CONNEXIN 43 ANTISENSE MORPHOLINO TRANSFECTION MEDIATED VIA METAFECTENE PRO DISRUPTS NEURAL DEVELOPMENT

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INTRODUCTION:

Earlier work in our laboratory on development of the *Xenopus* frog midbrain and hindbrain showed that connexin 43 (Cx43) expression is spatially and temporally regulated, suggesting that Cx43 expressing cells may play a critical role in early morphogenesis and patterning events that set up hindbrain and midbrain neural circuitry (Katbamna et al, 2004). Additionally, work by others indicated that Cx43 is expressed during development of the earliest components of the nervous system, at the time the neural folds, brain and eye vesicles form, and is also expressed during formation of the head mesenchyme and branchial arches (Landesman et al, 2003). During mouse development, Cx43 transcripts are associated with eye, ear, and branchial arch formation, and are present as a gradient in the midbrain/hindbrain junction area, as well as in the developing telencephalon (Ruangvoravat and Lo, 1992). In humans, the Cx43 gene is located within the candidate chromosome region for the oculodentodigital dysplasia locus, associated with abnormalities including ocular, nasal, and dental dysmorphisms, neurodegeneration, and conductive deafness (Paznekas et al, 2003). Although Cx43 is known to mediate gap junctional communication among cells during development, it also plays a role in cell adhesion during migration of neurons along radial glial fibers during formation of the mammalian cortical plate (Elias et al, 2007). Thus, to better define the roles Cx43 transcripts play during craniofacial and brain development, we injected Cx43 antisense oligonucleotide-morpholino with the transfecting reagent metafectene PRO in the developing *Xenopus*. Three days after injections we performed morphological assessment and Cx43 immunostaining to assay the phenotypes produced by these acute knock down experiments.

MATERIALS AND METHODS:

Materials:

Metafectene PRO, a polycationic liposomal transfection reagent, was obtained from Biontex laboratories GmbH (Munich, Germany). Human chorionic gonadotropin (HCG) and MS222 were purchased from Sigma (St. Louis, MO). Cx43 antisense morpholinos (Cx43-mo: TTCCTAAGGCACTCCAGTCACCCAT) and standard control antisense morpholinos (SC-mo) labeled with fluorescein were purchased from Gene Tools (Philomath, OR).

Embryo injections:

Xenopus embryos were obtained by injecting adult frogs with HCG. Fertilized eggs were separated and maintained in 10% Steinberg's solution at 15^{o}C until injections. Embryos were staged according to Nieuwkoop and Faber (1994). Stages 17/18 through 23/24 embryos were dejellied with fine forceps, placed in small wells made in agarose plates and injected with

antisense morpholinos. Pilot experiments with fluorescein-labeled SC-mo were used to optimize the volume and dispersion of the complexed morpholino when injected at the hindbrain/midbrain primordium immediately after neural tube closure. Morpholinos were complexed with metafectene PRO at reagent:morpholino ratios of 2µl:1µg and 0.5µl:1µg to yield final morpholino concentrations of 0.5 µg/µl and 2 µg/µl, respectively. Fifty-five nl of the complexed morpholino was injected in the area of the developing *Xenopus* hindbrain/midbrain primordium. A total of 55 animals were injected with Cx43-mo; 19 other embryos were injected with SC-mo and 10 embryos received metafectene PRO only. The animals were transferred to agarose dishes with 25% Steinberg's solution for 2-3 hours to facilitate healing and then moved to 10% Steinberg's solution until assay at stages 46/47 (3 days post-injection). All experiments were conducted in accordance with the Institutional Animal Care and Use Committee at Western Michigan University.

Whole mount assays, immunocytochemistry and H & E staining:
At stages 46/47, animals were euthanized with an overdose of MS222 and fixed overnight at 4^{o}C in Bouin's solution. Each specimen was embedded in paraffin and 20 µm serial sections were cut and stained with Cx43 antibody as described previously (Katbamna et al., 2004). Cx43-stained serial sections of each specimen were examined and photographed with a digital camera using a differential interference contrast light microscope. Photographed slides were then restained with routine H & E for further evaluation of the structures.

RESULTS:

Of the 55 animals with Cx43-mo, 18 (33%) showed abnormalities of the eyes, brain and/or craniofacial structures. Fifteen of the 18 animals were sectioned and stained to decipher structural changes. Of the 15 sectioned animals, regardless of stage injected and/or dose level, 93% (14/15) showed eye anomalies that ranged from acorn shape (compared to the normal spherical shape) to retinas with duplicated layers (Figure 1); 67% (10/15) showed brain abnormalities including reduced forebrain and diencephalon with one showing supernumerary tissue that appears to be a duplication of the anterior cerebellum (Figure 2); 27% (4/15) showed reduced ear structures, whereas all animals showed abnormal disposition and/or lack of normal face and head cartilage (Figure 1).

