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Insulin-like Growth Factor 2 Rescues Aging-Related Memory Loss in Rats

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Abstract

Aging is accompanied by declines in memory performance, and particularly affects memories that rely on hippocampal-cortical systems, such as episodic and explicit. With aged populations significantly increasing, the need for preventing or rescuing memory deficits is pressing. However, effective treatments are lacking. Here we show that the level of the mature form of insulin-like growth factor 2 (IGF-2), a peptide regulated in the hippocampus by learning, required for memory consolidation and a promoter of memory enhancement in young adult rodents, is significantly reduced in hippocampal synapses of aged rats. By contrast the hippocampal level of the immature form proIGF-2 is increased, suggesting an aging-related deficit in IGF-2 processing. In agreement, aged compared to young adult rats are deficient in the activity of proprotein convertase 2, an enzyme that likely mediates IGF-2 post-translational processing. Hippocampal administration of the recombinant, mature form of IGF-2 rescues hippocampal-dependent memory deficits as well as working memory impairment in aged rats. Thus, IGF-2 may represent a novel therapeutic avenue for preventing or reversing aging-related cognitive impairments.

Keywords

insulin-like growth factor-2; proprotein convertase; hippocampus; memory; rat; working memory

1. Introduction

Cognitive functions, and in particular long-term memory formation, storage and retrieval, are impaired in aged populations (Balota et al., 2000; Cansino, 2009; Koen and Yonelinas, 2014). Not all types of memories are equally affected by the aging process; for example, implicit (procedural) learning, semantic memory and verbal skills seem largely spared, whereas episodic/declarative memories, spatial memories, attention, and working memory

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are consistently impaired (Balota et al., 2000; Kausler, 1994). Thus, hippocampal- and cortical-dependent memories seem to be particularly vulnerable to impairments occurring with aging. This functional loss has been found in humans as well as in non-human animals (Gallagher and Pelleymounter, 1988; Rapp et al., 1997; Small et al., 2011). Moreover, in aged human subjects, memory performance remains unimpaired after brief, post-training delays, but gradually decreases as time passes, in agreement with a compromised hippocampal-dependent consolidation process (Mitrushina et al., 1991; Pace-Schott and Spencer, 2011). Using contextual conditioning paradigms, a similar decay of memory over extended post-learning delays has been observed in aged relative to young adult rodents (Foster and Kumar, 2007; Gold et al., 1982; Winocur, 1988). Studies in aged animals show that memory deficits are associated with hippocampal synaptic alterations suggesting that mechanisms of hippocampal memory consolidation, the process by which memories become stabilized and long lasting over time (Alberini, 2009; Dudai, 2004; Squire and Alvarez, 1995), are compromised (Burke and Barnes, 2010; Driscoll et al., 2003; Morrison and Baxter, 2012; Foster 2012; Yassa et al., 2010a, 2010b). During consolidation, memories are labile and their strength can be modulated (Alberini et al., 2012; Bambah-Mukku et al., 2014; Bekinschtein et al., 2007); therefore, targeting hippocampal mechanisms of consolidation might be an effective strategy for improving memory functions in aged individuals (Alberini and Chen, 2012; Stern and Alberini, 2013).

Using inhibitory avoidance (IA) in young adult rats, we have identified several components of gene expression cascades regulated in the dorsal hippocampus following learning and required for memory consolidation (Arguello et al., 2013; Chen et al., 2012; Milekic and Alberini, 2002; Taubenfeld et al., 2001; Garcia-Osta et al., 2006). Among these, the expression of insulin-like growth factor 2 (IGF-2 or IGF-II) increases during the first 24 hours following training, and this increase is essential for memory consolidation; in fact, its expression knockdown causes memory impairment (Chen et al., 2011). Furthermore, hippocampal or systemic administration of recombinant IGF-2, but not of another member of the IGF family, IGF-1, significantly enhances the retention and persistence of hippocampal-dependent memories in young adult rats and mice (Chen et al., 2011; Stern et al., 2014a, 2014b), as well as of hippocampal long-term potentiation (Chen et al., 2011). In contrast, IGF-1 may ameliorate memory deficits in aged animals (Markowska et al., 1998; Deak and Sonntag, 2012), suggesting distinct effects and/or mechanisms of IGFs on memory performance and deficit rescuing.

In the adult brain, IGF-2 is the most abundantly expressed among the insulin-like peptides, with the highest levels of mRNA expression found in myelin sheaths, leptomeninges, microvasculature, and the choroid plexus (Logan et al. 1994; Rotwein et al. 1988; Russo et al., 2005). In addition, relative to other brain regions, high levels of expression of IGF-2 protein and of its high affinity IGF-2 receptor (IGF-2R) are observed in the adult hippocampus and cortex (Couce et al., 1992; Fernandez and Torres-Alemán, 2012; Hawkes and Kar, 2004; Logan et al., 1994; Ye et al, 2015), brain regions essential for memory consolidation. Notably, both IGF-2 mRNA and mature protein levels are lower in the hippocampus of aged compared to young adult mice (Kitraki et al., 1993; Park and Buetow, 1991; Uddin and Singh, 2013; Pascual-Lucas et al., 2014). Because the effect of IGF-2 as a memory and synaptic plasticity enhancer is robust and very long-lasting, occurs via IGF-2R

and not via IGF-1R, and targets multiple domains of hippocampal-dependent memory (Chen et al., 2011; Stern et al., 2014a), here we sought to determine the expression of IGF-2 and IGF-2 receptor (R) in aged rats and whether IGF-2 treatment is effective in preventing age-related memory deficits.

