cDNA was in a pSGEM vector (a modified pGEMHE vector) between EcoRI and XhoI restriction sites. cDNAs encoding human $\alpha 9$ and human $\alpha 10$ subunits, in the pGEM-11Zf(+) vector, were generously provided by L. Lustig (University of California San Francisco, San Francisco, CA). The cDNAs encoding human subunits were subsequently inserted into the pSGEM vector between EcoRI and XhoI restriction sites. The oligonucleotides

encoding the 5' leader sequence of alfalfa mosaic virus RNA4 (AMV) were synthesized at the University of Utah core facility. The sequence of the synthesized oligonucleotides was as follows: sense- 5' GGGTTTTTATTTTAAATTTTCTTTCAAATAC-TTCCACCG 3'; antisense-5' AATTCGGTGGGAAGTATTT-GAAAGAAAATTAATAAAAAACCCGC 3'. The oligonucleotides were diluted in 10 mM Tris-Cl, pH 8.5 to a final concentration of 107 μ M for sense oligonucleotide and 80 μ M for antisense oligonucleotide. 20 μ L of each oligonucleotide was mixed in an annealing reaction tube. The annealing reaction was as follows: exposure to 95°C for 10 minutes followed by cooling to 25°C over a period of 45 minutes. The annealed oligonucleotide was ligated into MCS of pSGEM vector between the SacII and EcoRI restriction sites.

cRNA synthesis

The NheI enzyme was used to linearize the vector encoding human $\alpha 9$ and human $\alpha 10$ subunits. *In vitro* transcription was performed using the mMessage mMachine T7 kit (Ambion, Austin, TX). The reaction was followed by DNase treatment. The cRNA was purified with a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). The cRNA concentration was determined by measuring absorbance at 260 nm on an Epoch spectrophotometer.

Oocyte isolation and injection

The isolation of the oocytes was performed as previously described [53]. Briefly, stage IV–V oocytes were isolated from anesthetized adult frog. The oocytes were kept at 17°C in ND96 (96 mM NaCl, 1.8 mM CaCl₂, 2.0 mM KCl, 1.0 mM MgCl₂, 5 mM HEPES, pH 7.1–7.5) supplemented with antibiotics (50 U/mL penicillin, 50 μ g/mL streptomycin, 50 μ g/mL gentamicin). The oocytes were injected with 50.6 nL of cRNA and incubated for 1–3 days before recording. The amount of cRNA injected into each oocyte varied in different experiments. To compare levels of expression of human and rat $\alpha 9\alpha 10$ receptors, 3.3 ng cRNA of each nAChR subunit was injected into individual oocytes. To compare the level of expression of human receptors formed from subunits injected at different ratios, 4.4 ng cRNA of each nAChR subunit was injected into individual oocytes when a ratio of (1) is indicated and 22 ng cRNA was injected when a ratio of (5) is indicated. For all other experiments, 14.4–32 ng cRNA of each subunit was injected.

Two-electrode voltage clamp recording

ACh-gated currents were recorded from oocytes as previously described [53]. Briefly, an oocyte was placed in \sim 30 μ L chamber (4 mm diameter \times 2 mm deep) fabricated from Sylgard and gravity-perfused with ND96 at a constant flow rate (\sim 2 mL/min). The oocyte's membrane potential was held at -70 mV using an OC-725B two-electrode voltage clamp amplifier (Warner Instrument Corp., Hamden, CT). To evoke I_{ACh} , the perfusion of ND96 was replaced for one-second with ND96 containing 100 μ M ACh; such a pulse of ACh was applied once per minute. The peak of the ACh-gated current (I_{ACh}) was measured and the average of six consecutive I_{ACh} responses served as the control current response.

To minimize potential batch-to-batch variability, oocytes from the same isolation were used to compare the expression of receptors formed from unmodified and modified nAChR subunits. Furthermore, all recordings for a given comparison were performed on the same day.

Data analysis

Data are expressed as mean \pm SEM. Statistical comparisons between two groups were done using Student's t-tests, and those between multiple groups were done using ANOVA test with Tukey's post-hoc comparison.

Results

Human $\alpha 9\alpha 10$ nAChRs express poorly in *X. laevis* oocytes

Previous investigations of human and rat $\alpha 9$ -containing receptors reported difficulties in the expression of human $\alpha 9$ -containing receptors [38], [39]. Consistent with these reports, when cRNAs encoding human $\alpha 9$ and human $\alpha 10$ subunits of nAChRs were co-injected into oocytes at a 1:1 molar ratio, 100 μ M ACh produced small currents (Fig. 1A top), which on average were 30 ± 3 nA (Fig. 1B). Currents of this low magnitude are difficult to utilize for medium throughput pharmacological testing. In contrast, co-injection of rat $\alpha 9$ and rat $\alpha 10$ subunits yielded large currents (Fig. 1A bottom) with an average amplitude of 8067 ± 1638 nA (Fig. 1B). The difference in functional expression between rat $\alpha 9\alpha 10$ and human $\alpha 9\alpha 10$ nAChRs might be due to the inefficient translation of the human $\alpha 9$ or human $\alpha 10$ subunit or both, and this was explored in experiments described below.

