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N-METHYL-D-ASPARTATE RECEPTOR 1 CHANGES IN THE PIGLET BRAINTEM AFTER NICOTINE AND/OR INTERMITTENT HYPERCAPNIC-HYPOXIA

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Abstract—Prone sleeping and cigarette smoke exposure are two major risk factors for the sudden infant death syndrome (SIDS). Utilizing piglet models of early postnatal nicotine and/or intermittent hypercapnic-hypoxia (IHH) exposure, we tested the hypothesis that these exposures, separately or combined, increase *N*-methyl-D-aspartate (NMDA) receptor 1 (NR1) expression in the brainstem medulla. We also tested for gender-specific effects. Three piglet exposure groups were compared against 14 controls; 1, nicotine [*n*=14], 2, IHH [*n*=10], and 3, nicotine+IHH [*n*=14], with equal gender proportions in each group. Non-radioactive *in situ* hybridization and immunohistochemistry were performed for NR1 mRNA and protein expression, respectively, and were quantified in seven nuclei of the brainstem medulla. NR1 mRNA was significantly increased in the gracile and inferior olivary nucleus (ION) after nicotine exposure, in five of seven nuclei after IHH exposure, and in three of seven nuclei after nicotine+IHH. The increased mRNA changes were accompanied by increased protein only in the ION after IHH and nicotine+IHH (*P*=0.019, and *P*=0.008 respectively). By gender, control females had greater NR1 mRNA than males in the dorsal motor nucleus of vagus (*P*=0.05) and for protein in the ION (*P*=0.02). This gender difference was maintained after nicotine exposure in the ION with additional gender differences observed including greater mRNA in the cuneate nucleus (*P*=0.04) and nucleus of the spinal trigeminal tract (*P*=0.03) of males compared with females. Overall, more changes occurred at the mRNA level than protein, and IHH exposure induced more changes than nicotine or nicotine+IHH exposures. Together, these findings suggest that hypercapnic-hypoxic exposures (modeling prone sleeping or sleep apnea) are more likely to induce NMDA receptor changes in the developing brainstem than nicotine exposure alone.

Key words: cigarette smoke, medulla, mRNA, NR1, protein, SIDS.

Sudden infant death syndrome (SIDS) is a major cause of post-neonatal deaths in many developed countries. There is no known cause for SIDS but rather, several factors have been identified that increase the risk for SIDS. These include sleeping in the prone position (especially for the first time) and pre- and postnatal tobacco exposure. SIDS deaths also affect males more than females and have a peak incidence at 2–4 months of age (SIDS and KIDS Online, 2000).

Many factors suggest that SIDS infants have an underlying brainstem abnormality affecting arousal or respiratory control mechanisms that causes an increased likelihood of death when combined with other risk factors. As such, one line of study has aimed to identify receptor systems that are abnormal in the brainstem of SIDS compared with non-SIDS infants; the glutamatergic system being of interest to our laboratory. We previously found increased *N*-methyl-D-aspartate receptor 1 (NR1) expression in several brainstem medullary nuclei of SIDS infants compared with non-SIDS infants (Machaalani and Waters, 2003a). However, in that study, we could not assess associations with clinical risk factors because of the small cohort size.

We have since developed and used piglet models to investigate the contribution of the risk factors on *N*-methyl-D-aspartate (NMDA) receptor changes during the postnatal period. The piglet models include exposure to: 1, acute (1 day) intermittent hypercapnic-hypoxia (IHH), a model aimed to mimic the conditions of prone sleeping and/or obstructive sleep apnea (Waters and Tinworth, 2001), 2, nicotine, developed to mimic postnatal second hand smoke exposure (Machaalani et al., 2005), and 3, combined exposure to both postnatal nicotine and IHH. This study used those piglet models to determine the effects of nicotine and IHH, separately or combined, on NR1 mRNA and protein expressions in select nuclei of the piglet brainstem caudal medulla. Results were compared between controls and exposed piglets with subgroup analyses by gender. The hypothesis tested was that NR1 expression would increase in the medullary nuclei after nicotine and IHH exposures, and that the increase would be additive for the group with the combined exposure.

