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Research report

Habituation-induced neural plasticity in the hippocampus and prefrontal cortex mediated by MMP-3

John W. Wright[a,b,c,*], Peter C. Meighan[b,c], Travis E. Brown[b,c], Roberta V. Wiediger[a], Barbara A. Sorg[b,c], Joseph W. Harding[a,b,c]

[a] Department of Psychology, Washington State University, Pullman, WA 99164-4820, United States
[b] Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, WA 99164-6520, United States
[c] Program in Neuroscience, Washington State University, Pullman, WA 99164-6520, United States

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ABSTRACT

Head-shake response (HSR) habituation was presently used to investigate the phenomena of spontaneous recovery and neural plasticity. Independent groups of rats were presented with five consecutive habituation sessions separated by inter-session intervals (ISIs) of 2, 24 or 72 h. At the conclusion of testing hippocampus and prefrontal cortex tissue samples were collected for determination of matrix metalloproteinase-3 (MMP-3:stromelysin-1) expression as a marker of neural plasticity. The results indicated that by the fifth session the 2 h ISI group showed no spontaneous recovery, the 72 h ISI group revealed nearly complete spontaneous recovery; while the 24 h ISI group showed intermediate recovery. MMP-3 expression in the hippocampus and prefrontal cortex was elevated in the 2 and 72 h ISI groups, but not in the 24 h group. A second experiment utilized 7-day osmotic pumps to intracerebroventricularly infuse an MMP-3 inhibitor for 6 days. The animals were then tested on the seventh day using the 2 h ISI protocol. Delivery of the MMP-3 inhibitor facilitated spontaneous recovery, thus compromising the animal's ability to appropriately habituate. This effect was accompanied by a significant inhibition of hippocampus and prefrontal cortex MMP-3 expression. These results suggest that elevations in hippocampus and prefrontal cortex MMP-3 expression contribute to this simplest form of learning and may be a mechanism underlying spontaneous recovery.

1. Introduction

Habituation is characterized by the gradual waning of a behavioral response to repeated stimulation and is considered the simplest form of learning [21,44]. This decrement in response strength cannot be attributed to sensory adaptation or motor fatigue, but is thought to involve neural plasticity within the central nervous system [8]. Habituation has been documented across many species for several response systems ranging from the gill-withdrawal reflex in *Aplysia* [9] and tap withdrawal or chemotaxic response in the nematode *Caenorhabditis elegans* [4,41], to acoustic startle response in rats and mice [37,43] and feeding in humans [13]. The hippocampus (reviewed in [11,23,27,28,38,39]) and prefrontal cortex [49,51] have been implicated in the control of inhibitory processes, particularly habituation, and were the focus of this investigation.

The head-shake response (HSR) consists of a rapid rotation of the head about the anterior to posterior axis in response to a mild air stimulus applied to the ear [2]. This response follows a decreasing negatively accelerated function of stimulus frequency such that the higher the rate of stimulus presentation, the faster the rate of habituation. Following habituation the HSR spontaneously recovers as a function of the inter-session interval (ISI), reaching approximately 85–90% of its original response strength following 24 h of rest [33,49].

Our laboratory has utilized altered levels of matrix metalloproteinases (MMPs) as markers of neural plasticity in an effort to understand the role of these proteinases in the central mediation of spatial learning and habituation. The MMP family consists of zinc-dependent endopeptidases initially released from neurons and glia as inactive zymogens, but become activated once outside the cell (reviewed in [14,42]). MMPs are important contributors to the process of neural plasticity because they degrade the extracellular matrix (ECM), thus permitting synaptic restructuring (reviewed in [10,12,14,31]). The proteolytic activity of MMPs is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) designed to inhibit the active forms of MMPs by forming tight non-covalent complexes with them (reviewed in [5,24]). Our laboratory

* Corresponding author at: Department of Psychology, PO Box 644820, Washington State University, Pullman, WA 99164-4820, United States. Tel.: +1 509 335 2329; fax: +1 509 335 5043.
E-mail address: wrightjw@wsu.edu (J.W. Wright).

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recently reported elevations in brain MMP-3 contingent upon the acquisition of new hippocampal-dependent memories [30,36,47]. Therefore, we hypothesized that MMP-3 expression may also be important to the phenomena of habituation and spontaneous recovery.

