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Central administration of angiotensin IV rapidly enhances novel object recognition among mice

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Abstract

Angiotensin IV (Val¹-Tyr²-Ile³-His⁴-Pro⁵-Phe⁶) has demonstrated potential cognitive-enhancing effects. The present investigation assessed and characterized: (1) dose-dependency of angiotensin IV's cognitive enhancement in a C57BL/6J mouse model of novel object recognition, (2) the time-course for these effects, (3) the identity of residues in the hexapeptide important to these effects and (4) the necessity of actions at angiotensin IV receptors for pro-cognitive activity. Assessment of C57BL/6J mice in a novel object recognition task demonstrated that prior administration of angiotensin IV (0.1, 1.0, or 10.0, but not 0.01, nmol, i.c.v.) significantly enhanced novel object recognition in a dose-dependent manner. These effects were time dependent, with improved novel object recognition observed when angiotensin IV (0.1 nmol, i.c.v.) was administered 10 or 20, but not 30, min prior to the onset of the novel object recognition testing. An alanine scan of the angiotensin IV peptide revealed that replacement of the Val¹, Ile³, His⁴, or Phe⁶ residues with Ala attenuated peptide-induced improvements in novel object recognition, whereas Tyr² or Pro⁵ replacement did not significantly affect performance. Administration of the angiotensin IV receptor antagonist, divalinal-Ang IV (20 nmol, i.c.v.), reduced (but did not abolish) novel object recognition; however, this antagonist completely blocked the pro-cognitive effects of angiotensin IV (0.1 nmol, i.c.v.) in this task. Rotorod testing demonstrated no locomotor effects for any angiotensin IV or divalinal-Ang IV dose tested. These data demonstrate that angiotensin IV produces a rapid enhancement of associative learning and memory performance in a mouse model that was dependent on the angiotensin IV receptor.

Keywords

angiotensin II(3–8); cognition; drug discovery; learning and memory; novel object recognition; renin-angiotensin system

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1. Introduction

The increased prevalence of neurological disorders (Brookmeyer et al., 2007; Small et al., 1997) highlights the urgent need for new drugs to treat cognitive decline. Although research has focused on known deficiencies associated with Alzheimer's disease, clinical studies of cognitive enhancement with cholinesterase inhibitors (Birks, 2006; Raina et al., 2008) or antioxidants (Nunomura et al., 2007) have been disappointing, prompting the search for new pharmacological options.

Initially identified as a potent vasoconstrictor (De Bundel et al., 2008; Wright et al., 1993; Yang et al., 2008), intracerebroventricular (i.c.v.) administration of angiotensin II (Ang II) has been found to enhance the cognitive performance of rats, facilitating acquisition of conditioned avoidance (Baranowska et al., 1983; Braszko, 2002) and delayed object recognition (Braszko, 1996). These effects were independent of actions at Ang II's native receptor (AT₂) or AT₁ receptors (Baranowska et al., 1983; Braszko, 2002), and were attributed to actions of natural Ang II metabolites, including the hexapeptide, angiotensin IV (Val¹-Tyr²-Ile³-His⁴-Pro⁵-Phe⁶; Ang IV). In fact, Ang IV may possess the greater therapeutic potential as Ang IV is observed to bind with high affinity in sites associated with mnemonic performance, including the pyramidal layer of the hippocampus (CA1, CA2, CA3), dentate gyrus, basal nucleus of Meynert, and throughout the neocortex of guinea pigs (Harding et al., 1992; Miller-Wing et al., 1993; Wright et al., 1993), rats (Roberts et al., 1995), and humans (Chai et al., 2000). Consistent with this, central administration of Ang IV induced cognitive improvements equivalent to Ang II in rats (Braszko et al., 1988), and the blockade of AT₄ receptors produced deficits in spatial learning (Wilson et al., 2009; Wright et al., 1999). Agonism of AT₄ receptors has been reported to promote hippocampal long-term potentiation (LTP; Kramár et al., 2001; Wayner et al., 2001), spatial learning (Lee et al., 2004; Wright et al., 1999), and the restoration of spatial cognitive function following a variety of central insults (Stubley-Weatherly et al., 1996; Wright et al., 1996, 1999; Pederson et al., 2001).