2. Materials & Methods

2.1 Animals

Young adult (4 months) and aged (26 months) male Fischer 344 × Brown Norway (FBN) F1 hybrid rats were obtained from the National Institute on Aging's colony at Harlan (Indianapolis, IN) and Charles River (Frederick, MD). This hybrid rat strain is widely used for aging studies as they offer several advantages as an aging model (Lipman et al., 1996). Animals were singly housed, permitted *ad libitum* access to food and water, and maintained on a standard 12h light-dark cycle. All rats used in the experiments were monitored daily for health parameters by the experimenters and the animal care staff, and were weighed at least 3 times per week to confirm health status and appropriate levels of food and water intake. Experiments were performed during the light phase of the cycle. Rats were allowed to acclimate to the experimental facility for a minimum of 7 days, and were then handled for 3 minutes (min) per day for 5 days prior to beginning experimental manipulations. All protocols were approved by the Institutional Animal Care and Use Committee at New York University, and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Surgery and intra-hippocampal injections

Cannulation and injections were carried out as described previously (Chen et al., 2011; Stern et al., 2014b). Rats were anesthetized with ketamine (65 mg/kg)-xylazine (7.5 mg/kg) i.p. and stainless-steel guide cannulae (22-gauge; Plastics One) were stereotactically implanted to bilaterally target the hippocampus (relative to Bregma A/P: −4.0 mm, M/L: +2.6 mm, D/V: −2.0 mm in young adult or −2.3 mm in aged to account for increased skull thickness). Cannulae were secured in place with anchor screws and dental cement, and dummy stylets were inserted to maintain patency. Rats were returned to their home cages and allowed to recover from surgery for a minimum of 7 days. Following behavioral training, rats received bilateral hippocampal injections using 28-gauge needles extending 1.5 mm beyond the tips of the guide cannulae, connected to a 10-μL Hamilton syringe by polyethylene tubing. Injections were delivered at a rate of 0.33 μL/min using an infusion pump (Harvard Instruments), and needles were left in place for 2 min to allow dispersion of the solution. Rats received 250 ng of recombinant mouse IGF-2 (catalog number 792-MG, R&D Systems) in a volume of 1 μL per hemisphere, or an equal volume of vehicle solution (sterile phosphate buffered saline with 0.1% bovine serum albumin). To establish the correct cannula placements, rats were euthanized and their brains were post-fixed with 10% buffered formalin. Coronal sections (40 μm) were cut through the hippocampus and examined under a light microscope. Rats with incorrect placements were excluded from subsequent analyses.

2.3 Inhibitory avoidance (IA)

Inhibitory avoidance training and testing were carried out as described previously (Chen et al., 2011; Stern et al., 2014b). The IA chamber (Med Associates, Inc) consisted of a rectangular Perspex box divided into a safe compartment and a shock compartment. The safe compartment was white and illuminated with a house lamp and the shock compartment was black and remained dark. Foot shocks were delivered via the grid floor of the shock chamber with a constant current scrambler circuit. The apparatus was located in a sound-attenuated, dimly lit room. During training sessions, each rat was placed in the safe compartment facing the lit wall of the chamber, away from the door. After 10 sec, the guillotine door separating the light and dark compartments opened, allowing the rat access to the shock compartment. The door closed within 2 sec of the rat entering the shock compartment, and a 2 sec foot shock of either 0.6 or 0.9 mA, as specified, was administered. Rats remained in the shock compartment for 10 sec. The animals were transported to a different room and received a bilateral intra-hippocampal injection of IGF-2 or vehicle, and then returned to their home cage. Rats were tested for memory retention at the designated time point(s). Tests for IA memory consisted of placing the rat back in the safe compartment and recording its latency to cross to the shock compartment. Foot shock was not administered during retention tests, and each test session was terminated after 900 sec. Testing was carried out blind to treatments.

2.4 Object location test

Rats were trained and tested in a square open field arena with clear Plexiglas walls and floor (42 cm × 42 cm × 30 cm) located in a dim room. Visual cues were provided within the box and on the walls of the room. The walls of the box were covered with white and black paper. One black and one white wall also contained symbols (circle and square) to create four unique walls. Behavior was recorded with a video camera positioned approximately 1.5 m above the arena. Rats were first habituated (Hab) to the arena for 5 min each day for 2 consecutive days. The next day, training consisted of exposing the rats to 2 identical objects constructed from Mega Bloks® secured to the floor of the arena. Rats were initially placed facing a wall, away from the objects, and were allowed to explore the arena and objects for 5 min. Rats then received a bilateral intra-hippocampal injection of IGF-2 or vehicle as described for IA experiments, and were returned to their home cage. Rats were tested for retention of object location memory 4 hours (Test 1) and 1 day (Test 2) following training. Across both tests, one object remained in the same location as that of training, while the second object was moved to a novel location. Rats were placed in the arena facing the same direction as during training, and were allowed to explore for 5 min. The arena and objects were sprayed with 70% ethanol and cleaned between sessions. Video files were coded and scrambled. An experimenter blind to treatment scored the total time that rats spent actively exploring each object on each session. A rat was considered to be exploring an object when their nose was oriented towards the object and located within ~2 cm of the object (Ennaceur and Delacour, 1988). Memory was measured as the percentage of time spent exploring the object in the novel location compared to the stationary object.

2.5 Spontaneous alternation test

This test was employed to investigate spatial working memory, which is hippocampal-dependent (Lalonde, 2002). Rats were tested using a four-arm, plus-shaped maze (arms: 46 cm long, 14 cm wide, 8 cm tall) constructed of white plastic. The method was a modification of Newman et al. (2011). The maze was located on a table in the center of a room surrounded by visual cues outside of the maze. Animals received a bilateral intra-hippocampal injection of IGF-2 or vehicle 20 min before the start of the test. During the test, rats were placed in the start arm and allowed to freely explore the maze for 20 min. Behavior was recorded with a video camera positioned approximately 1.5 m above the arena and coded following the testing. An experimenter blind to the treatment recorded the number and sequence of arm entries. An alternation was defined as when the rat visited each of the four arms within five choices. For example, A,C,B,C,D was considered an alternation but A,C,B,C,A was not an alternation. Percent alternation was defined as the ratio of correct over total alternations $(\text{total arm entries} - 4) \times 100$.

