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pSer40 TYROSINE HYDROXYLASE IMMUNOHISTOCHEMISTRY IDENTIFIES THE ANATOMICAL LOCATION OF C1 NEURONS IN RAT RVLM THAT ARE ACTIVATED BY HYPOTENSION

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Abstract—Identification of neurons, and their phenotype, that are activated in response to specific stimuli is a critical step in understanding how neural networks integrate inputs to produce specific outputs. Here, we developed novel mouse monoclonal antibodies of different IgG isotypes that are specific to tyrosine hydroxylase (TH), and to tyrosine hydroxylase activated at its serine 40 position (pSer40TH), in order to assess changes in the activity of phenotypically identified cardiovascular neurons using fluorescence immunohistochemistry. We find that the proportion of C1 pSer40TH-positive neurons in the central and medial region of the rat rostral ventrolateral medulla (RVLM) increases dramatically following hydralazine treatment, whereas phenylephrine treatment does not significantly change the pSer40TH/TH ratio in these regions compared to control. This finding suggests that there is a mediolateral topology associated with the activation of C1 neurons following baroreceptor loading or unloading. Overall, we conclude first, that our newly characterized monoclonal antibodies are specific, and selective, against TH and pSer40TH. Secondly, that they can be used to label TH and pSer40TH immunoreactive neurons simultaneously, and thirdly that that they can be used to identify the activation state of catecholamine synthetizing neurons after physiological stimuli. Finally, we find that there is basal level of activation of TH neurons in the lateral, central and medial regions (~70%, 30% and 45%, respectively) of the C1 area, but that following unloading of the baroreceptors there is a marked increase in activation of central (~80%) and medial (~90%) C1 neurons in the RVLM.

Key words: tyrosine hydroxylase, phosphorylated tyrosine hydroxylase, RVLM, C1 neurons, IgG subtype-specific monoclonal antibodies, baroreflex.

INTRODUCTION

The rostral ventrolateral medulla (RVLM) is a critical area in the central control of blood pressure (BP) (Pilowsky et al., 2009). With the development of modern immunohistochemical and tract-tracing techniques, it was recognized that bulbospinal neurons that are inhibited by baroreceptor activation (Miyawaki et al., 1995) and hence termed 'sympathoexcitatory' (BS-SE) in the RVLM are extremely heterogeneous in terms of their neurotransmitter content, receptor expression, axon myelination (Schreihofer and Guyenet, 1997; Verberne and Sartor, 2004; Burke et al., 2011), and many other features. In 1974 (Hökfelt et al., 1974), it was demonstrated that in RVLM, all tyrosine hydroxylase (TH) neurons also contained phenylethanolamine-N-methyltransferase (PNMT; adrenaline synthesizing). Subsequently, it was discovered that these same PNMT cells (also termed C1 neurons) projected to the spinal cord where they synapsed exclusively – albeit in very small numbers – with sympathetic preganglionic neurons (Guyenet et al., 1989). Ablation or inhibition of the RVLM causes a catastrophic fall in arterial BP and sympathetic nerve activity (SNA) (Pilowsky et al., 1985; Sun and Reis, 1996; Horiuchi and Dampney, 1998). Depletion of C1 cells in RVLM does not significantly alter arterial pressure or generation of sympathetic vasomotor tone (Schreihofer and Guyenet, 2000); interestingly, depletion of catecholamine neurons does attenuate the pressor and sympathoexcitatory responses to stimulation of the RVLM. Functional identification of baroreceptor inhibited bulbospinal RVLM neurons using morphological criteria has until now been limited to the use of protein product of the feline osteosarcoma oncogene 'c-fos' (Fos) histochemistry combined with other markers (Minson et al., 1996b; Spirovski et al., 2012). A disadvantage of these approaches is that

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Abbreviations: BP, blood pressure; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Fos, protein product of the feline osteosarcoma oncogene 'c-fos'; IgG, immunoglobulin G; IHC, immunohistochemistry; mAbs, monoclonal antibodies; MAP, mean arterial pressure; MATF, Monash Antibody Technology Facility; PBS, phosphate-buffered saline; PFA, paraformaldehyde in 0.1 M phosphate buffer; PNMT, ph enylethanolamine-N-methyltransferase; pSer40TH, tyrosine hydroxylase phosphorylated at Ser40; pSer40THIgG$_{2a\kappa}$, pSer40TH antibody – IgG$_{2a\kappa}$ isotype; RVLM, rostral ventrolateral medulla; SNA, sympathetic nerve activity; TCS, tissue culture supernatants; TH, tyrosine hydroxylase; THIgG$_{1\kappa}$, TH antibody – IgG$_{1\kappa}$ isotype.

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there is heterogeneity of cell types in the RVLM, and the use of Fos on its own is not sufficient to parse out the different groups of cells.

Here, we investigate the possibility of using monoclonal antibodies (mAbs) directed toward TH, and mAbs directed toward TH that has been phosphorylated at the serine 40 residue (pSer40TH), a site that is known to increase TH activity at least 20-fold in most cases (Dunkley et al., 2004). Our objective was to determine if the ratio of phosphorylated to non-phosphorylated neurons would be useful in identifying activity changes in central cardiovascular catecholaminergic neurons in the RVLM.