In the present investigation, we assessed the dose-response relationship of Ang IV-enhanced learning and memory using a truncated version of the novel object recognition task. We have previously demonstrated that shortened paradigm parameters reduce the ability of control C57BL/6J mice to recognize a novel object (Carey et al., 2009). We hypothesized that central administration of Ang IV would enhance novel object recognition in a dose- and time-dependent manner under these conditions, independent of effects on locomotion, and pharmacological blockade of the AT₄ receptor would attenuate these enhancements. Moreover, although others have demonstrated that the valine¹ residue in Ang IV is essential for function of the peptide, whereas the proline⁵ and phenylalanine⁶ residues are not (Benoist et al., 2011; De Bundel et al., 2008; Sardinia et al., 1994), we conducted a complete alanine scan of the hexapeptide to assess the necessity of each residue for Ang IV-mediated enhancement of cognition.

2. Materials and Methods

2.1. Animals, housing and animal assurance

Subjects were 390 adult (approximately 70 days of age), male C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a temperature- and humidity-controlled room at the Torrey Pines Institute for Molecular Studies (Port Saint Lucie, FL) vivarium on a 12:12 h light / dark cycle (lights off at 19:00 h) with *ad libitum* access to food and water.

All animal experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Eighth edition, revised 2011). Consistent with these guidelines, ongoing statistical testing of data collected was used to minimize the number of animals used, within the constraints of necessary statistical power. All methods used were pre-approved by the Institutional Animal Care and Use Committee at the Torrey Pines Institute for Molecular Studies (Port Saint Lucie, FL).

2.2. Intracerebroventricular administration technique

Per the modified method of Haley and McCormick (1957), i.c.v. injections were directly made into the lateral ventricle as described by our group and others (Toll et al., 2012; van Heek et al., 1997; Zadina et al., 1997; Zhu et al., 1999). Briefly, mice were lightly anesthetized with isoflurane, a small incision was made in the scalp, and an injection was made 2 mm lateral and 2 mm caudal to bregma, at a depth of 3 mm. The volume of all i.c.v. injections was 5 μ L, using a 10 μ L microliter syringe (Hamilton Co., Reno, NV, USA). The syringe was left in place for 10 s to allow for diffusion of the infusate. Note that mice do not require a suture following i.c.v. drug administration and the quick (<1 min) recovery time from anesthesia occurs within the drug pretreatment times. We have previously compared this i.c.v. drug administration method to intraperitoneal administration and have found no impairment of novel object recognition (Paris et al., 2011).

2.3. Peptide Synthesis

Angiotensin IV (VYIHPF), and Ang IV variants with alanine substitution at each position (V1A, Y2A, I3A, H4A, P5A, and F6A) were obtained from GenScript (Piscataway, NJ, USA). The putative AT₄ antagonist, divalinal-Ang IV [V Ψ (CH₂-NH₂)YV Ψ (CH₂-NH₂)HPF; Krebs et al., 1996] was obtained from AnaSpec (San Jose, CA, USA). Synthetic peptides were diluted to concentration in 0.9 % sterile saline.

2.4. Behavioral Assays

2.4.1. Rotorod testing—In Experiment 1, locomotion was assessed in the rotorod task, which was conducted as previously described (Paris et al., 2011). Briefly, mice were trained to balance on an immobile rotorod (3 cm in diameter and suspended approximately 46 cm high; San Diego Instruments, San Diego, CA, USA) for 30 s. Mice were then assessed on the rotating rotorod across three fixed speed trials (30 s max. latency at 10 rpm), two fixed speed trials (180 s max. latency at 10 rpm), and two accelerated speed trials (180 s max. latency at 0 – 20 rpm). After each trial, the latency to fall from the rotorod was recorded. The last of these trials was utilized as a baseline measure of rotorod performance. One hour later, mice were administered drug and were assessed in accelerated speed trials (180 s max. latency at 0 – 20 rpm) over a 30 min period. Increased latencies to fall indicate increased motor performance.

2.4.2. Novel Object Recognition—Mice were assessed in a novel object recognition paradigm as previously described (Carey et al., 2009, 2012; Paris et al., 2011). Novel object recognition is a cognitive behavioral assay that is dependent on the activity of the frontal cortex and hippocampus (Broadbent et al., 2004; Ennaceur et al., 1997). The testing paradigm consists of 3 phases (two acquisition trials followed by one retention trial; each separated by an inter-trial interval; Carey et al., 2009, 2012; Paris et al., 2011). The duration of the phases and inter-trial intervals influence performance, as detailed below. In general, in the first phase, mice explore two objects (cube playing dice; 16 $\times$ 16 $\times$ 16 mm) that are evenly placed at opposing ends of a clean mouse housing cage (16 $\times$ 24 $\times$ 12 cm). Object exploration was operationally-defined as the duration of time mice spent physically contacting the object with a part of the body other than the tail and when mice were facing

the object (within 0.5 cm of the object) and actively exploring it (via sniffing or physical manipulation) as previously described by Carey et al. (2009). In Phase II, one of the familiar objects is displaced (moved from a centralized position at the end of the cage into close proximity to the wall). In the last phase, one of the familiar objects is replaced with a novel object (a marble; 1.25 cm diameter).

