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Strain differences in the effects of Angiotensin IV on mouse cognition

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ABSTRACT

Angiotensin IV has been shown to improve learning and memory in rodents. Strain dependent variation in murine behaviour, aminopeptidase activity and inhibitory effect of Angiotensin IV, structural variation in insulin regulated aminopeptidase (IRAP) and aminopeptidase N (ApN) and expression of the encoding genes were explored. Strain differences in the behavioural response to Angiotensin IV were observed, where CD mice were refractory. All strains showed inhibition of aminopeptidase activity by Angiotensin IV but CD mice displayed reduced endogenous aminopeptidase activity. No differences in the coding sequence of IRAP or ApN were found. RT-PCR analysis showed no difference in IRAP expression between strains but an increased expression of ApN was observed in CD mice. The lack of cognitive response of CD mice to Angiotensin IV cannot be explained through variation within IRAP sequence nor expression but the results highlight a potential role for ApN in the effects of Angiotensin IV.

1. Introduction

A positive effect of Angiotensin IV on learning and memory has been demonstrated previously: Harding et al. (1986) suggested that the effects of Angiotensin II on cognition may be due to the generation of metabolites such as Angiotensin IV, while Braszko et al. (1988) demonstrated that Angiotensin IV considerably improved learning of conditioned avoidance responses and facilitated recall of passive avoidance behaviour in rats. Memory facilitation by dose dependant intracerebroventricular (i.c.v.) Angiotensin IV in passive avoidance conditioning was also supported by later work of Wright et al. (1996) and Tchekalarova et al. (2001).

It has now been shown in rats that the cognitive effects of Angiotensin II occur after its conversion to Angiotensin IV (Braszko, 2006). Interestingly, LVV-hemorphin 7, an Angiotensin IV analogue, has also been shown to promote robust enhancing effects on spatial learning and facilitates memory retention and retrieval (Lee et al., 2004), while chronic i.c.v infusion of the AT_4 agonist norleucine^1-Angiotensin IV facilitates the rate of acquisition in the water maze, which is blocked by the AT_4 antagonist divalinal (Wright et al., 1999). Des-phe6-Ang IV (i.c.v.) shows similar effects to Angiotensin IV on passive and conditioned avoidance, and both improve novel object recognition (Braszko, 2004). Further evidence demonstrates the cognitive effects of Angiotensin IV and its analogues are the reversal of scopolamine- (Albiston et al., 2004; Pederson et al., 1998; Pederson et al., 2001), ischaemia- (Wright et al., 1996) and knife lesion-induced deficits in the water maze (Wright et al., 1999).

The definitive mechanisms underlying the effects of Angiotensin IV on cognition are unclear, and a matter of some ongoing debate. Albiston et al (2001, 2003) speculated that the cognitive effect of Angiotensin IV results from its inhibitory effects on the insulin regulated amino peptidase (IRAP) preserving *in vivo* substrates oxytocin and vasopressin, both of which have effects on learning and memory in their own right (De Weid et al., 1987; Lew et al., 2003). Wright et al. (2008a), however, argue that the concentrations of Angiotensin IV and the onset time of cognitive response are incongruent. In rats Angiotensin IV displays some degree of inhibition of IRAP, typically at μM concentrations (Stragier et al., 2007), while the physiological and indeed behavioural effects are seen at nM concentrations (Braszko et al., 2004).

ApN forms a critical step in the formation of Angiotensin IV and is found extensively in the brain and spinal cord. It acts on Angiotensin III to form Angiotensin IV and is also capable of cleaving N-terminal residues from Angiotensin IV itself (Ardaillou and Chansel, 1997). In this way it is capable of both Angiotensin IV catabolism and metabolism.

We previously reported that subcutaneous administration of Angiotensin IV to mice enhances recall in the novel object recognition test, although there were strain differences (Gard, 2008). The aim of this study was to test the hypothesis that Angiotensin IV acts via inhibition of IRAP-activity and that observed strain differences in mice are due to inherent differences in endogenous IRAP-activity or function.

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2. Materials and methods

2.1. Protocol

Colonies of CD, DBA_2 and, C57BL/6, henceforth referred to simply as C57 mice, were reared under identical conditions. The effect of Angiotensin IV on novel object recognition was determined in adult male mice of each strain. Aminopeptidase activity of isolated brain tissue of untreated individuals of each strain was determined, as was the ability of Angiotensin IV to inhibit enzyme activity. The gene sequences encoding the catalytic and zinc binding domains for IRAP and ApN in CD mice were determined and homology compared to the reference C57 strain (Accession *id*: IRAP: Q8C129, ApN: PB7449 Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bay Harbor, Maine http://www.informatics.jax.org 2008). The expression of IRAP and ApN was explored across all strains.

2.2. Animal husbandry

Male mice of each of strain (CD, DBA_2 and C57, 19–30 g) were bred and reared in-house under identical conditions, consisting of housing in North Kent M1/M2 cages on flake sawdust bedding in an air conditioned room (approx 19 °C ± 1 °C; humidity 50% ± 10%) under a 14/10-h light/dark cycle, commencing 07:00 to 21:00. The subjects had free access to food and water. All behavioural experiments were conducted between 11:00 and 15:00 h.

2.3. Humane treatment of animals

All behavioural experiments were licensed under the UK Scientific Procedures (Animals) Act, 1986.

