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Activation of $\alpha 2A$ -Containing Nicotinic Acetylcholine Receptors Mediates Nicotine-Induced Motor Output in Embryonic Zebrafish

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Abstract

It is well established that cholinergic signaling has critical roles during central nervous system development. In physiological and behavioral studies, activation of nicotinic acetylcholine receptors has been implicated in mediating cholinergic signaling. In developing spinal cord, cholinergic transmission is associated with neural circuits responsible for producing locomotor behaviors. In this study, we investigated the expression pattern of the $\alpha 2A$ nAChR subunit as evidence from others suggested it could be expressed by spinal neurons. In situ hybridization and immunohistochemistry revealed that the $\alpha 2A$ nAChR subunits are expressed in spinal Rohon-Beard (RB) neurons and olfactory sensory neurons in young embryos. In order to examine the functional role of the $\alpha 2A$ nAChR subunit during embryogenesis, we blocked its expression using antisense modified oligonucleotides. Blocking the expression of $\alpha 2A$ nAChR subunits had no effect on spontaneous motor activity. However, it did alter the embryonic nicotine-induced motor output. This reduction in motor activity was not accompanied by defects in neuronal and muscle elements associated with the motor output. Moreover, the anatomy and functionality of RB neurons was normal even in the absence of the $\alpha 2A$ nAChR subunit. Thus, we propose that $\alpha 2A$ -containing nAChR are dispensable for normal RB development. However, in the context of nicotine-induced motor output, $\alpha 2A$ -containing nAChRs on RB neurons provide the substrate that nicotine acts upon to induce the motor output. These findings also indicate that functional neuronal nAChRs are present within spinal cord at the time when locomotor output in zebrafish first begins to manifest itself.

Keywords

morpholino; behavior; Rohon-Beard neuron; spinal cord

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INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are distributed throughout the developing vertebrate central nervous system (Gotti and Clementi, 2004) and although much is known about the receptor structure and response kinetics (reviewed in Kalamida et al., 2007), there is still a need to better understand their functional role in behavioral processes. In the developing vertebrate spinal cord, cholinergic neurotransmission is associated with neuronal circuits producing locomotion which suggests the presence of nAChRs in spinal neurons (chick, Hanson and Landmesser, 2003; mouse, Zagoraïou et al., 2009; *Xenopus*, Perrins and Roberts, 1995; lamprey, Quinlan et al., 2004; zebrafish, Thomas et al., 2009). In *Xenopus*, motoneurons receive cholinergic excitation from other adjacent motoneurons or interneurons (Perrins and Roberts, 1995). In mice, when choline acetyltransferase activity is abolished, locomotor output is impaired (Myers et al., 2005).

The relatively simple spinal cord organization and well-characterized locomotor behaviors of zebrafish have garnered much attention in the recent years with researchers moving rapidly toward using locomotor output as a diagnostic read-out in large-scale genetic or chemical screens (Granato et al., 1996; Petzold et al., 2009). Exposure of zebrafish embryos to nicotine or even the simple act of removing the embryo from its protective chorion can activate a rhythmic motor output (Saint-Amant and Drapeau, 1998; Thomas et al., 2009). This indicates that an organized circuit is present very early in development which can produce a rhythmic locomotor output when appropriately activated by an excitatory drive. Since zebrafish embryos respond to nicotine even in the absence of supraspinal inputs (Thomas et al., 2009), we hypothesized that spinal neurons associated with the production of motor output would express functional nAChRs.

To investigate the role of specific nAChRs in modulating behavioral responses, a description of the spatiotemporal expression pattern of subunit and receptor subtypes was required before probing for functional nAChRs. Recent studies in zebrafish have provided important information regarding mRNA expression profiles of the $\alpha 2$, $\alpha 4$, $\alpha 6$, $\alpha 7$ and $\beta 3$ nAChR subunits during embryogenesis (Zirger et al., 2003; Ackerman et al., 2009). Based on those described mRNA expression patterns, we focused on the $\alpha 2A$ subunit (initially described as $\alpha 2$ but has now been renamed to $\alpha 2A$) because it was shown to localize to distinct neurons within spinal cord (Zirger et al., 2003). In mammalian systems, the nAChR $\alpha 2$ mRNA is localized in 19% of dorsal spinal neurons (Cordero-Erasquin et al., 2004), in small-to-medium size cells in the rat ventral spinal cord (Ishii et al., 2005), and in human fetal lumbar motor neurons (Keiger et al., 2003).

In this study, we first investigate the expression profile of the $\alpha 2A$ nAChR subunit in embryonic zebrafish. We then used nicotine-induced motor output as a diagnostic tool to probe for functional nAChRs in the developing zebrafish spinal cord. Using behavioral analysis in conjunction with morpholino antisense technology and anatomical methods, we show that the zebrafish Rohon-Beard (RB) neurons express the $\alpha 2A$ nAChR subunit, which are likely incorporated into functional receptors. We propose that RB neurons can be directly activated by nicotine to increase motor output in zebrafish embryos.

MATERIALS AND METHODS

Zebrafish maintenance

Animal protocols were approved by the Louisiana State University, Oregon State University, and University of Wisconsin-Milwaukee Institutional Animal Care and Use Committees. Adult wildtype (EkkWill) and transgenic (Tg(*isl2b*):GFP) zebrafish were maintained at 28°C with a lighting schedule of 14 h light and 10 h dark. Embryos were collected immediately after spawning from individual pairs and then placed into 100 mm Petri dishes containing embryo medium prior to microinjection procedures. For some experiments (only when indicated), embryos were raised in embryo medium containing 0.002–0.0045% phenylthiourea (Sigma, St. Louis, MO, USA) after 24 hours post fertilization (hpf) to prevent pigment formation. The Tg(*isl2b*):GFP zebrafish line was kindly provided by the late Chi-Bin Chien and Andrew Pittman at the University of Utah, School of Medicine.

Morpholino design

Morpholino antisense oligonucleotides (MOs) were synthesized by Gene Tools (Philomath, OR, USA). One MO was designed to target the predicted translation site of the nAChR $\alpha 2$ subunit and had the following sequence: 5'-GGATTTCGCCATGTCCAGCGTC-3'. A second MO that targeted the splice junction site at the exon2-intron2 boundary was also synthesized and had the following sequence: 5'-ATGCAAAGTATCAACTTACCACATC-3'. The $\alpha 2A$ MOs were fluorescently tagged with fluorescein at the 3' end. A standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a control and was not tagged with fluorescein. For all morpholino experiments, a stock MO was diluted to 3 mM in 1× Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6).

In the initial phases of this project, non-injected control embryos were compared to control MO-injected embryos and very similar results were obtained for both groups of embryos (Supplement Fig. 1B, left). Once we confirmed that injection of the control morpholino was not having any overt effects on the embryo, we focused on comparisons between control MO and $\alpha 2A$ MO-injected embryos. In addition, two different $\alpha 2A$ morpholinos were initially used, a splice block (SB) morpholino and a translation block (TB) morpholino. Both of them consistently produced the same behavioral phenotype (Supplement Fig. 1B, right; and Supplement Fig. 2B) as described in detail below. After demonstrating that both of these morpholinos produced very similar results, we used the splice block morpholino as its efficacy can be confirmed by RT-PCR along with antibody labeling. All the experiments presented herein were performed using the splice blocking $\alpha 2A$ MO unless otherwise noted. For simplicity, it will be referred to as $\alpha 2A$ MO from here onward.

Morpholino microinjections

Microinjection needles were pulled on a horizontal micropipette puller (Flaming/Brown P-97, Sutter Instruments, Novato, CA, USA) using fire-polished, filamentous borosilicate glass (outer diameter of 1.2 mm, Sutter Instruments). Morpholino solutions were prepared

fresh prior to the microinjection procedure. Control MO was injected at 0.25–1 mM in 0.1% phenol red (Sigma) and 125 ng/μL rhodamine-conjugated dextran. Fluorescently tagged α2A MOs were injected at 0.1–2 mM in 0.1% phenol red. Each solution was loaded into a micropipette needle and was then injected in the yolk stream of 1–2 cell stage zebrafish embryos. Following microinjection of the morpholinos, the embryos were individually screened at 5–6 hpf looking for fluorescence at the animal pole using epi-fluorescence and a 10x objective on an inverted microscope (Zeiss Axiovert 200M) using minimum light intensity (See Supplement Fig. 1A). Embryos lacking fluorescence were removed and thus not used in subsequent experiments.