The constant object in this study was always a centralized playing die and the target object in Phase III was a marble. Previous work has shown that neither playing dice nor marbles present as a more salient cue to C57BL/6J mice, and that these objects are inter-changeable in this protocol, with no significant difference in performance when a playing die or marble was used as the novel object ($F(1,33) = 0.316$; $p = 0.578$, one-way ANOVA), and no significant interaction of stimuli $\times$ phase ($F(2,100) = 0.96$; $p = 0.39$, two-way ANOVA) as previously reported in Carey et al. (2009).

In each phase, the amount of time mice spent exploring each object was recorded. Time spent exploring each object was calculated as a recognition index: [time spent investigating target object / (time spent investigating constant object + time spent investigating target object) $\times$ 100]. A significant increase in percentage of time spent with the novel object in Phase III is considered an index of enhanced learning and memory performance (Carey et al., 2009, 2012; Paris et al., 2011).

2.4.2.1. Standard Novel Object Recognition (10 min trials): In Experiment 3A, mice were assessed in a standard length novel object recognition paradigm, where the phases of the testing paradigm are each 10 min long, each separated by a 10 min inter-trial interval (Carey et al., 2012; Paris et al., 2011). In control C57BL/6J subjects, these timing parameters typically yield a significant increase from ~50 % exploration of the target object in Phases I and II to ~70 – 80 % exploration with the target novel object in Phase III (Carey et al., 2009, 2012; Paris et al., 2011), indicative of positive learning and memory performance. Only Experiment 3A, which evaluated possible AT₄ receptor antagonist-mediated impairment of novel object recognition, utilized the standard task parameters (10 min trials with 10 min inter-trial intervals).

2.4.2.2. Truncated Novel Object Recognition (5 min trials): In Experiments 2 – 4 (excluding Expt. 3A) mice were assessed in a novel object recognition paradigm utilizing truncated parameters. The truncated novel object recognition paradigm employs shorter (5 min) acquisition and retention trials and shorter (1 min) inter-trial intervals, presumably weakening the consolidation of the memory trace for the novel object (Genoux et al., 2002). As previously reported, utilization of these parameters resulted in an insignificant difference in the amount of time that control mice spent investigating the novel object across phases (Carey et al., 2009), suggestive of negative learning and memory performance. Given the goals of the present study, we utilized this truncated version of the novel object recognition task in Experiments 2, 3B and 4 to evaluate Ang IV-mediated enhancement of learning and memory performance.

2.5. Experimental schedule and drug dosing

2.5.1. Experiment 1: Locomotor effects of central angiotensin IV or divalinal-Ang IV—Locomotor behavior was assessed via performance in the rotorod task. Following rotorod training (described in detail above), mice were administered central vehicle (sterile saline, 0.9%, i.c.v.; $n = 16$), Ang IV (0.01, 0.1, 1.0, or 10 nmol, i.c.v.; $n = 8$ / group), or divalinal-Ang IV (20 nmol, i.c.v.; $n = 8$). Mice were immediately assessed for rotorod performance at 0, 10, 20, and 30 min, post-administration.

2.5.2. Experiment 2: Dose- and time-dependent effects of central angiotensin IV on novel object recognition

—To assess dose-dependent effects of Ang IV on novel object recognition, prior to Phase I of the task (described in detail above), mice were unilaterally administered (i.c.v.) sterile saline (0.9 %; n = 15), or one of four doses of Ang IV (0.01 nmol, n = 17; 0.1 nmol, n = 20; 1.0 nmol, n = 23; 10 nmol, n = 20). Mice began testing in Phase I of the task 10 min later.

To assess the duration of Ang IV action for effects on novel object recognition, the pretreatment time between drug administration and testing in Phase I of the novel object recognition task was manipulated. Mice were administered central Ang IV (0.1 nmol, i.c.v.) and began testing in Phase I of the novel object recognition task 20 (n = 15) or 30 (n = 16) min later. As a negative control, another group of mice received i.c.v. administration of vehicle (sterile saline, 0.9 %; n = 16) and were assessed in the novel object recognition task 30 min later. All groups were compared to the previous performance of mice that had received Ang IV (0.1 nmol, i.c.v.; n = 20) and had been tested for object recognition 10 min following treatment.