Functional expression of $\alpha 9$ versus $\alpha 10$ subunits In order to assess the influence of $\alpha 9$ vs

$\alpha 10$ subunits on the functional expression of $\alpha 9\alpha 10$ nAChRs, we injected cRNA encoding subunits from different species (i.e., rat *versus* human) at a 1:1 ratio. When human $\alpha 9$ was co-expressed with rat $\alpha 10$, the current amplitude was invariably low in all three batches of oocytes tested, averaging from 5 ± 1 nA to 50 ± 15 nA (Fig. 2 and Table 1). When rat $\alpha 9$ was co-expressed with human $\alpha 10$, the current was readily detectable (Fig. 2 and Table 1) and at a level similar to that seen after co-injection of rat $\alpha 9$ with rat $\alpha 10$ subunits (Fig. 1A bottom and Fig. 1B); the average current amplitude ranged between 732 ± 155 nA and 9755 ± 596 nA, depending which of three batches of oocytes was used. There are at least two possible reasons for the low functional expression: A) rat $\alpha 10$ co-expressed with human $\alpha 9$ produced functionally impaired receptors or B) human $\alpha 9$ subunits are not translated efficiently in oocytes.

Inefficient translation of the human $\alpha 9$ subunit appears to limit assembly of functional human $\alpha 9$ /human $\alpha 10$ receptors

When cRNAs encoding human $\alpha 9$ and human $\alpha 10$ subunits were co-injected at a 1:1 ratio, the I_{ACh} rarely reached 1 μ A with the average response equal to 142 ± 23 nA. Oocytes injected with a 5:1 ratio had currents averaging 5171 ± 748 nA. Injections at a 1:5 ratio produced oocytes with low average I_{ACh} amplitude equal to 6.5 ± 3.9 nA (Fig. 3 and Table 2). Thus, more abundant cRNA for the $\alpha 9$ subunit leads to substantially enhanced functional expression of $\alpha 9\alpha 10$ nAChRs. This increased functional expression suggests that translation of the human $\alpha 9$ subunit is likely a limiting factor in the assembly of $\alpha 9\alpha 10$ receptors.

AMV insertion and expression of human $\alpha 9$ -containing nAChRs

Previous investigators have shown that incorporation of 5'UTR of the *Xenopus laevis* β -globin gene facilitates the *in vitro* translation of different proteins in oocytes and other expression systems [50], [51], [54]. In pGEMHE and pSGEM vectors the 5' leader

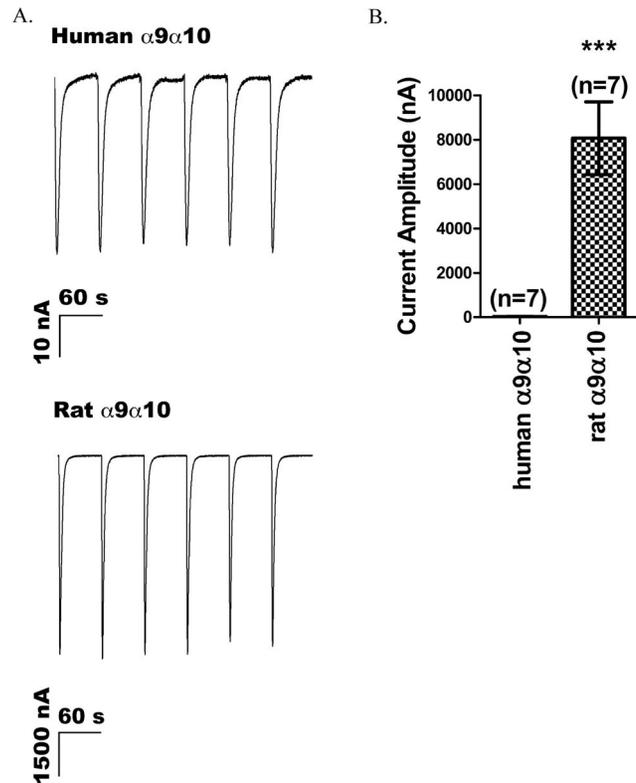


Figure 1. Comparison between the levels of exogenous expression of rat and human $\alpha 9$ -containing nAChRs in *X. laevis* oocytes. ACh-gated currents were measured in voltage-clamped oocytes as described in Methods. (A) Representative traces from an oocyte injected with human $\alpha 9$ and human $\alpha 10$ cRNA (top) and rat $\alpha 9$ and rat $\alpha 10$ cRNA (bottom). Robust currents were observed with rat cRNA; but only small currents were observed with human cRNA. (B) Comparison of the averaged current responses evoked by 100 μ M ACh applications from oocytes expressing human $\alpha 9\alpha 10$ and rat $\alpha 9\alpha 10$ receptors. The mean current amplitude was 30 ± 3 nA ($n = 7$ oocytes) for human $\alpha 9\alpha 10$ and 8067 ± 1638 nA ($n = 7$) for rat $\alpha 9\alpha 10$, $p < 0.005$. Error bars indicate SEM.
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sequence of the receptor subunit includes the 5'UTR of *X. laevis* β -globin, restriction sites of the vector's multiple cloning site, and the native 5'UTR of the subunit.