2.6 Hippocampal protein extracts and western blot analysis

Total and synaptoneurosomal (SN) extracts were prepared as previously described (Bambah-Mukku et al., 2014; Chen et al., 2011, 2012). Briefly, dorsal hippocampi were dissected in ice-cold cortical dissection buffer (2.6mM KCl, 1.23mM sodium phosphate monobasic, 26mM sodium bicarbonate, 5mM kynurenic acid, 212mM sucrose, 10mM dextrose, 0.5mM CaCl₂, 1mM MgCl₂). Tissue was collected in 1 mL ice-cold synaptoneurosome buffer (10mM HEPES, 2mM EDTA, 2mM EGTA, 0.5mM DTT, 0.5mM PMSF, 2mM NaF, 1mM sodium orthovanadate, 1mM benzamidine, 1μM microcystin) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and homogenized using a motor-driven glass-Teflon pestle. 200 μL of this homogenate was collected (total extract) and 0.2M NaCl was added prior to 30 min incubation on ice and 30 min centrifugation at 4°C, 16000xg. The resulting supernatant was collected, aliquoted, and stored at -80°C for subsequent analyses. The remaining hippocampal homogenate was used to prepare SN extracts by adding 1 mL of synaptoneurosome buffer and passing this volume first through a double layer of nylon mesh filter (100μm), then through a single layer of nitrocellulose filter (5μm). Resulting extracts were centrifuged 10 min at 4°C, 1000xg and pellets were resuspended in synaptoneurosome buffer containing 1% Triton X-100, 0.1% SDS and 0.1% sodium deoxycholate, aliquoted, and stored at -80°C. Protein concentrations were determined with the BioRad protein assay (BioRad Laboratories). 20–50 μg of protein were resolved on 10%, 15% or 4–20% gradient polyacrylamide gels, based on the molecular weight of the protein of interest, and transferred to small pore (0.2μm) low-fluorescence PVDF membranes (Thermo Scientific) or Immobilon-FL membranes (Millipore). Membranes were blocked and incubated with primary antibodies according to manufacturers' recommendation [anti-IGF-2 (1:500, Abcam), anti-IGF-2 receptor (1:1000, Cell Signaling Technology), anti-PC2 (1:1000, Abcam)]. After incubation overnight at 4°C, membranes were washed and treated with anti-rabbit or anti-mouse fluorescent-conjugated secondary antibodies (Li-Cor) for 1 h at room temperature. All blots were probed with anti-β-actin (1:10000, Santa Cruz) as a loading control. B-actin levels were confirmed to not change in hippocampal extracts of young adult vs. aged rats normalized to total proteins/sample detected by Blue Comassie staining. Membranes were washed and fluorescent signal was visualized using an Odyssey CLx

scanner (Li-Cor). Densitometric analyses were performed using ImageStudio software (Li-Cor). For competition assays, 100-fold molar excess of recombinant mouse IGF-2, mouse IGF-1 (catalog number 791-MG, R&D Systems), recombinant human proIGF-2 or human proIGF-1 (catalog number AHU100 and CU100 respectively, Cell Sciences) was co-incubated with primary antibodies.

2.7 Proprotein convertase 2 (PC2) activity assay

Hippocampal PC2 enzymatic activity was analyzed using a protocol adapted from Tang and colleagues (2009). Proteins were extracted from dorsal hippocampi as described in *Hippocampal protein extracts and western blot analysis*. 20 µg of total protein extract was incubated with 200 µM L-PyroGLu-Arg-Thr-Lys-Arg-7-Amino-4-methylcoumarin (pERTKR-AMC, R&D Systems) in 50mM sodium acetate (pH 5.0), 100mM NaCl, 1mM CaCl₂, and 0.5% Tween®-20 with or without 2µM of PC2-specific inhibitor Pro-7B2 (156–186)/CT Mouse Peptide (7B2/CT, Phoenix Pharmaceuticals, Inc.) in a total reaction volume of 100 µL. Fluorescent intensity from 7-Amino-4-methylcoumarin (7-AMC) release was measured using an Infinite® 200 PRO plate reader (Tecan Group Ltd.; λ_{ex} = 360 nm, λ_{em} = 480 nm). PC2-specific activity was calculated as the activity inhibited by 7B2/CT, using free 7-AMC as a standard.

2.8 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 21 and GraphPad Prism 6. Western blot data were analyzed with independent-samples *t* tests (effect of age), with Welch's correction in cases of unequal variances between groups. PC2 enzyme activity was analyzed with an independent-samples *t* test (effect of age). IA training data were analyzed with two-way analysis of variance (ANOVA). Preliminary screening of IA test data for normality (Kolmogorov-Smirnov goodness-of-fit test) revealed that latency scores across tests were negatively skewed toward the arbitrary cut-off latency of 900 sec. For this reason, test data were rank transformed (Conover and Iman, 1981) before analyzing with repeated measures (effects of repeated tests, age, and IGF-2) or two-way ANOVA (effects of age and IGF-2), followed by non-parametric Moses tests (non-transformed data) or Tukey HSD tests (rank-transformed data) comparing individual groups. One sample *t* tests were used to determine changes in novel object location preference compared to chance (50%). Object location (percent preference and total times spent exploring each object location) and spontaneous alternation (percent alternation and total arm entries) data were analyzed with two-way ANOVAs followed by Tukey HSD post-hoc tests. Effects were considered statistically significant when $p < 0.05$. For experiments in which multiple comparisons were employed to compare individual groups, alpha levels were Bonferroni-corrected based on the number of comparisons performed.

3. Results

3.1 Decreased mature form of IGF-2 and increased proIGF-2 in hippocampal synapses of aged rats

To determine whether the expression of IGF-2 and/or IGF-2R in the hippocampus is altered by aging, total and synaptoneurosomal (SN) protein extracts were prepared from dorsal

hippocampi of untrained (naïve) young adult and aged Fischer 344 × Brown Norway F1 (FBN) hybrid rats. SN preparations were validated (not shown) by enriched expression of PSD-95, and glutamate receptor subunits GluA2 and GluN2A, relative to total protein fractions as described in Chen et al. (2011, 2012). Relative quantitative western blot analyses were carried out on both total and SN extracts.

The levels of precursor and mature forms of IGF-2 were assessed using a rabbit polyclonal antibody raised against full-length IGF-2 (Abcam, catalog number ab9574). In the first set of experiments, we confirmed the specificity of this antibody. The antibody recognized bands at the predicted molecular weight of preproIGF-2 (22 kDa) and proIGF-2 (20 kDa), as well as of processed, mature IGF-2 (15 kDa). Recombinant mouse IGF-2 peptide consisting of a portion of the mature form of IGF-2, but not recombinant mouse IGF-1 peptide, competed the bands corresponding to both mature and precursor forms of IGF-2 in total and SN extracts (Fig. 1a). Recombinant human proIGF-2, but not proIGF-1, peptide competed bands corresponding to proIGF-2 in SN extracts and partially competed those of total extracts (Fig. 1a).

