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Research report

Quantitative analysis of binding parameters of [^{3}H]*N*-methylscopolamine in central nervous system of muscarinic acetylcholine receptor knockout mice

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Abstract

We have studied binding parameters (K_d, B_{max}) of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) in various brain regions and spinal cord of wild-type (WT) and muscarinic acetylcholine receptor (mAChR) subtype (M_1–M_5) knockout (KO) mice. In the M_1–M_4 KO mice, the number of [^{3}H]NMS binding sites (B_{max}) was decreased throughout the central nervous system (CNS) with significant regional differences. Our results collectively suggest that M_1 receptor was present in a relatively high density in the cerebral cortex and hippocampus, and the densities of M_1 and M_4 subtypes were highest in the corpus striatum. M_2 receptor appeared to be the major subtype in the thalamus, hypothalamus, midbrain, pons-medulla, cerebellum and spinal cord. These findings may contribute significantly not only to the further understanding of the physiological roles of mAChR subtypes in the central cholinergic functions, but also to the development of selective therapeutic agents targeting specific subtype.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Acetylcholine receptors: muscarinic

Keywords: Muscarinic acetylcholine receptor subtype; Knockout mice; CNS distribution

1. Introduction

Muscarinic acetylcholine receptors (mAChRs) in the central nervous system (CNS) are involved in a number of important physiological functions, including the control of extrapyramidal locomotor activity and higher cognitive processes such as learning and memory. Molecular cloning studies have revealed the existence of five distinct mAChR subtypes (M_1–M_5). Among these subtypes, M_1, M_3 and M_5 receptors are usually coupled to the $G_{q/11}$ protein which activates of phospholipase C, whereas the M_2 and M_4 subtypes are mainly coupled to the $G_{i/o}$ protein which inhibits adenylate cyclase activity and activates potassium channels resulting in hyperpolarization.

The distribution of mAChR subtypes in CNS has been investigated mainly by pharmacological studies with relatively subtype-selective agents and also by molecular, immunohistochemical and immunoprecipitational studies with measurement of the expression of mRNA levels and

Abbreviations: mAChR, muscarinic acetylcholine receptor; CNS, central nervous system; WT, wild type; KO, knockout; [^{3}H]NMS, [*N*-methyl-^{3}H]scopolamine methyl chloride; K_d, apparent dissociation constant; B_{max}, maximal number of binding sites

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receptor protein [2–4,14,28,30]. Studies on the distribution of mRNAs by in situ hybridization provide clear evidence for the distribution and identity of neurons that synthesize the receptor subtypes. However, the identification of regional distribution of pharmacologically relevant CNS mAChR subtypes has proven a difficult task. The difficulty may be primarily due to the lack of ligands endowed with a high degree of receptor subtype selectivity and to the fact that most tissues or organs express multiple mAChRs. In this situation, it may be one of the most promising ways to utilize mouse lines deficient in specific mAChR receptor genes (M_1–M_5) generated by gene targeting technology [19,30].

In the present study, we have measured specific [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) binding in various brain areas of wild-type (WT) and mAChR subtypes (M_1–M_5) knockout (KO) mice. The advantage of our technique is that it allows the direct estimation of physiologically or pharmacologically relevant receptors, and it can be adapted to investigate the interaction of acetylcholine and selective agonists and antagonists with mAChR subtypes. Our data have confirmed that there is a significant regional difference in the distribution of mAChR subtypes throughout the CNS.

2. Materials and methods

2.1. Materials

[*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 3.03 TBq/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). All other chemicals were purchased from commercial sources.

2.2. Animals

The generation of WT, M_1KO, M_2KO, M_3KO, M_4KO and M_5KO mice was described previously [13,17,18,22,23]. The genetic background of the mice used in this study was a mixture of the 129/SvJ and C57BL/6J strains. The littermate wild-type mice are often used as controls when analyzed KO mice originated from two different strains. However, this can be problematic for some reasons: (1) requirement of more number of mice because of significant phenotypic variance reflecting differences in genomic sequences, (2) no reproducibility of the results at a later time because of impossibility to obtain animals with the same genetic backgrounds and (3) difficulty in interpretation of the results when comparing phenotypes of multiple mutant lines. In order to overcome these issues, it is recommended to establish congenic lines by multiple backcrosses to inbred strains. In the present study, most of mice used were more than N7 generation of the C57BL/6J strain. Accordingly, it is considered that the WT mice used in this study are reasonably similar to all of the five mutant lines in their genetic backgrounds. Mouse genotyping was performed by PCR analysis of mouse tail DNA. Male mice at 3–8 months of age were used in this study. Mice were housed with a 12 h light–dark cycle and fed laboratory food and water ad libitum. This study was conducted according to guidelines approved by the Experimental Animal Ethical Committee of University of Shizuoka and University of Tokyo.

2.3. Tissue preparation

Male mice of WT, M_1KO, M_2KO, M_3KO, M_4KO and M_5KO were exsanguinated by taking the blood from the descending aorta after intraperitoneal administration of pentobarbital (40 mg/kg), and the brain was then perfused with cold saline from the aorta. The whole brain and spinal cord were excised, and the former was further divided into the cerebral cortex, corpus striatum, hippocampus, hypothalamus, thalamus, midbrain, pons-medulla and cerebellum according to the procedure described previously by Glowinski and Iversen [8]. The hypothalamuses from three mice were pooled for a determination, because of the scantiness of the tissue weight. Each brain region and spinal cord were homogenized by a Kinematica Polytron homogenizer in 19 volumes of ice-cold 30 mM Na^+/HEPES buffer (pH 7.5) and the homogenates were then centrifuged at 40,000×*g* for 20 min. The resulting pellet was finally suspended in the buffer for the binding assay. Protein concentrations were measured by the method of Lowry et al. [16].

2.4. Muscarinic receptor binding assay

The binding assay for mAChR was performed using [^{3}H]NMS as previously described [6]. The crude membrane fractions (60–180 μg protein) of mouse brain region and spinal cord were incubated with different concentrations (0.03–1.0 nM) of [^{3}H]NMS in 30 mM Na^+/HEPES buffer (pH 7.5). Incubation was carried out for 60 min at 25 °C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel, Gaithersburg, MD, USA) through Whatman GF/B glass fiber filters, and the filters were then rinsed two times with 3 mL of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight by immersion in scintillation fluid (2 L toluene, 1 L Triton X-100, 15 g 2,5-diphenyloxazole, 0.3 g 1,4-*bis*[2-(5-phenyloxazolyl)]benzene), and the radioactivity was determined by a liquid scintillation counter. Specific [^{3}H]NMS binding was determined experimentally from the difference between counts in the absence and presence of 1 μM atropine. All assays were conducted in duplicate.

2.5. Data analysis

Analysis of [^{3}H]NMS binding data was performed as previously described [31]. The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]NMS were estimated by Rosenthal analysis of the