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**Tryptophan hydroxylase immunoreactivity is altered by the genetic variation in postmortem brain samples of both suicide victims and controls**

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**Running title:** TPH gene polymorphism affects TPH immunoreactivity

**Key words:** tryptophan hydroxylase (TPH); serotonin 2A receptor (5HT2A receptor);

polymorphism; suicide; postmortem brain study; western blot; receptor binding assay

**Several lines of evidence suggest that a partly genetically controlled serotonergic dysfunction is involved in the biological pathogenesis of suicide. In this study, we measured tryptophan hydroxylase (TPH) immunoreactivity as a pre-synaptic maker, and serotonin receptor 2A (5HT2A receptor) density as a post-synaptic marker in the serotonergic system in 10 postmortem brains of suicide victims. We also examined whether TPH gene polymorphisms (A218C and A-6526G polymorphisms) could affect TPH immunoreactivity and 5HT2A receptor gene polymorphism (A-1438G polymorphism) could affect 5HT2A receptor density in 28 postmortem brain samples. No significant differences were found in TPH immunoreactivity or 5HT2A receptor density between suicide victims and controls. The AA genotype of the A218C polymorphism of the TPH gene showed higher TPH immunoreactivity along with lower 5HT2A receptor density than did any other genotypes in the postmortem brains of both suicide victims and controls. Our findings suggest that the A218C polymorphism of the TPH gene can be expected to provide new insights not only for neurobiological studies of suicide, but also for research into the behavioral characteristics that may be associated with serotonergic dysfunction.**

Abnormalities in the serotonergic system have been found in suicides[1]. A reduced concentration of 5-hydroxyindoleacetic acid, the principal metabolite of serotonin, has been identified in the cerebrospinal fluid of suicide attempters[2,3]. A blunted prolactin response to fenfluramine, which indicates central serotonergic hypofunction[4], has also been demonstrated in suicide attempters[5]. These findings seem to be independent of psychiatric diagnoses and to correlate with the lethality of suicide attempts.

Many, although not all, postmortem studies of suicide victims have reported an increase in 5HT2A receptor density in the prefrontal cortex of suicide victims [6-9]. It is

thought that these findings may result from mechanisms such as adaptive or compensatory 5HT2A receptor up-regulation secondary to reduced pre-serotonergic neurotransmission which might be under genetic control. These conclusions are based on the results of studies of family, twins and adoption, which suggest that genetic factors may be involved in the etiology of suicidal behavior[10,11]. Recently, Turecki et al. [12] have reported that the A–1438G polymorphism of the 5HT2A receptor gene significantly affects the 5HT2A receptor in the prefrontal cortex (Brodmann's area 9), but another study was unable to replicate their results[13]. In an association study, Du et al. reported that the C allele of the T102C polymorphism, which is almost completely in disequilibrium with the A–1438G polymorphism [14], was associated with suicidal ideation in major depressive disorders[15], although no association was found between these polymorphisms and suicidal behavior itself [12,16,17].

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in serotonin biosynthesis and has a major function in regulating the serotonergic system. However, TPH activity as a pre-synaptic serotonergic marker has not been measured in the postmortem brain of suicide victims. On the other hand, the polymorphisms of the TPH gene, mainly the A218C polymorphism, have been studied to determine whether the TPH gene is involved in suicide, but the results have been inconsistent [18-25].

To investigate the involvement of serotonergic dysfunction in suicide victims, we measured TPH immunoreactivity as a pre-synaptic maker, and 5HT2A receptor density as a post-synaptic marker in the serotonergic system in the prefrontal cortex (Brodmann's area 9) of postmortem brains of suicide victims and controls. In addition, to clarify genetic involvement in serotonergic functioning, we examined whether the A–6526G polymorphism or the A218C polymorphism of the TPH gene could affect TPH immunoreactivity and whether the A–1438G polymorphism of the 5HT2A receptor gene could affect 5HT2A receptor density.