As shown in Figure 1b and c, levels of mature IGF-2 in total hippocampal extracts did not change with aging [Independent samples *t* test: $t(16)=0.47$, $p=0.64$]; however, in the SN extracts of aged relative to young adult rats the levels of mature IGF-2 was significantly reduced [Independent samples *t* test: $t(34)=3.04$, $p=0.005$]. Conversely, the level of the precursor form proIGF-2 was increased in both total and SN extracts of aged relative to young adult rats [Fig. 1b and c; Independent samples *t* test: total: $t(16)=2.20$, $p=0.04$; SN: $t(32)=2.37$, $p=0.02$].

The levels of IGF-2R, assessed with two independently generated antibodies [(Cell Signaling Technology, catalog number CS-D3V8C (shown), and antibody 293 produced in Dr. Richard G. MacDonald's laboratory (Clairmont and Czech, 1991), not shown)] did not differ between young adult and aged rats in either total or SN hippocampal protein extracts [Fig. 1d; Independent samples *t* test, Cell Signaling antibody, total extract: $t(16)=0.12$, $p=0.91$; SN: $t(16)=0.52$, $p=0.61$].

Together, these data indicate that, while hippocampal IGF-2R levels do not change with age, the level of the mature form of IGF-2 protein is significantly decreased in the hippocampal synapses of aged rats. Conversely, the level of IGF-2 precursor forms significantly increases in both total and synaptic extracts, suggesting an aging-related impairment in IGF-2 processing.

3.2 Activity of the proprotein convertase 2 (PC2) is reduced in the hippocampus of aged rats

IGF-2 is post-translationally processed by members of the subtilisin-related proprotein convertase (PC) family of enzymes (Duguay et al., 1998; Qiu et al., 2005). Members of the PC family process precursor proteins at basic residues in immature secretory granules, within the Golgi apparatus, within endosomes, at the cell surface, or in the extracellular matrix (Seidah and Prat, 2012; Smeekens, 1993). These enzymes are responsible for the activation, and sometimes inactivation of target polypeptides (Lindberg, 1991; Seidah, 2011;

Seidah and Prat, 2012). Thus, we next explored the hypothesis that the altered proIGF-2 precursor/mature IGF-2 ratio in aged dorsal hippocampus could result from decreased peptide processing due to altered expression and/or activity of proprotein convertase 2 (PC2), the PC family enzyme most highly expressed within neurons and neurosecretory cells of the central nervous system (Cullinan et al., 1991; Schäfer et al., 1993; Winsky-Sommerer et al., 2000). Levels of PC2 were assessed with western blot analyses in both total and SN hippocampal protein extracts, and PC2 enzymatic activity was determined in total hippocampal extracts. As reported in Figure 2a, levels of PC2 did not change with age in either total or SN extracts [Fig. 2a; Independent samples *t* test, total: $t(16)=1.85$, $p=0.08$; SN: $t(16)=1.05$, $p=0.31$]. However, PC2-specific enzymatic activity was significantly attenuated in aged relative to young adult rats [Fig. 2b; Independent samples *t* test, total: $t(12)=9.53$, $p=1.52 \times 10^{-5}$]. Thus, aging is accompanied by a significant decrease of PC2 enzymatic activity in the rat hippocampus, without a change in the total PC2 protein levels.

3.3 IGF-2 administered in the hippocampus immediately after training rescues aging-related inhibitory avoidance memory impairment

IGF-2 has been reported to enhance memory retention and persistence in young rats and mice (Chen et al., 2011; Stern et al., 2014a, 2014b). Here we determined whether IGF-2 is effective in preventing aging-related memory deficits. Young adult and aged FBN hybrid rats were implanted with bilateral cannulae targeting the dorsal hippocampus, trained in IA, and bilaterally injected with vehicle or recombinant IGF-2 immediately after training. To assess the effect of IGF-2 on IA memory retention over time, a group of rats was injected immediately after training and tested at multiple time points (Fig. 3a). Latencies (shown as box plots) to cross to the dark, shock side of the IA chamber during training (acquisition) were not affected by age (Independent samples *t* test: $p=0.45$). For several of the time points at which IA memory was assessed, a number of rats showed strong IA memory such that ceiling latencies (i.e., 900 sec) were recorded and test data distributions were positively skewed. To account for this, non-parametric analyses were used to compare test data between experimental groups at each time point. For the 9 post hoc tests following overall analysis, significance levels were corrected to $p<0.005$. A repeated measures ANOVA across testing revealed a significant main effect of IGF-2 treatment [$F(1,27)=5.39$, $p=0.03$]. All rats showed strong IA memory at 1 day after training (median latency for each group = 900 sec); post hoc comparison of young vs. aged vehicle-injected rats revealed a trend toward lower latencies in the aged group (Moses test for extreme values: $p=0.01$). Further, young and aged rats that received IGF-2 showed a trend toward higher latencies, relative to their vehicle-injected counterparts (Moses tests: young veh vs IGF-2: $p=0.02$, aged veh vs IGF-2: $p=0.04$). All groups of rats had high IA latencies when tested again 14 days after training (median latency for each group = 900 sec); however, at this time point, aged vehicle-injected rats showed a significant memory impairment relative to young vehicle-injected rats, reflecting age-related memory deficits at this longer post-training time point (Moses test: young veh vs aged veh: $p=0.003$). The memory strengthening effect of IGF-2 persisted across tests at 14 days after training in young IGF-2-injected rats (Moses test: young veh vs IGF-2: $p=0.005$), and showed a trend towards memory strengthening in aged rats (Moses test: aged veh vs IGF-2: $p=0.04$) and in young rats tested once more 28 days after training (Moses test: young veh vs IGF-2: $p=0.05$). Given that IA memory remained quite strong in

all groups across all time points, a test for context generalization of conditioned fear was performed 1 day after the final test of IA memory. Rats were placed in a modified IA chamber that contained visual, tactile, and odor cues distinct from those present in the original training chamber. Both young adult and aged rats did not generalize to the new context. No generalization was found in either young adult or aged rats. Only a tendency toward fear context generalization was observed in aged vehicle-injected rats, but this effect was attenuated in aged rats that had received IGF-2 immediately after training (Fig. 3a).

