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The distribution of muscarinic M1 receptors in the human hippocampus

Elizabeth Scarr[a,b], Myoung Suk Seo[a], Timothy Douglas Aumann[c], Gursharan Chana[d], Ian Paul Everall[b,e], Brian Dean[a,*]

[a] The Molecular Psychiatry Laboratory, The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Victoria, Australia
[b] The Psychiatric Neuropathology Laboratory, the Department of Psychiatry, The University of Melbourne, Victoria, Australia,
[c] The Adult Neurogenesis Laboratory, The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Victoria, Australia
[d] The Integrative Biological Psychiatry Laboratory, Centre for Neural Engineering, The University of Melbourne, Parkville, Victoria, Australia
[e] The Department of Psychiatry, the North West Mental Health, Royal Melbourne Hospital, Parkville, Victoria, Australia

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ABSTRACT

The muscarinic M1 receptor plays a significant role in cognition, probably by modulating information processing in key regions such as the hippocampus. To understand how the muscarinic M1 receptor achieves these functions in the hippocampus, it is critical to know the distribution of the receptor within this complex brain region. To date, there are limited data on the distribution of muscarinic M1 receptors in the human hippocampus which may also be confounded because some anti-muscarinic receptor antibodies have been shown to lack specificity.

Initially, using Western blotting and immunohistochemistry, we showed the anti-muscarinic M1 receptor antibody to be used in our study bound to a single 62 kDa protein that was absent in mice lacking the muscarinic M1 receptor gene. Then, using immunohistochemistry, we determined the distribution of muscarinic M1 receptors in human hippocampus from 10 subjects with no discernible history of a neurological or psychiatric disorder.

Our data shows the muscarinic M1 receptor to be predominantly on pyramidal cells in the hippocampus. Muscarinic M1 receptor positive cells were most apparent in the deep polymorphic layer of the dentate gyrus, the pyramidal cell layer of cornu ammonis region 3, the cellular layers of the subiculum, layer II of the presubiculum and layer III and V of the parahippocampal gyrus. Positive cells were less numerous and less intensely stained in the pyramidal layer of cornu ammonis region 2 and were sparse in the molecular layer of the dentate gyrus as well as cornu ammonis region 1. Although immunoreactivity was present in the granular layer of the dentate gyrus, it was difficult to identity individual immunopositive cells, possibly due to the density of cells.

This distribution of the muscarinic M1 receptors in human hippocampus, and its localisation on glutamatergic cells, would suggest the receptor has a significant role in modulating excitatory hippocampal neurotransmission.

1. Introduction

The role of the cholinergic system in cognitive processes, such as attention and information processing, is well established (Carruthers et al., 2015). Importantly, it has been shown that muscarinic and nicotinic receptors play synergistic roles in controlling cognitive processes such as working memory (Ellis et al., 2006) with nicotinic receptors modulating phasic activity important in detecting cues and muscarinic receptors controlling tonic activity which is important for attentional control (Recent review: (Demeter and Sarter, 2013)). In addition, the cholinergic system within the septohippocampal pathway has been suggested to play a role in acquisition related to short term memory and recognition (Klinkenberg et al., 2011). Data from muscarinic M1 receptor knock out mice (*Chrm1*$^{-/-}$) suggests that the muscarinic M1 receptor has a critical role in memory processes occurring in the hippocampus (Anagnostaras et al., 2003), reinforcing the role

* Corresponding author at: Head of the Biological Psychiatry and Mental Health Division and the Molecular Psychiatry Laboratory, The Florey Institute for Neuroscience and Mental Health, 30 Royal Parade, Parkville, Victoria 3052, Australia.
E-mail address: brian.dean@florey.edu.au (B. Dean).

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of the hippocampal muscarinic M1 receptor in maintaining aspects of cognitive abilities in humans and other mammals.

Until recently, the high degree of structural homology at the orthosteric binding site on the five muscarinic receptors has meant it has not been possible to develop drugs specific for individual muscarinic receptors (Kruse et al., 2014). However, drugs have now been synthesised that are highly selective for, if not specific to, each individual muscarinic receptor (Conn et al., 2009). The use of such drugs in human cognitive paradigms has emphasised the role of the muscarinic M1 receptor in maintaining cognitive functioning (Nathan et al., 2013). This hypothesis is supported by preclinical studies showing muscarinic M1 receptor agonists are effective at modulating behavioural paradigms that require hippocampal engagement (Bradley et al., 2010; Vanover et al., 2008). Given the growing interest in targeting the muscarinic M1 receptor to try and modulate behaviours under the control of the hippocampus (Uslaner et al., 2013), we decided to determine the distribution of the muscarinic M1 receptor in the human hippocampus.

There have been studies on the muscarinic M1 receptor in the human CNS but these results must be treated with caution as some of the anti-muscarinic receptor antibodies used may lack specificity (Jositsch et al., 2009). Hence we began our study by obtaining data to support the specificity of the antibody to be used for our studies on the muscarinic M1 receptor.