Our aims in this study were first, to develop and characterize mouse mAbs of different immunoglobulin G (IgG) subclasses to TH, and to pSer40TH. Secondly, to determine that both antibodies exclusively stain catecholaminergic neurons, and thirdly, to establish the extent to which the antibodies could be used to determine increases or decreases in phosphorylation states in catecholaminergic neurons in different regions of the RVLM following baroreceptor loading (phenylephrine) or unloading (hydralazine). If this was the case, we believe that such an approach would enable the identification of TH neurons that were activated in different functional states.

EXPERIMENTAL PROCEDURES

Reagents

Chemicals were from Sigma–Aldrich (NSW, Australia) unless otherwise stated.

Peptide synthesis

Peptide sequences, corresponding to TH and pSer40TH were identified and validated using NCBI BLAST protein database. TH sequence: *C-PRFIGRRQSLIEDARK*; pSer40TH sequence: *C-PRFIGRRQpSLIEDARK.* Peptides were produced at >70% purity using standard peptide chemistry by C S Bio Co (CA, USA).

mAb production

mAbs were generated at the Monash Antibody Technology Facility (MATF, Melbourne, https://platforms.monash.edu/matf/). Briefly, mice were immunized intraperitoneally with a synthetic peptide, corresponding to the antigen of interest (TH or pSer40TH). Following immunization, the mouse producing the largest amount of the relevant antibody was selected for fusion. Hybridoma cells were produced by fusing SP2/0-Ag14 myeloma cells with mouse spleen cells in the presence of polyethylene glycol and then grown in azaserine hypoxanthine containing medium. Hybridoma cells were screened for reactivity with the antigen by microarray (Antigen MicroArray (AMA), MATF) followed by enzyme-linked immunosorbent assay (ELISA) of any IgG microarray-positive clones. The most responsive ELISA clones were expanded and subcloned by two rounds of serial dilution. Two stable clonal lines that secreted antibodies specific for each antigen were established. The isotype of the monoclonal antibody being produced was determined by a commercially available assay kit (IsoStrip, Roche, USA). The TH antibody was found to be $IgG_{1\kappa}$ ($THIgG_{1\kappa}$). The pSer40TH antibody was found to be an $IgG_{2a\kappa}$ ($pSer40THIgG_{2a\kappa}$).

Hybridoma cell culture

Newly established hybridoma cell lines were first cultured in Dulbecco's modified Eagle's medium (DMEM, JRH Biosciences) supplemented with 200 mM GlutaMAX (Invitrogen, VIC, Australia), 50 μg/mL gentamicin (Gibco, Invitrogen, VIC, Australia) and 20% fetal bovine serum (FBS). Cells were split every 3–4 days and seeded at 1×10^5 cells/mL in 75 cm^3 culture flasks for two weeks. Serum content of the medium was gradually decreased to 10%. Antibody-containing tissue culture supernatants (TCS) were collected after two weeks of culturing cells in 10% FBS-supplemented DMEM. Small aliquots of TCS were frozen and stored at −20 °C for future use.

ELISA

The TCS were tested against each target antigen by ELISA. Briefly, 96-well plates were coated with the peptide of interest (TH or pSer40TH) at a concentration of 4 μg/mL and blocked with 3% bovine serum albumin in phosphate-buffered saline (100 mM phosphate buffer, 0.9% NaCl, pH 7.4; PBS). Undiluted hybridoma TCS was added to the wells followed by the addition of a secondary antibody (donkey anti-mouse IgG (H + L), alkaline phosphatase labeled). A chromogenic reaction was initiated by addition of p-nitrophenylphosphate substrate, and stopped by addition of a stop solution (5 M sodium hydroxide). Absorbance was measured at 405 nm; clones were considered positive if the absorbance readings were more than triple than the negative control (hybridoma cell media).

Double-labeled immunofluorescence labeling of rat brainstem sections

Mouse mAbs produced by hybridoma cells were used for immunohistochemical analysis of free floating rat brain sections. Different IgG isotypes of the primary mAbs in conjunction with IgG-isotype specific secondary antibodies conjugated to two different fluorophores allowed for simultaneous detection of TH and pSer40TH in the tissue. Prior to incubation with the primary mAbs, brainstem sections were washed 3 × 30 min in PBS containing 0.3% Triton X-100 on an orbital shaker (Heidolph Instruments, Germany) at room temperature. Sections were then transferred to the solution containing 1:100 dilution of mouse mAbs against TH ($THIgG_{1\kappa}$) and pSer40TH ($pSer40THIgG_{2a\kappa}$) described above in Tris-PBS with 0.1% thimerosal, 0.3% Triton X-100 and 10% normal donkey serum, and incubated on an orbital shaker at 4 °C for 72 h. Following incubation with the primary mAbs, brainstem sections were washed with PBS containing 0.3% Triton X-100 (3 × 30 min) and incubated with Cy5-conjugated $IgG_{1\kappa}$-specific