Reverse transcription polymerase chain reaction (RT-PCR)

Embryos injected at the 1–2 cell stage with antisense oligonucleotide morpholino directed against the nAChR α2A subunit (α2A MO) or control morpholino (control MO) were collected at 24 and 48 hpf. Embryos (> 20) were directly immersed into TRI reagent (Invitrogen, Carlsbad, CA, USA) and RNA was isolated as described previously (Tanguay et al., 1999). Reverse transcription reactions were carried out using 1 μg of total RNA and the Superscript II Reverse Transcriptase Kit as per the manufacturer's instructions (Invitrogen). Each 25 μl PCR reaction contained 4 μl cDNA; 0.75 μl 10 mM dNTP; 2.5 μl 10X PCR buffer (Fermentas, Glen Burnie, MD, USA); 2 μl 25 mM MgSO₄; 2 μl of 3.75 μM of each primer (CATCATGTCTGACCACTGTACAGC; GAGCCGAACCTTCATCTTGCAG) for nAChR-α2A; 0.25 μl 500 U Taq DNA Polymerase (Fermentas); and 13.5 μl of nuclease-free water. The reactions were run in a PCR Express Thermal Cycler (Thermo Hybaid) at the following conditions: 95°C for 3 min, then 95°C for 20 s, 62°C for 30 s, and 72°C for 2 min, for a total of 30 cycles followed by 72°C for 5 min. The PCR products were resolved by electrophoresis through a 2% agarose gel and visualized by ethidium bromide staining.

Nicotine experiments

The (–)-nicotine used in this study was purchased from Sigma (St. Louis, MO, USA, catalog # N3876-5ml) and stock solutions were made fresh daily as needed in distilled water. The stock solution was diluted in embryo medium (pH 7.2) to obtain the desired final concentration (3–300 μM; most experiments were performed at 60 μM). The 60 μM concentration was used because it reliably evoked a large magnitude motor output even when the embryo was in the chorion.

Behavioral analysis

Embryos were placed in embryo media in 35 or 50 mm Petri dishes and videotaped with a Cohu video camera mounted to a Zeiss Stemi 2000-C dissecting microscope. The baseline spontaneous motor output (spinal musculature bends) for control and α2A MO-injected embryos (referred to as α2A morphants from here on) while in their chorions was recorded for 3–5 minutes. The embryos were then transferred into nicotine solution (60 μM, majority of experiments) while still in their chorions and the motor output was recorded again for 3–5 minutes. The motor activity was quantified as the number of bends in a one-minute epoch (bend rate) at different developmental time points between 20–28 hpf (embryos analyzed: non-injected controls, n=179; control MO, n=263; α2A TB MO, n=71; α2A SB MO, n=339). Behavior at each developmental time point was reproduced at least twice. The

number of embryos reported in the legends of the behavioral figures is for that particular experiment. Two individuals interchangeably performed the behavioral quantification and one of the two evaluators was always blinded for the quantification.

For the dechoriation experiments, 22 or 24 hpf embryos (while in their chorions) were placed in nicotine solution (60 μ M) for only one minute and their motor output was recorded. Following the one minute nicotine exposure, they were quickly transferred to fresh embryo media for a 20 min wash period. Then they were manually dechorionated using fine forceps and their motor output was videotaped for 3 minutes. The number of bends per minute was quantified at 22 and/or 24 hpf (3 experiments, non-injected control, n=16; control MO, n=26; α 2A MO, n=26). For the titration experiments of the α 2A MO the baseline bend rate was recorded for 3 minutes followed by nicotine application for another 3 minutes. We calculated across all embryos the mean baseline bend rate (B) over the 3-minute epoch and the mean nicotine response (N) at the first minute following nicotine application for control MO (B_{CON} , N_{CON}) and for α 2A MO-injected embryos (B_{MO} , N_{MO}). For any given α 2A MO concentration injected, the nicotine response as a percentage (%) of control MO was calculated as follows: $((N_{MO} - B_{MO}) / (N_{CON} - B_{CON})) \times 100$.

The embryo's response to tactile stimuli (touch response) was also assessed by mechanically probing the embryo trunk at 31 hpf for a total of 10 trials (time between trials ~5–10 seconds). Responses were scored as follows: 0, no response (no trunk bend); 1.0 normal response (trunk bend). The behavioral scores from the 10 trials were summed for each fish and the average touch response was taken from all the fish to obtain a touch response index (4 experiments, non-injected control, n=25; control MO, n=39; α 2A MO, n=46). All behavior experiments were carried out at ~25–26°C.

Generation of zebrafish α 2A nAChR antibody

The human nAChR α 2 nucleotide sequence was queried in GenBank to identify the zebrafish nAChR α 2A ortholog (accession number NM_001040327). ClustalW2 was used to align the known human and zebrafish nAChR and the α 2A subtype specific peptide (FMRRPEPEKKPKKTA) was designed which spanned positions 357–371 of the zebrafish protein. The KLH-conjugated peptide was injected into rabbits to generate a zebrafish-specific anti-nAChR α 2A polyclonal antibody (referred to as α 2A from here on) (Antibodies Inc., Davis, CA). Details on the antibody production follow.

The peptide was conjugated to KLH using sulfo SMCC from Sigma (Lot number 439-1RG3). This particular conjugation works by 'activating' an NH₂ group on the carrier which then is covalently linked to the sulfhydryl group on the cysteine of the peptide. After conjugation, the peptide was injected subcutaneously into the rabbit. Prior to the injection, pre-bleed serum was collected from the rabbit. On day 1; 500 μ g of conjugate in Complete Freund's Adjuvant /PBS was injected in the animal. On day 14, a boost injection with 300 μ g of conjugate in Incomplete Freund's adjuvant /PBS was given and another boost injection as above was given on day 21. On day 21, a test bleed was collected and this test bleed was analyzed in immunohistochemistry (IHC) and compared to the pre-bleed serum; also with IHC analysis. The animal was boosted again on days 35 and day 49 and a production bleed was collected. That production bleed was again tested in our IHC protocols. On day 106, a

last boost injection was given and the final production bleed was collected on day 114 of the production scheme.

Characterization of the $\alpha 2A$ antibody epitope

In this section and in the subsequent Table 1, we exclusively confer with the genome database terminology for nicotinic acetylcholine receptors (Chrn). However, in the following sections of the study, nicotinic acetylcholine receptors will be mainly referred to as nAChRs. The ninth, and most current, integrated assembly of the zebrafish genome (Zv9; http://ensembl.org/Danio_rerio) contains 17 neuronal-type nicotinic acetylcholine receptor (Chrn) transcripts: Chrn $\alpha 1$ (ENSDART20261), Chrn $\alpha 2a$ (ENSDART33947), Chrn $\alpha 2b$ (ENSDART79613), Chrn $\alpha 3$ (ENSDART149976), Chrn $\alpha 4$ (ENSDART104115), Chrn $\alpha 5$ (ENSDART21372), Chrn $\alpha 6$ (ENSDART31546), Chrn $\alpha 7$ (ENSDART134733), Chrn $\alpha 9$ (ENSDART140706), Chrn $\alpha 10$ (ENSDART12872), Chrn $\beta 2a$ (ENSDART2532), Chrn $\beta 2b$ (ENSDART41625), Chrn $\beta 3a$ (ENSDART74678), Chrn $\beta 3b$ (ENSDART50037), Chrn γ (ENSDART123966), Chrn δ (ENSDART15391), Chrn ϵ (ENSDART136428). The amino acid sequence of all 17 Chrn transcripts was BLAST searched against the Chrn $\alpha 2A$ epitope with low stringency to check for related sequence outside the epitope region. The search indicated the region was unique. Alignment of the 17 Chrn genes, restricted to residues flanking and including the Chrn $\alpha 2A$ epitope, was performed using the T-Coffee algorithm (<http://www.tcoffee.org/Projects/tcoffee/>) and ClustalW2-Phylogeny algorithm (<http://www.clustal.org/>).

mRNA synthesis and injection

The zebrafish cDNA sequence for the nAChR $\alpha 2A$ gene was cloned into the pCRTM 4Blunt-TOPO plasmid. Messenger RNA was transcribed *in vitro* using a modified protocol with the mMachine mMessage[®] T3 Kit (Life Technologies). Briefly, the plasmid encoding the chrna2 gene was linearized with Not I, and 0.4 μ g of linearized plasmid was used in the mMachine mMessage reaction. The reaction was incubated at 37 °C for 1.5 hours, and mRNA was precipitated with the addition of 1.25 volumes of 7.5M LiCl and incubated at -20 °C for 30 minutes. The mRNA was then centrifuged at 14,000 x g for 10 minutes, and the pellet washed 5 times with 1 mL of 75% EtOH and then dissolved into nuclease-free water. The 8.17 μ g/ μ L of capped $\alpha 2A$ nAChR mRNA stock was diluted in nuclease-free water to a working concentration of 200 ng/ μ L. A total amount, ranging between 0.3–0.6 ng, was co-injected with either the $\alpha 2A$ MO or the control MO into individual embryos at the 1–2 cell stage.

