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The N-Methyl-D-Aspartate Receptor in Heart Development: A Gene Knockdown Model Using siRNA

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Abstract

Antagonists of the N-methyl-D-aspartate receptor (NMDAR) may disrupt the development of the cardiac neural crest (CNC) and contribute to conotruncal heart defects. To test this interaction, a loss-of-function model was generated using small interfering RNAs (siRNA) directed against the critical NR1-subunit of this receptor in avian embryos. The coding sequence of the chicken NR1-gene and predicted protein sequences were characterized and found to be homologous with other vertebrate species. Analysis of its spatiotemporal expression demonstrated its expression within the neural tube at pre-migratory CNC sites. siRNA targeted to the NR1-mRNA in pre-migratory CNC lead to a significant decrease in NR1 protein expression. However, embryo survival and heart development were not adversely affected. These results indicate that the CNC may function normally in the absence of functional NMDAR, and that NMDAR antagonists may have a complex impact upon the CNC that transcends impairment of a single receptor type.

Keywords

NMDA receptor; electroporation; siRNA; NR1 subunit; heart defects; homocysteine; chicken embryo

INTRODUCTION

Congenital heart defects (CHD) are the most common congenital defect seen in the human population; with an incidence of approximately 10/1000 live births [1,2]. As a group these defects account for approximately 10% of the infant mortality and nearly 50% of the deaths resulting from malformations [3]. Nearly 25% of all CHD involve the conotruncus, a portion of the outflow tract that is composed of a mixed mesenchymalmyocardial structure called the *truncus arteriosus* and the mainly myocardium-containing *conus cordis*. Although these defects are common and our ability to recognize and treat individuals with CHD has increased over the past several decades, their etiology remains largely unknown due to the morphological complexity of the developing heart and the contributions from different cell types that are necessary for normal heart development, all of which are under strict spatiotemporal regulation

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from numerous genes. As a result of its complexity and strict molecular regulation there are a number of environmental conditions that may disrupt the normal ontogeny of the heart.

One environmental condition that has shown to impact negatively upon development and has garnered considerable attention is a folate deficiency [4-11]. The association between inadequate folate and abnormal development has been recognized for several decades, and there are several biochemical and/or molecular mechanisms that have been identified that may contribute to the observed abnormalities. Obviously the folate cycle, methyl group availability, nucleic acid synthesis and thus mitosis, all can be adversely impacted when folate is scarce. Additionally, an inadequate source of methyl groups may lead to gene hypomethylation. Alternatively, it has been hypothesized that the increase in homocysteine that accompanies folate insufficiency is the actual risk factor. We [12-14] and others [15,16] have shown that hyperhomocysteinemia is associated with conotruncal defects in the chicken embryo model, while recent evidence indicates that maternal hyperhomocysteinemia also may be associated with conotruncal defects in humans [17]. In fact it appears that hyperhomocysteinemia, rather than hypomethylation, is a more important risk factor in the development of heart defects [18].

The mechanisms or cellular pathways that are disrupted by homocysteine that may obstruct normal heart development are not well-understood. We have tested the hypothesis that homocysteine may affect heart development by interfering with the cardiac neural crest *via* its ability to perturb the N-methyl-D-aspartate receptor (NMDAR). This receptor regulates a calcium channel and is a subclass of the ionotropic glutamate receptors, where depending upon cellular conditions homocysteine may act as either an antagonist or an agonist [19]. The NMDAR is critically important for normal neural development, neuronal survival and synaptic plasticity [20-23]. Given its key role in neurogenesis, and the fact that normal development of the conotruncus depends upon neural crest cells that arise from the neural tube, then migrate into and populate the outflow tract [24], we began to test the hypothesis that the function of this receptor plays a critical role in the development of the outflow tract [25]. We found that the teratogenic effect of homocysteine was mitigated in the presence of NMDAR agonists [13]. Additionally, other well-characterized NMDAR antagonists including those that block the glycine binding site of the NMDAR, as does homocysteine, perturbed neural crest-related heart development [26]. Furthermore, homocysteine alters the differentiation, cell cycle progression and migration of neural crest cells [16,27]. Although the NMDAR is expressed in avian CNC cells *in vitro* [28] and at critical times during early development *in vivo*, it is not expressed in the mouse embryo until mid-gestation [29,30], and as predicted by our hypothesis, the mouse embryo is not susceptible to hyperhomocysteinemia-induced conotruncal defects [30].

Therefore, we decided to test the role of reduced NMDAR availability during the early stages of avian heart development by using siRNAs to reduce the expression of the NMDAR NR1-subunit in the CNC. Three distinct NMDAR-subunits (NR1, NR2, and NR3) have been identified, which confer differential activity. Most of these receptors are heteromeric proteins composed of the NR1-subunit that possesses a glycine binding site and the NR2 subunit that contains the glutamate binding site [31-34]. However, among these subunits the NR1 is uniquely required for NMDAR function, so its impaired expression will necessarily limit NMDAR function. Using these techniques, the current studies demonstrated that the reduced expression of the NMDAR NR1-subunit specific to the pre-migratory CNC did not have any obvious negative effect upon heart development.