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Abbreviations: BDNF, brain-derived neurotrophic factor; BR, blocking reagent; CUN, cuneate nucleus; DB, digoxigenin buffer; DDB, digoxigenin detection buffer; DMNV, dorsal motor nucleus of the vagus; GRAC, gracile nucleus; IHC, immunohistochemistry; IHH, intermittent hypercapnic-hypoxia; ION, inferior olivary nucleus; nAChR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartate; NR1, *N*-methyl-D-aspartate receptor 1; non-RISH, non-radioactive *in situ* hybridization; NRS, normal rabbit serum; NSS, normal sheep serum; NSTT, nucleus of the spinal trigeminal tract; NTS, nucleus of the tractus solitarius; OD, optical density; PBS, phosphate-buffered saline; SIDS, sudden infant death syndrome; SSC, standard saline citrate; XII, hypoglossal nucleus.

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EXPERIMENTAL PROCEDURES

Animal exposures

Mixed-breed miniature piglets (n=52) were randomly allocated to one of the following groups; 1) controls (n=14), 2) nicotine (n=14), 3) IHH (n=10) and 4) combined nicotine+IHH (n=14). Equal numbers of males and females were studied in each group. Ethical approval was obtained from the University of Sydney Animal Ethics Committee, and all experiments conformed to the international guidelines on the ethical use of animals. The number of animals used was kept to a minimum, and animals were continuously monitored to ensure good health and no suffering.

Piglets in the control and nicotine groups underwent surgery 0–2 days after birth for the implantation of an osmotic minipump containing sterile water (controls) or nicotine (nicotine groups), where the nicotine dose was 2 mg/kg/day. The method of implantation and nicotine release has been described in detail previously (Machaalani et al., 2005).

Piglets in the IHH groups were exposed to cyclic hypercapnia hypoxia (HH=8% O_2/7% CO_2 balanced with N_2) for a total of 48 min, 6 min of HH alternating with 6 min of air, and this was carried out on the day immediately prior to killing. Detailed methodology has been provided previously (Waters and Tinworth, 2001, 2003).

Piglets in the combined group underwent surgery at 0–2 days, for the implantation of an osmotic minipump containing nicotine and were also exposed to IHH on the day prior to killing.

Brain tissue and serum collection

As previously documented (Machaalani et al., 2005), blood and urine samples were collected for cotinine analyses at the same time as the piglet brains, after the piglets were killed with an overdose of pentobarbitone at age 13–14 days. Piglets implanted with an osmotic mini-pump had urine and blood samples collected. The blood was centrifuged and serum extracted, and the serum and urinary samples were analyzed for cotinine using gas chromatography mass spectrometry (GCMS) with selected ion monitoring. The whole brain, down to the spinomedullary junction, was removed fresh then fixed in 10% formalin for 15 days. After fixation, the brainstem was detached from the rest of the brain and cut into 4 mm slices, and then fixed for a further 5 days in 10% formalin. Slices were washed in 70% ethanol for 6 h and processed to paraffin over 3 days and then paraffin embedded. The closed medulla was cut in transverse 7 μm sections utilizing a rotary microtome, and sections were mounted on slides coated with 3-aminopropyltriethoxysilane and stored in dust free conditions.

Non-radioactive *in situ* hybridization (non-RISH) for NR1 mRNA

The non-RISH method employed was as reported previously (Machaalani and Waters, 2002). In brief, synthetic oligonucleotide NR1 antisense (5′ CTC CTC CTC CTC GCT GTT CAC CTT GAA CCG GCC GAA GGG GCT GAA 3′) and sense (5′ TTC AGC CCC TTC GGC CGG TTC AAG GTG AAC AGC GAG GAG GAG GAG 3′) probes (Gibco BRL, Melbourne, Australia), were labeled by 3′-tailing with digoxigenin-11-dUTP according to the manufacturers' instructions using the DIG Oligonucleotide tailing kit (Roche Diagnostics GmbH, Mannheim, Germany, Cat. # 03353583910).

