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TERATOGENIC EFFECTS OF PYRIDOXINE ON THE SPINAL CORD AND DORSAL ROOT GANGLIA OF EMBRYONIC CHICKENS

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Abstract—Our understanding of the role of somatosensory feedback in regulating motility during chicken embryogenesis and fetal development in general has been hampered by the lack of an approach to selectively alter specific sensory modalities. In adult mammals, pyridoxine overdose has been shown to cause a peripheral sensory neuropathy characterized by a loss of both muscle and cutaneous afferents, but predominated by a loss of proprioception. We have begun to explore the sensitivity of the nervous system in chicken embryos to the application of pyridoxine on embryonic days 7 and 8, after sensory neurons in the lumbosacral region become post-mitotic. Upon examination of the spinal cord, dorsal root ganglion and peripheral nerves, we find that pyridoxine causes a loss of neurotrophic tyrosine kinase receptor type 3-positive neurons, a decrease in the diameter of the muscle innervating nerve tibialis, and a reduction in the number of large diameter axons in this nerve. However, we found no change in the number of Substance P or calcitonin gene-related peptide-positive neurons, the number of motor neurons or the diameter or axonal composition of the femoral cutaneous nerve. Therefore, pyridoxine causes a peripheral sensory neuropathy in embryonic chickens largely consistent with its effects in adult mammals. However, the lesion may be more restricted to proprioception in the chicken embryo. Therefore, pyridoxine lesion induced during embryogenesis in the chicken embryo can be used to assess how the loss of sensation, largely proprioception, alters spontaneous embryonic motility and subsequent motor development.

Key words: pyridoxine, proprioception, DRG, motor neurons, chicken embryo, TrkC.

INTRODUCTION

Studies on chicken embryos have been instrumental for gaining an understanding of the development of motor activity during embryogenesis (Bekoff et al., 1975; O'Donovan and Landmesser, 1987; Sharp et al., 1999; Bradley, 2001; Bradley et al., 2005). However, we do not have a clear understanding of how sensory information modulates spontaneous embryonic movement. It has been shown that sensory synapses are present in the lumbosacral spinal cord as early as embryonic day (E) 7 in the chicken embryo (Lee et al., 1988; Davis et al., 1989) and that peripheral stimulation can initiate reflexic activity starting at this time (Oppenheim, 1972). Additionally, behavioral observations and manipulations have suggested that somatosensory feedback may be used to modify embryonic leg and wing motility as early as E9 (Bradley, 1997; Sharp et al., 1999). To verify that sensation can modulate embryonic motility, it is important to develop a model that alters or eliminates specifically a defined population of somatosensory neurons.

It has been know since the 1940s that large dosages of pyridoxine, the essential vitamin B_6, cause a significant peripheral neuropathy in adult mammals resulting in severe ataxia (Antropol and Tarlov, 1942). The effects of pyridoxine toxicity have been demonstrated in humans (Schaumburg et al., 1983), dogs (Antropol and Tarlov, 1942), cats (Stapley et al., 2002; Pearson et al., 2003), guinea pigs (Xu et al., 1989) and rats (Antropol and Tarlov, 1942; Xu et al., 1989; Helgren et al., 1997). Pyridoxine lesion in mammals is characterized by the loss of sensory neurons with large diameter axons. This sensitivity to pyridoxine is present in both skin and muscle innervating populations. Despite the loss of cutaneous sensory neurons, pyridoxine lesion results predominantly in a loss of proprioception from a behavioral perspective. Interestingly, pyridoxine does not appear to be toxic to motor neurons. Despite the lack of a mechanistic understanding of pyridoxine toxicity, pyridoxine overdose has been used in cats to study the role of sensation in response to postural perturbations (Stapley et al., 2002) and recovery from peripheral injury (Pearson et al., 2003).

It should be noted that pyridoxine is often used to ameliorate nausea during pregnancy in humans (up to 100 mg/day). The use of pyridoxine during pregnancy is somewhat controversial, but Shrim et al. (2006) have

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Abbreviations: c, corrected; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; E, embryonic day; NT-3, neurotrophin-3; PBS, phosphate-buffered saline; s.d., standard deviation of the mean; TrkC, neurotrophic tyrosine kinase receptor type 3; uc, uncorrected; vs., versus.

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shown that pyridoxine usage during pregnancy does not give rise to major malformations in new born children. However, more subtle neurological effects, such as a reduction in proprioception, were not examined.