In situ hybridization

To localize $\alpha 2A$ nAChR transcripts in embryonic zebrafish, *in situ* hybridization (Thisse and Thisse, 2008) was performed. The $\alpha 2A$ nAChR probes were prepared using linearized plasmids (pCR4-TOPO vector, Invitrogen). Forward 5' CAT GTC TGA CCA CTG TAC '3 and reverse 5'CTG CTC TTG ATG TGC TTC'3 primers were used to amplify the target gene used for probe synthesis. The genes shha and MyoD served as positive control probes due to their known expression at the notochord and medial fast muscle cells, respectively (the Zebrafish Model Organism Database, ZFIN). The embryos were raised in

phenylthiourea at a final concentration of 0.0045% at 24 hpf to inhibit the formation of pigmentation, and the solution was changed once daily. In situ hybridization was performed according to the Thisse protocol with minor changes. Briefly, up to 100 similar aged embryos were fixed overnight in 20 ml of 4% paraformaldehyde. Embryos were then dehydrated in MeOH for 15 minutes at room temperature and stored at -20°C in fresh MeOH until use. All subsequent rehydration, washings, and probe and alkaline phosphatase-conjugated antibody incubations, were conducted in separate mesh bottomed baskets in 24-well plates. Plates were sealed to eliminate evaporation during long incubations. Embryos were transferred to 1.5-mL microtubes for NBT/BCIP (Roche) staining. FastRed staining (Sigma, catalog# F4648) was performed according to the Raymond lab protocol (http://mcdbs.lsa.umich.edu/labs/praymond/dbl_label.html). Briefly, following overnight anti-DIG incubation, embryos were washed 2x30 minutes in FastRed buffer pH 8.2 (0.1M Tris-HCL, 0.4M NaCl). One FastRed tablet was dissolved for every 2 ml of buffer. Embryos were incubated in 1 ml stain solution at room temperature, in the dark for 1.5 to 2 hours and then washed twice in PBST (PBS containing 0.1% Tween 20).

In several experiments, the in situ hybridization assay was combined with the immunohistochemistry protocol described below to localize mRNA to individual cells. The in situ procedure was performed first and then followed by the IHC using the antibody anti-Hu (Pineda et al., 2006). Embryos were either visualized with a Zeiss Axiovert200 with a rhodamine filter set (for FastRed reaction) or for the NBT/BCIP, with a Nikon SMZ 1500 microscope. All data presented are from the in situ experiments performed using the FastRed detection method.

Immunohistochemistry

Whole mount IHC was performed as previously described (Menelaou et al., 2008; Menelaou and Svoboda, 2009; Svoboda et al., 2001). The monoclonal antibodies zn8, previously known as zn5, (1:500), znp1 (1:250), F59 (1:250), zn12 (1:500) were obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa) and were used to reveal primary motoneuron axons (Melancon et al., 1997), slow muscle fibers (Devoto et al., 1996), and RB neurons (Metcalf et al., 1990), respectively. The monoclonal antibodies anti-Hu (referred to as Hu from here on, Invitrogen) and anti-acetylated tubulin (referred to as aat from here on, Sigma) were used to reveal RB neurons and their peripheral processes, respectively. The polyclonal anti-nAChR $\alpha 2\text{A}$ (1:500) was generated by Antibodies Incorporated (Davis, CA, USA). For assessing nAChR $\alpha 2\text{A}$ knockdown using IHC, control MO-injected embryos and $\alpha 2\text{A}$ morphants were processed in the same tube to ensure consistent antibody labeling in all the animals. Tail clippings were performed to distinguish the control MO-injected from the $\alpha 2\text{A}$ morphants. For the peptide blocking experiments, the conjugated peptide that was used to generate the anti-nAChR $\alpha 2\text{A}$ antibody was pre-incubated with the nAChR $\alpha 2\text{A}$ antibody at 4°C . This peptide block “cocktail” was then used for IHC protocols on 30 hpf - 48 hpf zebrafish embryos. Fluorescent secondary antibodies, goat anti-mouse Alexa 546 or 488 (Molecular Probes, Eugene, OR, USA), were used at a 1:1000 dilution and goat anti-rabbit Alexa 546 or 488 at a 1:5000 dilution to reveal primary antibody labeling.

α -bungarotoxin labeling

Tetramethylrhodamine conjugated to α -bungarotoxin (α -btx) (Molecular Probes) was used to identify AChR clustering at neuromuscular junctions (Menelaou and Svoboda, 2009). Briefly, MO-injected embryos were processed for immunohistochemistry and then incubated in α -btx (10 μ g/mL) for 90 minutes before image analysis.

Dorsal mounting in agarose

Zebrafish embryos were incubated in 0.002% phenylthiourea at 24 hpf to prevent pigment formation and at 48 hpf they were individually mounted in 1.25% agarose (Fisher Scientific, Fair Lawn, NJ) with their dorsal side up. Agarose blocks were then placed into 12-well plate dishes containing PBST until image acquisition. Individual agarose blocks with embedded embryos were placed on a glass bottom Petri dish and imaged with fluorescent microscopy.

Image acquisition and morphological analysis

Images were obtained using either a 20x dry (0.6 N.A. or 0.8 N.A.) or 40x oil (1.3 N.A.) objective mounted on a Zeiss Axiovert 200M inverted microscope equipped with epifluorescence, an ORCA-ER digital camera (Hamamatsu, Japan), and a Zeiss ApoTome. For motoneuron axon analysis, the 20x dry objective (0.6 N.A.) was used to acquire single focal plane images. Image stacks (step size interval 0.5–0.8 μ m) from representative non-injected embryos, control MO-injected embryos and α 2A morphants were taken using the 40x oil (N.A. 1.30) objective and the ApoTome. Quantification of motoneuron morphology using the *znpl* antibody in non-injected embryos, control MO-injected and α 2A morphants (see Table 2) was performed on single focal plane images acquired using the 20x images. For assessing α 2A labeling in MO-injected embryos, single focal images using the 20x dry objective were first acquired for the control MO-injected embryos for each experiment (7 experiments analyzed between 24 and 33 hpf). Images of the α 2A MO embryos were acquired at the same exposure settings as the control MO-injected embryos to qualitatively determine the knockdown of expression using fluorescence intensity. For presentation purposes, serial stacks (step size interval 0.5–0.8 μ m) were obtained using the 40x oil objective and those stacks were then projected as a 2D image.

Serial stacks through dorsal spinal cord from dorsally embedded zebrafish were acquired for RB neuron migration analysis in 48 hpf wildtype fish. RB migration distances were obtained using AxioVision 4.7.1 (Carl Zeiss). First, the midline of spinal cord was determined by taking half of the total distance between the most lateral left and right boundaries of spinal cord. Then, the distance from the center of each RB nuclei to the midline was measured. The migration distances for at least ten RB cells were measured in each embryo. Image analysis focused on the 4–6 segments that span the yolk sac extension. Image stacks were reconstructed using Imaris 5.7.2 volume-rendering software (Bitplane Inc., Saint Paul, MN, USA). Images were cropped using Photoshop 7.0 (Adobe, San Jose, CA, USA) and CorelDraw Graphics Suite 12 (Ottawa, Ontario, Canada) was used to create cartoon diagrams and to help organize the figures. Photomicrographs of lateral views are presented with rostral to the left and dorsal to the top. Photomicrographs of dorsal views are presented with rostral to the top.

The results obtained from anatomical analysis of the control MO-injected were indistinguishable from non-injected controls and were also very consistent with results previously reported in the literature for *znp1* (Melancon et al., 1997; Menelaou et al., 2008), α -btx (Behra et al., 2002), F59 (Behra et al., 2002; Menelaou et al., 2008), *aat* and anti-Hu labeling (Svoboda et al., 2001). Thus, we present representative photomicrographs pertaining to the anatomy of control MO-injected embryos (n=63) and α 2A morphants (n=76), but not the non-injected controls (n=95).

Statistical Analysis

All values are reported as means $\pm$ standard error of the means (SEM). One way analysis of variance (ANOVA) for repeated measures analysis (with Holm Sidak post hoc test) was performed to test for significance in SigmaStat3.5 for all the behavioral responses. A Mann-Whitney *U* test was used for comparisons of non-parametric values and Student *t*-tests were used for comparisons of normally distributed values as indicated. Statistical significance was assigned if the *p* value was <0.05 .

RESULTS

Embryonic α 2A nAChR subunit expression

α 2 nAChR mRNA was previously detected in zebrafish spinal cord as early as 24 hpf using in situ hybridization (Zirger et al., 2003). Here we show the presence of α 2A nAChR mRNA in olfactory neurons and in spinal cord as early as 19–20 hpf (Fig. 1A and 1B). As positive controls, in situs were performed with probes for the *MyoD* gene and the sonic hedgehog gene (not shown). In spinal cord, the α 2A nAChR subunit mRNA expression pattern was primarily confined to dorsal-lateral cells that were likely RB neurons. This was confirmed by combining the in situ mRNA detection with antibody labeling for RB neurons (Fig. 1B). Early in development, RB neurons exhibited robust α 2A nAChR mRNA expression and the labeling signal was strong, very similar to the strong olfactory mRNA signal. Some mRNA expression was also detected in mid spinal cord where the FastRed signal appeared as tiny dots, but these mRNA signals were not as robust as the ones observed in RB neurons.

RB neurons are among the earliest born sensory neurons in embryonic zebrafish (Bernhardt et al., 1990). They are characterized by their large cell body and dorsal position within spinal cord. They extend rostral and caudal axonal projections within spinal cord and they extend peripheral processes which exit dorsal spinal cord to innervate the skin (Clarke et al., 1984). RB neurons are the primary sensory neurons that function prior to when the dorsal root ganglia neurons are born (An et al., 2002) and are known to mediate locomotive behaviors, such as the response to touch (Ribera and Nusslein-Volhard, 1998). During a touch response, mechanosensory channels are activated in the skin and this ultimately activates the RB neurons.