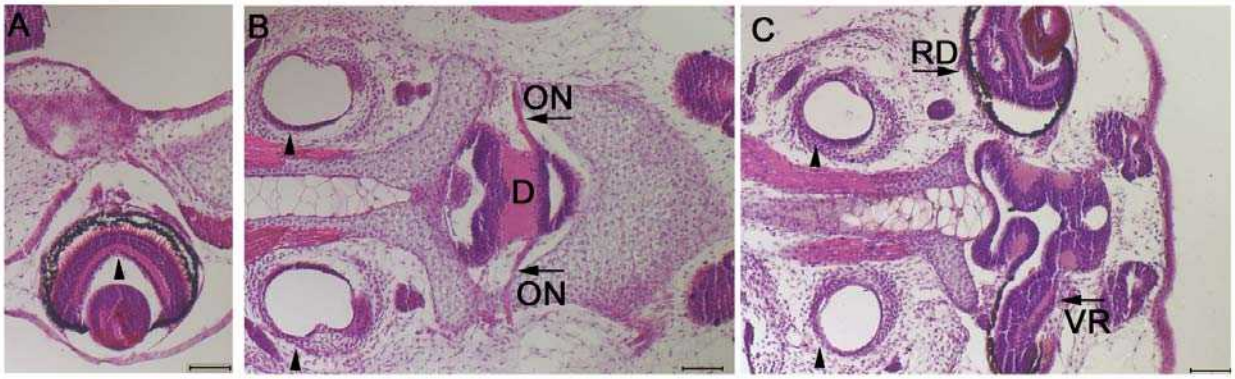

Figure 1: H & E sections showing eye, brain and ear anomalies in a specimen (C) along with comparable controls (A and B); A) normal left eye showing intact retinal layers (arrowhead); B) normal diencephalon (D) with exiting optic nerves (ON) anterior to normal ears (arrowheads); C) retinal duplication (RD) in the right eye, failure of separation of the migrating ventral retinal (VR) tissue from the diencephalon in the left eye, and bilaterally reduced ears (arrowheads) of 2 µg/µl dose animal (scale bar: 100 µm).

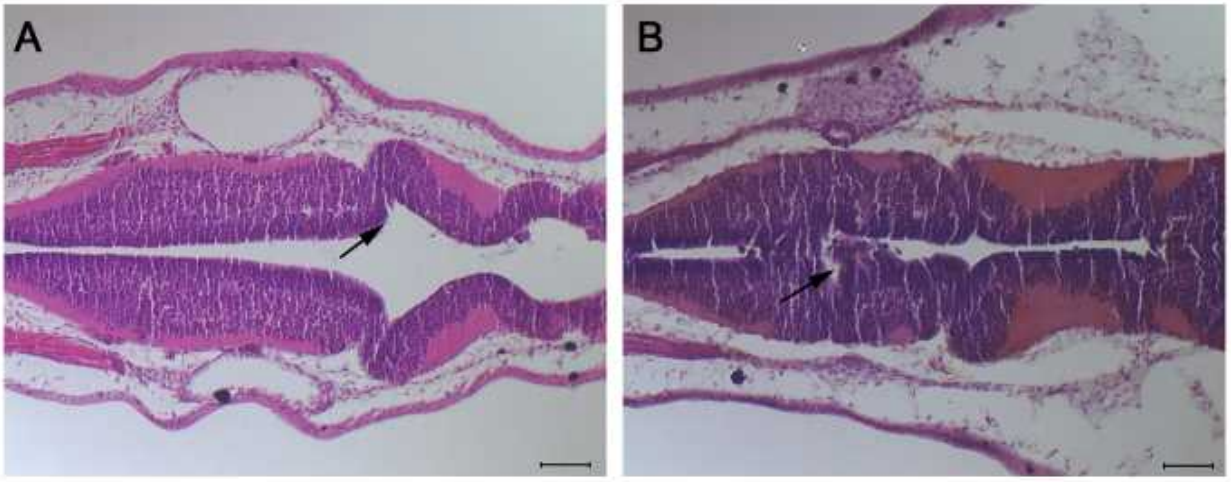

Figure 2: H & E stained sections showing A) a normal cerebellum (arrow) in a metafectene injected control animal and B) an apparent supernumerary hindbrain/midbrain junction (arrow) including anterior cerebellum in a Cx43 morpholino (2 µg/µl) injected animal (scale bar: 100 µm).

CONCLUSIONS:

Cx43 antisense morpholino injected into the newly closed neural tube in the presumptive midbrain/hindbrain region between stages 17/18 and 23/24 produced developmental abnormalities of the face, eyes and brain structures. Metafectene PRO effectively transfected *Xenopus* embryos producing acute Cx43 knock downs.

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