Plant viruses use host translational machinery for replication. RNAs of many plant viruses possess efficient translation enhancers [55] that can be used in order to improve the translation of recombinant proteins or expression of receptors in heterologous systems. Among such enhancers are untranslated regions from different viral RNAs. The 5'UTR from alfalfa mosaic virus RNA4, the 3'UTR of brome mosaic virus and the 5'leader of tobacco mosaic virus were shown to be able to enhance the mRNA translation of foreign proteins [56], [57], [58], [59], [60].

In an attempt to improve the translation of human $\alpha 9/\alpha 10$ we modified the 5'leader sequence of human $\alpha 9$ and human $\alpha 10$ subunits by introducing the 5'UTR of RNA4 of alfalfa mosaic virus (AMV) into the multiple cloning site of the pSGEM vector (Fig. 4B) between SacII and EcoRI sites, after the 5'UTR of β -globin and in front of the nAChR subunit.

The AMV incorporation improved the functional expression of human $\alpha 9$ homomeric receptors by 37- to 101-fold, and the human $\alpha 9\alpha 10$ heteromeric receptors by 41- to 250-fold, depending on the batch of oocytes used (Fig. 5 and Tables 3 and 4).

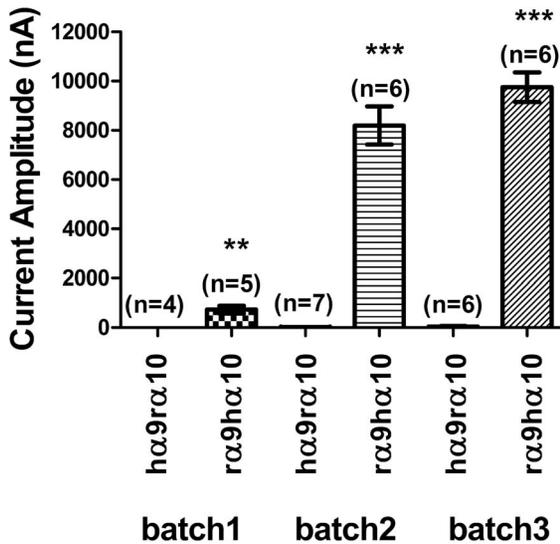


Figure 2. Comparison between the level of expression of human $\alpha 9$ /rat $\alpha 10$ (h $\alpha 9$ r $\alpha 10$) and rat $\alpha 9$ /human $\alpha 10$ (r $\alpha 9$ h $\alpha 10$) receptors. Receptors assembled from injection of cRNAs encoding subunits from different species have different levels of functional expression. h $\alpha 9$ r $\alpha 10$ nAChRs were expressed with low efficiency compared to r $\alpha 9$ h $\alpha 10$. Results from three batches of oocytes are shown. All oocytes of a given batch were injected on the same day and recordings performed 2 days later. Values of mean current amplitudes are given in Table 1. ** $p < 0.01$. Error bars indicate SEM. doi:10.1371/journal.pone.0064655.g002

Despite the variability in the expression levels of human $\alpha 9$ and $\alpha 9\alpha 10$ receptors, which is also commonly observed for other nAChRs, the large improvement in expression was highly reproducible.

Discussion

In this study, we determined that the functional expression of human $\alpha 9$ subunits of nAChRs in *X. laevis* oocytes depended on the composition of its 5' untranslated region. By introducing the 5' leader sequence of alfalfa mosaic virus RNA4 into the multiple cloning site of the pSGEM vector just preceding the coding region of human $\alpha 9$ or $\alpha 10$ subunits, we created a vector that gave ~70-fold higher expression levels of $\alpha 9$ homomeric receptors and ~80-fold higher expression levels of $\alpha 9\alpha 10$ heteromeric receptors compared to those achieved with unmodified vectors.

Since the early demonstration that mRNA encoding nicotinic receptors from *Torpedo californicus* electric organ could produce functional receptors when it is injected into oocytes of *X. laevis* [61], [62], oocytes have frequently been used as an exogenous expression system to study the pharmacology of nAChRs. In most cases, the receptor subunits assemble into functional receptors [36], [63], [64]. However, sometimes the cRNA injected into oocytes fails to yield functional receptors. For example, when cRNA encoding the $\alpha 6$ subunit is co-injected with cRNA encoding the $\beta 2$ or $\beta 4$ subunit, there is little or no detectable ACh-gated current [65]. In our laboratory, the unmodified cRNA of human $\alpha 9$ nAChRs failed to produce functional receptors. Other authors also reported difficulties in expressing human $\alpha 9$ -containing nAChRs [38], [39]. The ability of cRNAs of rat $\alpha 9$ and human $\alpha 10$ subunits, but not those of human $\alpha 9$ and rat $\alpha 10$, to form receptors with high levels of functional expression suggests that human $\alpha 9$ is a limiting factor in the assembly of functional receptors.