2.5.3 Experiment 3: Effects and actions at angiotensin IV receptor for novel object recognition

—The necessity of the AT₄ receptor for Ang IV-induced improvements in novel object recognition was investigated. In order to assess potential decrements in object recognition performance, mice were assessed in the non-truncated version of the task (each of the three phases lasts 10 min with a 10 min inter-trial interval; Carey et al., 2009, 2012). In this version of the task, mice demonstrate typical object recognition, spending greater time with the novel object in Phase III of the task (Carey et al., 2009, 2012). Prior to Phase I, mice were unilaterally administered sterile saline (0.9 %, i.c.v.; n = 8) or the AT₄ antagonist, divalinal-Ang IV (20 nmol, i.c.v.; n = 20). Mice began testing in Phase I of the task 30 min later.

To elucidate the AT₄ receptor as a target for Ang IV's mnemonic-enhancing effects, mice were then assessed in the truncated version of the novel object recognition task (5 min trials with 1 min inter-trial intervals) utilized in Experiments 2 and 4. Fifty min prior to Phase I, mice were unilaterally administered saline vehicle (0.9 %, i.c.v.) or divalinal-Ang IV (20 nmol, i.c.v.). Ten min prior to Phase I, mice received a second drug administration of saline vehicle (0.9 %, i.c.v.) or Ang IV (0.1 nmol). This yielded three i.c.v. groups: (1) saline/saline (n = 15), (2) divalinal-Ang IV/saline (n = 12), and (3) divalinal-Ang IV/Ang IV (n = 24). Performances were compared to those of mice that had received Ang IV (0.1 nmol, i.c.v.; n = 20) in Experiment 2.

2.5.4. Experiment 4: Alanine scan of angiotensin IV for effects on novel object recognition performance

—To assess the important residues for Ang IV's cognitive-enhancing activity, alanine was separately substituted for each of the six amino acids present in the Ang IV peptide, with one of the six analogs administered at 0.1 nmol, i.c.v. to naïve mice: V1A (n = 20), Y2A (n = 16), I3A (n = 15), H4A (n = 16), P5A (n = 15), or F6A (n = 16). Peptide effects on novel object recognition were compared to that of Ang IV (0.1 nmol, i.c.v.) as tested in Experiment 2 and mice administered saline (0.9 %, i.c.v.; n = 15). Mice were assessed in Phase I of the novel object recognition task 10 min following drug administration.

2.6. Statistical Analyses

Data obtained in the rotorod task (Expt. 1) and the standard (Expt. 3A) and truncated (Expts. 2, 3B, and 4) versions of the novel object recognition paradigm were analyzed via separate repeated measures analyses of variance (ANOVA) with drug treatment condition as the

between-groups factor, and time (Expt. 1) or trial phase (Expts. 2 – 4) as the within-groups factors. For all interactions, results of simple main effects and simple main effect contrasts are presented following significant interactions. Where appropriate, Fisher's Protected Least Significance Difference *post-hoc* tests were utilized to assess group differences. Effects were considered significant when $p < 0.05$. All data are presented as mean $\pm$ SEM for the treatment condition.

3. Results

3.1. Experiment 1: Central administration of angiotensin IV or divalinal-Ang IV did not influence locomotor activity on the rotorod

Neither administration of Ang IV (0–10 nmol, i.c.v.), nor the AT₄ antagonist, divalinal-Ang IV (20 nmol, i.c.v.), significantly influenced locomotor behavior on the rotorod task compared to administration of vehicle (saline 0.9%, i.c.v.; Fig. 1). There was a significant main effect of time [$F(4,200) = 25.01$, $p < 0.05$] wherein all mice spent more time on the rotorod prior to falling at 10 ($p = 0.001$), 20 ($p < 0.0001$), or 30 ($p < 0.0001$) min post-administration (but not 0 min) compared to their respective baseline performance.

