Int J Mol Epidemiol Genet 2011;2(1):51-55
www.ijmeg.org /ISSN1948-1756/IJMEG1008003

Brief Communication

Strain differences and the role of AT_1 receptor expression in anxiety

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Received August 26, 2010; accepted December 20, 2010; Epub December 26, 2010; published January 1, 2011

Abstract: This study investigated strain specific differences to the anxiolytic response to losartan focusing on genetic variation that may influence such responses. This included: AT_1 receptor sequence variation, angiotensin II receptor associated protein (ATRAP) and receptor expression between strains. Sequencing of exon 3 of $AT_{1a}R$ revealed no differences between BKW mice (*n=6)* and C57 and DBA_2 strains (*n=3).* Comparisons of AT^1 expression do show significant differences, whereby BKW mice showed the highest levels of expression and DBA_2 mice intermediate levels when compared to the C57 strain. Sequencing of sections of the Angiotensin receptor associated protein (ATRAP) identified a non-synonymous point mutation- (T/C) transversion (position 109-161) (SNP id = rs13467517) resulting in a Valine → Alanine (V157A) amino acid change in the BKW and DBA_2 strains. Our results indicate that the previously reported strain dependent effects are not due to variation in $AT1_a$ receptor sequence. Differences in AT_1 gene expression levels between strains, which mirror their anxiety phenotype, are observed. This is coupled with a non-synonymous single nucleotide polymorphism in ATRAP, a negative regulator of AT_1 signalling.

Keywords: AT_1 receptors, anxiety, anxiolytic, strain differences, angiotensin receptor associated protein (ATRAP), losartan

Introduction

The AT_1 receptor antagonists are reported to exert their anti-stress and anti-anxiety properties by modulating, in part, the HPA axis and three interacting cortical systems: CRF, GABA Type A receptors ($GABA_A$), and noradrenaline [2]. Stimulation of AT_1 receptors in the paraventricular nucleus (PVN) by angiotensin (Ang II) increases CRF production and is important for the induction of anxiety; indeed, CRF_1 receptor antagonists have been shown to decrease stress-induced anxiety [6].

The inhibition by AT_1 receptor antagonists is sufficient to block stress-induced changes in CRF_1 receptors and restores the inhibitory effect of the cortical $GABA_A$ system [7]. Importantly the CRF and GABA systems are tightly interconnected within the paraventricular nucleus, an area which contains high numbers of AT_1 receptors and where losartan has been shown to cross the blood brain barrier and exert its effects [8,9].

Ang II can also potentiate neurotransmission; hence, blockade of the Ang II system with specific antagonists may hyperpolarise the membrane potential of sympathetic neurons, resulting in normalisation of their activity. Further, AT_1 receptor facilitates catecholamine release throughout the AT_1 pre-synaptic receptor mechanism and blockade of this via losartan may have a beneficial anti-anxiety effect [10]

There is however, within-strain and inter-strain variation in the responses of rodents to various paradigms of anxiety which highlights an 'order' of anxiety, whereby some mice strains show higher levels of anxiety compared with others. Some strains, such as the C57BL/6, have been described as non-emotive, while the contrary has been described for DBA_2, BALB/C and A/J mouse strains [11,12]. Observations of C57BL/6J and BALB/C also show that the former are non-anxious, while mice the latter are

much less active and generally more anxious [13]; suggesting that performance in the EPM and LD is markedly influenced by genetic variation between strains.

The angiotensin receptor associated protein (ATRAP/AGTRAP) is known to act as a negative regulator of AT_1 receptor signalling and is reported to potentiate Ang II signalling effects. Indeed, overexpression of ATRAP results in a marked decrease of agonist induced AT_{1A} mediated activation of phospholipase C [14], reduces the number of AT_1 receptors at the cell surface [15] and negatively regulates promoter transcription and protein synthesis.

In this study strain differences to the anxiolytic response to losartan were investigated in mice focusing on areas of genetic variation that may influence strain specific responses. This includes: AT_1 receptor sequence variation, the angiotensin II receptor associated protein (ATRAP) and receptor expression differences between strains.

Materials ans methods

Animal treatment and husbandry

All behavioural experiments were licensed under the UK Scientific Procedures (Animals) Act, 1986. Male mice of each of strain (BKW, DBA_2 and C57, 19-30g) 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 19ºC +/- 1ºC; 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.

PCR

PCR amplification was carried out using a Hybaid touchdown thermal cycler using standard thermal cycling conditions. PCR products were then electrophoresed on a 2% Agarose gel for 1 hour at 80V, stained and visualised using ethidium bromide. DNA samples were sequenced using the forward primer used for initial PCR by Cogenics UK (Primer details available upon request).

Mice were sacrificed and had their brains immediately removed. Hypothalamic brain tissue was used to isolate total RNA using the guanidine isothiocyanate enhanced TRIzol method (Invitrogen, UK). RNA integrity was determined spectrophotometrically and only RNA with a A_{260} \A_{280} ratio above 1.8 was used for reverse transcription. 1ug of RNA was treated with Turbo DNase I (Ambion) before being reverse transcribed using BIO-RAD Iscript RT PCR kit with random oligo(dT) primers.

