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Hippocampal $G\alpha_{q/11}$ but not $G\alpha_o$-coupled receptors are altered in aging

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ABSTRACT

Normal aging may limit the signaling efficacy of certain GPCRs by disturbing the function of specific Gα-subunits and leading to deficient modulation of intracellular functions that subserve synaptic plasticity, learning and memory. Evidence suggests that $G\alpha_{q/11}$ is more sensitive to the effects of aging relative to other Gα-subunits, including $G\alpha_o$. To test this hypothesis, the functionality of $G\alpha_{q/11}$ and $G\alpha_o$ were compared in the hippocampus of young (6 months) and aged (24 months) F344 × BNF_1 hybrid rats assessed for spatial learning ability. Basal GTPγS-binding to $G\alpha_{q/11}$ was significantly elevated in aged rats relative to young and but not reliably associated with spatial learning. mAChR stimulation of $G\alpha_{q/11}$ with oxotremorine-M produced equivocal GTPγS-binding between age groups although values tended to be lower in the aged hippocampus and were inversely related to basal activity. Downstream $G\alpha_{q/11}$ function was measured in hippocampal subregion CA1 by determining changes in $[Ca^{2+}]_i$ after mAChR and mGluR (DHPG) stimulation. mAChR-stimulated peak change in $[Ca^{2+}]_i$ was lower in aged CA1 relative to young while mGluR-mediated integrated $[Ca^{2+}]_i$ responses tended to be larger in aged. GPCR modulation of $[Ca^{2+}]_i$ was observed to depend on intracellular stores to a greater degree in aged than young. In contrast, measures of $G\alpha_o$-mediated GTPγS-binding were stable across age, including basal, mAChR-, $GABA_BR$ (baclofen)-stimulated levels. Overall, the data indicate that aging selectively modulates the activity of $G\alpha_{q/11}$ within the hippocampus leading to deficient modulation of $[Ca^{2+}]_i$ following stimulation of mAChRs but these changes are not related to spatial learning.

1. Introduction

G-protein coupled receptors (GPCRs) interact with a variety of Gα-subunits and effectors, giving rise to considerable diversity in signal transduction and resulting in the modulation of a variety of cellular processes including cell excitability, kinase activity, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), neurotransmitter release and gene expression. GPCRs transduce extracellular signals via an associated G-protein heterotrimer that includes a Gα-subunit bound to a GDP molecule under resting conditions. In response to neurotransmitter binding, the transmembrane receptor protein undergoes a conformal change that drives a GTP-exchange reaction at the Gα-subunit. The active GTP-bound Gα-subunit can then modulate the activity of effector proteins until the GTP is hydrolyzed back to GDP by the Gα-subunit's intrinsic enzymatic activity, thus terminating signaling action. Acetylcholine, glutamate and γ-amino butyric acid (GABA), each interact with a subset of GPCRs, but the consequences for neural activity are subtype-dependent. M1 muscarinic acetylcholine receptors (mAChR) and Group I metabotropic glutamate receptors (mGluR), including mGluR1 and mGluR5, couple to $G\alpha_q$ and $G\alpha_{11}$ that stimulate phospholipase C (PLC) to catalyze the formation of inositol phosphates (IP) and diacylglycerol (DAG) and subsequently releases intracellular Ca^{2+} stores (ICS) via inositol triphosphate receptors (IP3Rs; reviewed in Caulfield and Birdsall, 1998; Bordi and Ugolini, 1999). This signaling cascade is distinct from GPCRs, including M2 mAChRs and $GABA_B$ receptors ($GABA_BRs$), that couple to $G\alpha_o$ and $G\alpha_i$ to inhibit adenylyl cyclase and limit neurotransmitter release (reviewed in Caulfield and Birdsall, 1998; Chalifoux and Carter, 2011).

Aging is associated with progressive cognitive decline as well as increased risk for neurodegenerative disorders such as Alzheimer's disease (AD). Therapeutic interventions would offer the greatest

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benefit if administered at the earliest indication of cognitive impairment, but the biological basis for this impairment must be sufficiently characterized to optimize therapeutic efficacy. Naturally occurring rodent models can assess the effects of normal aging on neural substrates and behavior without confounds stemming from neuropathological disease. Using these rodent models, mAChR-mediated phosphoinositide (PI) turnover has been reported as impaired (Ayyagari et al., 1998; Chouinard et al., 1995; Nicolle et al., 1999) or enhanced (Parent et al., 1995; Tandon et al., 1991) in the aged hippocampus. Similar disagreement is apparent in studies of Group I mGluR signal transduction (Nicolle et al., 1999; Parent et al., 1995). Comparatively less is known about the integrity of M2 mAChR- or $GABA_BR$-stimulated signaling in the aged hippocampus, but compounds that block either M2 mAChRs or $GABA_BRs$ enhance learning and memory in aged rats (Froestl et al., 2004; Lasarge et al., 2009; Quirion et al., 1995). However, it is unclear if these benefits are derived from reversing age-related changes to GPCRs or indirectly promoting postsynaptic activity by facilitating neurotransmitter release.