MATERIALS AND METHODS

Chicken Eggs/Embryo Staging

Fertile pathogen-free White Leghorn eggs were obtained from SPAFAS (Roanoke, IL) and incubated at 38°C in a humidified incubator with programmed turning as described previously [13,30,35]. Embryos were staged according to the Hamburger and Hamilton method, simplified subsequently as “HH stage” [36].

Cloning of the chicken NR1 cDNA

Total RNA was isolated from embryonic (E) 17 chicken embryos using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. The NR1 cDNA was cloned using the Rapid Amplification of cDNA Ends or RACE strategy and the GeneRacer kit (Invitrogen). Briefly, 3 µg of total RNA was initially treated with calf intestinal phosphatase followed by tobacco acid pyrophosphatase to leave a 5'-phosphate for ligation. An RNA oligomer was ligated to the 5'-end of the mRNA, creating a priming site for the GeneRacer 5' PCR primers that were provided with the kit. Reverse transcription (RT) was performed using SuperScript II reverse transcriptase. For the 3'-end NR1 cDNA synthesis, an oligo-dT-based primer (GeneRacer Oligo dT) was used for first-strand cDNA synthesis. To synthesize the 5'-end of the NR1 cDNA, a gene-specific primer (5'-CACCAGCCACACGTAGCCGGAGCCCGTCA-3') was used in separate RT reactions, designed with the Primer Express Software v.2 (Applied Biosystems), based on the known alignment of the duck NR1 cDNA with equivalent human, mouse, and rat sequences (GenBank accession numbers: duck- D83352; human- D13515; rat- L08228; mouse- D10028) using the CLUSTALW algorithm (www.ebi.ac.uk/clustalw). To amplify the 5'- and 3'-ends of the NR1 cDNA, expressed sequence tags (EST) from the BBSRC UMIST chicken EST database were screened and aligned using the duck NR1 sequence. The following primers were used in the touchdown PCR reactions using Platinum Taq DNA Polymerase: for 3'-NR1 cDNA end amplification, a gene-specific forward primer (5'-TGCCTTCATCTGGGACTCGGCGGTGCTG-3') and a reverse primer of known sequence (GeneRacer 3'); for 5' NR1 cDNA end amplification, a forward primer of known sequence (GeneRacer 5') and a reverse gene-specific primer (5'-GGTGTTAGGTGATCGTTGGGAGCAGGAGGGTGACT-3'). Reaction products were isolated from the agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and subcloned into a pCR II-TOPO vector (Invitrogen). This vector was transfected into TOP10 One Shot *E. Coli* cells. Plasmid DNA was purified using the QIAprep Spin Miniprep kit (QIAGEN) and cDNA inserts were sequenced on an ABI Prism 3700 sequencing platform (Applied Biosystems). The 5'- and 3'-end sequences of the chicken NR1 cDNA were used to design primers to amplify the remaining NR1 cDNA sequence, using the forward 5'-TCCAAGGCTGAGAAAGTGCTGCAG-3', and reverse 5'-AACGCCAGCTGCATCTGCTTCTCC-3' primers. NR1 sequences obtained from multiple PCR and sequencing replicas were mapped using the Sequencher software (Gene Codes). NR1 cDNA translation was performed with the JustBio translation tool (www.justbio.com). Multiple sequence alignments of the mRNA and protein sequences of the duck, human, mouse and rat (GenPept accession numbers: duck BAA11898; human-BAA02732; mouse-BAA00920; rat-NP_058706) were performed using the CLUSTALW algorithm.

Temporal Expression of the NR-1 subunit of the NMDAR

RT-PCR was performed at various HH stages relevant to CNC cell development. Whole embryos at HH stages 8, 14, 18, heads isolated from embryos at HH stages 28 and 34, and positive-control brains from E17 embryos, were placed in RNAlater (Ambion) and stored at -20°C until further processing. Total RNA was isolated as described above. RT (0.15 µg/µl per reaction) was performed using SuperScript II reverse transcriptase, and either the GeneRacer oligo-dT primers or random hexamers (IDT, IA). Short-distance (SD)-PCR was

used to evaluate the expression of the NR1 subunit and to verify the presence of the NR1 protein at HH stage 10. For this, PCR primers for the chicken NR1 cDNA were designed to amplify a highly conserved, 424-base pair (bp) region of the NR1 sequence using the rat NR1 cDNA sequence as a reference [37]. Primers were also designed to amplify a 181-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment, which was used as a positive control (forward 5'-GGACACTTCAAGGGCACT GT- 3', and reverse 5'-TCTCCATGGTGGTGAA GACA-3'). Reaction products were analyzed on a 1% agarose gel and verified by sequencing.