Although α 2A nAChR subunit mRNA was detected early in development, it does not necessarily mean that translated proteins are present. We designed an antibody against the zebrafish α 2A nAChR subunit and found that the translated protein was expressed in

structures such as the olfactory epithelium, spinal cord, and muscle in young embryos (Fig. 1C and 1D; muscle not shown). The olfactory system is comprised of the peripherally derived olfactory sensory neurons and the olfactory bulb of the central nervous system (Sato et al., 2005). These sensory neurons extend processes into the olfactory bulb and the mature olfactory system responds to chemical environmental cues that mediate a variety of behavioral responses (Vitebsky et al., 2005). In the developing zebrafish embryo, differentiation of the olfactory placode occurs very rapidly (Hansen and Zeiske, 1993). Consequently, the olfactory epithelium matures quickly (from 24 to 36 hpf) as more $\alpha 2A$ immuno-positive cells were detected with the antibody (Fig. 1C).

In spinal cord, the $\alpha 2A$ subunit protein was detected very early in development. In wildtype embryos, the antibody labeled cells in the dorsal-lateral aspect of spinal cord (data not shown) similar to the $\alpha 2A$ nAChR mRNA expression pattern. Antibody labeling in Tg(*isl2b*:GFP) embryos, a transgenic line in which RB neurons express green fluorescent protein (GFP), confirmed the identity of these $\alpha 2A$ immunoreactive neurons as RB cells in the dorsal spinal cord (Fig. 1D). Since the GFP-positive cells in the head region (trigeminal ganglion) of the *isl2b* embryos did not appear immuno-positive for the $\alpha 2A$ subunit (Fig. 1E), we conclude that the $\alpha 2A$ subunit protein was specifically expressed in RB neurons and that the labeling was not related to potential microscopy artifacts associated with the GFP signal. However, the $\alpha 2A$ subunit protein was not detected in mid spinal cord with the antibody at these early developmental stages. This is in contrast with the mRNA expression profiles, but one possible explanation for the labeling discrepancy is that even though $\alpha 2A$ mRNA was present in these mid spinal cells at these early stages of development, it may have not yet been translated to protein. On the other hand, we cannot exclude the possibility that the protein levels may have just been too low to detect with the antibody at these early developmental stages.

Is this antibody specific for the $\alpha 2A$ nAChR subunit? To date, 17 neuronal-type nicotinic acetylcholine transcripts have been identified in the integrated assembly of the zebrafish genome (Zv9; http://ensembl.org/Danio_rerio). The amino acid sequence of all 17 these transcripts was aligned against the $\alpha 2A$ epitope with low stringency to check for related sequence outside the epitope region. The search indicated the region was unique. The $\alpha 2A$ epitope region was most closely related to Chrn $\alpha 2b$, Chrn $\alpha 1$, Chrn $\alpha 3$ and Chrn $\alpha 5$, presented in order from closest to least related (Table 1). The five sequences were divergent with only one conserved residue and one conserved substitution. While there was very limited potential for the anti- $\alpha 2A$ antibody to cross-react with the homologous $\alpha 2B$ epitope, there was no potential for the anti- $\alpha 2A$ antibody to cross-react with any other nAChR proteins (Table 1).

Our attempts to further confer antibody specificity with western blot analysis proved unsuccessful. This is not too surprising, as some commercially available antibodies also do not work well in western blot experiments even though they work well in IHC experiments. As an alternative, we pre-incubated the antibody at 4°C with the conjugated peptide that the antibody was designed against. We then performed IHC with this “cocktail” on 30 hpf - 48 hpf zebrafish embryos. The antibody signal that was routinely detected on the RB neurons

and olfactory epithelium at these developmental time-points was significantly reduced in intensity in these peptide blocking experiments (Fig. 1F and 1G).

Blocking $\alpha 2A$ nAChR expression *in vivo* by morpholino antisense oligonucleotides

In order to examine the potential functional role of $\alpha 2A$ -containing nAChRs, we used antisense morpholino (MO) oligonucleotides (Draper et al., 2001; Nasevicius and Ekker, 2000). We designed an $\alpha 2A$ splice blocking MO to disrupt splicing at the exon2-intron2 boundary of the 6-exon nAChR- $\alpha 2A$ transcript, thus resulting in exon 2 skipping during nAChR $\alpha 2A$ mRNA processing *in vivo*. Reverse transcription polymerase chain reaction analysis (Fig. 2A) and immunohistochemistry (Fig. 2B–E) were used to assess knockdown success of the $\alpha 2A$ subunit following $\alpha 2A$ MO microinjection. RT-PCR analysis in 24 and 48 hpf zebrafish confirmed that injection of the $\alpha 2A$ MO disrupted effective splicing of its native RNA. This was revealed by the shifted fragment (~450 bp) when compared to its control spliced counterpart (~590 bp) (Fig. 2A).

Immunohistochemistry was used to assess the efficacy of the $\alpha 2A$ MO in blocking the $\alpha 2A$ subunit protein expression and also let us confirm the specificity of the $\alpha 2A$ antibody. At 33 hpf, olfactory sensory neurons from wildtype zebrafish embryos injected with the control MO exhibited strong $\alpha 2A$ protein expression (Fig. 2B, left). RB neurons also expressed $\alpha 2A$ protein (Fig. 2C, left; Fig. 2D). Injection of the $\alpha 2A$ MO significantly reduced $\alpha 2A$ protein expression in both olfactory neurons (Fig. 2B, right) and RB neurons (Fig. 2C, middle and right; Fig. 2E). The absence of $\alpha 2A$ immunoreactivity was not simply due to an absence of RB neurons as RB cells were still detected by zn12; a marker of RB neurons (Fig. 2D and E). These results indicate that the $\alpha 2A$ MO successfully blocked the expression of the nAChR $\alpha 2A$ subunit protein *in vivo*.

$\alpha 2A$ -containing nAChRs and potential role(s) in mediating nicotine-induced motor output

Zebrafish embryos exhibit spontaneous activity in the form of musculature bends of the trunk which begins at ~17 hpf. At this age, primary motoneuron axons make their initial contacts with muscle fibers (Melancon et al., 1997; Saint-Amant and Drapeau, 1998). This spontaneous motor output peaks at 19–20 hpf and gradually decreases over time (Saint-Amant and Drapeau, 1998; Thomas et al., 2009). The zebrafish embryonic motor output can be modulated by cholinergic agonists, such as acetylcholine and nicotine (Thomas et al., 2009). When zebrafish embryos are exposed to exogenous cholinergic agonists, a rhythmic bending of the musculature occurs that resembles swimming (Thomas et al., 2009). This increased rhythmic motor output is likely mediated through the activation of nAChRs located on spinal neurons since absence of the brain structures does not affect spontaneous or agonist-induced motor activity (Saint-Amant and Drapeau, 1998; Downes and Granato, 2006; Thomas et al., 2009). However, the specific neural substrates and specific nAChRs underlying these behavioral phenotypes are not yet known. Therefore, we took advantage of our ability to knockdown the nAChR $\alpha 2A$ subunit expression *in vivo* to determine if $\alpha 2A$ -containing nAChRs were involved in mediating the nicotine-induced motor output.

We first evaluated the consequences of knocking down $\alpha 2A$ protein expression on the baseline spontaneous activity rates in zebrafish embryos at various developmental stages

(Fig. 3A). Embryonic motor activity was videotaped between three and five minutes and the bend rates of the trunk musculature (number of bends per minute) were plotted (Fig. 3A). The rates of spontaneously occurring muscle bends at different developmental times in the $\alpha 2A$ morphants were similar to the rates of spontaneously occurring muscle bends in control MO-injected embryos (Fig. 3A). Those rates were in accord with studies that have characterized this activity in zebrafish embryos (Saint-Amant and Drapeau, 1998; Thomas et al., 2009).

Since $\alpha 2A$ MO injection did not reduce the baseline spontaneous activity in zebrafish embryos, we were then in the position to examine whether $\alpha 2A$ -containing nAChRs were involved in modulating other embryonic motor behaviors. We adopted the nicotine exposure paradigm of Thomas et. al, 2009 which was shown to reliably increase the musculature bend rates in zebrafish. However, because all of the experiments in the current study were performed with the embryo in its protective chorion, we evaluated various nicotine concentrations (3–300 μM) to determine a concentration that could produce a robust output (Supplement Fig. 2A). Three micromolar nicotine failed to elicit a motor output in zebrafish embryos. Exposure to 30, 60 and 300 μM nicotine was able to elicit a motor output. However, the magnitude of the response in embryos exposed to 30 μM nicotine while in the chorion was small, thus we opted to use the next highest concentration of 60 μM . The spontaneous activity of 25 hpf MO-injected embryos was monitored for three minutes and then transferred in embryo medium containing 60 μM nicotine (Fig. 3B). Control MO-injected embryos exhibited an increase in their musculature bend rate when exposed to nicotine. In contrast, $\alpha 2A$ morphants exposed to nicotine displayed a much lower bend rate compared to stage matched control MO-injected embryos (Fig. 3B). When the bend rates of individual 25 hpf control MO-injected embryos and $\alpha 2A$ morphants at the first minute of nicotine exposure were plotted (Fig. 3C, embryos at minute 4 in 3B), six out of seven $\alpha 2A$ morphants had bend rates below the lowest control MO-injected embryo bend rate (Fig. 3C, indicated by dashed line). In some experiments in which $\alpha 2A$ morphants exhibited reduced bend rates upon nicotine exposure, IHC was subsequently performed to confirm the absence of $\alpha 2A$ protein. Protein expression was absent or greatly reduced in RB neurons, olfactory neurons, and muscle.

