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Maternal dietary supplementation with omega-3 polyunsaturated fatty acids confers neuroprotection to the newborn against hypoxia-induced dopamine dysfunction

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ABSTRACT

Introduction: Up to 84% of prematurely born infants suffer hypoxic, anoxic, and ischemic insults. Those infants with subsequent behavioral, motor or cognitive dysfunction represent 8–11% of all live births. Yet, no interventions employed during pregnancy attenuate risk of morbidity in those at-risk infants. Dietary supplementation with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) has been shown to reduce stroke-induced neuropathology in rat models emulating this adverse clinical event. To extend those studies we sought to determine whether maternal dietary supplementation with ω-3 PUFAs would confer neuroprotection against hypoxia-induced neurochemical dysfunction in newborn rat pups exposed to repetitive hypoxic insults.
Methods: We provided pregnant rats with either a ω-3 PUFA enriched diet or else a standard rat chow diet. At postnatal day 7, pups were assigned randomly to either repetitive hypoxic insults or repetitive bursts of room air. On postnatal day 12, pups were sacrificed and brain dopamine levels characterized.
Results: Baseline brain dopamine levels did not differ between rat pups born to dams who received ω-3 PUFA enriched versus standard rat chow diets. Rat pups born to dams maintained on normal diets, who were exposed to five days of repetitive hypoxic insults, experienced a 57% reduction in striatal dopamine levels accompanied by significant apoptosis. In contrast, ω-3 PUFA-enriched newborn pups experienced no loss in striatal dopamine levels, and only minimal apoptosis.
Conclusions: Our findings suggest that it may be feasible to confer neuroprotection against hypoxia-induced dopamine dysfunction to newborns likely to experience hypoxic insults. This could significantly improve the outcomes of those 8–11% of newborns who would otherwise experience hypoxia-induced behavioral, motor and cognitive dysfunction.

1. Introduction

Hypoxic insults occurring during the perinatal period are among the leading causes of permanent brain dysfunction and remain a serious public health concern [1–3]. Most hypoxic insults typically occur in the setting of unambiguous clinical compromise, such as placental dysfunction, prolonged labor, or cardiorespiratory resuscitation [4,5]. However, other more insidious mechanisms, such as apnea of prematurity, which is a common occurrence in prematurely born infants [6–9], can induce repetitive hypoxic insults. Between 10% and 13% of all infants are born prematurely [10,11]. Apnea with concomitant hypoxic insults will afflict 78% of those born at 26–27 weeks gestation, 54% born at 30–31 weeks, and 7% born at 34–35 weeks [6].

Regardless of whether children who are born prematurely or at term, perinatal hypoxic insults are associated with diminished academic performance and other manifestations of executive dysfunction [12–16]. Perturbed function within neural networks subserving arousal and/or vigilance is also seen; infants with a history of apnea of prematurity require more intensive stimuli to be awakened from sleep [17]. Dampened autonomic dysfunction, manifesting as higher resting heart rates with reduced heart rate

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variability, has also been reported [18]. Yet despite these adverse outcomes attributed to perinatally-occurring hypoxic insults, there has been little progress towards developing clinical interventions that can be initiated during pregnancy to confer neural resiliency to at-risk newborns [19]. The absence of such interventions undermines progress towards mitigating the morbidity and mortality associated with these adverse perinatal events.

An emerging body of literature suggests that omega-3 polyunsaturated fatty acids (ω-3 PUFAs) confer neural resiliency against a number of insults [20–23]. Yet, there remains a paucity of data describing whether dietary supplementation with ω-3 PUFAs can confer neuroprotection against hypoxic insults occurring in their newborns. To overcome this barrier, this study was designed to characterize the extent that maternal prenatal dietary supplementation with ω-3 PUFAs will confer neuroprotective resiliency to the newborn against hypoxia-induced dopamine dysfunction, one neurotransmitter system that is exquisitely vulnerable to such insults [24,25].

2. Material and methods

All studies were performed at *Emory University under an IACUC approved protocol (170-2003).* Pregnant rats received dietary supplementation with ω-3 PUFAs by adding menhaden fish oil 15% weight by weight (w/w) (Sigma-Aldrich) into standard rat chow mix, to achieve a total daily dose of 3.5–4.0 g. The ω-3 PUFA enriched diet was initiated on day 1 of pregnancy (confirmed by presence of vaginal plug) and continued through 12 days postdelivery of their pups. Therefore, ω-3 PUFA enriched pups received both pre- and postnatal (via maternal colostrum) dietary enrichment with ω-3 PUFAs. Control rats pups were born to dams that were maintained on a standard rat chow diet during pregnancy and thereafter.