In Situ Hybridization (ISH)

Riboprobe synthesis was initiated from total RNA isolated from HH stage 10 and HH stage 15 embryos as described previously (12). Antisense and sense riboprobe were synthesized from the NR1 cDNA-containing plasmid that was linearized with Spe I and Not I, respectively. Antisense and sense templates were treated with proteinase K 200 µg/ml and SDS 0.5% for 30 minutes at 50°C, isolated by phenol:chloroform and ethanol precipitation. The purified linearized templates were then transcribed by T7- (antisense) and SP6- (sense) RNA polymerases. The transcription mixture (20 µl) included 1 µg of linearized template cDNA per reaction, NTPs at 5 mM each for the SP6 reaction (7.5 mM for T7 reaction), with UTP:digoxigenin -UTP molar ratio of 1:3, 10× reaction buffer (Ambion) 2 µl, enzyme mix (SP6 or T7, Megascript, Ambion) 2 µl, and nuclease-free water. The reaction mixture was incubated for 6 hours at 37°C followed by a DNase I (Ambion) treatment for 15 minutes at 37°C. Riboprobes were then extracted with phenol:chloroform and precipitated with isopropanol. The antisense and sense riboprobes were maintained as a pellet in isopropanol at -20°C until used for ISH. For ISH all steps were performed at room temperature unless specified. HH Stage 10 and 15 embryos were collected in ice-cold PBS, and fixed in 4% paraformaldehyde overnight at 4°C. Embryos were dehydrated by successive ethanol baths (70%, 95%, and 100%), and toluene (×2, 30 minutes each), and embedded in paraffin. 10-µm-thick transverse sections were cut and mounted on poly-L-lysinated slides, air-dried overnight, and stored desiccated at 4°C until used for ISH. Sections were rehydrated and post-fixed with 4%-paraformaldehyde and incubated in 0.1% active DEPC-PBS. Endogenous peroxidase activity was inhibited by incubation in 0.3% H₂O₂ in methanol. Sections were then rinsed in nuclease-free water and equilibrated in 5× SSC (NaCl 0.75 M, Na-Citrate 0.075 M). Sections were then prehybridized for 2 hrs at 58°C in the hybridization mix (formamide 50%, 5× SSC, pH 7.5, salmon sperm DNA 50 µg/ml) and then 24 hrs at 58°C in the presence of either antisense or sense RNA probes (500 µg/ml). After incubation, sections were washed with increasing stringency of SSC at 65°C and equilibrated in TBS (Tris-HCl 0.1 M, pH 7.5, NaCl 0.15 M). Slides were then blocked in TNB solution (Tris-HCl 0.1 M, pH 7.5, NaCl 0.15 M, and 0.5% blocking reagent and then incubated with anti-digoxigenin, peroxidase-coupled antibodies (1:100; anti-digoxigenin-POD, Fab fragments, Roche), for 30 minutes. Sections were then rinsed in TNT buffer (Tris-HCl 0.1 M, pH 7.5, NaCl 0.15 M, Tween 20, 0.05 [v/v]), and incubated with TSA Plus Direct-Cyanine 3 (Cy3) solution (NEN Life Science) according to the manufacturer's protocol. Slides were washed in TNT buffer, mounted in Vectashield medium with 4',6-diamidino-2-phenylindole (DAPI, Vector) counter-stain for nuclear localization, coverslipped, visualized under a microscope equipped with epifluorescence (Axiovert 135, Carl Zeiss), and photographed with a digital camera (Magnifier, Optronics).

Small interfering RNA (siRNA) techniques

For the bilateral transfection of the neural tube with siRNA duplexes were assessed for both the efficacy of the NR1 knockdown, embryo survival and CHD frequency. Embryos were placed randomly into one of 4 experimental groups: [1] *control, wild-type* (CWT), embryos untreated; [2] *control, no transfection* (CNT), where the embryos underwent all procedures except transfection; [3] *control, transfection* (CT), embryos transfected with a reporter vector containing an enhanced-green-fluorescent-protein (pCAX-EGFP, kindly provided by Dr.