Tissue sections were deparaffinized in xylene and taken through a series of ethanol to DEPC H_2O. Sections were then microwaved (Sharp 700W, Japan) in Tris–EDTA buffer (1 mM EDTA, 1 mM sodium citrate, 2 mM Tris; pH 9.0) for 14 min, left to cool for 10 min, washed in DEPC H_2O, washed in phosphate-buffered saline (PBS) and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were washed in PBS, a hydrophobic barrier was drawn around the sections and then they were rinsed in PBS. Sections were prehybridised in 4× standard saline citrate (SSC)/50% formamide for 2 h at 37 °C, rinsed in 2× SSC and hybridized in 50 ng antisense probe in 100 μl hybridization buffer (18% formamide, 2× SSC, 1× Denhardt's solution, 10% dextran sulfate, 50 mM dithiothreitol (DTT), 250 μg/ml yeast t-RNA, 100 μg/ml polyadenylic acid and 500 μg/ml denatured and sheared salmon sperm DNA). Hybridization was carried out overnight at 37 °C. Following hybridization, sections were stringently washed for 15 min each in 2× SSC (once at room temperature), 2× SSC (twice at 37 °C), 1× SSC (once at 37 °C), and then in 1× SSC and 0.5× SSC (once at room temperature). Immunohistochemical detection for NR1 mRNA was then performed by washing sections in digoxigenin buffer (DB) (33 mM maleic acid, 50 mM NaCl, pH 7.5), incubating in blocking solution (10% w/v blocking reagent [BR] from Roche Diagnostics, 2.5% normal sheep serum (NSS) in DB) for 1 h at 37 °C, and then in anti-DIG-alkaline phosphatase antibody (1:250 dilution in DB, containing 10% BR and 1% NSS) for 2 h at room temperature. Sections were then washed in DB and in digoxigenin detection buffer (DDB) (33 mM Tris, 33 mM NaCl, 50 mM $MgCl_2$, pH 9.5), and color was developed overnight at room temperature in a humidified dark box by incubating sections with a mixture of 35 μl nitroblue tetrazolium (NBT), and 45 μl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 10 ml DDB. The color reaction was stopped by washing in Tris buffer and distilled H_2O, and sections were then mounted in Aquamount. Negative controls to test non-specific hybridization included a labeled sense probe, unlabeled antisense probe and no probe where only the hybridization mixture was added.

Immunohistochemistry (IHC) for NR1 protein

IHC for NR1 protein was performed over two days, and carried out at room temperature unless otherwise stated. This procedure was slightly modified from our previous report (Machaalani and Waters, 2002). After deparaffinization in xylene, rehydration in ethanols to water, and antigen retrieval by microwaving in Tris–EDTA buffer on high for 12 min, sections were quenched for endogenous peroxidase activity in 3% hydrogen peroxide (H_2O_2)/50% methanol in PBS for 30 min and then proceeded to immunostaining. Immunostaining was performed using a goat polyclonal antibody raised against a peptide mapping at the carboxyl terminus of the NR1 of human origin (sc-1467, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were incubated with 10% normal rabbit serum (NRS) in PBS for 30 min and then in NR1 antibody (diluted 1:200 in 1% NRS) overnight at 4 °C. Sections were washed in PBS, incubated in biotinylated rabbit anti-goat IgG secondary antibody for 30 min (Vector Laboratories Inc., Burlingame, CA, USA), and then in avidin–biotin–horseradish peroxidase (Vectastain ABC kit; Vector laboratories Inc., Cat. # PK-4000), and color developed through the addition of DAB. Sections were then washed and counterstained with Harris' hematoxylin, differentiated in acid alcohol and immersed in Scott's Blue solution. The sections were taken through a graded series of alcohol, cleared in two changes of xylene, mounted in DPX and coverslipped. Negative controls composed of sections where the primary NR1 antibody were replaced with 1% NRS.

Quantitative analyses

The following seven nuclei of the brainstem caudal medulla were quantified; hypoglossal nucleus (XII), dorsal motor nucleus of the vagus (DMNV), nucleus of the solitary tract (NTS), gracile nucleus (GRAC), cuneate nucleus (CUN), inferior olivary nucleus (ION) and nucleus of the spinal trigeminal tract (NSTT). The boundaries of the seven nuclei were defined by referring to Fig. 17 from the *Human Brainstem Atlas* of Paxinos and Huang (1995). The boundaries of the nuclei were also determined in an adjacent tissue section stained with hematoxylin and eosin.