The embryonic spinal circuit producing locomotion in zebrafish is most likely established at a very young age (~19 hpf) (Downes and Granato, 2006). We examined the nicotine-induced swim-like behavior in MO-injected embryos during early stages of development. Between 20–22 hpf, the nicotine-induced motor output was almost completely abolished in $\alpha 2A$ morphants (Fig. 4, shown at 20 and 22 hpf). At later time-points (~23 hpf and after), nicotine did elicit a motor output in the $\alpha 2A$ morphants (Fig. 4, shown at 23 and 28 hpf). However, even at those later times, the nicotine-induced output was still significantly reduced when compared to control MO-injected embryos (latest point analyzed was 28 hpf). Moreover, there was a dose dependency effect for the $\alpha 2A$ MO and its ability to significantly reduce the nicotine-induced motor output. When titrated out, we observed small but consistent reductions in nicotine-induced motor outputs when injecting 0.25 mM morpholino into the embryo. As the $\alpha 2A$ MO concentration was increased, the nicotine-induced motor output was decreased (Supplement Fig. 2Ci, 2Cii). At the highest concentration of morpholino used, we did not observe any morphological phenotypes

typically associated with “off-target” effects. The ability of the $\alpha 2A$ MO to completely abolish the motor output in young embryos suggested that that $\alpha 2A$ -containing nAChRs were most likely mediating the nicotine phenotype. However, it could be that other nAChRs were present, but were not just activated by 60 μM nicotine. When 23 hpf $\alpha 2A$ morphants were exposed to 300 μM nicotine, the nicotine induced motor output was still abolished (Supplement Fig. 2B). Taken together, these results suggest that the nicotine-induced motor behavior in zebrafish may be mediated exclusively through the activation of $\alpha 2A$ -containing nAChRs early in embryogenesis.

Lastly, mRNA rescue experiments were performed to demonstrate the specific linkage between $\alpha 2A$ subunit expression knockdown and the observed reduction in motor output upon nicotine exposure. Co-injection of synthetic $\alpha 2A$ mRNA with the $\alpha 2A$ MO restored the embryos ability to respond to nicotine as those embryos exhibited bend rates very similar to bend rates of control MO-injected embryos (Fig. 5A and B). The mRNA rescue also restored $\alpha 2A$ subunit expression in olfactory (Fig. 5C) and RB neurons (Fig. 5D) as confirmed by immunohistochemistry utilizing the $\alpha 2A$ antibody.

Nicotine versus non nicotine-induced motor output in embryonic zebrafish

The reduction in the nicotine-induced motor behavior in $\alpha 2A$ morphants can potentially be attributed to defects in either the input (spinal neurons) or output elements (motoneurons and muscle) associated with the motor behavior. To determine the locus of the defect in the young embryos (i.e. 20–22 hpf), we needed a reliable, non-nicotinic approach to activate a motor output. The act of dechoriation is one way to elicit motor output in early life stage embryos. Zebrafish remain in their chorions during embryonic stages of development, but if dechorionated, an increase in their musculature bend rate occurs (Saint-Amant and Drapeau, 1998; Thomas et al., 2009). Even though the exact mechanism underlying this robust motor output following dechoriation is largely unknown, dechoriation can be reliably used to elicit a motor output in the absence of pharmacological agonists. If embryos were not able to move upon dechoriation, that would suggest that either the motoneurons or muscle were compromised, or that cholinergic-mediated excitation within spinal cord itself (Perrins and Roberts, 1995), was somehow disrupted.

The experimental protocol is shown in figure 6A. Embryos injected with an $\alpha 2A$ MO while still in the chorion exhibit reduced motor output following nicotine exposure at 22 hpf (Fig. 6B, minute 4). The muscle bend rates of those same embryos returned to baseline after a wash period. The embryos were then quickly dechorionated. Upon dechoriation, they exhibited increased musculature bend rates similar to control MO-injected embryos (Fig. 6B, minutes 27–30). Since the $\alpha 2A$ morphants still produce robust motor outputs following the act of dechoriation indicates that the elements associated with the output side of the motor behavior (motoneurons and/or muscle) are not compromised in the absence of the nAChR $\alpha 2$ subunit expression.

Based on the findings from the dechoriation experiment, we predicted that the components comprising the output side of the motor behavior would not be affected by $\alpha 2A$ subunit knockdown. The first component analyzed was the distribution of postsynaptic muscle nAChRs within the myotomes. We analyzed the post-synaptic muscle nAChR

distribution in $\alpha 2A$ morphants with α -btX staining. In the 26 hpf embryos shown, the postsynaptic nAChR distribution in muscle fibers was indistinguishable from the control MO-injected embryos (Fig. 6C). This finding also confirms the specificity of the $\alpha 2A$ MO as the morpholino did not disrupt the formation of the muscle specific nAChRs.

The morphological development of primary motoneurons and muscle in zebrafish embryos was then analyzed. At 30 hpf, there were no differences in pathfinding of primary motoneuron axons (Fig. 6D). Moreover, the morphology of slow muscle fibers was indistinguishable between control MO-injected embryos and $\alpha 2A$ morphants (Fig. 6E). These findings demonstrate that the reduction of the nicotine-induced motor output in $\alpha 2A$ morphants was not likely due to alterations in the output elements of the circuitry that produce embryonic motor behavior. They also suggest that upstream input elements within spinal cord associated with producing the nicotine-induced swim-like behavior are most likely affected by $\alpha 2A$ nAChR expression knockdown. Affecting those upstream elements would likely result in the reduced motor output when embryos are exposed to nicotine.

Knocking down $\alpha 2A$ nAChR subunit expression does not alter Rohon-Beard neuron development or function

In addition to the swim-like motor behavior, zebrafish embryos possess a distinct behavioral response to tactile stimulation referred to as touch response. The mechanosensory RB neurons mediate this touch response after 27 hpf (Ribera and Nusslein-Volhard, 1998). RB neurons have peripheral processes that innervate the skin and are sensitive to mechanical stimulation through mechanosensory terminals. Following tactile stimuli, RB neurons relay excitation from the skin to the contralateral spinal cord to generate contralateral muscle contraction (Ribera and Nusslein-Vollhard, 1998). This is achieved through activation of CoPA (commissural primary ascending) interneurons (Downes and Grant 2006; Gleason et al., 2003) which project to contralateral spinal cord and activate cells on the opposite side of the spinal cord. This ultimately results in the activation of the contralateral musculature via the excitation of motoneurons. Activation of this spinal circuit by RB neurons results in a left-right touch-evoked coiling (before 27 hpf) and touch-evoked swimming (following 27 hpf) (Saint Amant and Drapeau, 1998; Downes and Granato, 2006).

Since RB neurons express the nAChR $\alpha 2A$ subunit very early in development (Fig. 1B and D), it seemed logical to evaluate touch sensitivity in embryos injected with control and $\alpha 2A$ MO. Any differences in their sensitivity to touch would indicate possible defects in RB neuron excitability, connectivity, or mechanosensation. Tactile stimulation to the trunk at ~31 hpf can induce a robust tail flip away from the stimulus site (Fig. 7A, left; shows an $\alpha 2A$ morphant). $\alpha 2A$ morphants did not exhibit a significant reduction in their ability to respond to touch when compared to control MO-injected embryos (Fig. 7A, right).

We then examined aspects of RB neuron anatomy and development, focusing on RB neuron peripheral processes and RB neuron migration. RB cells extend peripheral processes into the skin along a stereotypical ventral-lateral trajectory and these processes can be easily visualized within the skin using α -tubulin immunoreactivity (Svoboda et al., 2001; Paulus et al., 2009). By 48 hpf, the tubulin distribution within the peripheral processes of RB neurons is typically fragmented, and this fragmentation serves as a marker of RB neurons that have

entered into programmed cell death (Svoboda et al., 2001). RB neurons in $\alpha 2A$ morphants had peripheral processes exhibiting tubulin fragmentation just like the RB neurons in control MO-injected embryos (Fig. 7B).

During early stages of development (18–30 hpf), RB neurons are present in two bilateral rows in the dorsal and lateral-most aspect of spinal cord (Fig. 7C). RB cells enter a migratory phase (~36 hpf) to the midline forming an almost single row of cells by 48 hpf (L.T. Paul and Svoboda, unpublished observations). We sought to determine if knocking down expression of the $\alpha 2A$ nAChR subunit disrupted RB neuron migration. RB neurons were labeled with the Hu antibody in wildtype embryos. We also used the Tg(*isl2b*:GFP) line of zebrafish which expresses GFP in RB neurons (Fig. 7D) to monitor RB neuron migration. RB cells did in fact reach the midline by 48 hpf in $\alpha 2A$ morphants when compared to control MO-injected embryos (see Table 2 for quantification). These findings indicate that blocking the expression of the $\alpha 2A$ nAChR subunit did not impact RB neuron morphology or their normal function in mediating the touch response.