Krull) and negative-control siRNA; [4] and knockdown (K), embryos were transfected with both the EGFP and an siRNA containing vector targeted against NR1-mRNA (siRNA-NR1). In groups 2, 3 and 4, the embryos were incubated for 29-33 hours, HH stage-9, 7-8 somites, or the initial stages of CNC migration. Prior to their undergoing the transfection procedure, embryos were inspected to ensure they were morphologically normal and of the proper developmental stage; all other embryos were discarded. To assess siRNA knock-down efficacy, embryos from the CT- and K-groups were incubated for a total of 3.5 days, when the NR1 protein was analyzed by immunoblotting. Negative-control and siRNA-NR1 and pCAX-EGFP were transfected into selected areas by electroporation, following methods described previously [38-41]. In summary, eggs were windowed and the embryos staged. Part of the vitelline membrane was removed along a line parallel to the long axis of the embryo at the edge of *area pellucida*. For all groups the injection solution included pCAX-EGFP 2 mg/ml, carboxymethylcellulose 2 mg/ml, and phenol-red 0.1% v/v in nuclease-free water. For groups CT and K, negative-control siRNA, and siRNA-NR1 were added at a final concentration of 500 µg/ml, respectively. The transfection solution (pL) was delivered into the neural tube lumen, between the second and third somite, by a micropipette oriented cranially in the saggittal plane, and attached to a pressure injector (Picospritzer III, Pneutronics). For groups CT and K an electrode pair (Parallel Fixed Platinum Electrodes, Protech International) was applied above the blastoderm horizontal and parallel to the embryo on each side of the neural tube, pointing in a cranial-to-caudal direction. This setting allowed for adequate electrode coverage of the entire neural tube region corresponding to the site premigratory CNC, *i.e.* from the otic placodes to the caudal border of somite 3. After circuit resistance was adjusted to 0.5-0.8 kOhms test solutions were injected into the neural tube and the electroporation current applied (4 square wave pulses, 50V, 50 ms). After electroporation, 0.05-0.2 ml of Ringer's with penicillin 100 UI/ml and streptomycin 100 µg/ml were pipetted over the embryo. Eggs were then sealed using 3M Magic scotch tape, and incubated until they were harvested.

siRNA Selection

The chicken NR1 mRNA (GenBank accession number: AY510024) was used as the template for siRNA-NR1 design using the HiPerformance Design Algorithm (Novartis). Candidate siRNA target sequences were then compared with the human NMDAR NR1-mRNA (GenBank accession number: NM_000832) to select siRNAs homologous to the inferred constitutive exon sequences of the GRIN1 gene. The two highest-ranking siRNA duplexes were selected, representing the best putative combination of knockdown potency and specificity. Targeted siRNA-NR1 sequences 5'-CACCGACGGGTAGAATTCAA-3', and 5'-ACGCATGTCTATATATTCTGA-3' were homologous to the NR1 subunit mRNA nucleotide positions 1008-1028, and 375-395, respectively. A proprietary siRNA duplex with the target sequence 5'-AATTCTCCGAACGTGTACGT-3' (QIAGEN) was used as a negative control in the NR1 silencing experiments.

Embryo survival and CHD rate assessment

Embryos were examined under a dissecting scope after 6.5 days total incubation time (~5 days after electroporation), corresponding to HH stage 30. This time point follows completion of the major stages of heart looping, and the septation of *truncus arteriosus*/distal *conus*, thus allows for the diagnosis of the main categories of cardiac outflow defects. Embryo survival was defined by the demonstration of the heart beat. Embryos were evaluate for the presence and types of CHD as previously described [13,35].

Immunoblotting

After 3.5 days (~50-55 hours after electroporation), embryos that were selected randomly for groups CT and K were evaluated for viability and staged. Samples were obtained from

surviving embryos HH stage 21-22 with preserved landmarks (otic placodes and somite borders) and symmetrical neural tube. Embryo segments between the otic vesicles and the inferior border of third somite were dissected out using fine tungsten needles. Each of these neural tube segments were trimmed of surrounding tissue and placed into T-PER (Tissue Protein Extraction Reagent; Pierce). The tissue was then homogenized, centrifuged at 10000 rpm for 5 minutes and supernatants mixed with Laemmli buffer (Tris-HCl 62.5 mM, pH 6.8, SDS 2%, glycerol 25%, Bromophenol Blue 0.01% with added dithiothreitol 350 mM). For each lysate, total protein was determined by BCA Protein Assay Kit (Pierce). Protein samples loaded onto gels were derived from three embryos *per* group at equal protein concentrations. For blotting, 15 µg of protein from each of the experimental chicken samples or from rat brain extract (positive control) was loaded onto a 7.5% SDS-PAGE resolving gel. After electrophoresis proteins were transferred to nitrocellulose membranes in transfer buffer (Tris 25 mM, glycine 192 mM, methanol 20% [v/v]) with constant 40 V. Membranes were rinsed with TBS-T (Tris 20 mM, NaCl 500 mM, pH 7.4, Tween 20, 0.1% [v/v]), and blocked with TBS-T and 2% ECL Advance Blocking Agent (Amersham). Membranes were incubated with primary mouse monoclonal antibodies against NR1 (Synaptic Systems) and β -actin (Sigma) diluted in blocking solution at 1:10000 and 1:5000, respectively. Membranes were incubated with primary antibodies for one hour at room temperature, rinsed and then incubated with a secondary, peroxidase-linked, antibody (Amersham) at 1:5000. Blots were then rinsed and exposed to ECLTM Advance Western Blotting Detection Kit (Amersham).

