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Brain Research 1063 (2005) 105 – 113
BRAIN RESEARCH
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Research Report

Validation of a selective serotonin 5-HT_{2C} receptor antibody for utilization in fluorescence immunohistochemistry studies

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Accepted 24 September 2005
Available online 7 November 2005

Abstract

Although radioligand binding studies have shown that the serotonin 5-HT_{2C} receptor (5-HT_{2C}R) is widely expressed throughout the brain, more detailed knowledge of 5-HT_{2C}R distribution within different neuronal populations will aid in understanding the mechanisms through which this receptor acts. Double-label immunohistochemical procedures can be utilized to examine the localization of receptors within specific neuronal populations. In order to conduct such studies, however, it was first necessary to examine the utility and specificity of two commercially available anti-5-HT_{2C}R antibodies [from Santa Cruz (SC) and BD PharMingen (PH)]. In male Sprague–Dawley rats, both antibodies produced widespread immunoreactivity (IR) throughout the brain area chosen for study, the ventral tegmental area, which is the origin of the dopamine mesocorticoaccumbens "reward" pathway. Co-labeling with the SC and PH 5-HT_{2C}R antibodies demonstrated that IR for the two antibodies largely overlapped. However, SC 5-HT_{2C}R IR was more concentrated within IR cell bodies and was more consistent among assays than the PH 5-HT_{2C}R IR. Thus, the SC 5-HT_{2C}R antibody was chosen for subsequent studies. When examined in 5-HT_{2C}R knockout vs. wild-type mice, the SC 5-HT_{2C}R antibody produced widespread IR in wild-type, but not 5-HT_{2C}R knockout, mice. In addition, 5-HT_{2C}R-IR was not present in either native CHO cells, known to be devoid of 5-HT_{2A}R or 5-HT_{2C}R, or in CHO cells transfected with the 5-HT_{2A}R. Thus, these studies suggest that the SC 5-HT_{2C}R antibody produces reliable staining selective for 5-HT_{2C}R vs. 5-HT_{2A}R in rodent brains and is therefore suitable for use in future immunofluorescence 5-HT_{2C}R localization studies.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Serotonin receptors

Keywords: 5-hydroxytryptamine receptor; 5-HT_{2C} knockout mouse; CHO cell; Ventral tegmental area; Immunofluorescence

1. Introduction

The serotonin (5-HT)$_{2C}$ receptor (5-HT_{2C}R), one of 15 known 5-HT receptor subtypes [7], is a seven transmembrane G-protein ($G\alpha_{q/11}$) coupled receptor that has been implicated in a number of physiological and psychological conditions including schizophrenia, obsessive compulsive disorder, obesity, anxiety, depression, and addiction [4]. Studies have demonstrated that 5-HT_{2C}R mRNA and protein are widely expressed throughout the brain [1,5,14], including the origin [ventral tegmental area (VTA)] and termination sites [nucleus accumbens (NAc) and prefrontal cortex (PFC)] of the DA mesocorticoaccumbens or "reward" pathway. However, little is known of the particular neuronal populations upon which the 5-HT_{2C}R exerts its actions. As such, studies examining the distribution of 5-HT_{2C}R protein in different neuronal populations throughout the brain are necessary in order to fully understand the mechanisms of action of this receptor.

A widely utilized method for determining receptor localization within various cell types is the use of double-label immunohistochemistry techniques, which require

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doi:10.1016/j.brainres.2005.09.050

utilization of an antibody that specifically recognizes the receptor. Unfortunately, none of the commercially available anti-5-HT_{2C}R antibodies have been extensively utilized in standard immunohistochemical procedures. As such, the effectiveness of the antibodies and their specificity for the 5-HT_{2C}R in vivo are uncertain and should be validated before the antibodies are employed for such studies. Thus, prior to initiating detailed localization studies, we sought to analyze the utility and validity of two commercially available anti-5-HT_{2C}R antibodies for use in fluorescence immunohistochemical studies: a goat polyclonal anti-5-HT_{2C}R antibody that is directed toward a 19 amino acid sequence at the N-terminus of the receptor (SC 5-HT_{2C}R; Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal anti-5-HT_{2C}R antibody that is directed toward amino acids 384–459 of the C-terminal end of the 5-HT_{2C}R (PH 5-HT_{2C}R; BD PharMingen, San Diego, CA). While each of these antibodies has demonstrated an ability to detect selective changes in 5-HT_{2C}R protein expression in the VTA to a similar extent via Western blot analysis (Bubar, Thomas and Cunningham, unpublished observations), to our knowledge, only the PH 5-HT_{2C}R antibody has been exploited for immunohistochemical analysis in the brain [8].