Table 1. Comparison of the functional expression of receptors following co-injection of cRNA for subunits of different species.^a

Oocyte Batch #	Receptor	Mean current amplitude (nA)	SEM	n
1	h $\alpha 9$ r $\alpha 10$	5	1	4
1	r $\alpha 9$ h $\alpha 10$	732	155	5
2	h $\alpha 9$ r $\alpha 10$	21	7	7
2	r $\alpha 9$ h $\alpha 10$	8200	774	6
3	h $\alpha 9$ r $\alpha 10$	50	15	6
3	r $\alpha 9$ h $\alpha 10$	9755	596	6

^aGraphical representations of these results are provided in Fig. 2. doi:10.1371/journal.pone.0064655.t001

There are several possible factors that can influence the level of functional expression of nAChRs in the *X. laevis* oocyte system. First, the cRNA composition might prevent or interfere with efficient translation. For example, formation of secondary structures may take place that prevent efficient binding of cap-binding proteins and initiation of translation [44]. The nucleotide sequence just preceding the start codon is important for translation initiation. In eukaryotes, the optimal sequence surrounding the start codon is GCCA/GCCaugG [66]. If the purine at the -3 position is changed to a pyrimidine, the efficiency of translation initiation might be reduced. Second, a high G+C content of

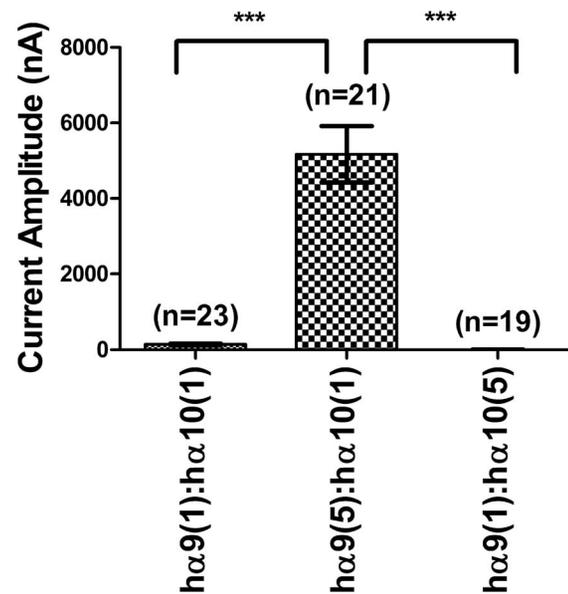


Figure 3. Comparison of functional receptor expression following injection of different ratios of receptor subunit cRNA. Differing subunit ratios of cRNA were injected into oocytes and the resulting levels of expression of functional receptors were compared. Recordings were performed 2 days after injection. The data from oocytes of four different batches were combined to determine the mean current amplitudes. Values are given in Table 2. A one-way ANOVA test with Tukey's post-hoc comparison indicated a significant difference between h $\alpha 9$ (1):h $\alpha 10$ (1) vs. h $\alpha 9$ (5):h $\alpha 10$ (1), $p < 0.001$, and between h $\alpha 9$ (5):h $\alpha 10$ (1) vs. h $\alpha 9$ (1):h $\alpha 10$ (5), $p < 0.001$. There was no significant difference between h $\alpha 9$ (1):h $\alpha 10$ (1) and h $\alpha 9$ (1):h $\alpha 10$ (5), $p > 0.05$. doi:10.1371/journal.pone.0064655.g003

Table 2. Comparison of the functional expression of receptors upon co-injection of different ratios of cRNA for specific subunit.^a

Receptor	Mean current amplitude (nA)	SEM	n
h $\alpha 9(1)$:h $\alpha 10(1)$	142	23	23
h $\alpha 9(5)$:h $\alpha 10(1)$	5171	748	21
h $\alpha 9(1)$:h $\alpha 10(5)$	6.5	3.9	19

^aGraphical representations of these results are provided in Fig. 3.
doi:10.1371/journal.pone.0064655.t002