Densitometry

Immunoblot films (Kodak BioMax Light Film, Sigma) were scanned using a Bio-Rad GS-800 Densitometer. Digital immunoblot photographs were analyzed using the Quantity One 1-D analysis software (Bio-Rad). For each lane of interest, the NR1 and β -actin bands were defined and the background removed using a local background subtraction method. For each lane of interest, differences in loading were accounted for by calculating the ratio of NR1 to β -actin band intensities.

Statistical analysis

All statistical calculations were performed using the Stat $\times$ act V.8 statistical software (Cytel). The significance level was set at 0.05. Survival and CHD frequencies were analyzed using a nonparametric test (Fisher-Freeman-Halton). Pair-wise comparisons were performed after correction for multiple comparisons using the Bonferroni method. NR1 knockdown efficiency was analyzed by comparing the relative band intensity ratios calculated with a paired t-test with a two-tailed distribution.

RESULTS

CLONING AND EXPRESSION PATTERN OF THE NR1 SUBUNIT

The chicken sequence of the NR1-subunit cDNA had not been cloned previously, therefore, applying a RACE-based strategy, we generated a 3820-bp cDNA fragment (Lie and Rosenquist, GenBank accession number AY510024), which contained a 2898-base pair coding sequence that encodes a predicted 965- amino acid protein (GenPept accession number AAR98574). Compared to the rat NR1 sequence, the cloned chicken NR1 cDNA lacked both an N1-cassette and the equivalent sequence for exon-3, and displayed poor homology at the 3'-end. Instead, the 3'-end of the chicken NR1 cDNA, or putative C2 cassette, aligned to the duck NR1 sequence with high homology. The predicted chicken NR1 protein sequence was >99% homologous to the duck protein, where the only difference was a histidine (His) to asparagine (Asn) substitution at position 371 (Figure 1). The degree of homology between the duck, human, rat, and mouse mRNA and their protein sequences of the N1-lacking NR1 isoforms are presented in Table 1.

EXPRESSION PATTERN

To determine the timing for our subsequent siRNA electroporation experiments, we initially assessed the temporal expression of the NR1-subunit in samples obtained from whole embryos at stages HH 8, 14, and 18, heads isolated from embryos staged HH 28, and 34, as well as control brains from E17 embryos. NR1 expression was confirmed at all stages (Figure 2). Additionally, the spatial expression of the NR1 gene during CNC delamination and migration was characterized using fluorescence ISH on transverse sections of embryos at HH stages 10 and 15, respectively. The method used in these studies allowed signal detection at the cellular level while retaining structural integrity of the tissue by the use of paraffin-embedded sections [42]. In the negative-control NR1-sense treated sections, as expected, no expression was detected. In the younger embryos (HH stage 10) when the neural crest is in the early stages of emigrating from the neural tube the antisense probes failed to detect NR1 expression. Sections from later staged embryos (HH stage 15) when the neural crest are migrating the NR1 mRNA signal was detected but appeared to be confined principally to the neural tube and mainly to the perinuclear region of these cells (Figure 3).

IN VIVO ELECTROPORATION

Distribution of EGFP—Approximately a third of the embryos that were treated with the pCAX-EGFP plasmid expressed EGFP bilaterally (Figure 4). As expected, the neural basal lamina confined the transfection to the neural epithelial cells, as indicated by the lack of fluorescence in the somites or surrounding mesoderm (Figure 4). EGFP expression was not observed in embryos that were treated with plasmid but were not treated by electroporation. Fluorescence of representative embryos 36 and approximately 72 hours after electroporation is shown in Figures 5-7. It is obvious from these images that the transfection can be targeted to the CNC and that these manipulations do not appear to alter the migration patterns of these cells since fluorescent cells can be seen moving toward the outflow tract at appropriate stages of development (Figures 6, 7). These images also demonstrate the variability in fluorescence among embryos.

Embryo Survival—Preliminary experiments showed that electroporation, by itself, resulted in a high rate of embryo mortality. By day 8 (HH stage 34) or 6.5-7 days after treatment, from the total of 200 embryos that were subjected to electroporation, only 3 survived. Therefore a more useful collection time-point was chosen for embryo survival and CHD rate analysis. For these and the remaining studies embryos were subjected to electroporation and then incubated 6.5 days (HH stage 30), a time-point where the number of embryos surviving was relatively high and the early stages of heart development could be assessed accurately.