3.2. Experiment 2: Angiotensin IV enhanced novel object recognition in a dose- and time-dependent manner

Central administration of Ang IV influenced novel object recognition performance in a dose-dependent manner (Fig. 2A). There was a significant interaction between Ang IV concentration and novel object recognition phase [$F(8,180) = 2.90$, $p < 0.05$]. Simple main effects found that i.c.v. Ang IV significantly increased the percentage of time that mice spent investigating the novel object in Phase III, compared to the time spent investigating the target object in Phase I, when administered 0.1 [$F(1,19) = 21.28$, $p = 0.0002$], 1.0 [$F(1,22) = 10.02$, $p = 0.005$], or 10.0 nmol [$F(1,19) = 11.39$, $p = 0.003$] Ang IV, but not 0.01 nmol Ang IV or saline. In Phase III, mice administered Ang IV (0.1 nmol, i.c.v.) spent a significantly [$F(4,90) = 6.64$, $p < 0.05$] greater percentage of time investigating the novel object than did saline-administered controls ($p = 0.03$), whereas those administered Ang IV (0.01 nmol, i.c.v.) spent a significantly reduced amount of time investigating the novel object compared to all other groups ($p_{\text{saline}} = 0.02$; $p_{\text{AngIV 0.1 nmol}} < 0.0001$; $p_{\text{AngIV 1.0 nmol}} = 0.001$; $p_{\text{AngIV 10.0 nmol}} = 0.0003$).

Ang IV administration also potentiated novel object recognition performance in a time-dependent manner (Fig. 2B). There was a significant interaction between pretreatment time and novel object recognition phase [$F(6,126) = 2.15$, $p = 0.05$]. Simple main effects indicated that a 10 min [$F(1,19) = 21.28$, $p = 0.0002$], or 20 min [$F(1,14) = 4.92$, $p = 0.04$], pretreatment with Ang IV (0.1 nmol, i.c.v.) significantly increased the percentage of time that mice spent investigating the novel object in Phase III, compared to the time spent investigating the target object in Phase I. Neither 30 min pretreatment with saline or Ang IV (0.1 nmol) significantly influenced the percentage of time mice spent investigating the novel object. There was a notable stepwise reduction in novel object recognition performance when Ang IV (0.1 nmol, i.c.v.) was administered 20 min prior to testing versus 30 min prior to testing. Mice administered Ang IV (0.1 nmol, i.c.v.) 10 min prior to testing spent a significantly [$F(3,63) = 4.00$, $p < 0.05$] greater percentage of time investigating the novel object compared to saline-administered controls ($p = 0.002$) or those administered Ang IV (0.1 nmol, i.c.v.) 30 min prior to testing ($p = 0.03$). As well, mice administered Ang IV (0.1 nmol, i.c.v.) 20 min prior to testing spent a significantly greater percentage of time investigating the novel object than did saline-administered controls ($p = 0.047$); albeit, to a notably lesser extent.

3.3. Experiment 3: Angiotensin IV receptor blockade attenuated novel object recognition and Angiotensin IV effects to enhance cognitive performance

Central administration of the AT₄ antagonist, divalinal-Ang IV, significantly interacted with novel object recognition performance [$F(2,52) = 3.46, p < 0.05$] (Fig. 3A). In the standard (non-truncated) novel object recognition task, saline-administered controls significantly increased the percentage of time that they spent investigating the novel object in Phase III, compared to the time spent investigating the target object in Phase I [$F(1,7) = 14.73, p = 0.006$]. This effect was also observed among mice administered divalinal-Ang IV (20 nmol, i.c.v.); albeit, to a lesser extent [$F(1,19) = 11.59, p = 0.003$]. In Phase III of the task, saline-administered mice spent a significantly [$F(1,26) = 7.49, p < 0.05$] greater percentage of time investigating the novel object than did their divalinal-Ang IV-administered counterparts ($p = 0.01$).

In the truncated novel object recognition task, divalinal-Ang IV administration significantly interacted with testing phase [$F(6,134) = 3.30, p < 0.05$] to block the mnemonic-enhancing effect of Ang IV (Fig. 3B). Simple main effects found that Ang IV (0.1 nmol, i.c.v.) significantly increased the percentage of time that mice spent investigating the novel object in Phase III, compared to the time spent investigating the target object in Phase I, [$F(1,19) = 21.28, p = 0.0002$], but this was not observed in any other group. In Phase III, mice administered Ang IV (0.1 nmol, i.c.v.) spent a significantly [$F(3,67) = 9.56, p < 0.05$] greater percentage of time investigating the novel object than did those administered saline ($p = 0.01$), divalinal-AngIV alone ($p = 0.0002$), or divalinal-AngIV + AngIV ($p < 0.0001$).

