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# The nicotinic acetylcholine receptors of zebrafish and an evaluation of pharmacological tools used for their study

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ABSTRACT

Zebrafish (*Danio rerio*) have been used to study multiple effects of nicotine, for example on cognition, locomotion, and stress responses, relying on the assumption that pharmacological tools will operate similarly upon molecular substrates in the fish and mammalian systems. We have cloned the zebrafish nicotinic acetylcholine receptor (nAChR) subunits and expressed key nAChR subtypes in *Xenopus* oocytes including neuronal ($\alpha4\beta2$, $\alpha2\beta2$, $\alpha3\beta4$, and $\alpha7$) and muscle ($\alpha1\beta1_b\varepsilon\delta$) nAChR. Consistent with studies of mammalian nAChR, nicotine was relatively inactive on muscle-type receptors, having both low potency and efficacy. It had high efficacy but low potency for $\alpha7$ receptors, and the best potency and good efficacy for $\alpha4\beta2$ receptors. Cytisine, a key lead compound for the development of smoking cessation agents, is a full agonist for both mammalian $\alpha7$ and $\alpha3\beta4$ receptors, but a full agonist only for the fish $\alpha7$, with surprisingly low efficacy for $\alpha3\beta4$. The efficacy of cytisine for $\alpha4\beta2$ was somewhat greater than typically reported for mammalian $\alpha4\beta2$. The ganglionic blocker mecamylamine was most potent for blocking $\alpha3\beta4$ receptors, least potent for $\alpha7$, and roughly equipotent for the muscle receptors and the $\beta2$-containing nAChR. However, the block of $\beta2$-containing receptors was slowly reversible, consistent with effective targeting of these CNS-type receptors in vivo. Three prototypical $\alpha7$-selective agonists, choline, tropane, and 4OH-GTS-21, were tested, and these agents were observed to activate both fish $\alpha7$ and $\alpha4\beta2$ nAChR. Our data therefore indicate that while some pharmacological tools used in zebrafish may function as expected, others will not.



## 1. Introduction

The nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction was the first molecular mediator of electro-chemical synaptic transmission to be studied in detail and ultimately isolated and cloned. Muscle-type nAChR are pentameric assemblies of subunits identified as $\alpha1$, $\beta1$, $\delta$, and $\varepsilon$ or $\gamma$, with each receptor containing two $\alpha1$ subunits. The mediators of nicotine's effects in the brain and autonomic nervous system were subsequently identified [1]. These neuronal nAChRs are also pentameric ligand-gated cation channels. They mediate synaptic transmission in autonomic ganglia and modulate synaptic and cell function in the central nervous system. Individual genes code for subunits of two general classes, based on the presence or absence of a key structural element in the $\alpha1$ subunit of muscle-type receptors, a pair of adjacent cysteines in the ligand-binding subdomain. Neuronal nAChR beta subunits lack this feature. Functional neuronal nAChR subunits can be further classified into two major subfamilies: homomeric receptors with $\alpha7$ and $\alpha9$ subunits that may function without beta subunits, and heteromeric receptors which are assemblies of $\alpha$ ($\alpha2$–$\alpha6$) and $\beta$ ($\beta2$–$\beta4$) subunits [2]. Heteromeric receptors constitute the high-affinity binding sites for nicotine in the nervous system.

Neuronal nAChRs are often located presynaptically in the CNS and modulate release of important neurotransmitters such as norepinephrine, serotonin, gamma aminobutyric acid, glutamate, and dopamine [3]. Signaling through nAChRs regulates and influences neural functions including several aspects of cognition and is involved in pathways mediating drug dependence and addictive behaviors [2]. nAChRs are also involved in schizophrenia, attention deficit hyperactivity disorder, Alzheimer's, Tourette's, Parkinson's, autism, and epilepsy [2].

Neuronal nAChRs have been studied in animal models including non-human primates, rats, and mice. Although the specific nAChR subtypes involved in many of the various functions and diseases described above are not known for certain, studies of nAChR

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knockout mice have provided valuable leads, especially in regard to nicotine addiction. In many cases the in vivo pharmacology described in animal studies has been validated through the in vitro study of cloned receptor subunits in expressions systems such as the *Xenopus* oocyte [1]. As an outcome of these many research studies, numerous clinical studies have also been made or are presently underway for the indications noted.

Recently zebrafish (*Danio rerio*) have also been used to study the role of nAChRs in several behaviors including locomotor and stress responses, and cognitive and exploratory behaviors [4]. Zebrafish can be used for the study of nAChRs' role in normal development and the effects of nicotine on developing embryos [5]. This new information about zebrafish nAChRs, and the advantages of the zebrafish system, provide an opportunity to develop and test therapeutic agents targeted to neuronal nAChRs. Zebrafish have been used in some behavioral assays similar to those used with other vertebrates. Five-day-old zebrafish possess locomotor and simple sensory capabilities, while older zebrafish exhibit additional behaviors, such as feeding and escape [5]. Zebrafish are amenable to relatively high throughput screening approaches to test compounds for effects on learning, memory, and anxiety. Zebrafish have been used to examine the anxiolytic effects of nicotine [6], spatial discrimination learning [7], and delayed spatial alternation [8]. Zebrafish are being used to screen for potential neuroprotective compounds in a model of Parkinson's [9]. The advantages of zebrafish for pharmaceutical screening [10,11] can be exploited to complement existing cell culture and mouse studies to test and develop new cholinergic therapeutic compounds.

We have cloned eight zebrafish neuronal nAChR cDNAs ($\alpha$2, $\alpha$3,$\alpha$4, $\alpha$6, $\alpha$7, $\beta$2, $\beta$3, $\beta$4) [12,13] and zebrafish muscle nAChR subunit cDNAs [14]. These are largely expressed in regions analogous to structures in mammals. In order to interpret the behavioral studies and to lay the groundwork for possible use of zebrafish for high throughput screening, and as a model to study nAChRs, the basic pharmacological properties of the major nAChR subtypes present in zebrafish should be determined.