AT_1-R and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was analysed using the $2^{(\Delta\Delta Ct)}$ method [16]. Primers and probes were designed using Perlprimer [17] and were developed to span Intron/Exon boundaries and to ensure specificty to AT_{1a}, by avoiding regions of high similarity and designing primers in the 5' untranslated region. The primers and probe were synthesised by MWG Biotech; *n*=3 in triplicate in all cases. Confirmation of product specificity by size was established by melt curve analysis and where agarose gel electrophoresis revealed single specific PCR products.

The initial real-time PCR resulted in greater variance across replicates than anticipated. For this reason a subsequent duplication was performed. However, the nature of the $\Delta\Delta Ct$ analysis does not lend itself readily to the simple addition of data from two independent observations, hence a simple Bayesian approach was employed, following the procedure outlined in Box & Tiao [18] and Quinn & Keough [19]. The mean and variance in $\Delta\Delta Ct$ values from the first round of real-time PCR were used to set up the prior distribution, which was assumed to be normally distributed. The standardised likelihood function was normally distributed with the mean $\Delta\Delta Ct$ value of the second round of real-time experiments and variance equal to the squared standard error of this mean. The mean of the posterior distribution is

$$\bar{\theta} = \frac{1}{\frac{1}{s_0^2} + \frac{n}{s^2}} \left(\frac{1}{s_0^2} \bar{\theta}_0 + \frac{n}{s^2} \bar{y} \right)$$

, s_0^2 is the estimate of the prior variance, s^2 is the second sample variance, $\bar{\theta}_0$ is the mean of the prior distribution and $\bar{y}$ is the mean of the second sample. The variance of the posterior distribu-

$$\bar{\sigma}^2 = \frac{1}{\frac{1}{s_0^2} + \frac{n}{s^2}}$$

tion is .

Data analysis and statistical comparisons

Statistical comparisons were performed using Minitab 15 (Minitab Solutions). Bayesian analysis and RT-PCR calculations were analysed using Microsoft Excel (Microsoft). *P<0.05* was considered statistically significant

Results

AT_1 sequencing

Sequencing of exon 3 of $AT_1{}^{\Delta\Delta}R$ which contains the entire open reading frame (MGI:87964) revealed no differences between BKW mice (*n*=6) and the reference C57 and DBA_2 strains (*n*=3) . Comparisons of the sequencing data using ClustalW alignment showed that AT_1 R sequence was identical between strains.

AT_1 RT-PCR expression analysis

The posterior probability distributions of mean $^{\Delta\Delta}$Ct values are given in **Figure 1**. Comparisons of C57 and BKW show significant differences between strains, represented by the non-overlapping 95% confidence intervals. In summary C57 shows significantly different AT_1R expression levels to the BKW strain.

ATRAP- Functional mutation A-V

Sequencing of ATRAP hydrophilic domains identified a non-synonymous point mutation resulting in (T/C) transversion at position 157 in the hydrophilic tail domain (position 109-161) (SNP id = rs13467517). This results in a Valine → Alanine (V157A) amino acid change in the BKW and DBA_2 strains (**Figure 2** and **3**).

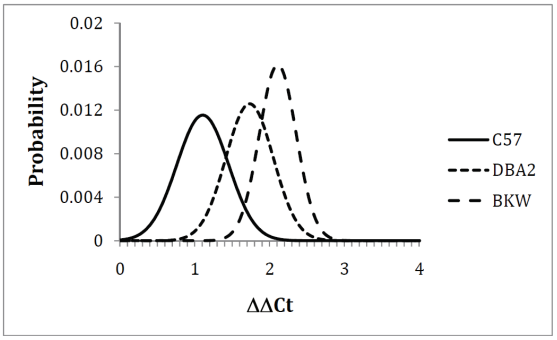

Figure 1. Posterior probability distributions for the mean DDCt values across strains.

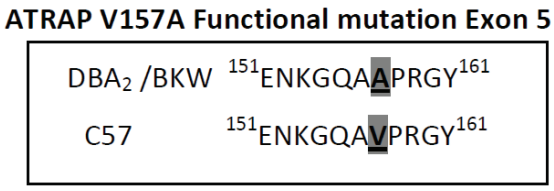

Figure 2. Diagrammatic representation of ATRAP V-157-A mutation in C57, DBA_2 and BKW mice. BKW and DBA_2 mice differ from C57 strains resulting in a Valine to Alanine substitution at position 157. (*n*=6).