3.4. Experiment 4: Substitution of alanine for valine¹, histidine⁴, or phenylalanine⁶ abolished angiotensin IV mediated enhancement of novel object recognition performance

There was a significant interaction between drug treatment and novel object recognition phase [$F(14,250) = 1.81, p < 0.05$] (Fig. 4). Substitution of alanine for valine¹ (V1A), a residue reportedly critical for Ang IV function (Benoist et al., 2011; De Bundel et al., 2008; Sardinia et al., 1994), histidine⁴ (H4A), or phenylalanine (F6A) abolished the pro-cognitive effects of Ang IV (Fig. 4). However, Ang IV analogs that had alanine replacement of tyrosine² (Y2A) [$F(1,15) = 8.39, p = 0.01$], isoleucine³ (I3A) [$F(1,14) = 7.46, p = 0.02$], or proline⁵ (P5A) [$F(1,14) = 6.37, p = 0.02$] retained significant enhancement of the percentage of time spent with the novel object in Phase III compared to time spent with the target object in Phase I (Fig. 4). In Phase III of the task, only Ang IV (0.1 nmol, i.c.v.) significantly [$F(7,125) = 4.08, p < 0.05$] enhanced the percentage of time spent with the novel object compared to saline-administered controls ($p = 0.01$). Ang IV-cognitive enhancement was also significantly greater than that achieved with V1A ($p < 0.0001$), I3A ($p = 0.02$), H4A ($p = 0.002$), and F6A ($p < 0.0001$) in Phase III.

4. Discussion

The present findings upheld the hypotheses that central administration of Ang IV improves novel object recognition in a dose- and time-dependent manner. The utilization of a truncated novel object recognition task to deliberately impair learning and memory performance has been demonstrated previously with C57BL/6J mice (Carey et al., 2009), and is thought to reflect poor quality mnemonic encoding when opportunities for consolidation are reduced during the shorter inter-trial intervals (Genoux et al., 2002). While saline-administered controls did not demonstrate a significant shift in the percentage of time spent investigating the novel (vs. familiar) object (~60 %; indicating a lack of retention), mice administered Ang IV (0.1 nmol, i.c.v) or higher doses spent a significantly greater percentage of time with the novel object (~70 – 80 %; indicating a shift towards retention). Ang IV (0.1 nmol) was the most efficacious dose, consistent with prior reports (Kramár et

al., 1997; Lee et al., 2004; Pederson et al., 2001; Wright et al., 1993), and procognitive effects occurred rapidly within a 10 – 20 min drug pretreatment time (diminishing notably in a graded manner at 20 or 30 min pretreatment). An inverted U-shape dose-response was also evident with Ang IV (0.01 nmol) demonstrating a significant reduction in the recognition index compared to saline administration, and Ang IV (1.0 and 10.0 nmol) demonstrating a significant enhancement from each group's respective training performance (in Phase I) which was not greater than the performance of control mice in the retention trial (in Phase III). These data extend prior findings in rats, wherein infusions of Ang IV have been demonstrated to enhance object recognition and long-term memory using a 24 hr inter-trial interval to aide consolidation for cognitive tasks (Braszko, 2004; Braszko et al., 1988; Gard, 2008). Ang IV effects in rats have likewise been demonstrated to influence performance in learning tasks that occur over days (Benoist et al., 2011; Braszko, 2004; Braszko et al., 1988). Consistent with other studies of Ang IV's effects on working memory (Braszko et al., 2008; De Bundel et al., 2009, 2010), the present findings offer further evidence that Ang IV has rapid, dose-dependent effects to enhance cognitive performance of mice.

The procognitive effects of Ang IV are likely centrally mediated, as Ang IV demonstrated a rapid onset after i.c.v. administration in the present study and, as a hexapeptide, is not thought to be readily transported across the blood-brain-barrier (Chua et al., 2004). Although some procognitive effects following systemic administration of Ang IV have been observed in different strains of mice (Gard, 2008; Golding et al., 2010), Ang IV is rapidly metabolized in circulation with a half-life of ~10 sec (Handa et al., 2000). Given the conserved distribution of Ang IV in brain regions associated with mnemonic performance, this peptide has important therapeutic potential for cognitive enhancement. As such, additional attempts to identify analogs that are more amenable to systemic administration and central nervous system penetration seem justified. In support, recent attempts to stabilize the Ang IV peptide against rapid metabolism via the substitution of norleucine for valine¹ resulted in high-affinity binding at presumed AT₄ sites and a peptide-induced increase in hippocampal synaptic density *in vitro* which lasted for days without a loss of spatial memory performance (Benoist et al., 2011; De Bundel et al., 2008). Similarly, cleaving the proline⁵-phenylalanine⁶ residues from the C-terminus of Ang IV maintained *in vitro* hippocampal enhancements in synaptic spine density as well as *in vivo* spatial water maze performance, although, these effects were smaller compared to the full sequence peptide (Benoist et al., 2011). Our systematic alanine scan demonstrated that substitution of valine¹ with inert alanine completely attenuated Ang IV's procognitive effects, consistent with prior reports (Benoist et al., 2011; De Bundel et al., 2008; Wright et al., 1999), as well as alanine substitution for histidine⁴ or phenylalanine⁶. Alternatively, replacement of tyrosine², isoleucine³, or proline⁵ reduced Ang IV's mnemonic efficacy but did not attenuate it. The differential results produced from these alanine scan analogs contribute to previous work and suggest future modifications to increase “drugability,” such as substitution of D-amino acids or cyclization, as has been observed with other rapidly-degraded peptides (Aldrich and McLaughlin, 2009).

