Vivo-morpholino knockdown of aquaporin 1 (AQP1) protein expression in American eel (*Anguilla rostrata*) gastrointestinal tract

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Vivo-morpholinos are artificial nucleic acids that can block the protein production from a specific gene. This study used Vivo-morpholinos to try to 'knockdown' (reduce) the amount of aquaporin 1 water channel protein to be able to see differences in function with or without the aquaporin 1 protein.

In common with Japanese eels (*Anguilla japonica*)[1], in the American eel, aquaporin 1 (AQP1) water channel protein is expressed in the apical membrane of tall columnar surface epithelia cells in the rectum but is largely found in the endothelium of blood vessels in the middle of the tissue in the intestine itself. In the rectum, AQP1 is thought to play a role in water absorption in marine eels that drink seawater to mitigate the effects of dehydration caused by the hyper-osmotic external environment[2]. However due to a lack of specific inhibitors of the AQP1 water channel, it has so far proved impossible to directly demonstrate the actual function of this protein in rectal water absorption. In an attempt to determine the role of AQP1, eels were injected with AQP1-specific vivo-morpholinos in order to 'knockdown' AQP1 protein expression as a prelude to investigations into the effect of this on rectal water absorption.

Moropholinos are artificial molecules with a structure that mimics that of nucleic acids. Their structure is such that they form hydrogen bonds with specific RNA sequences. They are used to either block translation of mRNAs (as in this study) or disrupt proper exon splicing to prevent production of the correct protein. The 'vivo' portion is an addition designed to allow the vivo-morpholinos to enter cells.

Freshwater eels were anesthetized in 500 ppm MS-222 until unresponsive and intraperitoneally (i.p.) injected with vivo-morpholinos (Gene Tools LLC; 1.25 μmoles/kg fish weight) dissolved in phosphate buffered saline (PBS; n = 5 fish) or PBS alone (control fish; n = 9 fish). This regimen was performed one day before eels were transferred to seawater and was then carried out for a further five days. The following day, fish were anesthetized and sacrificed, and the intestine and rectum dissected out. The surface epithelium (and underlying non-muscle tissue) of each tissue was scraped off using a microscope slide and this was homogenized using a syringe and 16 gauge needle, in the presence of buffer containing protease inhibitor cocktail (Research Products International). A crude membrane extract was prepared by

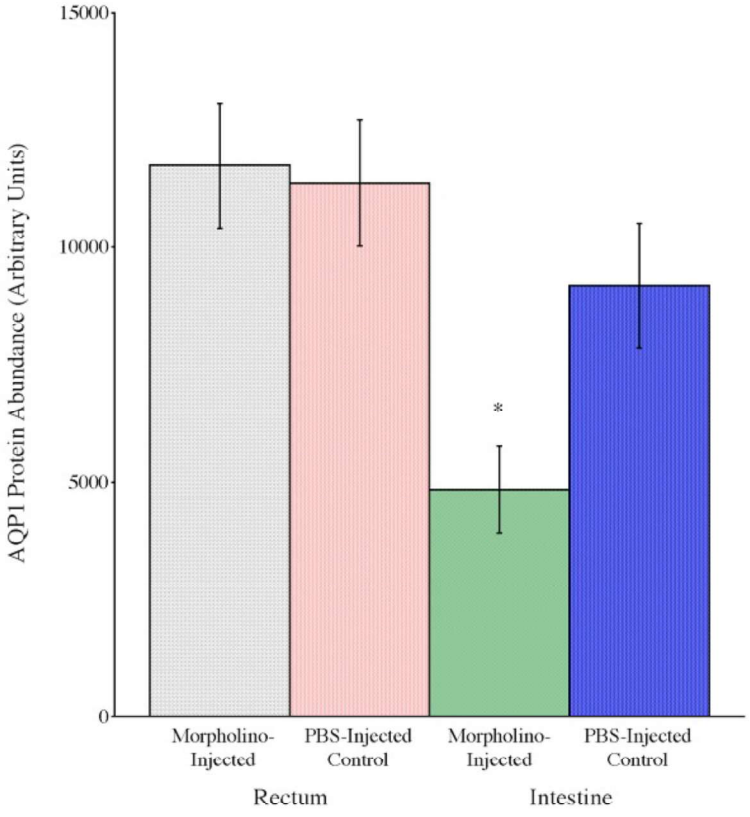

Figure 1. Quantification of Western blots of AQP1 protein expression in eel (*Anguilla rostrata*) gastrointestinal tract in vivo-morpholino knockdown or control fish. Eels were injected into the peritoneal cavity with either vivo-morpholino dissolved in PBS (n = 5 fish) or PBS alone (controls; n = 9 fish) for six consecutive days. Statistical significance: t-test where *p<0.05. Eels were transferred from freshwater to seawater after day one of the procedure. Error bars represent ± standard error of the mean (SEM).

filtration through cheesecloth, followed by ultracentrifugation at 50,000*g* in a Type 70 rotor (Beckman). Crude membrane pellets were re-suspended again in buffer containing protease inhibitor cocktail and frozen in aliquots. The protein concentration in each crude membrane extract was measured using a BCA kit micro assay (Pierce). 100 μg of rectal or 300 μg of intestinal crude membrane extract protein was heat-denatured (99˚C) and run on a Laemmli-SDS poly-acrylamide (10%) reducing gel, which was subsequently electro-blotted onto a PVDF membrane as part of a Western blot procedure. Blots were processed using a custom-made rabbit-anti-eel AQP1 polyclonal primary antibody[3] and a goat-anti-rabbit alkaline phosphatase linked secondary antibody and NBT-BCIP enzyme substrate (Pierce). Western blots were quantified using a gel documentation and analysis system (Syngene) and statistically analyzed using a Statsview software package (Abacus Concepts) and a 2-tailed unpaired t-test.

Results (Fig 1) showed no effect of injection with the AQP1-specific vivo-morpholinos in the rectum, compared to control fish. However, in the intestine a significant decrease in AQP1 expression was found following AQP1-specific vivo-morpholino injection. This suggests that the vivo-morpholinos are showing a limited amount of penetrance into the tissue, as they were able to block the translation of AQP1 protein in the endothelial cells of blood vessels in the middle of the underlying intestinal tissue, but were presumably unable to diffuse far enough (from the peritoneal cavity) to reach the luminal surface epithelial cell layer (the furthest layer into the tissue) in the rectum. Consequently, unless a better way is found to deliver the vivo-morpholinos to the target cells, they will be of limited use for future studies of this type.

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