Given the complex relationship between GPCRs and associated signal transduction mechanisms, this study presents findings from a series of comparative pharmacological analyses designed to determine whether aging selectively impairs receptor-stimulated activation of $G\alpha_{q/11}$ leading to insufficient modulation of subsequent neural responses within the hippocampus of young adult and aged rats that were characterized for spatial learning. First, this study used mAChR and $GABA_BR$ agonist-stimulated [^{35}S]guanosine-5′-O-(3-thio)triphosphate (GTPγS)-binding to assess functional coupling of these receptors to specific Gα-subunits that were biochemically verified using an immunocapture scintillation proximity assay (SPA). Subsequently, activity downstream of mAChRs or Group I mGluRs was examined by measuring agonist-stimulated changes to $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Subjects

Male, Fischer 344 × Brown Norway F_1 hybrid rats were obtained from the National Institutes of Aging rodent colony maintained by Harlan–Sprague–Dawley, Inc., (Indianapolis, IN, USA) and were 6 months of age (young; $n = 21$) or 24 months of age (aged; $n = 47$) at the time of behavioral training. All animals were housed in a facility approved by the International Association for the Assessment and Accreditation of Laboratory Animal Care at Wake Forest University School of Medicine. The Institutional Animal Care and Use Committee of Wake Forest University approved all protocols described in these studies.

2.2. Behavioral testing in Morris water maze

Rats were behaviorally characterized using a standardized place-learning task developed to optimize detection of age-associated changes in spatial learning (Gallagher et al., 1993). Rats trained 3 trials a day for 8 days to navigate to a submerged platform using spatial cues surrounding the maze. Rats were placed into the water at one of four equally spaced start positions in a counterbalanced order and allowed 90 s to locate the platform after which time they were guided to the platform. Rats remained on the platform for 30 s before transfer to a holding cage for 30 s. Every sixth trial (i.e. the third trial on days 2, 4, 6 and 8) was a probe where the platform was lowered and inaccessible during the first 30 s of the trial then subsequently raised for escape. Following place-training, rats received a single session of 6 cued trials, escaping to a visible black platform extending 2 cm above the water surface, to assess sensorimotor function and motivation. Data were acquired via a video camera mounted above the maze connected to a digital video recorder and computer running Ethovision software (Noldus, Leesburg, VA, USA). Cumulative distance and average distance from platform assessed training and probe trial performance, respectively. Values from the second, third and fourth probe trials were summed to produce a "proximity score", a graded measure summarizing individual performance (Gallagher et al., 1993; Bizon et al., 2009; Nieves-Martinez et al., 2012).

2.3. Hippocampal microdissection and membrane preparation

Approximately 2 weeks after the completion of behavioral testing, rats were decapitated, the brain removed, and hippocampi dissected on an ice-cold plate. 1 mm-thick transverse sections were made through the septal–temporal axis using a tissue chopper and sub-dissected into dentate gyrus (DG; including the hilus), CA3 (including CA2) and CA1 regions. Regions from both hippocampi were pooled, frozen on dry ice and stored at −80 °C until used for membrane preparation as described in McQuail et al. (2012). Membrane protein content was measured using the Pierce bicinchoninic acid assay kit (Rockford, IL, USA) and aliquots were stored at −80 °C until used for GTPγS-binding assays.