The present investigation examined the following questions: (1) does the pattern of habituation and spontaneous recovery change with repeated HSR sessions varied over three ISIs? (2) Are there changes in MMP-3 expression in the hippocampus and prefrontal cortex following five habituation sessions? (3) Does pretreatment with an MMP-3 inhibitor (MMP-3i) alter the response strength and temporal pattern of subsequent habituation and spontaneous recovery?

2. Materials and methods

The protocols used in these studies were approved by the Washington State University Institutional Animal Care and Use Committee and conformed to the guidelines for the care and use of laboratory animals as required by the National Institutes of Health (NIH Publication #80-23).

2.1. Animals

Male Sprague–Dawley rats (300–350 g, breeding stock derived from Taconic, Germantown, NY) were adapted to a 12 h light/dark cycle initiated at 0600 h in an American Association for the Accreditation of Laboratory Animal Care-approved vivarium at a temperature of 21 ± 1 °C. The animals were housed in pairs and provided water and food (Harlan Teklad F6 Rodent Diet, Madison, WI) *ad libitum*, except the night prior to surgery when food was removed.

2.2. Behavioral testing

The apparatus was patterned after that used by Askew et al. [2] and consisted of an elevated platform measuring 7.5 cm × 16 cm. This surface was positioned on a 0.9 m tall wooden column that allowed the subject freedom of movement during testing. The surface of the platform was covered with 1 cm square wire mesh cloth surrounded by plywood sloping down and outward, forming a collar 17 cm × 24 cm at its extreme to discourage escape attempts. The base of the wooden column pivoted 360°, thus permitting the experimenter to compensate for movements by the animal and maintain a face-to-face orientation. The test room was painted black with ambient light set at the minimum level (5.7 Fc) necessary to score responses in order to reduce spatial cues.

2.2.1. Experiment 1

Three independent groups of rats (16 per group) were tested over five habituation sessions separated by fixed ISIs of 2, 24, or 72 h. HSRs were elicited by a hand held tube (orifice diameter = 0.5 mm) that provided a continuous stream of air that was oscillated across the center of the subject's left ear at an approximate rate of 3 cycles/s. The air tube was held 1–1.5 cm from the animal's ear. The intensity of the air stream was set at 5–6 cm displacement of a 40 cm column of 95% ethanol in a 0.5 mm inside diameter U-shaped manometer (inside diameter = 0.5 mm).

The standard habituation session consisted of a 5 min undisturbed adaptation period on the test stand, followed by 24, 15-s stimulus presentations. Each stimulus presentation was separated by a fixed 15-s inter-trial interval (ITI). An IBM-compatible computer signaled the intervals to the experimenter by displaying the 15-s trial in green numbers, and the 15-s ITI in red numbers (Turbo Pascal® 7.0 Borland International, Inc., Scotts Valley, CA). The experimenter recorded the number of HSRs following each trial. The first session for all animals was conducted between 0600 and 1100 h. Subsequent sessions (total of 5) were conducted following the designated ISI of 2, 24 or 72 h. At the conclusion of each session, the rat was removed from the test stand and placed back into its home cage. Upon completion of the fifth session the animals were euthanized by decapitation at 5 min, 2 or 6 h post-session, with four rats randomly selected from each group per time point. The brains were immediately removed and dissected on a glass culture dish filled with crushed ice. The prefrontal (anterior 1 cm) and hippocampal cortices were frozen in liquid nitrogen and stored at −70° C until assayed for MMP-3 levels by Western blotting. The times required to dissect the hippocampus and prefrontal cortex were approximately 90 and 30 s, respectively, for a total dissection time following euthanization of about 2 min. Home cage control animals (12 rats) were handled equivalently to the other groups but did not experience habituation trials. Four of these animals were randomly selected and euthanized along with members of each ISI group. Tissue samples were collected as described above.