NR1 Knock-Down with siRNA—NR1 protein expression was confirmed in the neural tube of embryos at HH stages 21-22, or day 3.5 of incubation. For these experiments, neural tubes were isolated from untreated embryos and the expression of the NR1 protein assessed by immunoblot analysis. The specificity of the antibodies against the NR1 protein and β -actin were confirmed using rat brain extract as a positive control. Additionally these antibodies have been shown previously to cross-react with their respective chicken proteins [43]. Results from these studies demonstrated an approximate 115 kD band representing the chicken NR1-subunit protein, which is of similar molecular weight as the control rat brain (Figure 8). When embryos were subjected to electroporation with the siRNA to the NR1-subunit there was a significant reduction in its protein expression in these embryos compared to those embryos that were given only the control siRNA (Figures 8 and 9). Densitometry performed on these gels demonstrated that the siRNA-NR1 treatment led to an average reduction of approximately 35% in the expression of the NR1 protein (Figure 9). This degree of reduction was within the predicted range of protein suppression based upon the typical result of siRNA knock-down in other

models. Based upon these published outcomes of siRNA knock-down, this repression was predicted to be sufficient to inhibit the cellular function of the targeted protein [44-50].

EMBRYO SURVIVAL AND PHENOTYPE WITH NR1 KNOCK-DOWN

Although the process of electroporation had a negative impact upon embryonic survival, this was not exacerbated by the knock-down of the NR1 protein (Table 2). Hearts were evaluated according to our published methods [35], and no abnormalities were observed in the embryos that received the siRNA-NR1. These results indicated that NR1 knockdown was not associated with an increased frequency of heart defects in this embryo model (Table 3).

DISCUSSION

The general purpose of these experiments was to characterize further the role of the NMDAR during heart development. This was based upon our earlier data demonstrating that pharmacological NMDAR antagonists were associated with neural crest-derived conotruncal heart defects in the chicken embryo model [13,26], and that the NMDAR is expressed robustly in these embryos, as demonstrated by immunoblotting and quantitative PCR analyses [30]. While the expression of the NMDAR has not been studied in the early human embryo, most of the compounds that were identified in the large-scale *Baltimore-Washington Infant Study 1981-1989* [51] as being associated with major conotruncal and other cardiovascular malformations also had the ability to act as NMDAR antagonists. Thus the functional role of the NMDAR during early development is a topic of ongoing interest.

A typical next step in advancing knowledge of the functional role of a given protein is to analyze the effect of its knockout in a mouse model; however, this receptor is not expressed in the mouse embryo during the early stages of development that are known to be important in the migration of the CNC [30]. As an alternative, we designed the current study to apply siRNA technology to the functional knock-down of the NMDAR in the pre-migratory CNC of the chicken embryo model.

The function of this receptor is completely dependent upon the presence of the NR1 subunit, which is the rationale for targeting the siRNA to this protein. However, since the available information on the chicken NR1 gene and protein was limited, it was important that this lack in our knowledge be addressed by performing preliminary studies to determine its gene and protein structure and sequences as well as its spatiotemporal expression during the early stages of development. In general, the NR1 isoform that was isolated in the current study was homologous to previously published sequences for both mammalian and avian species [20, 52]. This isoform, however, lacked the N1 cassette, or rat exon 5, but contained both C1 and C2 C-terminal cassettes; all three of these cassettes have been reported in other isolated chicken NR1-isoforms [52-54]. The predicted encoded protein from this isoform contains 965 amino acids, and the predicted 21 amino acid deletion that corresponds to the N1 cassette [55]. The structural differences observed between the isoform in the current investigation and those described previously are not necessarily unexpected. The clones from the various studies were obtained from different tissues and alternative splicing of the N1-cassette is regulated differently among different tissues during development [56,57].

The significance of the Asn for His substitution at position 371 in the chicken NR1 sequence, when compared to the duck sequence, is unknown. However, this amino acid substitution occurs within the N-terminal domain which could alter the assembly dynamics between the NR1 or with NR2/NR3 subunits. Although the homology appears to be very similar across species for the majority of the NR1 sequence it is divergent in the distal 3'-end, downstream of the C1 cassette. In fact this diversity between the current isoform and the rat NR1 sequences suggests a different mechanism of receptor trafficking in the chicken as opposed to mammals,

which has been proposed previously [48]. This structural disparity between the species may explain the different ontogeny of the chicken NR1, compared with that of the mouse [30].

The NR1 mRNA was observed almost exclusively within the neuroepithelium of the neural tube which was the target tissue of the siRNA transfections. Immunoblots of the neural tube samples demonstrated that bilateral transfection of the siRNA to the NR1 transcript resulted in an approximate 35% reduction in the expression of the NR1 protein. This reduction is comparable to the suppression obtained in other studies by siRNA techniques and has been shown to be sufficient to have a measurable effect upon the function of the targeted protein [44-50,58]. Although great care was taken to ensure that these samples were as pure as possible, there remains the potential that they were contaminated with trace amounts of mesenchyme or other tissues that did not express NR1. Therefore, the observed 35% reduction could represent an underestimation of the true decrease of NR1 expression in the premigratory CNC. Furthermore, the post-treatment embryos were collected 48hr or more after siRNA treatment, and although we do not know the specifics of the post-siRNA treatment kinetics of NR1 expression, we do know that siRNA-induced suppression is not permanent, thus it may be assumed that the cells that had responded to siRNA were resuming normal NR1 expression.