In this study we have expressed zebrafish neuronal $\alpha4\beta2$, $\alpha2\beta2$, $\alpha$7, and $\alpha3\beta4$ nAChRs and a muscle nAChR, $\alpha1\beta1b\varepsilon\delta$, in *Xenopus* oocytes and determined the $EC_{50}$s for acetylcholine, nicotine, and cytisine and the $IC_{50}$ of mecamylamine for each subtype. We have also conducted preliminary tests of additional $\alpha$7-selective compounds on each subtype (alone or with ACh) to determine whether these compounds may act similarly in zebrafish compared to other animal models.

## 2. Materials and methods

### 2.1. Zebrafish maintenance

Zebrafish colonies were maintained at 28 °C in stand-alone self-circulating systems following the guidelines of IACUC at the Ohio State University and the NIH/NIAAA.

### 2.2. Cloning of zebrafish nAChR cDNAs

RNA was isolated from zebrafish embryos using Trizol (Invitrogen, Eugene, OR). We first used polymerase chain reaction (PCR) with degenerate PCR primers in combination with 5′ and 3′ RACE (First Choice RLM RACE (Ambion, Austin, TX) to isolate partial 5′ and 3′ cDNAs encoding zebrafish neuronal nAChR $\alpha$4, $\alpha$2, $\beta$2, $\alpha$7, $\alpha$3, and $\beta$4 subunit cDNAs. The primers were based on conserved TM3 and TM 4 sequences present in human, rat, mouse, bovine, and chick nAChRs. Alignments were made and PCR primers designed to the most conserved regions. Information in the Sanger Centre Zebrafish Genome database (http://www.sanger.ac.uk) was also used to design gene-specific 5′ and 3′ RACE primers used in the cloning of some of the cDNAs. Reverse transcription using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen) followed by PCR with Platinum *Pfx* polymerase (Invitrogen) was used to isolate full-length nAChR clones using primers designed based on information obtained from the 5′ and 3′ RACE clones. The cDNAs were then cloned into PCRII TOPO vectors and sequenced. The muscle type nAChR subunits $\alpha$1, $\beta$1b, $\varepsilon$, and $\delta$ were cloned as previously described [14].

After linearization and purification of cloned cDNAs, RNA transcripts were prepared in vitro using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX).

### 2.3. In situ hybridization

In situ hybridization was performed as described in Ikenaga et al. [15]. The probes for $\beta$1a and $\beta$1b were 430 bps and 491 bps long, respectively. Regions for hybridization including 3′UTR were selected based on the low homology. Specifically, probe sequences were amplified by performing PCR with GGGTTGTTTGGAAAATAGCCTCAGA and TAGCGTCCGTCCACAGAGAGTACAG for $\beta$1a, and GGACTGGCAGTATGTTGCTATGGTG and GGGTAATTAGGCAAACCATAGTATAATGA for $\beta$1b.

### 2.4. Quantitative PCR

Total RNA was purified from pools of 20 embryos/larvae for each time point, 1, 2, 3, 8, and 21 days post fertilization (dpf), using RNeasy Micro (Qiagen, Valencia, CA). Total RNA was DNAse treated with TURBO DNAse (Ambion, Austin, TX). Following treatment, first-strand cDNA was synthesized using iScript cDNA Sythensis Kit (BioRad, Hercules, CA). Primers and probes, containing a 5′-6-FAM and a 3′-Black Hole Quencher-1, were synthesized by Integrated DNA Technologies (Coralville, IA). Primers corresponding to sequences in neighboring exons and a probe encompassing the exon junction were selected in order to eliminate amplification from the genomic DNA. Primers and probes were designed as follows:

$\beta$1a: forward 5′-aacttactgcctcgctacttgggt-3, probe 5′-aggaaccagtggaggaagagccaaa-3′, reverse 5′-acagtgctctcgttatggcttcct-3′;
$\beta$1b: forward 5′-ttcgtgcggagtgaaggtgacata-3′, probe 5′-agaagtggatcttcaacatgccctgg-3′, reverse 5′-actctcgctaaagcctgtgtccaa-3′;
Elongation factor1-$\alpha$ (elf1-$\alpha$): forward 5′-ttgatgcccttgatgccattctgc-3′, probe 5′-attggaactgtacctgtgggtcgtgt-3′, reverse 5′-acaaccataccaggcttgaggaca-3′.

Elf1-$\alpha$ was used as an endogeneous control for all runs. TaqMan Fast Polymerase (Applied Biosystems, Carlsbad, California) was used in all experiments. Cycling conditions were as follows: an initial hold at 95 °C for 20 s, followed by 40 cycles at 95 °C for one second and at 60 °C for 20 s. Results are an accumulation of 3–4 biological repeats with 3 internal repeats per run. Negative controls were run with each sample set. All qPCR runs were carried out using the StepOnePlus Real-Time PCR system (ABI, Foster City, CA).

Absolute transcript copy numbers were determined using the standard curve method. Quantification of known amounts of target DNA was used to establish a comparative for unknown transcript levels of specific subunits. From the standard curve, quantification of the transcript number was determined. Numbers were normalized to the endogenous control (elf1-$\alpha$) to account for variations in concentration of starting templates. Normalized absolute numbers, from multiple runs, were plotted in Fig. 2C.

### 2.5. Expression of CFP-conjugated $\beta$1s in zebrafish myocytes

$\beta$1a and $\beta$1b clones were modified so that they had in-frame insertions of Cyan Fluorescent Protein (CFP) in the III-IV