Given that memory retrieval (via testing) early following training can lead to memory strengthening through reconsolidation (Inda et al., 2011), a second group of young adult and aged rats underwent cannula implant, IA training, and IGF-2 or vehicle injections, but were tested for memory retention only once, at 14 days after training. As shown in Figure 3b, memory retention at this time point was affected by both age and treatment. Aged rats had significantly lower latencies than young rats [two-way ANOVA, main effect of age: $F(1,33)=15.7$, $p=0.001$]. IGF-2 injections enhanced IA memory retention across age groups [main effect of treatment: $F(1,33)=12.6$, $p=0.001$, hence significantly improved age-related memory loss in the aged group [age $\times$ treatment interaction: $F(1,33)=15.1$, $p=0.001$]. With significance levels corrected for 3 comparisons ($p<0.02$), post hoc analyses confirmed a statistically significant decrease of test latencies in aged relative to young adult vehicle-injected rats (Moses test: $p=0.001$), and a statistically significant increase of test latencies in aged IGF-2-injected rats relative to their aged vehicle-injected counterparts (Moses test: $p=0.002$). To determine whether the memory-rescuing effect of IGF-2 extends to weaker memory traces, a group of rats underwent the same experimental procedures, but received a 0.6 mA footshock during training rather than the previously employed 0.9 mA footshock (Fig. 3c). IA memory retention from this training assessed 14 days later was also significantly decreased in aged relative to young adult rats [two-way ANOVA, main effect of age: $F(1,24)=10.9$, $p=0.003$] and significantly improved by IGF-2 injections [main effect of treatment: $F(1,24)=18.9$, $p=0.001$]. Post hoc analyses confirmed that IGF-2 administration specifically strengthened memory in both young adult rats (Tukey HSD post hoc test: $p=0.02$) and aged rats (Tukey HSD post hoc test: $p=0.03$).

To determine whether the memory strengthening effects of IGF-2 endured beyond 14 days after training, additional groups of rats were trained and, immediately after, bilaterally injected into the dorsal hippocampus with vehicle or IGF-2. The rats were then tested 28 days later (Fig. 3d). In both young adult and aged rats, IGF-2 produced memory enhancement [two-way ANOVA, main effect of treatment: $F(1,19)=8.81$, $p=0.008$]. For the 6 post hoc comparisons following this overall analysis, significance levels were corrected to $p<0.008$. The data analysis revealed that memory retention in the young adult animals injected with vehicle was reduced (median latency of young veh group = 200.8 sec) relative to earlier time points. Young IGF-2-injected rats showed higher median latencies than their vehicle-injected counterparts (median latency of young IGF-2 group = 900 sec), but this difference was not statistically significant (Moses test: $p=0.07$). However, aged IGF-2-injected rats tested 28 days after IA training showed higher median latencies than aged vehicle-injected rats, supported by a statistical trend (Moses test: $p=0.02$). This effect on aged rats by IGF-2 relative to vehicle treatment persisted and emerged as a statistically significant increase at testing given 7 days later (Moses test: $p=0.001$).

Together, these data indicate that IGF-2 administered in the dorsal hippocampus immediately after training ameliorates aging-related IA memory impairment.

3.4 IGF-2 administered into the dorsal hippocampus after training rescues aging-related impairments in object location and spontaneous alternation

We then sought to determine whether IGF-2 rescues aging-related impairment in non-aversive memory tasks, as well as in working memory. Toward this end, we employed object location and spontaneous alternation paradigms (Gerstein et al., 2013; Lalonde, 2002). The object location task measures the memory of an encountered object's spatial location on the basis of rodents' natural bias to explore novel stimuli and places (Dix and Aggleton, 1999; Ennaceur et al., 1997). This task is non-aversive and dorsal hippocampal-dependent (Barker and Warburton, 2011; Gaskin et al., 2009; Mumby et al., 2002; Warburton and Brown, 2015). During the object location training session, young and aged rats showed no preference for either of the two object locations [Fig. 4a; two-way ANOVA, main effects of age, treatment, and age $\times$ treatment interaction were not significant (p values >0.36)]. Immediately after training, rats received a bilateral injection of vehicle or recombinant IGF-2 in the dorsal hippocampus. Four hours later, the rats were tested (Test 1). Vehicle-injected young rats had significant memory as shown by the preferential exploration of the moved object ($64.41 \pm 2.72\%$) over chance [one samples t test comparing young veh *vs.* chance (50%), $t(12)=3.683$, $p=0.002$], whereas the vehicle-injected aged rats explored the objects at near chance level ($53.76 \pm 2.99\%$; one samples t test comparing aged veh *vs.* chance (50%), $p>0.39$), revealing an aging-related memory impairment ($p<0.05$). This memory deficit of the aged rats was fully rescued by IGF-2 ($72.78 \pm 3.23\%$, $p<0.001$). Additionally, in line with previous findings (Chen et al., 2011; Stern et al., 2014a and 2014b), IGF-2 administration, compared to vehicle, enhanced the memory in young rats ($75.43 \pm 2.04\%$, $p<0.05$) [Fig. 4a; two-way ANOVA, main effect of age: $F(1,44)=5.69$, $p=0.02$ and treatment: $F(1,44)=29.08$, $p<0.001$, followed by Tukey HSD post-hoc tests]. At test 2, given 24 hours following training, the young and aged vehicle-injected rats showed no significant memory over chance level (young veh: $52.37 \pm 2.59\%$, aged veh: $54.61 \pm 2.76\%$; one samples t test comparing young veh and aged veh *vs.* chance (50%), $p>0.26$). In contrast, IGF-2 injected rats still had a significantly higher memory in the young group (young IGF-2: $65.21 \pm 2.33\%$, $p<0.05$) but not in the aged group (aged IGF-2: $50.90 \pm 5.08\%$, $p>0.91$) [Fig. 4a; two-way ANOVA age $\times$ treatment interaction: $F(1,44)=5.99$, $p=0.0184$, followed by Tukey HSD post-hoc comparisons]. The total time spent exploring the objects did not differ across experimental groups during either training or test 1 [Fig. 4b; two-way ANOVA, main effects of age, treatment, and age $\times$ treatment interactions ($p>0.20$)]. However, at test 2, the aged rats showed significantly less exploration time in both the vehicle (young veh: 21.75 ± 2.94 sec, aged veh: 10.33 ± 2.07 sec, $p<0.05$) and IGF-2 (young IGF-2: 28.81 ± 3.67 sec, aged IGF-2: 10.34 ± 1.36 sec, $p<0.001$) groups as compared to young adult rats [Fig. 4b; two-way ANOVA main effect of age: $F(1,44)=31.62$, $p<0.001$, followed by Tukey HSD post-hoc tests].