DISCUSSION

$\alpha 2A$ nAChR subunits are components of functional nAChRs during early embryogenesis

The nicotine-induced behavioral response in this study was used as a tractable endpoint to investigate potential disruptions in cholinergic signaling mechanisms in developing zebrafish. Since the behavioral response is reliably activated by nicotine, we reasoned that functional nAChRs were mediating it. We focused on potential subunits which could be incorporated into functional nAChRs early in embryogenesis and more importantly, which cells were expressing those functional nAChRs, that when activated by nicotine, would result in an increased motor output. Even if the subunits were in place at the cellular level, were those subunits forming functional receptors?

Functional neuronal nAChR assembly requires five subunits, either as a combination of alpha and beta subunits or five alpha subunits (Karlin, 2002). The precise repertoire of functional nAChR assembly in the zebrafish spinal cord still remains to be explored. However, when all the receptor subunits are spatially, temporally and physiologically described only then we will begin to gain insights into the role and function of cholinergic transmission in the developing spinal cord.

Here, we focused on the expression profile of the $\alpha 2A$ nAChR subunit using $\alpha 2A$ specific probes for RNA localization and a zebrafish antibody designed against the $\alpha 2A$ subunit. We identified specific cell types that expressed the $\alpha 2A$ nAChR subunit and hypothesized that those cell types could potentially assemble functional nAChRs incorporating the $\alpha 2A$ subunit. Our findings revealed that $\alpha 2A$ subunit is expressed very early in development by RB neurons and olfactory sensory neurons. Our findings are in accord with previous reports in zebrafish in which the $\alpha 2A$ transcript was localized in the olfactory placode and in the spinal cord (Zirger et al., 2003). We previously reported that RB neurons also express the $\beta 2$ subunit (Welsh et al., 2009). In another study, it was demonstrated that RB express the $\alpha 6$ subunit transcript as early as 24 hpf (earliest time point analyzed in that study, Ackerman et al., 2009). Our findings in combination with other reports on nAChR subunit expression

indicate that potential subunit partners required for nAChR assembly do exist on zebrafish RB neurons. The embryonic expression of the $\alpha 2A$ subunit by RB neurons reported in our study is significant because if partnered with an appropriate beta subunit, it could be part of a functional nAChR early in embryogenesis.

Zebrafish embryonic spontaneous activity and touch responses can occur in the absence of supraspinal inputs suggesting that these motor behaviors are intrinsic to neural networks within the spinal cord (Saint Amant and Drapeau, 1998). Moreover, decapitated embryos still exhibited an increased alternating motor output when exposed to nicotine further implicating the presence of functional nAChRs within spinal neurons (Thomas et al., 2009). Knocking down expression of the $\alpha 2A$ subunit with morpholino antisense oligonucleotides yielded different results depending upon which behavioral context was being analyzed. For example, blocking expression of the $\alpha 2A$ subunit had no effect on spontaneous activity; the musculature bend rates did not speed up nor did they slow down. However, in the context of the nicotine-induced behavior, a quite different story emerged. We found that nicotine-mediated increase in motor output was almost completely abolished in very young 20–22 hpf zebrafish embryos. One interpretation of this is that the $\alpha 2A$ subunit is part of a functional receptor with a beta counterpart at these early developmental stages and nicotine exposure activates this receptor to generate the motor output. If this was true, the results from these very young embryos suggest that the only alpha subunit available to form a functional nAChR would be the $\alpha 2A$ subunit. When its expression is knocked down, no functional receptors are formed, and the nicotine-induced output is completely abolished. Even when high concentrations of nicotine were used (300 μM); knocking down expression of the $\alpha 2A$ early in embryogenesis abolished the nicotine-induced motor output. This “all or none response”, when observed at the concentrations used in this study, further substantiates the idea that early in embryogenesis (< 23 hpf), the only alpha nAChR subunit available to partner and form a functional receptor with a beta subunit is the $\alpha 2A$ subunit.

At later stages of development (~23–28 hpf), a subtle nuance appears. $\alpha 2A$ morphants do respond to nicotine, but the overall nicotine-induced motor output is reduced. These results indicate that early in development, activation of $\alpha 2A$ -containing nAChRs essentially mediates the nicotine-induced swim-like responses and several hours later, the nicotine response is mediated in part by $\alpha 2A$ -containing receptors and likely by another nAChR not comprised of $\alpha 2A$ subunits.

The $\alpha 2A$ -containing nAChRs are functional early in development as revealed by the nicotine-induced behavioral assay. We also suggest that they are associated with input elements of the spinal circuitry that produces movement. For example, when embryos were injected with the $\alpha 2A$ morpholino, they moved as well as control MO-injected embryos. If functional receptors were located on interneurons within the spinal circuitry that relied on cholinergic transmission, the movement should be reduced in the $\alpha 2A$ morphants. In the touch response assay in older embryos (28–31 hpf), activation of RB neurons with tactile stimulation elicited what appeared to be normal touch responses. Thus, in this assay, the spinal circuitry appeared to function normally. Lastly, the anatomy of the output elements such as muscle specific nAChRs, primary motoneurons, and muscle fiber morphology appeared unaffected following $\alpha 2A$ MO injection.

Candidate spinal neurons mediating nicotine-induced motor output in embryonic zebrafish

We provided evidence that RB neurons express the nAChR $\alpha 2A$ subunit as early as 19–20 hpf. In *Xenopus*, RB neurons possess acetylcholinesterase activity, the catabolic enzyme for acetylcholine (Moody and Stein, 1988), which further supports that RB neurons might be cholinoreceptive in nature. RB neurons do not appear to receive direct synaptic inputs from other cells and in *Xenopus*, intracellular stimulation of a single RB neuron can elicit fictive swimming (Clarke et al., 1984). This important finding suggests that when RB neurons are presented with a drive/excitation, they could initiate a robust motor output. In the context of the experiments presented herein, that excitatory drive is essentially the application of exogenous nicotine. When applied to the embryo, nicotine can activate $\alpha 2A$ -containing receptors, presumably those located on RB neurons. If many RB neurons receive sustained excitatory drive (by the application of nicotine) through activation of their nAChRs, this would result in a dramatic increase in motor output. Even though injection of the $\alpha 2A$ MO dramatically reduced the nicotine-induced motor output in embryos, it had no apparent effect on the non-nicotine-induced motor output, the actual development of RB neurons, or their sensitivity to touch.

The findings from the dechoriation experiment (Fig. 6) are important for interpretation of our results and warrant further discussion. When $\alpha 2A$ morphants were exposed to nicotine, they did not move in response to nicotine exposure. However, when those same morphants were then removed from their protective chorion, they moved vigorously just like control MO-injected embryos. Moreover, the bends of the musculature occurred in a left-right fashion (Saint Amant and Drapeau, 1998). If interneurons associated with the spinal central pattern generator (CPG) express $\alpha 2A$ -containing nAChRs, knocking down $\alpha 2A$ expression should have diminished the $\alpha 2A$ morphants ability to move upon dechoriation. That ability to move in the normal, un-manipulated embryo is in part, produced by endogenous ACh release in spinal cord. Lastly, the mRNA expression and antibody data point to RB neurons as expressing abundant $\alpha 2A$ -containing nAChRs early in embryogenesis. Other spinal cells express $\alpha 2A$ mRNA as well, but not to the same levels that RB neurons do. Moreover, since no obvious labeling was observed in mid-ventral spinal cells with the $\alpha 2A$ antibody at early stages of development, it is likely that the $\alpha 2A$ mRNA was not yet translated into protein in these cells. If this were true, the assembly of functional nAChRs containing $\alpha 2A$ subunits by mid-ventral spinal cells could not take place. Hence, we did not consider these as being good candidate cells to be activated by exogenous nicotine and activate a motor output, especially at time-points less than 23 hpf of age. Based on all of these findings, we propose a plausible spinal circuit for nicotine-induced motor output that places RB neurons at the top of the signaling cascade (Fig. 8). RB neurons, upon activation of their functional nAChRs (which contain $\alpha 2A$ subunits) by bath application of nicotine, provide sustained excitation to CoPA (commissural primary ascending) interneurons (Gleason et al., 2003) via glutamatergic synapses (Higashijima et al., 2004). CoPA interneurons send their axons to contralateral elements of the spinal CPG to provide commissural excitation (Hale et al., 2001; Gleason et al., 2003; Pietri et al., 2009). This activation can then excite the CPG network which would then activate motoneurons to release acetylcholine onto muscle fibers, thus producing muscle contractions.