2.4. GTPγS-binding and anti-G-protein scintillation proximity assay

All reagents were purchased from Sigma–Aldrich (Saint Louis, MO, USA), unless otherwise stated. GTPγS-binding reactions were conducted in triplicate in 96-well Opti-plates (PerkinElmer, Waltham, MA, USA). The reaction buffer contained 100 mM NaCl, 5 mM $MgCl_2$ in 50 mM HEPES (pH 7.4). To unmask $G\alpha_{q/11}$ activity, membranes were pre-treated with 10 mM *N*-ethylmaleimide for 30 min on ice (Salah-Uddin et al., 2008) and guanosine 5′-diphosphate concentration was 0.1 mM (Delapp et al., 1999; Porter et al., 2002). For the $G\alpha_o$ assay, GDP concentration was 50 mM (Delapp et al., 1999). Non-specific binding was determined in the presence of 10 μM GTPγS. Basal GTPγS-binding was measured in the absence of any experimental compounds. 100 μM oxotremorine-M or 300 μM baclofen (Tocris, Ellison, MO, USA) stimulated mAChR and $GABA_BRs$, respectively. These concentrations were selected to produce maximal GTPγS-binding based upon prior studies examining total or Gα-subunit specific GTP-exchange in rodent brain (Delapp et al., 1999; McQuail et al., 2012; Porter et al., 2002; Zhang et al., 2007). Ten micrograms of membrane protein was added to each well and equilibrated at room temperature for 30 min. GTPγS-binding was initiated by the addition of 500 pM [^{35}S]GTPγS (PerkinElmer) to a final volume of 200 μl/reaction. After 60 min, IGEPAL CA-630 was added to a final concentration of 0.3% (v/v) with agitation at +4 °C for 30 min. Anti-$G\alpha_{q/11}$ or anti-$G\alpha_o$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added at a final dilution of 1:100 and incubated for 1 h at +4 °C. Anti-IgG-coated scintillation proximity assay beads (PerkinElmer) were suspended in 25 ml of 50 mM HEPES and 50 μl added per well then incubated for 30 min at +4 °C. Plates were centrifuged and counted in a TopCount scintillating microplate reader (PerkinElmer). Basal GTPγS-binding (counts per minute; CPM) was determined by subtracting non-specific activity. Agonist-stimulated values were transformed to "percent over basal" [% = (stimulated-basal)/(basal)*100] to facilitate comparisons between age groups and subregions or "net CPM" values (i.e. basal subtracted from stimulated) for correlation with basal activity.

2.5. Hippocampal slice preparation and calcium imaging

Transverse hippocampal slices (250 μm) were prepared and loaded with Calcium Green-AM (Molecular Probes, Eugene, OR, USA) as described in Hampson et al. (2011). In contrast to intracellular injection, acetoxymethyl (AM) ester derivatives of fluorescent indicators allow for the labeling of multiple cells per slice/field of view. Imaging was performed on CA1 cells with an upright confocal microscope (Nikon, New York, NY, USA) equipped with a water-immersion objective, a Hamamatsu Orca-ER digital camera and an Ultraview spinning disc confocal system (PerkinElmer). Calcium Green emission images (500–600 nm) were acquired by laser excitation at 488 nm and sampled at 0.3 s intervals. A piezoelectric "stepping" motor advanced the objective through the focal plane to acquire 40 vertical slices (2.5 μm) per field, producing a complete three-dimensional image every 12 s. Slices were perfused with artificial cerebrospinal fluid (ACSF; 126 mM NaCl, 20 mM $NaHCO_3$, 5 mM KCl, 2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose in 20 mM HEPES [pH 7.4]) for 2 min to determine baseline fluorescence and then perfused with either 50 μM oxotremorine-M or 50 μM (*S*)-3,5-dihydroxyphenylglycine (DHPG; Group I mGluR agonist; Tocris Bioscience) in ACSF for 3 min. Drug perfusion was followed by 5 min ACSF washout. Each slice was then incubated with 50 μM cyclopiazonic acid (CPA; Tocris Bioscience) for 10 min to deplete intracellular calcium stores (Soler et al., 1998) and the drug stimulation protocol was repeated. Calcium imaging results were expressed as percentage change in cell soma fluorescence (ΔF) of baseline Calcium Green fluorescence (F_0; average of first 120 s of recording). This relatively quantitative approach is necessary because uptake of the indicator dye is dependent upon infiltration of the cell plasma membrane followed by cleavage of the dye from the conjugated acetoxymethyl group by endogenous esterases to facilitate cytosolic localization and retention within the cell. Peak change in fluorescence ($\Delta F/F_0$; i.e. the single greatest value observed during agonist administration) was obtained from each cell under all conditions to determine maximal response and the cumulative effect of agonist-stimulated change to $[Ca^{2+}]_i$ was evaluated by calculating area under the curve (AUC) using the integral of the response calculated in GraphPad Prism 5 software (La Jolla, CA, USA).

2.6. Statistical analyses

Data are presented as the mean ± standard error. Independent- or paired-samples *t*-tests and repeated measures analysis of variance (RMANOVA) were performed using GraphPad Prism 5 software. RMANOVAs were followed with Bonferroni post hoc tests to evaluate significant differences while correcting for multiple