Next we examined the effects of IGF-2 on spontaneous alternation, a spatial working memory task that is hippocampal-dependent (Lalonde, 2002; Newman et al., 2011). Young adult and aged rats were bilaterally injected with IGF-2 into the dorsal hippocampus 20 min

before the start of the task. Vehicle-treated young adult rats exhibited a significantly higher percentage of correct alternations as compared to the vehicle-treated aged rats (young veh: $57.06 \pm 3.96\%$, aged veh: $39.68 \pm 6.53\%$, $p < 0.05$) revealing an aging-related deficit in working memory. IGF-2 significantly increased the percent of correct alternations in both the young adult (young IGF-2: $74.65 \pm 2.7\%$, $p < 0.05$) and aged rats (aged IGF-2: $63.34 \pm 4.03\%$, $p < 0.01$) [Fig. 4c; two-way ANOVA, main effect of age: $F(1,44)=10.03$, $p=0.002$ and treatment: $F(1,44)=20.72$, $p < 0.001$, followed by Tukey HSD post-hoc tests]. Additionally, the young adult animals exhibited a significantly higher number of total arm entries than the aged animals (young veh: 26.58 ± 2.1 , aged veh: 16.50 ± 1.24 ; $p < 0.01$). However, IGF-2 treatment compared to vehicle did not affect the number of total arm entries in either groups, hence a similar difference was found in the IGF-2-treated groups (young IGF-2: 30.08 ± 2.66 , aged IGF-2: 19.33 ± 1.64 , $p < 0.01$) [Fig. 4d; two-way ANOVA, main effect of age: $F(1,44)=27.58$, $p < 0.001$, followed by Tukey HSD post-hoc tests].

Together these data led us to conclude that IGF-2 administration in the dorsal hippocampus of rats significantly recues aging-related memory loss in spatial and working memory tasks. Additionally, IGF-2 significantly enhances the memories of these tasks in young adult rats.

4. Discussion

Age-related memory impairments are suggested to reflect failures of memory consolidation and storage at either the molecular or systems level. In fact, a major problem in memories of older individuals is increased forgetting and susceptibility to interference of recent memories (Balota et al., 2000; Burke et al., 2010; Gallagher and Pelleymounter, 1988; Kausler, 1994; Rapp et al., 1997). Given the current rise in the aged population and the significant impact of cognitive impairments associated with aging, there is great interest in identifying treatments that can prevent or treat these deficits.

Here we have provided evidence that in the dorsal hippocampus of aged rats there is likely a deficit in the post-translational processing of the mature form of the IGF-2 peptide, as levels of a mature form of IGF-2 were significantly decreased while levels of its precursor proIGF-2 were significantly augmented in synaptic extracts of aged dorsal hippocampi compared to those of young adults. Moreover, in agreement, in dorsal hippocampal extracts of aged rats, the activity of the processing enzyme subtilisin-like proprotein convertase PC2 was found to be significantly reduced. Finally, in line with the role of IGF-2 in memory consolidation and enhancement in young adult rats and mice, we found that delivery of recombinant IGF-2 to the dorsal hippocampus of aged rats significantly rescues deficits of aversive and non-aversive hippocampal-dependent memories, as well as of working memory.

IGF-2 has been shown to mediate consolidation and enhancement of many types of hippocampal-dependent memories (Agis-Balboa et al., 2011; Chen et al., 2011; Stern et al., 2014a, 2014b). Because IGF-2 crosses the blood brain barrier, it represents a potential therapeutic tool readily available for preclinical testing and clinical trials (Alberini and Chen, 2012; Stern and Alberini, 2013). Our present study using an acute administration of recombinant IGF-2 in rats significantly extends previous knowledge reporting that viral-mediated overexpression of IGF-2 in the hippocampus enhanced memory and promoted

dendritic spine formation in aged mice (Pascual-Lucas et al., 2014); first, because recombinant IGF-2 is more readily available and useful in therapeutic settings than brain viral expression, second because we show an effect in another species on aversive and non-aversive tasks, as well as on working memory, and, third, because we provide a mechanism for the decline in IGF-2 during aging, which may suggest a new target for enhancing the effects of IGF-2 and correcting the aging-related changes. In sum, we suggest that IGF-2 may represent an important target for prevention of numerous age-related pathologies. IGF-2 expression was also found to be decreased in the hippocampus of patients with Alzheimer's disease (AD) (Pascual-Lucas et al., 2014) and hippocampal viral-mediated overexpression of IGF-2 or intraventricular administration of IGF-2 in mouse models of AD rescued behavioral deficits, promoted dendritic spine formation, restored normal hippocampal functions and even decreased amyloid levels found in these models (Mellott et al., 2014; Pascual-Lucas et al., 2014). Thus, the role of IGF-2 in ameliorating memory deficits seems to target mechanisms involved in both aging and AD.

Notably, our results show that aging correlates with impaired hippocampal IGF-2 processing leading to decreased levels of mature IGF-2, and that a bilateral injection of IGF-2 significantly corrects the impairment. Because these two measures were conducted in separate groups of rats, to actually strengthen the conclusion that IGF-2 rescues rather than enhances aging-related memory deficits, it would be interesting to determine whether mature IGF-2 protein levels correlate with memory abilities in individual young and aged animals.

Furthermore, we noted that the FBN hybrid rats had higher IA memory latency overall, as compared to the Long Evans rats, which we had previously used to study the effects of IGF-2. This increased response to IA likely reflects rat strain differences, and is in agreement with previous reports showing differences in performance among FBN and other rat strains like Fischer 344, Brown Norway, and Wistar strains (van der Staay and Blokland, 1996). The higher IA latencies of both young and aged FBN rats may therefore have limited our ability to detect the memory-enhancing effects of IGF-2 after shorter post-training delays (i.e., as in Fig. 3a). Nevertheless, we did observe clear memory-enhancing effects of IGF-2 treatment on other non-aversive tasks, including object location and spontaneous alternation.