In the context of tactile stimulation, activation of these CPG elements will result in a left-right coiling of the musculature resembling swim-like behaviors (Downes and Granato, 2006; Saint-Amant and Drapeau, 1998). In a similar fashion, activation of $\alpha 2A$ -containing nAChRs on RB cells, the likely spinal neurons activated by nicotine, results in an increase in the motor output. Thus, RB neuron activation by tactile stimulation or by nicotine activation of nAChRs will produce a motor output.

Functional receptors on the RB neurons are likely present early in embryogenesis, but are the RB neurons themselves functional at this same developmental stage? The work of Saint-Amant and Drapeau (1998) suggest that they are even though they do not fire over shooting action potentials until about 27 hpf (Ribera and Nusslein-Vollhard, 1998). Tactile stimulation to 21 hpf zebrafish embryos that were coiling, increased the coiling rate. These experiments were somewhat confounded by the fact that the embryos were coiling vigorously as a result of dechoriation, but the implication is that the tactile stimulation was activating RB neurons which increased the coil rate even more, at least at 21 hpf. If one simply substitutes nicotine in place of the tactile stimulus as the mechanism for the excitatory drive at 21 hpf, an increase in the motor output would still occur. When $\alpha 2A$ subunit expression is blocked, RB neurons may not be able to form functional nAChRs rendering them unable to respond to exogenously applied nicotine and thus resulting in a diminished motor output in the presence of nicotine. They still however, would respond to touch as disrupting the nAChRs would not impinge on the RB neuron's ability to perceive and convey mechanoreceptor induced touch sensation. Importantly, our results provide no direct evidence that nAChRs expressed by RB neurons are involved in the touch-evoked behavior. Instead, a well-characterized motor output caused by nicotine exposure was utilized to probe for the localization of putative functional nAChRs. Our data suggests that RB neurons possess functional nAChRs very early in development. However, the role that these receptors play in the normal development and physiology of spinal RB neurons still needs to be identified.

Conclusion

We have demonstrated that nicotine can activate presumptive $\alpha 2A$ -containing nAChRs located on RB neurons to initiate a motor output very early in development. Based on our data, activation of nAChRs on RB neurons can produce a robust embryonic motor behavior through an active process. Although our results revealed that functional nAChRs are likely present on RB neurons, their role in the normal development of the organism remains somewhat of a mystery. It remains to be determined if there is a source of endogenous acetylcholine that could potentially activate RB neurons. One intriguing scenario that remains to be tested is that the release of such endogenous acetylcholine could be used to trigger programmed cell death in the RB cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

aat	anti-acetylated tubulin
α-btx	α-bungarotoxin
CoPA	commissural primary ascending
Chrn	nicotinic acetylcholine receptor
CPG	central pattern generator
GFP	green fluorescent protein
hpf	hours post fertilization
MO	morpholino
MN	motoneuron
nAChR	nicotinic acetylcholine receptor
RB	Rohon-Beard

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Figure 1. Expression of the zebrafish $\alpha 2A$ nAChR subunit in embryogenesis

A) In situ hybridization in a 20 hpf wildtype embryo using nAChR $\alpha 2A$ RNA probes. A photomicrograph of the head region viewed from the ventral side is shown. White arrows point to $\alpha 2A$ nAChR mRNA expression in the region of the olfactory epithelium. Note the size of the scale bar. B) Left, same as in A, but photomicrograph at the left now shows a lateral view of spinal cord. The white dashed line separates dorsal spinal cord, where there is robust $\alpha 2A$ nAChR mRNA expression, from ventral spinal cord where mRNA expression is weaker appearing as individual dots. Right, the mRNA expression is overlaid with anti-Hu signal in the same embryo. The large cells in dorsal spinal cord are the Rohon-Beard neurons. C) $2A$ nAChR subunit protein is expressed by olfactory sensory neurons as revealed by anti- $\alpha 2A$ immuno-staining at 24 and 36 hpf. D) Left, representative images from a 22 hpf Tg(*isl2b*:GFP) embryo shows a dorsal view of the spinal cord region to reveal the anti- $\alpha 2A$ immuno-staining in RB neurons. Right, merged image shows GFP positive RB neurons labeled with the $\alpha 2A$ antibody. E) Left, photomicrograph of a 22 hpf Tg(*isl2b*:GFP) embryo shows GFP expression in trigeminal neurons. Middle, image shows immuno-labeling (or absence of) with the zebrafish antibody designed against the nAChR $2A$ subunit. The GFP positive cells in trigeminal ganglion did not label with the antibody. Right,

merged image shows that immuno-staining with the $\alpha 2A$ antibody does not label trigeminal ganglion cells. F) Top, photomicrograph shows a 30 hpf embryo labeled with the $\alpha 2A$ antibody. White arrows point to RB neurons. Bottom, photomicrograph of a 30 hpf embryo obtained at the same exposure setting used to acquire the image in the top panel reveals immuno-staining with the $\alpha 2A$ antibody previously incubated with the $\alpha 2A$ peptide ($\alpha 2A$ block peptide). The labeling of dorsal cells in spinal cord has been greatly reduced. G) Left, photomicrograph of a 33 hpf embryo reveals anti- $\alpha 2A$ labeling in the olfactory epithelium. Right, photomicrograph of a 33 hpf embryo acquired with the same exposure setting used to acquire the image in the left panel. The embryo was incubated in the anti- $\alpha 2A$ block peptide “cocktail”. The labeling of the olfactory epithelium is greatly reduced. White dashed circle highlights the region of the olfactory epithelium. Scale bars, 20 μm .

Figure 2. $\alpha 2A$ MO blocked native mRNA splicing and abolished anti- $\alpha 2A$ subunit immunoreactivity in zebrafish embryos

A) An $\alpha 2A$ splice blocking MO was designed to target the exon2-intron2 boundary in the 6-exon nAChR- $\alpha 2A$ transcript. The MO is predicted to result in exon 2 skipping during nAChR $\alpha 2A$ mRNA processing *in vivo*. RNA from 24 and 48 hpf zebrafish were reverse-transcribed and amplified using $\alpha 2A$ subunit specific primers. The PCR products from control MO-injected embryos and $\alpha 2A$ morphant zebrafish predicted to amplify at ~590 bp and ~450 bp, respectively (yellow arrows point to 450 bp bands). B) Embryos (33 hpf) injected with control MO displayed nAChR $\alpha 2A$ immunoreactivity in olfactory sensory neurons but exhibited a substantial reduction in nAChR $\alpha 2A$ expression when injected with the $\alpha 2A$ MO. C) Control MO-injected embryos exhibited nAChR $\alpha 2A$ immuno-reactivity in RB neurons at 33 hpf (left, yellow arrowheads), whereas stage matched $\alpha 2A$ morphants (middle and right) lacked $\alpha 2A$ labeling. D) Double antibody labeling using zn12 and $\alpha 2A$ antibodies in 31 hpf control MO-injected embryos. E) Same as is D but for 31-hpf $\alpha 2A$ morphants. The zn12 antibody was used to label RB neurons in dorsal spinal cord to confirm the specific knockdown of the $\alpha 2A$ subunit expression specifically in RB neurons. Dotted line in B denotes the boundary of the olfactory sensory organ. Scale bars, 20 μ m.

Figure 3. Knockdown of $\alpha 2A$ nAChR subunit expression: Effects on embryonic spontaneous activity and nicotine-induced motor output

A) Top, video stills of a 25 hpf embryo in the chorion demonstrating embryonic spontaneous motor activity. The spontaneous motor output (bends per minute) was quantified for a 3–5-minute epoch (indicated as a dotted line, shown here for 3 minutes). Below, plot shows the baseline spontaneous activity from control MO-injected embryos and $\alpha 2A$ morphants across developmental stages. B) Top, experimental design performed for all the nicotine-induced experiments. The baseline spontaneous motor activity of zebrafish embryos was first recorded for 3 minutes (black line) and at the end of minute 3, the embryos were transferred to embryo medium containing 60 μM nicotine for another 3 minutes (grey line). Below,

representative plot shows the baseline spontaneous activity and nicotine-induced motor output from 25 hpf embryos injected with a control MO (black circles, n=6) and the α 2A MO (white circles, n=7). C) Bend rates from individual embryos were taken from minute 4 as indicated by the dashed circle in B. Dashed line marks the lowest bend rate produced by nicotine in control MO-injected embryos. α 2A morphants exhibited reduced bend rates in response to nicotine since most embryos had bend rates below the lowest control MO-injected bend rate. Asterisks denote significant differences between baseline and nicotine-induced bend rates (control MO, $p < 0.001$ and α 2A MO, $p < 0.05$; repeated measures ANOVA). ‡ denotes significance between control MO and α 2A MO nicotine responses ($p < 0.001$; repeated measures ANOVA).