The mechanisms by which Ang IV enhances cognitive performance are still a subject of investigation. While Ang IV has high affinity for a distinct binding site referred to as the AT₄ receptor, recent findings suggest that Ang IV may also have important actions at AT₁ receptors that mediate working memory (De Bundel et al., 2010). However, activation of AT₁ receptors (by Ang II itself) has been found to inhibit synaptic LTP (Wayner et al., 1993) and inhibit long-term depression (LTD; Tchekalarova and Albrecht, 2007). The present finding that pharmacological blockade of AT₄ receptors with the putative antagonist divalinal-Ang IV (Coleman et al., 1998; Krebs et al., 1996) significantly reduced object recognition supports a pro-cognitive mechanism for Ang IV that is mediated; albeit not exclusively, by AT₄ receptors. Additionally, pretreatment with divalinal-Ang IV completely

attenuated the procognitive effects of Ang IV in the truncated version of the novel object recognition task. As such, activation of these Ang IV-sensitive sites may underlie important therapeutic targets for cognitive enhancement. Although Ang IV activation of AT₄ receptors has been reported to increase cerebral blood flow ~30 % (Kramár et al., 1997), it possesses limited hypertensive actions as compared to its precursors (Ang II and Ang III; De Bundel et al., 2008; Wright et al., 1993; Yang et al., 2008) and the enhancement of cognition does not appear to be due to non-specific alterations in affective status (Braszko, 2004; Braszko et al., 1998) or changes in locomotor activity as measured presently.

Despite this evidence, the distinct identity of AT₄ binding sites remains to be fully elucidated. Ang IV binds with high affinity to insulin-responsive aminopeptidase (IRAP) and the hepatocyte growth factor receptor, c-Met (De Bundel et al., 2008; Kawas et al., 2011; Yamamoto et al., 2010). An interaction with IRAP may partly underlie Ang IV's procognitive effects. Ang IV and divalinal-Ang IV bind and inhibit IRAP *in vitro* (Lew et al., 2003; Stragier et al., 2007) and binding of iodinated Ang IV and its analog Nle¹-Ang IV co-localized with IRAP mRNA in the mouse brain (Albiston et al., 2001). Moreover, Ang IV may have multiple targets *in vivo*, as the Ang IV analog, norleual, competitively inhibits c-Met receptor binding *ex vivo* and *in vitro*, and demonstrates activity in human cells that are devoid of IRAP (De Bundel et al., 2008; Kawas et al., 2011; Yamamoto et al., 2010). Ang IV actions at one of these putative brain targets may also influence cognition via indirect modulation of other neurotransmitter systems. For instance, Ang IV-mediated improvement of conditioned avoidance in rats was blocked by co-administration of systemic dopamine 1-like receptor antagonist (Braszko, 2004), and selective dopaminergic lesions in amygdala and hippocampus attenuate Ang IV enhancement of passive avoidance (Winnicka et al., 1998) or object recognition (when lesions were aimed at nuclei in the nucleus accumbens; Winnicka et al., 1997). Although these findings do not invalidate the conclusions of the present data, they do suggest multiple mechanisms may underlie Ang IV-mediated mnemonic improvement.

The development of peptide compounds as novel therapeutics has been limited, as peptides are thought to be hindered by generally poor oral bioavailability and rapid metabolism in circulation, (Fonseca et al., 2009; Vlieghe et al., 2010). However, peptides potentially offer several advantages as drugs, including high activity, high specificity, low toxicity, and the minimization of drug-drug interactions (Marx, 2005). Although the delivery of peptides as therapeutic agents remains a challenge, the development of novel strategies for synthesizing peptides with increased stability, bioavailability, and efficacious cell penetration highlight opportunities for therapeutic development. The present findings suggest that the application of these methods to develop, and characterize, peptide analogs of Ang IV may prove a promising source of future therapeutics for cognitive disorders.