A reduction of 35% or more in the expression of a key protein following siRNA knock-down has been shown to be sufficient to have a measurable effect upon the function for a given protein. However, in this case, siRNA knock-down did not result in neural crest-related early developmental dysfunctions that produce conotruncal heart defects. Since the treated embryos did not express NR1 in the neural tube during the time of formation and initial migration of the CNC, these results do not support a key role for the function of the NMDAR during these processes. Although these results do not appear to be congruent with previous studies investigating the effects of NMDAR antagonists [26,35], pharmacologic, gene knock-down and knockout techniques all have limitations, and the result of none of these can necessarily be interpreted as the single correct result. For example, there is not a linear association between receptor binding affinity of the NMDAR agonists/antagonists, and embryo survival or susceptibility to heart or other defects [26]. There also may be similar, non-NMDA, receptors that play a critical role in the development of the heart or key differences in the pharmacodynamics of the various teratogenic compounds [13], which ultimately may explain the differences between these studies. It has been shown that antagonistic pharmacological effects may not be recapitulated in single gene knockouts, and knockouts of functionally important genes has sometimes yielded unexpected results that do not support results from the majority of the pharmacological experiments. For instance, antagonists of neuropeptide-Y are associated with the earlier onset of puberty, but there was no similar result in several neuropeptide-Y knockout mouse strains [59]. In another example, it is well-demonstrated that the effects of cannabinoids are mediated by the cannabinoid receptor type-1(CB1), but in the conditional CB1 knockout mouse, the effects of cannabinoids were not altered [60]. Thus, the teratogenic interaction among the NMDAR antagonists and other receptors is likely to be quite complex and may involve other, as yet, undiscovered signaling pathways.

In summary, the data obtained in the current experiments provide additional characterization of the mRNA sequence of the chicken NMDAR-NR1 subunit, particularly during the early stages of heart development. Our data complement the findings of others on the expression of this gene in the chicken. In addition, we provide a method for the use of siRNA-electroporation to generate specific gene knock-down models. In this case the method was restricted to assess the effects of gene expression on the premigratory CNC cell population. This method permits further dissection of the molecular events involved in the development and migration of these cells. While our data do not support a direct cause and effect between NR1 expression and neural crest-derived heart defects, normal heart development is the result of interactions among

various cell types and their spatiotemporal regulation by multiple genes. These results can be included in the larger menu of results that are available to design further studies.

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MSTMRLLLLLFFSSSFARAGCDPKIWNIGAVLSTKKHEQIFREAVNQANKRHGTWKLQLNATSVTHKPNA
 IQMALSVCEDLISSQVYAILVSHPPAPNDHLTPTPVSYTAGFYRIPVIGLITRMSIYSDKSIHLSFLRTVPPYS
 HQANVWFEMMRVFNWVNHVILVSDDH EGRAAQKLET LLEERESK**AB**KVLQFDPGTKNVTSLLEAKELE
 ARMILSASEDDAATVYRSAAMLNMTGSGYVWLVGEREISGNALRYAPDGVIGLQLINGKNESAHSIDAVA
 VVAQAVHDLFEKENITDPPRGCVGNTNIWKTGPLFKRVLMSKYSEGVTGRVEFNEDGDRKFANYSIMN
 LQNRKLQVQGIYNGSNVLTNDRKIWPGETEKPQGYQMSTKLKVTIHQEPFVYVKTQADGTCREEFTI
 NGDPVKKVFCTGPNETIPGRPTVALCCYGFCIDLLIRLAGVMNFTYEVHLVADGKFGTQERNNSNKEW
 NGMMGELLSGQADMI VAPLTINNERAQYIEFSKPFKYQGLTILVKKEIPRSTLDSFMQPFQSTLWLLVGLS
 VHVVAVMLYLLDRFSPFGRFKVNSEEEEDALTSSAMWFSWGVLNLSGIGEGAPRSFSARILGMVWAG
 FAMIIVASYTANLAFLVLD RPEERITGINDPRLRNPSDKFIYATVKQSSVDIYFRQVELSTMYRHMEKHN
 YESAAEAQAVRDNKLHAFIWDSAVLEFEASQKCDLVTTGELFFRSGFGIGMRKDSPWKQNVSLAILKSH
 ENGFMEDLDKTWVRYQECDSR SNAPATLTFENMAGVFMLVAGGIVAGIFLFI EIA YKRHKDARRKQMQL
 AFAAVNWVRKNLQ**DRKSGRAEPDPKKATFRSITSTLASSFKRRRSKDTPCRMPOECORDRLAPW**
SQDSPGKLPPHSERPSAHHHPSCASPRHIPTAPGGDACVPHRQLLQLE

Figure 1.

Protein sequence of the chicken NMDAR-NR1 subunit. Predicted translation of the NR1 cDNA (GenPept, accession number AAR98574). The single amino substitution between the duck and chicken is shown in **bold text**; C1 and C2 cassettes are indicated by *italicized* or *italicized* text, respectively.