2.2.2. Experiment 2

Three additional groups of rats (4 per group) experienced five habituation sessions separated by a fixed ISI of 2 h. Prior to behavioral testing each rat was prepared with a 7-day osmotic pump (Model 2001, Alza Scientific Products, Palo Alto, CA) that infused intracerebroventricularly (icv) at a rate of 1 μl/h. A control group received pumps filled with artificial cerebrospinal fluid (aCSF, 1 μl/h). Members of a second group received an MMP-3i at a dose of 1 μg/h (Ac–Arg–Cys–Gly–Val–Pro–Asp–NH_2, m.w. 686.8; #444218 Calbiochem, San Diego, CA in aCSF). A third group received MMP-3i at a dose of 5 μg/h. All pumps were primed in sterile 0.15 M NaCl at 38 °C for 6 h prior to placement. This preparation has been previously described in detail [50]. Following 6 days of infusion each animal was habituated over five sessions on the 2 h ISI protocol as detailed in Experiment 1. Two hours following the conclusion of behavioral testing correct placement of each icv guide cannula was confirmed by the icv injection of 5 μl of fast green dye via the chronic cannula immediately followed by decapitation, brain extraction onto ice, and visual confirmation of dye within the brain ventricles. At this time samples from the hippocampus and prefrontal cortex were frozen in liquid nitrogen and stored at −70 °C as previously described. These tissues were assayed for MMP-3 levels by Western blotting. Delay times following euthanization to tissue freezing were 4–5 min. All cannulae were appropriately placed and all pumps had nearly exhausted their contents.

2.3. Enzyme assay

The specificity of the MMP-3 inhibitor was determined utilizing fluorescence enzyme assays conducted in black 96-well plates according to the manufacturer instructions (Biomol International L.P., Plymouth Meeting, PA). Briefly, catalytic domains for MMP-1, -3, -8, -9 and -13 were used with the fluorogenic peptide substrate: Mca–Pro–Leu–Gly–Leu–Dpa–Ala–Arg–NH_2. Samples were pre-incubated with MMP-3 inhibitor for 30 min at 37 °C. Subsequently, the fluorogenic peptide substrate was added to the samples and fluorescence was measured at 10 min intervals for one h using a Perkin-Elmer plate reader. Background values obtained from each interval were subtracted from all samples and the data were normalized as percent of enzyme activity compared with activity in the absence of inhibitor.

2.4. Western blotting

Brain tissues were homogenized in ice-chilled radioimmunprecipitation buffer, pH 7.4 containing 50 mM Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3O_4 and protease inhibitors (1 mM PMSF, and 1 μg/mL each of Aprotinin, Leupeptin, and Pepstatin). The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C and the protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL). The protein samples were separated on a 7.5% SDS-PAGE gel and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with blocking solution (2.5% nonfat milk in TBS) for 20 min at room temperature and then incubated overnight at 4 °C with MMP-3 antibody (1:1000) in blocking solution. After three washings of 5 min each with 0.05% Tween 20-TBS (T-TBS), the membranes were probed with goat anti-mouse IgG (1:4000) conjugated to horseradish peroxidase in blocking solutions for 2 h. The membranes were further washed six times, 5 min each, with T-TBS and visualized by chemiluminescence methods. The densitometric analysis of the blots was performed using TotalLab Software (Phoretix, UK). The results were expressed as a fold increase in band density over density from home cage control rats matched for age, gender and handling, but not behaviorally tested.

2.5. Data analyses

2.5.1. Experiment 1

The 24 trials of each session were grouped into 8 blocks of 3 trials each, and the mean number of HSRs during each trial block was calculated for each animal. Data from the five sessions were separately plotted for each of the three ISIs. One-way ANOVAs for between measures were utilized to compare the number of HSRs measured during the first trial block of Session I across the three ISI groups. An ANOVA for within measures was used to compare the number of HSRs during the first trial block of each of the five sessions for each of the three ISI groups. Post hoc analyses were accomplished using Newman–Keuls tests with a level of significance set at $p < 0.05$.

The data sets concerned with pro- and active-MMP-3 levels were each analyzed using 3 (ISIs) × 4 (control + 3 delay times) ANOVA for each of the two brain structures. Again, Newman–Keuls post hoc tests were used to further evaluate significant effects ($p < 0.05$).

2.5.2. Experiment 2

The most important information regarding the specificity of the MMP-3 inhibitor concerned the IC_{50}s for MMP-3 and MMP-9 given that the latter has been shown to influence the neural plasticity associated with spatial memory [30], passive avoidance conditioning [36], and habituation [49]. The effects of this inhibitor on MMP-3 and -9 were compared using an independent measures *t*-test with a level of significance set at $p < 0.05$. The behavioral data sets were analyzed as described in Experiment 1. The data sets concerned with pro- and active-MMP-3 levels measured in the hippocampus and prefrontal cortex were evaluated using one-way ANOVA (groups: 0, 1, or 5 μg/μl/h). Significant effects were further analyzed using Newman–Keuls post hoc tests.