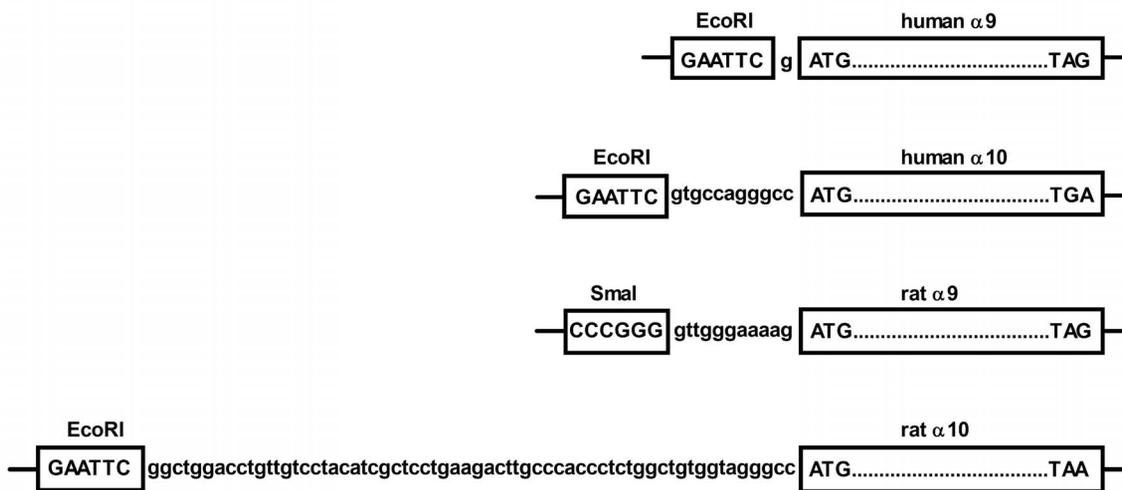
mRNA can halt efficient transcription and translation by formation of secondary structures. For example, the gene encoding human acetylcholinesterase (AChE) is highly G+C rich (65%) which results in the formation of a secondary structure in the 5' region [67] that serves as an attenuator of transcription. In addition, two highly homologous and highly G+C-rich genes encoding *Bungarus* and rat acetylcholinesterases have strikingly

different rates of transcription with approximately equal translation in oocyte functional tests [68]. The difference in the transcription rate is believed to be determined by the differences in the coding sequences.

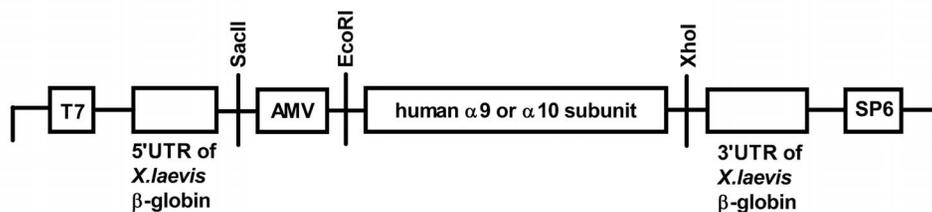
The G+C content of human $\alpha 9$ mRNA is 49 % for the gene-coding sequence compared to 51% for rat mRNA. $\alpha 10$ subunits are richer in G+C content with a 65% in the human and 59% in the rat subunit. Thus, the G+C content of human $\alpha 9$ is only slightly lower than its rat counterpart. Based on the relatively equal G+C composition of human and rat $\alpha 9$ mRNAs and high homology in nucleotide sequences of gene-coding regions it is unlikely that G+C content contributes to the low level of functional expression observed from unmodified human $\alpha 9$ subunit in our study.

The UTR is another factor influencing translational efficiency. It was shown to be important for the translation of different proteins in different expression systems. Mutations in the UTR affect the translation of aspartyl protease BACE1 protein and HT3A receptor [69], [70], [71]. When the 5'UTR of BACE1 is present, the protein, but not mRNA, level in transfected HEK293, COS7 and H4 cells is reduced as much as 90%. The inhibitory effect of the 5'UTR is due to the upstream open reading frame

A.



B.



AMV sequence: 5'-GTTTTTATTTTAAATTTCTTTCAAATACTTCCACC-3'

Figure 4. Comparison of the 5' untranslated regions in human $\alpha 9$, human $\alpha 10$, rat $\alpha 9$, and rat $\alpha 10$ subunits. (A) Native 5'UTRs of subunits are between the restriction site and the start codon. (B) The modifications made to the 5' untranslated region of human $\alpha 9$ and $\alpha 10$ subunits are shown. The 5'UTR of RNA4 of the alfalfa mosaic virus coat protein was inserted into the multiple cloning site of the pSGEM vector between SacII and EcoRI sites. The subunit-encoding sequence is between the EcoRI and XhoI sites.