Figure 2.

NR1 temporal expression analysis of chicken embryos. Short-distance (SD) PCR amplification and agarose gel image demonstrating NR1 expression in embryos staged from 8-34 hours. Expected amplicon length: NR1- 424 bp; GAPDH- 181 bp. L-ladder; O (oligo-dT)- or H (random hexamer)- based reverse transcription reaction; 8, 14, 18, 24, 34- stage Hamburger Hamilton; E- embryonic day.

Figure 3.

NR1 mRNA expression in stage HH 15 neural tube by fluorescence *in situ* hybridization. A. Paraffin-embedded transverse section through the embryo at the occipital level, hybridized with an antisense NR1 riboprobe. Sections were counterstained with DAPI (blue) for nuclear visualization. A. DIC image showing the neural tube and lumen (L) as well as the surrounding mesoderm (M). B. The same section as A with a TRITC filter, demonstrating Cy3 fluorescence limited to the neural tube. C. Image in B merged with an image of the same section seen with a DAPI filter. D. Magnified view of the inset in C: demonstrating the cytoplasmic localization of Cy3.

Figure 4. Stage HH 11 embryo 12 hours after transfection with pCAX-EGFP. A. DIC image of the neural tube viewed along its longitudinal axis. Lateral edges of the neural tube (stars) and somitic mesoderm (arrowheads) are shown, magnification (M) 40 $\times$. B. Equivalent confocal section at the same level. The neural tube is clearly marked by EGFP fluorescence, M 40 $\times$.

Figure 5.

Stage HH 18 embryo, 36 hours after bilateral neural tube transfection with pCAX-EGFP. A. Embryo examined *in ovo* under transmitted (left) and UV (right) light. B. Magnified view of the neural tube examined *ex ovo*. Trunk neural crest cells migrate in streams between the somites (stars) along the intersomitic and ventrolateral pathways (arrows), also a characteristic behavior for the migration of caudal CNC at earlier stages.

Figure 6. Stage HH 22 embryo, 50 hours after bilateral neural tube transfection with pCAX-EGFP. A. Lateral view of the CNC streams converging at the circumpharyngeal ridge (blue rectangle), from which cardiac neural crest populate the branchial arches 3, 4, and 6, and the outflow tract (oft). B. Higher-magnification dorsolateral view of the outflow tract being populated with CNC. The normal topography of the outflow tract in relation to the primitive ventricle (V) has been distorted for a better view of the former. A, B- Embryo examined under combined transmitted-UV light.

Figure 7.

Stage HH 24 embryo, three days after bilateral neural tube transfection with pCAX-EGFP. A. Lateral view of the embryo under transmitted light, showing the outflow tract (oft), ventricle (v), and venous pole (vp) of the heart (left). Combined transmitted-UV light image reveal neural tube and cardiac neural crest fluorescence (right). B. Magnified view of the inset in A. Late migrating CNC (arrows) reach the distal outflow tract.

Figure 8.

Immunoblotting of neural tube samples of embryos incubated for 3.5 days or stage HH 21-22. Left: Antibodies against the NMDAR-NR1 subunit and β -actin label single bands with molecular weights of 115 kD, and 42 kD, respectively in wildtype or control chicken embryos. Right: Immunoblotting of protein samples from CT and siRNA NR1 electroporated (K) embryos with antibodies against NR1 and β -actin. Rat brain served as the positive control.

Figure 9.

Relative protein expression of the NR1 subunit in the groups that were electroporated with either control siRNA (CT) or siRNA-NR1 (K). NR1 expression levels were based on densitometry analysis of the gels from individual embryos. Bars represent means of intensity-area value ratios of the NR1 and β -actin bands from each group. Error bars represent standard deviation(s) where N= the total number of embryos per group; e= number of repeated experiments. The data were analyzed by a two-tailed, paired t-test, $p=0.0282$ (* $p<0.05$)

Table 1

The comparisons between the chicken NR1 mRNA and protein sequences to other organisms

Organism	NR1 coding sequence Length (bp) /homology	NR1 protein Length (aa)/ homology
Chicken	2898	965
Duck	2898/95	965/99
Mouse	2817/83	938/94
Rat	2817/83	938/94
Human	2817/83	938/94

bp: base pair; aa: amino acid

Table 2

Embryo survival in the different treatment groups

Group	CWT	CNT	CT	K
Embryos				
Survivors	10	8	7	6
Dead	0	26*	29*	15*
% Survival	100	23*	19*	29*

* Significantly different than CWT $p < 0.001$

Table 3

Frequency of heart defects in the various treatment groups

Group	CWT	CNT	CT	K
Hearts				
CHD	0	1	1	0
Normal	10	7	6	6
% of embryos with CHD (CHD/ total number of survivors)	0 (0/10)	13 (1/8)	14 (1/7)	0 (0/6)