Given that previous studies from our lab reported significant effects of systemic treatments of IGF-2 in many types of short- and long-term memories processed by hippocampal-cortical systems (Stern et al., 2014a, 2014b), we suggest that IGF-2 should be readily tested in preclinical and hopefully then clinical studies. Expanding the regimen and potential routes of IGF-2 administration may further improve the already significant strengthening effect on different types of hippocampal-dependent memories. For example, multiple doses, slow release, and subcutaneous or intranasal administration are all parameters that should be further investigated for therapeutic translation.

IGF-2 has also been previously shown to rescue the impairment of adult hippocampal neurogenesis and working memory in the Dgcr8-deficient mouse model of 22q11.2 deletion-associated schizophrenia (Ouchi et al., 2013), hence an effect of IGF-2 on age-related memory impairment may result from an effect on neurogenesis. In the present study, we

have not investigated neurogenesis and further experiments will be needed to address this issue. However, based on previous reports with young adult rodents, the IGF-2-mediated memory enhancement was determined to occur via IGF-2 receptor (R) and not via IGF-1R; moreover, IGF-1 injected into the hippocampus did not produce memory enhancement (Chen et al., 2011; Stern et al., 2014b). Because neurogenesis is predominantly, if not exclusively, mediated by IGF-1R (O'Kusky and Ye, 2012), we speculate that, if, like in the young adult brain, the memory enhancing effect of IGF-2 in aged animals occurs via IGF-2R, it is likely that it is not resulting from neurogenesis-related mechanisms. In further support of this idea, despite observations of an overall reduction in the rate of neurogenesis in aged animals (Bizon and Gallagher, 2003, 2005; Bizon et al., 2004; Kuhn et al., 1996), individual aged animals with the lowest numbers of newborn hippocampal neurons actually show better spatial memory performance (Bizon and Gallagher, 2003, 2005), arguing against a role for decreased neurogenesis in age-related cognitive decline. Nevertheless, because the mechanism of action of IGF-2 depends on the specific cell types, cellular microenvironment, and developmental stage, at present, an additional effect of IGF-2 on neurogenesis cannot be excluded (Iwamoto and Ouchi, 2014). Furthermore, among the insulin group of peptides, IGF-2 may represent a most highly effective candidate for memory enhancement and for rescuing memory and synaptic plasticity impairments in both young and aged subjects. In fact, to our knowledge, the effect of insulin as a memory enhancer has been reported to be controversial (Clayson 1971; Schwarzberg et al. 1989; Kopf and Baratti 1996, 1999; Kopf et al. 1998; Moosavi et al. 2006). Moreover although insulin produces changes in proliferation of new cells and synaptic molecular pathways in aged animals, it enhances memory only in young but not aged populations, suggesting that it may protect against neuronal loss, but may not reliably improve memory performance in aging (Haas et al. 2015). IGF-1, to our knowledge, has not yet been found effective in enhancing memory in healthy conditions (Stern et al. 2014). However, studies in both humans and rodent models reported a rescuing effect of IGF-1, as well as insulin or IGF-2, on cognitive functions in pathological conditions, particularly in cerebrovascular alterations and its role in aging and AD (Liu et al. 2001, 2004; Carro et al. 2002; Fernandez and Torres-Aleman 2012; Torres-Aleman 2012; Johansson et al. 2013; Trueba-Saiz et al. 2013), suggesting that IGF-1 and IGF-2 act via distinct mechanisms. Although IGF-1 has been reported to improve memory deficits in aging the data are controversial (for excellent reviews of this subject see Deak and Sonntag 2012 and Sonntag et al. 2012). An interesting question that remains to be investigated is whether IGF-1 and IGF-2 may contribute complementary effects in rescuing aging-related cognitive deficits.

Decreased synaptic levels of mature functional IGF-2 protein in the hippocampus of aged rats provides a potential mechanism for the impairment in memory expression, consolidation and/or storage observed with aging, and for the rescuing effect of recombinant, mature IGF-2 delivered into the hippocampus at the time of learning. The decreased processing of proIGF-2 to mature IGF-2, paralleled by a deficit in PC2 activity in aged hippocampi, also indicates that IGF-2 may not be the only substrate affected by PC2 activity impairment with aging. The identification of a deficit in PC2 activity in the aged hippocampus is a novel and interesting finding, and begs the question of which additional substrates may be affected. PC2 and PC1/3 are the two proprotein convertases primarily expressed in neural and

endocrine cells, and are mostly localized within immature and dense-core secretory granules to process several polypeptide prohormones within the acidic regulated secretory pathway (Muller and Lindberg, 1999; Seidah and Prat, 2012; Winsky-Sommerer et al., 2000). Substrates of PC2 include proopiomelanocortin, pro-cholecystokinin, pro-somatostatin, β -endorphins, and α -melanocyte stimulating hormone (Benjannet et al., 1991; Billova et al., 2007; Cain et al., 2003; Galanopoulou et al., 1995; Marcinkiewicz et al., 1993). Further, PC2 together with PC1 generates insulin through endoproteolytic processing of preproinsulin (Baillyes et al., 1991; Seidah and Prat, 2012; Steiner et al., 1996). IGF-2 has been found to be processed by PC4 in other tissues (Qiu et al., 2005; Tani et al., 2008); in fact, this enzyme subtype is exclusively expressed in testicular germ cells in males and in the placenta and ovary in females (Gyamera-Acheampong and Mbikay, 2009; Seidah and Prat, 2012), and therefore it is unlikely that PC4 is responsible for IGF-2 processing in the brain. We suggest that in the brain PC2 and/or PC2 together with PC1 process precursor forms of IGF-2 into mature IGF-2, and identifying approaches that reduce PC2 deficits may also be an interesting and novel avenue for developing therapies against cognitive impairments.

Aging-related deficits include impairments in short-term, long-term, and working memories (Foster and Kumar, 2007; Gold et al., 1982; Newman et al., 2011; Winocur, 1988). With our experimental design we determined that IGF-2 is able to modulate the consolidation process of different memory tasks in both young and aged animals. In fact, a single post-training IGF-2 treatment resulted in a long-term enhancement of an aversive memory (IA) in both young and aged animals. However, with a non-aversive memory (object location), while an increase in performance was witnessed at the shorter time point tested (4 hours) for both young and aged rats, only the young group showed a persistent memory enhancement. The more transient effect of IGF-2 in aged vs. young rats could reflect deficits in mechanisms of consolidation occurring at baseline conditions in aging, which is in agreement with the reported increased forgetting and susceptibility to interference of recent memories (Balota et al., 2000; Burke et al., 2010; Gallagher and Pelleymounter, 1988; Kausler, 1994; Rapp et al., 1997). Because IGF-2 captures activity-dependent processes (Chen et al., 2011) it is possible that general deficits in the aged hippocampus may limit the persistence of the IGF-2 effect in aged subjects. However, different schedules and modalities of administration, as well as targeting different ages, may reveal more significant effects and may drive preclinical and clinical research.