2.4. Drugs

Angiotensin IV (Val-Tyr-Ile-His-Pro-Phe--OH) (batch number; S: 22–24/25, 0566586) was obtained from Bachem, (Germany). The stock solution of 1 mM was stored frozen and was diluted as required to one of three doses: 4.7 μg/kg, 47 μg/kg and 470 μg/kg.

2.5. Behavioural studies

The novel object recognition test was selected as a measure of learning and memory as it is not confounded by any effects of reward or punishment and investigates selected drug effects on memory consolidation. Object recognition was determined in mice using a modification of the method previously described for rats by Karwowska-Polecka et al. (1997).

For testing, mice were placed into an open field (34 × 60 cm), the floor of which was marked with a 3 × 6 grid and in which two identical solid and impermeable objects were positioned at one end of the field, each 10 cm from the adjacent walls. Mice were allowed to habituate for three min. One hour later they were again exposed to the same open field and objects, which had been cleaned thoroughly with 50% ethanol, for 3 min. The mice do not receive any drug treatment until after the second exposure. 24 h later the mice were again exposed to the cleaned field now containing one of the original objects and a novel (different size, shape and colour) object. The position of the novel object alternated left or right but using the same locations to counter-balance any place preference. Final trials were video recorded and time spent exploring each of the objects was recorded manually over 3 min, as was locomotor activity (line crossing). Exploration of the object was defined as sniffing, or approaching within 5 mm. Climbing or sitting on the object was not considered as exploration. Learning (Discrimination score, D) was quantified as the proportion of time spent exploring the novel object compared with the familiar object, taking into account possible confounding effects of changes in locomotor activity. The D-score was calculated as $(A-B)/(A+B)$ where A was the time spent exploring the novel object and B was the time spent exploring the familiar object.

The effects of Angiotensin IV (4.7, 47 or 470 μg/kg, s.c. in 10 ml/kg chosen to reflect previously effective doses in the rat) administered immediately after the second training trial, was assessed in comparison to saline controls; $n = 8$ in all cases.

2.6. Aminopeptidase activity assay

To determine aminopeptidase activity and the inhibitory effects of Angiotensin IV, a method based on the cleavage of L-leucine-p-nitroanilide into L-leucine and p-nitroaniline was employed (Stragier et al., 2007). The latter P-nitroaniline compound shows a characteristic absorption at 405 nm upon cleavage by aminopeptidases. Whole brains were removed immediately post mortem and placed in ice cold extraction buffer (50 mM Tris, 0.25 M Sucrose, 140 mM NaCl pH 7.5). This was followed by homogenization at full speed for one min with a Ystral homogeniser. Homogenate was centrifuged at 4000 g for 8 min at 4 °C. The supernatant was removed and centrifuged at 100,000 g for 40 min at 4 °C. The pellet was then washed and re-suspended in assay buffer (50 mM Tris, 140 mM NaCl pH 7.4). Protein concentration was assayed using Bradford reagent (Sigma-Aldrich, UK). 25 μg of membrane homogenate was incubated with varying concentrations of leucine-p-nitroanilide (Sigma-Aldrich UK). Angiotensin IV inhibition assays were carried out using 1.2 mM substrate aliquoted into 96 well plates at 37 °C with a range of Angiotensin IV concentrations. The absorbance was read every 10mins using an ASYS plate reader at 405 nm. K_m and IC_{50} values were estimated using non linear regression.

2.7. PCR

To explore possible gene sequence variation between strains, gene sequencing of IRAP Exons 6 and 7 and ApN Exons 6 and 7 was conducted. These exons were considered to be the most likely candidates for variation associated with Angiotensin IV inhibition because they code for the catalytic and zinc binding domains. DNA from animals was obtained from 10 to 20 mm of tail tissue and digested overnight at 56 °C in 20 μl Protinase K (Qiagen). DNA was subsequently extracted using Qiagen DNAeasy kit (Qiagen). 10 ng of DNA was used for PCR amplification in a Hybaid touchdown thermal cycler using primers detailed in Table 1, AmpliTaq Gold (Applied Biosystems) and the following thermal cycling conditions: stage 1: 94 °C for 2 min; stage 2: 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min for 35 cycles; stage 3: 72 °C for 10 min. PCR products were then electrophoresed on a 2% agarose gel for 1 h at 80 V. Bands were excised from the agarose and cleaned using the Qiagen QIAquick PCR cleanup kit (Qiagen). Purified DNA samples were ethanol precipitated and air dried at a concentration of 20 ng/100 bp. Samples were sequenced (Cogenics UK) using the forward primer.

2.8. RT-qPCR

To examine potential differences in basal levels of IRAP and ApN expression an RT-qPCR method was developed to explore relative

Table 1
Primer sequences for IRAP and ApN sequencing.

IRAP Exon 6 F	5′-ATT GCT GAT CGG GTC TTC CT-3′
IRAP Exon 6 R	5′-GGG CGT TTA TGA GAT GAG ACA-3′
IRAP Exon 7 F	5′-TCT TCA ATT AGC CTG ATA GTC CA-3′
IRAP Exon 7 R	5′-ATG TGG CTA TTC CCT CCT CA-3′
ApN Exon 5 F	5′-CAT GGA GGT AGT GCC AGG AT-3′
ApN Exon 5R	5′-GTC ACA GAC ACT CCC GCT CT-3′
ApN Exon 6 F	5′-GGT TGG ATG TGA TTA GTG ATG-G 3′
ApN Exon 6 R	5′-AGC TCA GAA GGG GTG TGT CA-3′