Beginning on postnatal (PN) day 7 and continuing through PN 12, both ω-3 PUFA-enriched and control newborn pups were assigned randomly to receive either repetitive hypoxic insults or bursts of compressed room air, as described below, for 2 h blocks of time. At the conclusion of each 2 h period, all pups were returned to the dam for a 45 min interval, for feeding and grooming. This sequence was repeated 2 more times each day, totaling three 2-hour sessions of repetitive hypoxic insults or bursts of compressed air. During PN 7–12, pups were exposed to either a total daily dose of 6 h of repetitive bursts of hypoxia or normoxia. On PN 12, at the conclusion of the protocol, pups were euthanized for neurochemistry. Our protocol for inducing repetitive hypoxic insults has been previously validated (*23*) to insure that it does not induce maternal separation-induced stress in newborn rat pups, which can evoke changes in neurochemistry [26–30].

2.1. Hypoxia-inducing system

Our hypoxia-inducing system consists of a clear Plexiglas chamber, solenoid valves, and compressed gas [24,25]. The internal environment within the system is warmed and humidified to the appropriate level for each postnatal day of age [31,32]. Attached to the hypoxia-inducing chamber is one gas cylinder containing 10% oxygen, 3% carbon dioxide, and nitrogen and a second gas cylinder containing only compressed air. After placing the rats into the chamber, a programmable timer opens the solenoid valve between the hypoxic gas cylinder and hypoxic chamber for a 20 s period, allowing introduction of the hypoxic gas mixture at a flow rate of 10 liters per minute, which provides 18.5 complete air exchanges per minute within the chamber. As the solenoid valve closes, the second solenoid valve, attached to the gas cylinder containing only compressed air, opens for 40 s. These alternate on and off to expose the rats to 20 s bursts of hypoxia, followed by 40 s bursts of room air, thereby inducing 60 hypoxic events per hour. This novel system allows for a user-selected frequency and duration of hypoxic insults, thereby permitting us to determine the specific hypoxic "doses" delivered. In addition to the hypoxia-inducing chambers, we also use identically constructed normoxic chambers. Solenoid valves of these chambers are attached to cylinders containing only compressed air, which cycled through the chambers at the same flow rates and frequency as gas flowed through the hypoxia-inducing chamber. The normoxia chamber acted as a control for rats receiving hypoxic gas and for any potential impact associated with maternal separation [26–29]. Additionally, the duplicate use of solenoid valves and a compressed gas source on the normoxia chamber allow further control of other factors such as sound, pressure changes, or temperature fluctuations, all of which are intrinsic to compressed gas sources.

2.2. HPLC assessment of brain tissue content of dopamine

Briefly, rats were decapitated and neural tissue was dissected from the precommissural striatum at coordinates previously described [24]. Dissected neural tissue was homogenized, centrifuged at 10,000 rpm for 10 min, and then filtered through a 0.22 μm filter. Samples were then placed in our refrigerated autosampler which injected them into our high performance liquid chromatography (HPLC) system. *Chromatography*. Homogenates were assayed for dopamine using HPLC with electrochemical detection as previously described [25]. Twenty microliter samples were injected from the autosampler onto a C18 reversed phase column maintained at 30 °C. De-gassed mobile phase was delivered at a flow rate of 0.3 mL/min. The electrochemical detection was performed by a GBC Antec Leyden VT03 electrochemical flow cell with a glassy carbon working electrode maintained at a potential of +0.60 V, relative to the reference electrode.

2.3. Immunohistochemistry for apoptosis

Rat pups were deeply anesthetized with a lethal dose of sodium pentobarbital, perfused transcardially with 0.9% heparinized-saline followed by a fixative of 4% paraformaldehyde. Brains are removed, equilibrated overnight in 30% sucrose, and sectioned on a freezing microtome at a thickness of 50 μm. Sections were collected in 0.05 M Tris-buffered saline containing 1% sodium azide. Adjacent series are processed for TH or Nissl substance using neutral red or thionin. For caspase-3 processing, sections were incubated 24–48 h at 4 °C with primary antibody polyclonal rabbit-anti-caspase 3 in diluent of normal goat serum, triton X-100 and TBS. Following incubation and three 5 min rinses in TBS, sections were incubated for 1.5–2 h in secondary biotinylated goat-anti-rabbit, rinsed in TBS and incubated in avidin-biotin-peroxidase complex in TBS and Triton X-100. Sections were then incubated in 0.05% 3,3 diaminobenzidine tetrachloride (DAB) and 0.01% hydrogen peroxide in 0.05 M Tris buffer for 5–10 min. The reaction was stopped by extensive rinses in TBS. Brain sections were mounted onto gelatin-coated slides, air dried, dehydrated in ethanol, cleared in xylene and coverslipped with DPX mountant using procedures employed within our laboratory [33].

2.4. Statistical approach and sample sizes

As our laboratory has recently observed male-female differences in hypoxia-induced dopamine cell dysfunction (unpublished findings), with males appearing to be more vulnerable than females, we used only male rat pups in this study. Power calculations that were based upon our preliminary studies suggested that no less than five male ω-3 PUFA enriched rats and five male