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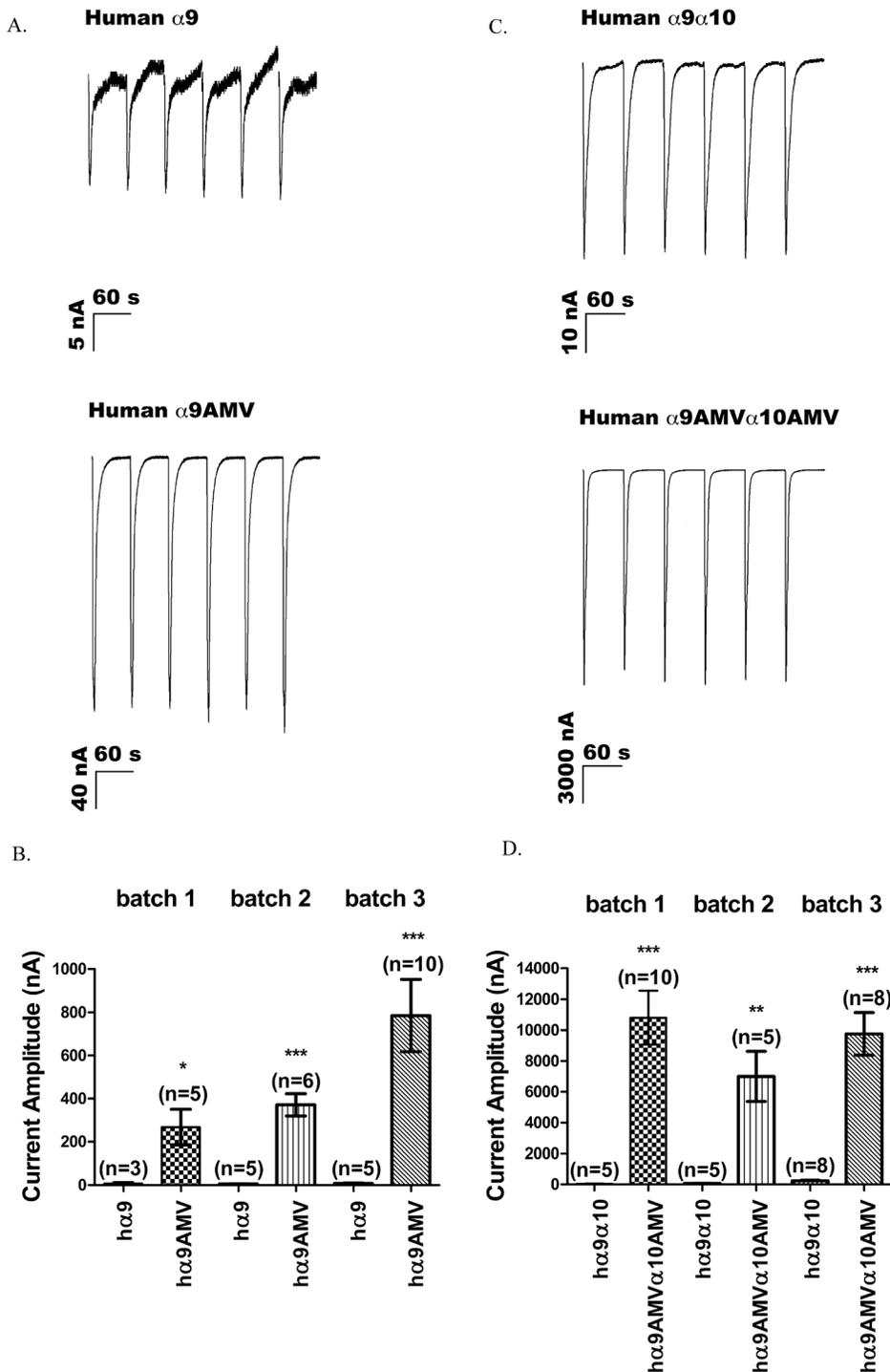


Figure 5. AMV improves the level of functional expression of $\alpha 9$ -containing nAChRs. (A) Representative traces and (B) comparisons of the levels of functional expression of homomeric human $\alpha 9$ receptors encoded by cRNA without (A, top) and with (A, bottom) AMV. The results are from three different batches of oocytes, each isolated from a different frog and recorded on 3rd day after injection, are presented. (C and D) Comparison of the level of expression of heteromeric receptors. Recordings were conducted on the second day after injection. Values for mean current amplitude are shown in Tables 3 and 4. * $p < 0.05$; ** $p < 0.01$. Error bars indicate SEM. doi:10.1371/journal.pone.0064655.g005

(uORF) [69], [71]. Due to their importance, the UTR regions are frequently modified to improve translation. For example, it became a common practice to include 5'- and 3'- UTRs of *Xenopus* β -globin into expression vectors to flank the gene-coding region [51]. UTRs of viruses have also been used to replace native

UTRs, which results in improved yields of translated proteins or improved functional expression of receptors. For example, the 5'UTR of tobacco mosaic virus enhances the translation of chloramphenicol acetyltransferase and β -glucuronidase in tobacco mesophyll protoplasts, *E. coli*, and *Xenopus* oocytes [56], [58], [59],

Table 3. Insertion of AMV improves the expression of human $\alpha 9$ homomeric receptors.^a

Oocyte Batch #	Receptor	Mean current amplitude (nA)	SEM	n
1	$\alpha 9$	7	4	3
1	$\alpha 9$ AMV	268	83	5
2	$\alpha 9$	5	1	5
2	$\alpha 9$ AMV	372	52	6
3	$\alpha 9$	8	2	5
3	$\alpha 9$ AMV	785	167	10

^aGraphical representations of these results are provided in Fig. 5.
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[72]. The facilitatory effect of the 5' leader is due to recruitment of eukaryotic initiation factor 4G indirectly via heat shock protein 101 [73].