To assess the utility of the 5-HT_{2C}R antibodies, we first examined the distribution of immunoreactivity (IR) produced by each of the antibodies in single-label fluorescence immunohistochemistry experiments conducted using brains from male Sprague–Dawley rats. We then examined whether the two antibodies were labeling similar sites by performing double-label fluorescence immunohistochemistry experiments combining the two 5-HT_{2C}R antibodies and comparing their patterns of immunoreactivity (IR). Due to concerns that the 5-HT_{2C}R antibodies may display cross-reactivity for the closely related 5-HT_{2A}R, specificity studies were conducted comparing 5-HT_{2C}R IR with IR produced by an anti-5-HT_{2A}R antibody. We first examined 5-HT_{2C}R and 5-HT_{2A}R IR in 5-HT_{2C}R knockout (KO) mice and their wild-type (WT) littermates to show that 5-HT_{2C}R IR is not detected in 5-HT_{2C}R KO mice, while 5-HT_{2A}R IR is unaltered. Finally, in order to demonstrate that the 5-HT_{2C}R antibody does not bind to the 5-HT_{2A}R, we examined 5-HT_{2C}R and 5-HT_{2A}R IR in Chinese hamster ovary (CHO) cells, which do not normally contain 5-HT_{2C}R or 5-HT_{2A}R [2], and CHO cells transfected with the 5-HT_{2A}R. Based upon the results of these studies, we identified an anti-5-HT_{2C}R antibody suitable for future fluorescence immunohistochemistry studies examining the distribution of 5-HT_{2C}R on different neuronal populations throughout the brain.

2. Methods

2.1. Brain tissue preparation

Naïve male Sprague–Dawley rats (n = 8; virus antibody-free; Harlan Sprague–Dawley, Inc., Houston, TX) were used. Rats were deeply anesthetized with pentobarbital (100 mg/kg, IP, Sigma) and perfused transcardially with phosphate buffered saline (PBS) followed by 3% paraformaldehyde in PBS. Brains were removed, blocked at mid-pons, and post-fixed for 2 h at room temperature (RT). Brains were then cryoprotected in 30% sucrose for 48 h at 4 °C, rapidly frozen on crushed dry ice, and stored at −80 °C until sectioning. Coronal sections (20 μm) containing the VTA (−4.8 through −6.5 from Bregma) were taken from rat brains using a cryostat (Leica CM 1850 at 20 °C) according to the atlas of Paxinos and Watson [12]. Free floating sections were processed as described below. All experiments conformed to the *NIH Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1986) and were approved by the UTMB Institutional Animal Care and Use Committee.

In addition, frozen brains from the 5-HT_{2C}R KO mouse on a C57Bl/6J background and WT littermates (n = 2/genotype) that had been previously fixed (4% paraformaldehyde/PBS) and cryoprotected (30% sucrose) were obtained from the laboratory of Dr. Lawrence Tecott at the

Table 1
List of antibodies employed in the experiments

Primary antibody	Dilution	Species	Catalog #	Supplier	Location
Anti-SC 5-HT_{2C}R	1:100	Goat	sc-15081	Santa Cruz Biotechnology	Santa Cruz, CA
Anti-PH 5-HT_{2C}R	1:300	Mouse	556335	BD PharMingen	San Diego, CA
Anti-5-HT_{2A}R	1:500	Rabbit	–	Dr. Bryan Roth, Case Western Reserve University	Cleveland, OH
Anti-Flag	1:1000	Mouse	F-3165	Sigma	St. Louis, MO
Anti-Flag	1:1000	Rabbit	F-7425	Sigma	St. Louis, MO
Secondary antibody	**Dilution**	**Fluorescent dye[a]**	**Catalog #**	**Supplier**	**Location**
Donkey anti-goat	1:2000	Alexa Fluor 488	A-11055	Molecular Probes	Eugene, OR
Donkey anti-mouse	1:2000	Alexa Fluor 555	A-31570	Molecular Probes	Eugene, OR
Donkey anti-rabbit	1:2000	Alexa Fluor 555	A-31572	Molecular Probes	Eugene, OR
Goat anti-mouse	1:2000	Alexa Fluor 488	A-11029	Molecular Probes	Eugene, OR
Goat anti-rabbit	1:2000	Alexa Fluor 555	A-21429	Molecular Probes	Eugene, OR

[a] Alexa Fluor 488 dye appears green; Alexa Fluor 555 dye appears red.