A PUTATIVE MUSCARINIC RECEPTOR (M4) FROM THE CEREBELLUM OF THE SPINY DOGFISH, SQUALUS ACANTHIAS.

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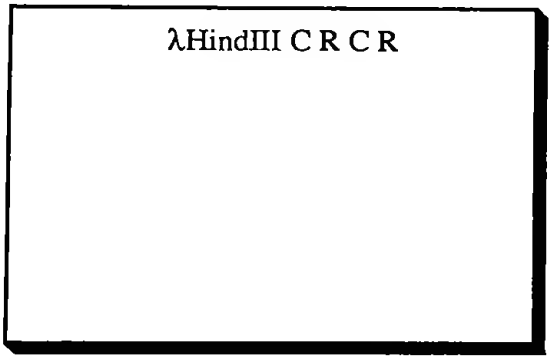
Previous studies have demonstrated that muscarinic receptor subtypes are expressed in specific regions of the central nervous sysytem. The M1 subtype has been localised to the cerebral cortex and corpus striatum, whilst the M2 subtype is expressed in the medulla-pons. The M4 receptor has a similar distribution to the M1 subtype, and one proposal is that they are particularly involved in the cognitive processes of learning and memory (Nathanson, N.M., Ann. Rev. Neurosci. 10:195-236) . As cognitive processes rely upon synaptic plasticity the receptor subtype heterogeneity may be a mechanism by which such plasticity can be achieved (Braun, et al, Biochem. Biophys. Res. Comm. 149:125-132, 1987). A single cell could vary its expression of several receptor subtypes, and thus control its response to a neurotransmitter. We have sought to apply the polymerase chain reaction (PCR) to isolate a dogfish cerebellar cDNA encoding a putative seven transmembrane domain G-coupled muscarinic receptor. The new sequence shows strong homology to the rat muscarinic M4 receptor, which has previously been shown by in situ hybridization to be abundant in the granule cell layer of the cerebellar cortex (Braun, et al, 1987). No muscarinic receptors could be identified by amplification of rectal gland cDNA, confirming previous physiological investigations.

RNA was extracted from the dogfish shark brain and rectal gland (1g of each organ). Tissues were immediately flash-frozen in liquid nitrogen upon removal from the animal to prevent ribonuclease action. Samples were polytron homogenized in lysis buffer and mRNA isolated (Fast-track mRNA extraction kit,InVitrogen,San Diego CA). First strand cDNA was synthesized from 1µg of mRNA (RT-PCR kit, InVitrogen). Approximately one tenth of the first strand cDNA was amplified with degenerate muscarinic receptor primers A and B (figure 1). The amplification profile was 35 cycles each of: dissociation at 95°C for 0.5 min, annealing at 30°C for 0.5 min, and extension at 60°C for 0.5 min. Control amplifications omitting input cDNA were simultaneously performed to exclude reaction contamination. Following the primary PCR, a second-round PCR was conducted on 1µl of the 50µl primary product using internal ("nested") muscarinic primers C and D (figure 1). The cycling profile was as for the first round PCR but limited to 20 cycles. No products were obvious following the primary amplification, yet a single 1kb band was clearly visible in brain cDNA after the secondary PCR (figure 2). Identical experiments performed using dogfish rectal gland cDNA consistently failed to amplify any products.

Figure 1: Conserved muscarinic receptor amino acid sequences within transmembrane domains (TM) II and VII to which redundant primers were designed.

Sense primer A:	5'.TVNNYY.3'	TM II
Sense primer C:	5'.CADLII.3'	TM II
Anti-sense primer B:	3'.CYALCN.5'	TM VII
Anti-sense primer D:	3'.GYWLCY.5'	TM VII

Figure 2: Agarose gel of the second round amplification of dogfish cerebellum (C), and rectal gland cDNA (R)..A single band of ~1Kb is amplified only from cerebellar cDNA.



The single amplified product was cloned into a plasmid vector (TA-cloning kit, InVitrogen), and transformed into competent E. coli cells. Recombinants were screened by blue/white selection, and mini-preparation DNA prepared for DNA sequencing. A modified alkaline lysis double-stranded sequencing protocol was used along with Sequenase 2.0™ enzyme and chain terminators (USB, Cleveland OH). Sequence information was analysed on an Apple Macintosh II using DNA STAR (LASERGENE™). By aligning the translated sequence with known muscarinic receptors from other species, the receptor appears most similar to the M4 type found in rats and humans. Using these methods, only partial sequence data is generated. To obtain the complete sequence of this new muscarinic receptor, future work will include screening a dogfish genomic library.

Figure 3: Amino acid alignment between the translated shark cerebellar putative muscarinic receptor and the rat M4 muscarinic receptor. Asterisks (*) indicate amino acid complete homology. Transmembrane domains (TM) are underlined.

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                    TM II                                   TM III
Shark Musc.CADLIIGVFSMNLYTVYIIKGYWPLGAVVCDLWLALDYVVSNASVMNLLIISLDRY
Rat M4     CADLIIGVISMNLFTTYIIMNRWALGNLACDLWLSIDYVASNASVMNLLVISFDRY
           ******** **** * ***   * **   *****  *** ********* ** ***

                              TM VI                          TM VII
Shark Musc.QVAAREKKVTRTIFAILLAFILTWTPYNVMVLISTFCDDCVPETVWVVGYWLCY
Rat M4     MSLIKEKKAAQTLSAILLAFIITWTPYNIMVLVNTFRDSCIPKTYWNLGYWLCY
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Within a short period of time (5-6 days) we were able to identify a new muscarinic receptor in the shark cerebellum and determined much of its sequence. We were also able to confirm what physiological evidence has suggested, that there are no muscarinic receptors expressed in the dogfish shark rectal gland. The reverse genetics techniques used proved to be highly efficient both in mimimizing the time required to obtain results and in conserving the number of animals used. We required only a single shark to obtain 1 gram samples from the brain and rectal gland.

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