5. Conclusion

Central administration of Ang IV dose-dependently enhanced learning and memory performance in a novel object recognition task, independent of locomotor effects. These effects occurred rapidly and were not observed after a 30 min drug pretreatment. Divalinal-Ang IV, a pharmacological antagonist of Ang IV binding sites, reduced (but did not abolish) novel object recognition and blocked Ang IV's procognitive effects. Ang IV action to improve object recognition may be partly independent of the tyrosine², isoleucine³, or proline⁵ residues. Overall, these data confirm the procognitive effects of Ang IV in the brain, suggesting potential therapeutic value in the treatment of cognitive disorders.

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Abbreviations

Ang	angiotensin
AT	angiotensin receptor
IRAP	insulin-responsive aminopeptidase
i.c.v.	intracerebroventricular
LTD	long-term depression
LTP	long term potentiation
SEM	standard error of the mean

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Highlights

- Cognitive-enhancing effects of i.c.v. angiotensin IV (Ang IV) characterized in mice
- Ang IV dose-dependently (0.1–10 nmol) enhances novel object recognition (NOR)
- Ang IV enhancement of NOR performance occurs rapidly, but of brief duration
- Ang IV receptor antagonist blocks Ang IV-mediated enhancement of NOR
- Alanine scan of Ang IV suggests three residues contributing to pro-cognitive core

Figure 1.

Central administration of angiotensin IV or divalinal-Ang IV did not significantly influence locomotor behavior in the rotorod test. Mice were centrally administered angiotensin IV (0.0, 0.01, 0.1, 1.0, or 10.0 nmol, i.c.v.) or divalinal-Ang IV (20 nmol, i.c.v.) and examined for locomotor activity 0, 10, 20, and 30 min post-administration.

Figure 2.

Central administration of angiotensin IV enhances cognitive performance in a (A) dose- and (B) time-dependent manner in the truncated novel object recognition paradigm (5 min trials with 1 min inter-trial intervals). Data represent the percentage of time mice spent investigating the novel object in Phases I – III of the task (A) 10 min following intracerebroventricular (i.c.v.) administration of saline or angiotensin IV (0.01, 0.1, 1.0, or 10.0 nmol) or (B) 30 min following i.c.v. administration of saline vs. 10, 20, or 30 min following i.c.v. administration of angiotensin IV (0.1 nmol). * indicates significantly different from respective performance in Phase I. † indicates significantly different from saline-administered controls in Phase III. ‡ indicates significantly different from all other groups in Phase III. ^ indicates significantly different from angiotensin IV- (0.1 nmol, i.c.v., 10 min pretreatment) administered mice in Phase III. Broken line indicates chance levels of investigation, ($p < 0.05$).

Figure 3.

Central angiotensin IV antagonism attenuates (A) standard novel object recognition (10 min trials with 10 min inter-trial intervals) and (B) Ang IV's effects to enhance cognition in a truncated novel object recognition paradigm (5 min trials with 1 min inter-trial intervals). Data represent the percentage of time mice spent investigating the novel object in Phases I – III of (A) the standard novel object recognition task 30 min following intracerebroventricular (i.c.v.) administration of saline or divalinal-Ang IV (20 nmol) or (B) the truncated novel object recognition task 30 min following i.c.v. administration of saline or divalinal-Ang IV (20 nmol) and 10 min following i.c.v. administration of angiotensin IV (0.1 nmol). * indicates significantly different from respective performance in Phase I. † indicates significantly different from saline-administered mice in Phase III. ‡ indicates significantly different from all other groups in Phase III. Broken line indicates chance levels of investigation, ($p < 0.05$).

Figure 4.

Valine¹, histidine⁴, and phenylalanine⁶, but not tyrosine², isoleucine³, and proline⁵, are critical residues for angiotensin IV's procognitive effects in a truncated novel object recognition paradigm (5 min trials with 1 min inter-trial intervals). Data represent the percentage of time mice spent investigating the novel object in Phases I – III of the task, 10 min following intracerebroventricular administration of saline, angiotensin IV (0.1 nmol), or an analog (0.1 nmol) with alanine-substituted for valine¹ (V1A), tyrosine² (Y2A), isoleucine³ (I3A), histidine⁴ (H4A), proline⁵ (P5A), or phenylalanine⁶ (F6A). * indicates significantly different from respective performance in Phase I. † indicates significantly different from saline-administered controls in Phase III. ^ indicates significantly different from angiotensin IV- (0.1 nmol, i.c.v.) administered mice in Phase III. Broken line indicates chance levels of investigation, ($p < 0.05$).