The alfalfa mosaic virus is an RNA virus consisting of three genomic RNAs and one subgenomic RNA (RNA4). RNA1 and RNA2 encode the replicase proteins P1 and P2, whereas RNA3 encodes viral movement protein (MP). RNA4 is 881-nucleotides long, with a 661-nucleotide long coding sequence that encodes a coat protein required for infectivity and replication of the virus [74]. The 5' UTR of RNA4 is 39-nucleotides long, uracil rich and was shown to be able to improve the translation of foreign proteins. Computer-based structure prediction as well as nuclease-sensitivity analysis indicate the unstructured character of the 5' leader sequence, which can facilitate cap-independent translation initiation [75]. This fact might be relevant if cap-dependent translation initiation of unmodified human $\alpha 9$ subunit is disrupted. The substitution of the native 5' UTR with a 37-base-pair AMV RNA4 leader was shown to improve the translation of several proteins [58], [72]. For example, *in vitro* translation of human interleukin 1 β and barley α -amylase improved as much as 35-fold [59]. Also, the introduction of AMV into the 5' leader of GABA_A receptors improved the expression of those receptors in *X. laevis* oocytes [72].

The 5' UTRs of human nicotinic receptors may be an important factor for receptor function, considering evidence from other systems suggesting that this region could have the regulatory elements important for translation initiation [69], [76], [77]. Many human nAChR subunits have upstream uATG repeats (uATGs) and upstream open reading frames (uORFs). For example, human $\alpha 9$ has an uORF with a length of 36 codons. uORFs are involved in translational regulation of oncogenes by suppressing the level of translation [78], [79]. It is believed that the uORF causes the small ribosomal subunit to stall and therefore halt translation initiation [80]. How the uORF affects the translation of the $\alpha 9$ subunit is an open question. When cRNAs encoding nicotinic receptor subunits are injected into oocytes at a 1:1 molar ratio, it is assumed that the two subunits will be translated with equal efficiencies so the amount of protein of the two subunits will also be produced in a 1:1 ratio. However, different receptor subunits might be translated with different efficiencies.

mRNA stability might be a contributing factor to the observed different levels of expression between unmodified human $\alpha 9$ -containing receptors and rat $\alpha 9$ -containing receptors. One of the factors that determines the stability of mRNA is located within 3'-end of mRNA. In particular, the poly(A) tail is required to ensure high functional stability of the mRNA as was shown for rabbit

Table 4. AMV improves the expression of human $\alpha 9\alpha 10$ heteromeric receptors.^a

Oocyte Batch #	Receptor	Mean current amplitude (nA)	SEM	n
1	h $\alpha 9\alpha 10$	43	6	5
1	h $\alpha 9$ AMV $\alpha 10$ AMV	10813	1739	10
2	h $\alpha 9\alpha 10$	60	11	5
2	h $\alpha 9$ AMV $\alpha 10$ AMV	6999	1627	5
3	h $\alpha 9\alpha 10$	237	41	8
3	h $\alpha 9$ AMV $\alpha 10$ AMV	9763	1379	8

^aGraphical representations of these results are provided in Fig. 5.
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globin protein [81], [82]. The 3' UTR of the human $\alpha 9$ subunit had a short (6 nucleotides) native 3' UTR, followed by the 3' UTR of *Xenopus* β -globin, followed by a poly(A) tail. In contrast, the modified construct incorporated the 5' UTR of the alfalfa mosaic virus RNA4. This addition may slow degradation of the mRNA.

Another factor that may contribute to the fast turnover of mRNA is an AU-rich region at 3'-untranslated region. Many RNA-binding proteins such as ELAV-like proteins (HuD, Hel-N1, HuC, HuR) bind to AU-rich regions at the 3'-untranslated region of RNA and prevent degradation of mRNA [83]. Human $\alpha 9$ as well as rat $\alpha 9$ subunit 3' UTRs have six non-overlapping AUUUA motifs separated by non-AU nucleotides in a U-poor region. In addition, they have one AAAUUUAAAA motif.

A second possibility for low expression level of receptors in oocytes is the lack of posttranslational modifications in the oocyte expression system. The possible posttranslational modifications of nAChRs include proteolytic cleavage, disulfide bond formation, glycosylation, palmitoylation, fatty acid acylation, phosphorylation, amidation, hydroxyproline, proline isomerization, etc. [84], [85], [86], [87]. The lack of functional expression of $\alpha 6$ -containing receptors is likely due to posttranslational mechanisms, insofar as functionality is achieved when the C-terminus of the $\alpha 6$ subunit is replaced with the C-terminus of an $\alpha 3$ subunit implying that important regulatory elements for efficient receptor function are located outside of ligand-binding domain [65].