The goal of this study was to begin to characterize the effects of pyridoxine administration on the peripheral nervous system of embryonic chickens to determine if pyridoxine lesion can be used to study the role of sensory feedback during embryogenesis. Since these studies are likely to take place on E9 or later after sensory synapses are formed, it was necessary to begin pyridoxine application prior to that time. However, it was important to begin administration of pyridoxine after sensory neurons become post-mitotic around E6 (Hamburger et al., 1981). This was to avoid the complication of additional cell divisions replacing neurons that had been killed by pyridoxine. Therefore, pyridoxine was administered on E7 and E8 and the effects on motor neurons and various sensory neuron types were assayed on E13 allowing time for the removal of pyridoxine-sensitive neurons.

EXPERIMENTAL PROCEDURES

Incubation and pyridoxine treatment

All procedures on chicken embryo were in compliance with the guidelines of the Southern Illinois University Institutional Animal Care and Use Committee.

White leghorn chicken eggs (Charles River Laboratories, Wilmington, MA, USA) were incubated on their sides in a forced-air incubator (99.5°F, 55% relative humidity) until E7. Eggs were then removed from the incubator and candled. A location free of underlying blood vessels, toward the pointed end of the egg, was marked. A fine file was used to create a groove in the shell at the marked location up to the shell membrane. For control embryos, 100 µL of 0.1 M phosphate-buffered saline (PBS: 25 mM $NaH_2PO_4{\cdot}H_2O$, 75 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) was then injected under the shell membrane through a 32-gauge syringe needle. Animals treated with pyridoxine were injected with 3.75 mg of pyridoxine hydrochloride (Sigma, Chemical Co., St. Louis, MO, USA) dissolved in 100 µm of PBS. This dosage was selected after a preliminary dose–response study (see Results). Holes were sealed with a small piece of label tape and the eggs were returned to the incubator. Injections were repeated on E8 after candling to ensure embryo viability.

Tissue collection

Eggs were removed from the incubator on E13 and cracked into a small glass dish. The membranes were quickly incised and the embryos decapitated. Then, the bodies were rinsed in PBS, pinned in small dissecting dishes and eviscerated. An incision through the ventral vertebrae of the lumbosacral region was made with a scalpel blade to allow for better penetration of fixative. The spinal column was then severed at the last thoracic segment and the lumbosacral spinal column, including dorsal root ganglions (DRGs), was removed and placed into fixative. For comparisons of skin and muscle innervating nerves, the lateral branch of the femoral sensory nerve and the lateral branch of the tibialis nerve were dissected and placed into fixative. Care was taken such that no axons from a given nerve would be lost due to variations in branch points.

Tissue fixation

Spinal columns isolated for the various assays were fixed differently. Tissue for neurotrophic tyrosine kinase receptor type 3 (TrkC) immunostaining and spinal morphology was emersion fixed in Bouin's fixative for two days at 4 °C and then washed in PBS (5×). Tissue for immunofluorescence was emersion fixed in 4% buffered paraformaldehyde (PBS, pH 7.2) overnight at 4 °C and then rinsed in PBS (3×). Nerves collected for cross-sectional analysis were emersion fixed in 2.5% glutaraldehyde for 3–4 h at 4 °C and then rinsed in Sorensen's phosphate buffer at 4 °C for 24 h. Tissue was then post-fixed in 1% osmium tetroxide (OsO_4) for two hours at room temperature and rinsed twice in distilled water.

TrkC immunocytochemistry

Spinal columns used for TrkC immunocytochemistry were dehydrated and embedded in paraffin, sectioned at 10 µm on a rotary microtome and mounted on slides. The slides were then examined to identify those that contained the third lumbosacral DRG. These slides were then deparaffinized and rehydrated.

The primary antiserum used (courtesy of Frances Lefcort, Montana State University) was generated previously against the extracellular domain of the chicken TrkC receptor and its specificity has been described (Lefcort et al., 1996). The standard ABC peroxidase method with diaminobenzidine visualization was used. Sections from two embryos, one control and one pyridoxine treated, were reacted simultaneously to control for variability in reaction intensity that can sometimes occur. Briefly, sections were treated with 0.5% hydrogen peroxide. Then, sections were placed in blocking solution (1% bovine serum albumin, 1% normal horse serum, 0.3% Triton X-100 in PBS) for one hour prior to reaction with the primary antiserum. Sections were incubated overnight at room temperature in the primary antiserum (diluted 1:1000 in blocking solution). Standard ABC protocols (Vector Labs) followed by diaminobenzidine reaction (12.5 mg/50 ml PBS with 30 µl H_2O_2) were used for visualization and slides were coverslipped with Permount.

Substance P and calcitonin gene-related peptide (CGRP) immunocytochemistry

Spinal columns for immunofluorescence were cryoprotected overnight in 20% sucrose (w/v) dissolved in PBS. Sections were cut at 14 µm on a cryostat and collected directly onto charged slides. Sections were collected alternately onto a series of three slides. Each series was examined under low power magnification and a series that contained