1.1. Materials and methods

1.1.1. Tissue collection and processing

Antibody Validation

Although the focus of our study was the human hippocampus, all validation experiments were completed using cortex because muscarinic M1 receptors are expressed at relatively high levels in that CNS region (Scarr et al., 2013). Frozen (Dean et al., 1999) and fixed tissue was used for these experiments.

Immunohistochemistry Human Hippocampus

Brain tissue was collected, following consent from the nearest next of kin, with the approval of the Ethics Committee of the Victorian Institute of Forensic Medicine. The tissue was sourced from the Victorian Brain Bank at the Florey Institute of Neuroscience and Mental Health. Details pertaining to the ten cases from whom tissue was collected and who had no history of psychiatric or neurological disorders are in Table 1.

Tissue for immunohistochemistry required the removal of the whole brain from the cranium, the brains were hemisected with the right hemisphere being fixed in 37% formaldehyde for at least two weeks before being processed for neuropathology using a standardised process. The slices (1 cm thickness) from the neuropathology cut were then stored in 10% neutral buffered formalin. The hippocampal blocks for this study were taken at the level of the lateral geniculate nucleus. After removal, the blocks were placed in phosphate buffered saline (PBS). The blocks were serially sectioned at 50 μm on a vibratome and stored in PBS containing 0.5% sodium azide at 4 °C.

Immunohistochemistry Mouse CNS

With permission of the Animal Ethics Committee of the Florey Institute for Neuroscience and Mental Health, CNS was removed from muscarinic M1 (*Chrm1*$^{-\backslash-}$) and M4 (*Chrm4*$^{-\backslash-}$) receptor knock out mice and wild type (C57BL/6-NTAC) mice (n = 5 per group), fixed as for human tissue, embedded in paraffin wax and serially sectioned at 7 um on a rotary microtome before being stored at room temperature. The sections used for this study were taken at the level of Bregma 1.18 mm (Franklin and Paxinos, 2008).

1.1.2. Antibody validation

The polyclonal anti-muscarinic M1 receptor antibody used in this study (mAChR-M1-Rb; batch Af340) was raised against amino acids 247–345 of the mouse muscarinic M1 receptor (NM_007698) (Narushima et al., 2007) and was purchased from Frontier Institute Co. Ltd. Hokkaido, Japan. The specificity of the antibody was explored by i) using Western blot and fresh frozen samples of cortex from *Chrm1*$^{-/-}$, *Chrm4*$^{-/-}$ and wild type mice plus a sample of human cortex and ii) immunohistochemistry using fixed brain tissue from *Chrm1*$^{-\backslash-}$ and wild type mice plus human cortex.

1.1.3. Western blotting

CNS tissue (~50 mg) from *Chrm1*$^{-\backslash-}$, *Chrm4*$^{-\backslash-}$, wild type mice and a human case were homogenised (10% w/v) into 10 mM Tris-HCl (pH 7.0) containing 320 mM Sucrose, 1 mM EDTA and 20 mM KCl. Homogenates were centrifuged at 1000 x *g* for 10 min at 4 °C and the supernatant recovered. Protein concentrations were determined using the Bio-Rad modified Lowry protein assay adapted for the microplate. The homogenates were diluted in reducing buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 100 mM dithiothreitol, 4% SDS, 0.0025% bromophenol blue) to give a final concentration of 1.5 mg/ml. Samples were denatured by heating at 55 °C for 30 min, briefly centrifuged and 20 μl of each sample loaded onto a polyacrylamide gel (4% loading, 10% stacking gel) alongside BioRad broad range molecular weight standards and separated using a constant voltage (150 V). The proteins were then transferred to nitrocellulose membranes overnight in Towbin transfer buffer using a constant current (40 mA). Loading and uniform transfer were confirmed using 0.1% Ponceau S in 3% trichloroacetic acid. The nitrocellulose was blocked for 1 h in antibody diluent (5% non-fat milk powder (NFMP) in Tris buffered saline containing 0.1% Tween-20; TTBS) then incubated with rabbit anti-CHRM1 antibody (Frontier Institute Co. Ltd; mAChR-M1-Rb; batch Af340; 1/100 in antibody diluent) overnight at 4 °C. This was followed by incubation with goat anti-rabbit antibody conjugated to horseradish peroxidise (1/2000 in TTBS: DAKO; P0448; batch

Table 1
Demographic and tissue collection information for the subjects whose hippocampal tissue was used in this study.

Subject ID	Age (Years)	Sex	Cause of Death	Post-mortem Interval (hours)	Brain pH
C1	36	M	Crush accident	42	6.46
C2	42	M	Cardiomegaly	63	6.34
C3	21	F	Myocarditis	58	6.03
C4	21	M	Acute epiglottitis	40	5.82
C5	25	M	Electrocution	24	6.42
C6	75	M	Cardiogenic shock	69.4	6.19
C7	52	M	Cardiomegaly	33.75	6.52
C8	66	F	Infrarenal atherosclerosis	49.25	6.44
C9	52	M	Ischaemic heart disease	50	6.78
C10	66	M	Coronary artery atheroma	71.75	6.47