Figure 4. Knockdown of $\alpha 2A$ nAChR subunit expression reduces nicotine-induced motor output during embryonic development

The baseline spontaneous motor activity while embryos still in their chorions was recorded for 3 minutes and at the end of minute 3, embryos were quickly transferred into embryo medium containing 60 μM nicotine. Representative plots of the spontaneous and nicotine-induced activity are shown from top to bottom at different developmental stages. Between 20–22 hpf, the nicotine-induced motor behavior (indicated by grey line) was almost completely abolished in $\alpha 2A$ morphants (20 hpf, $n=11$; 22 hpf, $n=16$) when compared with their stage matched control MO-injected counterparts (20 hpf, $n=14$; 22 hpf, $n=21$). At later stages of development (>23 hpf), $\alpha 2A$ morphants (23 hpf, $n=10$; 28 hpf, $n=12$) respond to

nicotine but their nicotine-induced motor output was still reduced when compared to control MO-injected embryos (23 hpf, n=8; 28 hpf, n=23). Asterisks denote significant differences between baseline and nicotine-induced bend rates ($p < 0.001$; repeated measures ANOVA). ‡ denotes significance between control MO and $\alpha 2A$ MO nicotine responses ($p < 0.001$; repeated measures ANOVA).

Figure 5. mRNA “rescue” of α2A nAChR subunit expression restores the nicotine-induced motor output

A) The baseline spontaneous motor activity of embryos injected with α2A MO alone or α2A MO + α2AChR mRNA (rescue) and while still in their chorions was recorded at 21 (α2A MO, n=8; mRNA ‘rescue’, n=6), 25 (α2A MO, n=9; mRNA ‘rescue’, n=10) and 28 hpf (α2A MO, n=8; mRNA ‘rescue’, n=6) for 3 minutes. At the end of minute 3, embryos were quickly transferred into embryo medium containing 60 μM nicotine (indicated by the grey line). The nicotine-induced bend rates are greatly reduced in α2A morphants when compared to embryos co-injected with α2A MO and α2AChR mRNA (‘rescue’). B) The bend rates of embryos injected with α2A MO + α2AChR mRNA (‘rescue’, n=9) are compared to embryos injected with the control MO + α2AChR mRNA (n=7). C) Photomicrographs of 30 hpf embryos in the region of the olfactory epithelium labeled with the α2A antibody. Left, embryo injected with control MO + α2AChR mRNA exhibits strong labeling with the antibody. Middle, embryo injected with α2A MO exhibits minimal, if any labeling with the α2A antibody. Right, α2A MO-injected embryo rescued with α2AChR mRNA exhibits good labeling with the α2A antibody. D) Photomicrographs of spinal cord in 30 hpf embryos labeled with the α2A antibody. Left, α2A MO-injected embryo rescued with α2AChR mRNA exhibits good labeling with the α2A antibody (RB neurons indicated

by white arrows). Right, embryo injected with the $\alpha 2A$ MO exhibits reduced labeling with the $\alpha 2A$ antibody. Asterisks denote significant differences between baseline and nicotine-induced bend rates ($p < 0.001$; repeated measures ANOVA). ‡ denotes a significance difference in the nicotine-induced bend rates of embryos injected with $\alpha 2A$ MO + $\alpha 2AChR$ mRNA and $\alpha 2A$ MO ($p < 0.001$; repeated measures ANOVA).

Figure 6. Output elements of the motor circuit are not altered by knockdown of α 2A nAChR subunit expression

A) Experimental design for the dechorionation experiment. The spontaneous motor activity was first recorded while embryos were still in their chorions for 3 minutes (black line). They were then transferred into embryo medium containing 60 μ M nicotine for 1 minute (grey line) and subsequently placed in fresh embryo media and allowed to wash for 20 minutes (dashed line). The spontaneous activity following the 20-min wash was recorded for 3 minutes and then the embryos were quickly dechorionated (red line, minute 27) and recorded for another 3 minutes. B) Representative plot of the motor activity as described in A. The nicotine-induced response at 22 hpf in α 2A morphants ($n=10$) was reduced, whereas, the response to dechorionation (red line, minutes 27–30) produced a large increase in the bend rate similar to control MO-injected embryos ($n=10$). C) Representative images of control MO-injected and α 2A morphants reveal post-synaptic muscle nAChRs using rhodamine-conjugated α -bungarotoxin (α -btx) at 26 hpf. D) Primary motoneuron axons at 30 hpf were labeled using znp1 and both control MO-injected embryos and α 2A morphants exhibited normal axonal trajectories (see Table 2 for quantification). E) Slow muscle fibers stained with F59 were not affected in the α 2A morphants when compared to control MO-injected embryos. Asterisks in B denote significant differences between baseline and nicotine-induced bend rates and between baseline and dechorionation bend rates ($p < 0.001$; repeated measures ANOVA). ‡ denotes significance between control MO and α 2A MO

nicotine responses ($p < 0.001$; repeated measures ANOVA). Scale bars, 20 μm in C and D; 40 μm in E.

Figure 7. Touch response and RB development following knockdown of nAChR α2A subunit expression

A) Video stills show a ~31 hpf α2A morphant that exhibits a touch response following tactile stimulation to their tail. Right, quantification of touch response (see materials and methods) from 31 hpf non-injected embryos, control MO-injected and α2A morphants showed that there was no significant difference. The number of embryos analyzed is indicated by N. B) Representative images of anti-acetylated tubulin (aat) labeling (lateral view) revealed that RB tubulin distribution in the peripheral processes is fragmented at 48 hpf in control MO-injected embryos and α2A morphants. C) Schematic illustration shows dorsal view of the spinal cord and the migration of RB neurons (yellow circles). Early in development, RB neurons are positioned bilaterally at the dorsolateral aspect of spinal cord (shown here at 24 hpf). Around 30–36 hpf, they enter a migratory phase (indicated by red arrows) to the midline (marked by red dashed line) forming an almost linear row of cells by 48 hpf. The grey shaded areas mark the yolk sac. Cartoon is not drawn to scale. D) Representative images of RB neurons (dorsal view) in 48 hpf *Tg(isl2b:GFP)* zebrafish injected with a control MO or α2A MO. See Table 2 for quantification of the RB neuron migration. The midline of the spinal cord is indicated by the dashed yellow line. Scale bars, 20 μm in B; 10 μm in D.

Figure 8. Rohon-Beard neurons are candidate spinal neurons for initiating the nicotine-induced motor behavior

A proposed cellular model shows the possible interaction between RB neurons and CPG elements that produce locomotor behaviors in zebrafish. Nicotinic activation of $\alpha 2A$ -containing nAChRs ($\alpha 2A$ *nAChRs) located on RB neurons will activate excitatory CoPA cells via glutamate release (highlighted by thick black lines). The CoPA interneurons will in turn provide excitation to contralateral interneurons of the CPG via glutamate release which in turn excite motoneurons to activate muscle. Dashed black line indicates the midline of

spinal cord. Abbreviations: CoPA: commissural primary ascending; CPG: central pattern generator; MN: motoneuron; RB: Rohon-Beard neuron.

Table 1

Epitope sequence alignments for the design of the $\alpha 2A$ antibody.

Chrn $\alpha 2a$	WVHSVFLDHIPRWLF---MRRPEPEKKPKK-T-----ANLSTSKSWFQQET
Chrn $\alpha 2b$	WVHSVFLDLIPRWLF---MRRPAPDSRRRQKLLLLQRQVPGMTARPANASLSTSTSWFRGQN
Chrn $\alpha 1$	WVRKIFIDTIPNMFFSTMKRPSQERQEK-RLFPADFDISDISGKMPASVTYHSPITKNPD
Chrn $\alpha 3$	WVRRVFLSLLPRVMF---MTRPEKQEVPAKQC-----SVHPPVSSKQTSVCRGPQ
Chrn $\alpha 5$	WVRRIFLHHLPRLLC---MRSHV-DRYAT-----SA--GTRTGGLGYGKSLSQDTN
	** : *: :*. : * :
* conserved residue, : conserved substitution, . semi-conserved substitution	

Amino acid sequence of the Chrn $\alpha 2A$ epitope that was chosen to be targeted by the Chrn $\alpha 2A$ antibody compared against the 4 most related Chrn subunits. The area highlighted by the grey box indicates the amino acid sequence that the Chrn $\alpha 2A$ antibody was targeted against.

Table 2

Morphology of motoneuron axon trajectories and RB neuron migration in morpholino injected zebrafish embryos.

<u>Normal Trajectories (%)</u>					
	Dorsal	Ventral	Total hemisegments	Fish (n)	
Primary motoneurons *					
Control MO	93.96±1.99	88.88±5.57	247	18	
α2A MO	96.86±1.20	96.36±1.38	258	18	
RB neurons **					
	<u>RB cell distance from midline (μm)</u>		<u>RB cells (n)</u>		<u>Fish (n)</u>
	wt	isl2b	wt	isl2b	wt isl2b
Control MO	3.05±0.14	2.73±0.19	157	67	12 7
α2A MO	2.95±0.13	2.85±0.26	193	43	16 3

* Primary motoneuron axon trajectories were evaluated in 28–30 hpf embryos using the znp-1 antibody. There were no significant differences in the dorsal and ventral trajectories (p-values > 0.05).

** RB neuron migration was assessed in 48-hpf wildtype (wt) and Tg(*isl2b*:GFP) (*isl2b*) fish. The Hu antibody was used in wildtype fish to label RB neurons. In *isl2b* transgenic fish RB express the green fluorescent protein. There were no significant differences in RB neuron migration distances between control and α2A MO injected embryos (p-values > 0.05). All values are reported as mean±SEM.