A third possibility is the lack of appropriate chaperones in oocytes. There are several chaperones described for nicotinic receptors such as BiP, calnexin, Erp57, and RIC3 [88], [89], which facilitate proper folding and improve functional expression of receptors. Nicotine exposure causes an upregulation of nicotinic AChRs in brain as well as *in vitro*, and a possible explanation of this effect is through the chaperoning by nicotine [90], [91], [92]. The RIC-3 is a chaperone that upregulates the expression of $\alpha 7$ nAChRs in oocytes [93], [94], [95], [96]. Interestingly, RIC-3 has no effect on the expression of $\alpha 9$ receptors [40], [97].

There are few reports of successful expression of $\alpha 9$ receptors in mammalian cells [98]. GH4C1 cell line derived from pituitary gland was successfully transfected with rat $\alpha 9\alpha 10$ receptors [41]. Here, the average ACh-evoked currents ranged between 16 pA to 300 pA. Also, an $\alpha 9$ /HT3a chimera, where the N-terminus of rat $\alpha 9$ was fused to the C-terminus of mouse HT3a receptor, produced functional receptors [99]. Mouse $\alpha 9\alpha 10$ receptors were successfully transfected into HEK293 cells [98]. The problem of the lack of expression of human $\alpha 9$ receptor in mammalian cell lines was addressed in several reports [40], [98], [99], [100]. It was shown that co-transfection of human $\alpha 9$ and $\alpha 10$ subunit with AChR-associated proteins rapsyn and chaperone RIC-3 in CL4

cells increased the cytosolic calcium level after application of 100 μ ACh but no measurements of ionic current from $\alpha 9$ -containing receptors were reported [100]. It is still an open question as to whether the lack of functionality in mammalian cells is due to inefficient transcription, translation, improper folding, and lack of chaperoning or posttranslational modifications or a combination of these.

In our current study, we observed the effect of the 5'UTR of the human $\alpha 9$ subunit on the expression of functional receptor. We conclude that the inefficient expression of human $\alpha 9$ -containing receptors can be improved by modifying 5'UTR of the cRNA encoding the subunit. It is possible that the initiation codon of the original unmodified subunit is in unfavorable form such that the small ribosomal subunit fails to associate with the RNA. By including the 5'UTR of RNA4 of alfalfa mosaic virus, we were able to construct an RNA, which when expressed in *X. laevis* oocytes, can be used to screen new ligands which bind to the $\alpha 9^*$ receptor (* denotes possibility of other subunits). The reasons for the poor ability of $\alpha 9$ receptors (both rat and human) to be expressed in the mammalian cells still remain to be explored.

Transcriptional and translational mechanisms are likely involved in regulation of human and rat $\alpha 9$ subunit expression in native tissues. In the rat adrenal medulla expression levels of $\alpha 9$, $\alpha 3$, and $\alpha 7$ subunits were determined by quantitative PCR [19] and the level was lowest for the $\alpha 9$ subunit. However, the same study showed that transcription of $\alpha 9$, but not $\alpha 3$ and $\alpha 7$ subunits, is upregulated in response to stress. Regulation of transcription and translation of nAChRs may also be relevant in the context of smoking. The concentration of nicotine in active smoker plasma can be 100 nM to 1 μ M. Chronic exposure to nicotine leads to activation and desensitization of nAChR subtypes including $\alpha 4\beta 2$

and $\alpha 7$. As a result, the level of expression of $\alpha 4\beta 2$ nAChRs is increased in the brain [101]. Smoking is also associated with carcinogenesis, and nicotine-derived metabolites NNK and NNN are considered carcinogenic in lung, breast, and bladder cancers. $\alpha 9$ receptors mediate cell proliferation of breast cancer cells, and increased $\alpha 9$ nAChR subunit mRNA levels were observed in breast tumor tissues [102]. Moreover, $\alpha 9$ -nAChR mRNA expression was higher in advanced-stage tumors. It was also shown that nicotine upregulates the mRNA as well as protein level for $\alpha 9$ receptors in breast tumor tissue [102]. The mechanism by which nicotine treatment leads to this upregulation remains elusive. $\alpha 9$ subunit expression seems to be important for cell proliferation, therefore, the mechanisms, whether transcriptional or translational, that control subunit expression might open exciting new avenues for control of tumorigenesis.

Our findings suggest the involvement of 5'-untranslated region in the efficient expression of human $\alpha 9$ -containing receptors in oocytes. It remains to be investigated whether 5'untranslated region contributes to the regulation of translation of $\alpha 9$ subunit *in vivo*.

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Author Contributions

Conceived and designed the experiments: OF JMM. Performed the experiments: OF. Analyzed the data: OF JMM. Contributed reagents/materials/analysis tools: JMM. Wrote the paper: OF JMM.

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