Our data also revealed that IGF-2 improved performance on a working memory task, which is consistent with previous findings from our laboratory showing that IGF-2 enhances, in addition to long-term memory also short-term and working memories in young mice (Stern et al., 2014a). Although the mechanisms by which short-term and working memories can be enhanced are still under investigation, these results suggest that IGF-2 may target different plasticity mechanisms in different brain regions, in accordance with the evoked behavioral activation.

The memory-rescuing effect of IGF-2 is an important finding in that, to date, there are no effective treatments available to combat or prevent aging-related cognitive impairments. The advantage offered by IGF-2 is that it is naturally produced, it crosses the blood-brain barrier (enabling systemic treatment) and enhances multiple aspects of impaired cognition,

including short-term, long-term and working memory functions, without affecting memory flexibility (Stern et al., 2014a). The significant effect of IGF-2 on age-related memory loss also implies that IGF-2 treatment could prove useful in targeting mechanistically-linked age-associated pathologies such as AD and age-related ischemia or dementias (Heininger, 2000). In conclusion, we propose that IGF-2 might be an important target for developing effective treatment to prevent or reverse cognitive impairments associated with aging.

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Highlights

- 1.** The mature form of insulin-like growth factor-2 is reduced in the hippocampus of aged rats.
- 2.** The activity of proprotein convertase 2 is decreased in the hippocampus of aged rats.
- 3.** Hippocampal administration of insulin-like growth factor 2 rescues aging-related memory loss.

Figure 1.

Decreased mature IGF-2 and increased proIGF-2 protein levels in the hippocampus of aged compared to young adult rats. (a) Western blot competition with recombinant mouse IGF-2 peptide (rmIGF-2), recombinant mouse IGF-1 peptide (rmIGF-1), recombinant human proIGF-2 peptide (rhproIGF-2), or recombinant human proIGF-1 peptide (rhproIGF-1). The band detected by anti-IGF-2 at 15 kDa is competed in dorsal hippocampal synaptoneurosomal (dHC SN) and total (dHC total) protein extracts by rmIGF-2 but not rmIGF-1, while the band detected at 20 kDa is competed by rhproIGF-2 but not rhproIGF-1. (b–d) Relative quantitative western blot analyses of dHC total or SN extracts. Data are expressed as mean percentage $\pm$ SEM of the average protein concentration found in the young adult rat group. Each densitometric value was normalized against β -actin detected on the same blot (loading control). Representative blots are shown above their respective grouped data. (b) 20 kDa proIGF-2 protein and 15 kDa IGF-2 mature protein in dHC total protein extracts (n=8–10/group) from young adult and aged rats. (c) 20 kDa proIGF-2 protein and 15 kDa IGF-2 mature protein in dHC SN extracts (n=17/group) from young adult and aged rats. (d) IGF-2 receptor (IGF-2R) levels in dHC total (n=9/group) and dHC SN extracts (n=9/group) from young and aged rats. * p<0.05, ** p<0.01.

Figure 2.

Significant decrease of proprotein convertase 2 (PC2) enzymatic activity in the hippocampus of aged rats. (a) Data are shown as mean percentage of the protein concentration found in young adult rats. Representative bands from relative quantitative Western blot analyses are shown above their respective data. PC2 protein levels in dHC total hippocampal protein extracts and dHC SN extracts (n=9/group). (b) PC2-specific enzymatic activity taken as picomoles of 7-Amino-4-methylcoumarin released from pERTKR-AMC per minute (pmol/min) inhibited by the PC2-specific inhibitor Pro-7B2 (156–186)/CT Mouse Peptide (n=7/group). ** p<0.01.

Figure 3.

Bilateral dorsal hippocampal IGF-2 injection given after IA training rescues aging-related long-term memory impairment. Experimental schedules are shown above their respective data. Injections (arrow) of vehicle or recombinant IGF-2 peptide immediately after training at specified shock intensities and tested at indicated time points. IA memory shown as median latencies expressed in seconds (s) (black bar within box) $\pm$ quartiles (box) and minimum/maximum values (whiskers). (a) IA memory was tested at 1 (Test 1), 14 (Test 2) and 28 days (Test 3) after training. 1 day after Test 3, generalization (Gen) was tested in a different context ($n = 7-9$ /group). (b) IA memory was tested at 14 days after training ($n = 8-11$ /group). (c) IA memory was tested at 14 days after training ($n = 7$ /group). (d) IA memory was tested at 28 days after training (Test 1), and tested again at 35 days after training (Test 2). Moses test between aged vehicle and IGF-2 groups at Test 2: $p=0.001$; $n = 10-11$ /group). Bonferroni-corrected non-parametric between-group comparisons or Tukey HSD tests are shown by * $p<0.005$, or * $p<0.05$, respectively.

Figure 4.

Bilateral dorsal hippocampal injection of IGF-2 rescues the impairments of object location and spontaneous alternation memory in aged rats. Experimental schedules of the assessment of spatial location and working memory across exploration and test days are listed above (a) and (c). Injections (arrow) of vehicle or IGF-2 immediately after training (a and b) or 20 min prior to testing (c and d). (a) Percent of time spent exploring the moved object for young adult and aged rats at training, test 1, and test 2. Data are expressed as means $\pm$ SEM (n=12 per group). (b) Means $\pm$ SEM of total exploration time (in seconds, s) of both familiar and novel object locations at training, test 1, and test 2 in young adult and aged rats (c) The percent of correct alternations, expressed as means $\pm$ SEM, for young adult and aged animals that received IGF-2 or vehicle (n=12 per group). (d) Total arm entries ($\pm$ SEM) during the 20 min spontaneous alternation test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significance levels for Tukey HSD post hoc tests. ## $p < 0.01$ significance for one sample t tests comparing each group to chance performance (50%). Dashed lines represent chance performance.