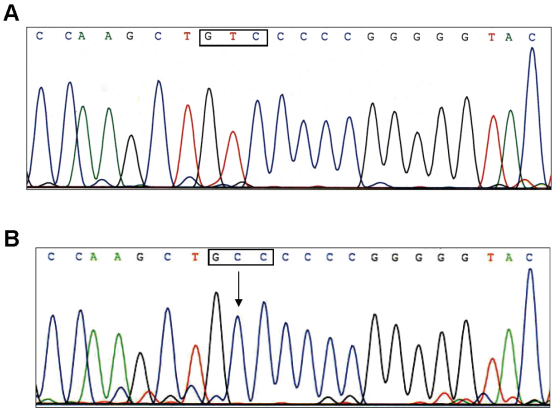

Figure 3. A. Representative sequence data of ATRAP in C57 strain. **B**. Representative sequence data of a mutation in ATRAP. DBA_2 and BKW strains show T/C base transversion.

Discussion

The anxiolytic effect of AT_1 antagonists has been demonstrated in rodents by several groups [3,4,5,10,20) although some effects are reported as strain specific. The results presented herein demonstrate that the previously reported strain dependent effects are not due to variation in $AT1_a$ receptor sequence. However, differences in AT_1 gene expression levels between strains are observed whereby BKW and DBA_2 strains show higher expression compared to C57, which mirrors their anxiety phenotype. This is coupled with a non-synonymous

single nucleotide polymorphism in ATRAP, a negative regulator of AT_1 signalling.

The findings in this study show that AT_1 receptor sequence is identical between strains and, while no differences in AT_1 receptor sequence were observed, RT-PCR expression analysis of hypothalamic AT^1 receptors revealed differences in expression: BKW mice showed the highest levels of expression and DBA_2 mice an intermediate level, when compared to the C57 strain

In previous studies by Gard et al, [5] BKW mice showed the highest anxiety-like behaviour in the L/D and EPM test followed by DBA_2 and C57. These observations are consistent with others where the C57 strain are reported as "low anxiety" with significantly lower anxiety than DBA_2 mice [12, 21] . The low level of anxiety in C57 mice and the higher levels of anxiety in DBA_2 and BKW mice mirror the expression levels of AT_1 receptors found in this study; suggesting that the more anxious strains exhibit higher levels of AT_1 receptor expression. Such differences in the protein level of AT_1 receptors would also be of interest as well as possible effects of post-translational modifications in these strains.

The previous strain dependent effect, whereby losartan is only effective in the BKW strain, may represent an effect due to the higher levels of anxiety inherently displayed by this strain, perhaps due to this increased AT_1 expression. Whether higher AT_1 expression in the BKW strain results in an anxious phenotype or whether increased anxiety is due to increased upregulation of AT_1 receptors remains unresolved; however overexpression of AT_1 receptors in mice lacking AT_2 receptors is linked with anxiety-like behaviour [22]. In contrast, the phenotype of $AT_1^{-/-}$ mice may also be of interest in determining the role of these receptors in anxiety.

The use of more than one strain, when testing the mechanism and the role of AT_1 antagonists, is not typical of this literature, possibly explaining why the correlation between AT_1 expression and an anxious phenotype may not have been identified previously.

It is interesting to note that in the previous study by Gard *et al*, [5] the C57 and DBA_2 strains showed similar contractile responses to Ang II, while the BKW strain showed a significant reduction. ATRAP is associated with reduced Ang II signalling and this study has identified that both DBA_2 and BKW mice posses a non-synonymous polymorphism (rs13467517) in this gene. ATRAP modifies Ang II receptor signalling *in vitro* and in DBA_2 and BKW mice the SNP results in a non-synonymous Valine to Alanine amino acid change at position 157 of the hydrophilic domain of the protein. The precise effect of this polymorphism is unknown, and is only observational but it is tempting to speculate that the higher expression of AT_1 receptors in DBA_2 and BKW, in contrast to C57 mice, is linked to this polymorphism.

At present the discrete localisation of ATRAP is unknown, although low levels have been identified in the brain [14]. Perhaps increased AT_1 receptor expression also results in increased ATRAP expression. Functional differences in ATRAP also provide a possible explanation of the strain differences observed in contractile effects of Ang II in isolated tissue. Despite showing no variation in receptor structure, contractile effects were reduced in BKW strains. Owing to the presence of the ATRAP-SNP in DBA_2 strains, it would be expected that the reduced contractile effect would also be observed in this strain. However, DBA_2 and C57 mice showed comparable contractile effects, perhaps due to signalling by another mechanism resulting in a compensational loss in signalling.

In agreement with previous studies AT_1 receptors appear to be implemented in anxiety like behaviour. The previously identified strain differences and the "order" of anxiety seen within and between strains may be attributed to variation in AT_1 expression levels, where more anxious animals show higher AT_1 expression. Whether the changes in AT_1 expression are mediated by the functional mutation in ATRAP, are due to posttranslational modifications or variability in protein levels or linked to SNPs in the promoter region was not specifically investigated in this study.

Importantly, current treatments focus on serotonin, dopamine and noradrenaline in the brain and their efficacy and response rate has not improved despite the development of newer drugs [23]. Data show that specific antagonism of AT_1 receptors reduces anxiety and therefore

suggests that levels of AT_1 expression have an important role. Whether AT_1 expression is the critical mediator of anxiety and whether higher AT_1 receptor expression is indicative of an anxious phenotype warrants further research.

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