The anti-TPH antibody stained a band on the gel corresponding to a molecular weight of ~55kDa (Figure 1). TPH immunoreactivity of the 10 suicide victims (mean ± S.D. = 126.8 ± 53.3 %) and 12 controls (100 ± 34.9 %) was not significantly different. No significant correlation was found either between age and TPH immunoreactivity in any of the subjects, both suicide victims and controls. Moreover, there was no significant difference in TPH immunoreactivity between male and female subjects who did or did not commit suicide (data not shown). TPH immunoreactivity tended to correlate with the postmortem delay ($\rho$= 0.37, p = 0.089, Spearman's rank order correlation).

$^{3}$H-ketanserin binding in the prefrontal cortex of the suicide victims (mean ± S.D. =81.3 ± 38.6 fmol/mg protein) was not different from that of the controls (70.5 $\pm$ 43.1 fmol/mg protein), and tended to be negatively correlated with age ($\rho$ = - 0.37, p = 0.094, Spearman's rank order correlation). For both the suicide victims and the controls, binding of $^{3}$H-ketanserin was not significantly different in male and female subjects. No significant correlation was found between the postmortem delay and $^{3}$H-ketanserin binding. There was a significant negative correlation, however, between TPH immunoreactivity and $^{3}$H-ketanserin binding (r = - 0.51, p = 0.014) (Figure 2).

Figure 2

The A218C polymorphism of the TPH gene had a significant influence on both TPH immunoreactivity and $^{3}$H-ketanserin binding (Figure 3). Individuals with the AA genotype of A218C polymorphism (n=8, 100.0 ± 36.9 %) showed significantly higher TPH immunoreactivity than those with the AC genotype (n=10, 55.1 ± 25.4 %) (z = - 2.40, p = 0.016,) and also a tendency to higher TPH immunoreactivity than those with the CC genotype (n=10, 69.0 ± 18.0 %) (z = - 1.69, p =0.091). Individuals with only the A allele (n=8, 100.0± 36.9 %) were characterized by a significant increase in TPH immunoreactivity as compared to those with any C allele (n=20, 62.0± 22.6 %) (z = - 2.33, p =0.019). Age and postmortem

Figure 3

delay were not significantly different among the genotypes of the A218C polymorphism of the TPH gene.

Individuals with the AA genotype (35.6 ± 33.5 fmol/mg protein) showed significantly lower $^{3}$H-ketanserin binding than those with the AC genotype (96.8 ± 33.4 fmol/mg protein) ($z = -2.84$, $p = 0.0045$) or the CC genotype (74.6 ± 28.9 fmol/mg protein) ($z = -2.40$, $p = 0.016$). Individuals with only the A allele (n=8, 35.34± 33.5 fmol/mg protein)) exhibited a significant increase in TPH immunoreactivity as compared to those with any C allele (n=20, 85.7± 32.4 fmol/mg protein)) ($z = -3.00$, $p = 0.027$).

On the other hand, neither the A–6526G polymorphism of the TPH gene nor the A-1438 polymorphism of the 5HT2A receptor gene had any affect on TPH immunoreactivity or $^{3}$H-ketanserin binding, respectively (data not shown). No significant differences between suicides and controls in genotype frequency were observed for the A218C polymorphism and the A–6526G polymorphism of the TPH gene and the A–1438G polymorphism of the 5HT2A receptor gene.

Ours is the first study to measure TPH immunoreactivity in postmortem brains of suicide victims and to investigate the extent of genetic involvement in the amount of TPH. The major finding of our study was that the AA genotype of the A218C polymorphism of the TPH gene showed higher TPH immunoreactivity than the AC or CC genotype in postmortem brain samples from both those who did commit suicide and those who did not. As far as we know, this is the first evidence that TPH activity may be genetically regulated by the A218C polymorphism of the TPH gene. Because this polymorphism is a single base transition situated in intron 7 [26], it is unlikely to affect the expression of TPH directly, but since it may feature linkage disequilibrium with unknown functional variations in a regulatory region of the TPH gene, it might affect the expression of the gene indirectly. In this study, we found that TPH immunoreactivity was not significantly altered in the prefrontal cortex of the suicide

victims. Previously we also found no association between the A218C polymorphism of the TPH gene and suicide victims[23]. These results together suggest that the A218C polymorphism does not play a major role in the biological suicidal tendencies, but seems to be involved in the serotonergic function.

There was no change in 5HT2A receptor density in the prefrontal cortex (Brodmann's area 9) of the suicide victims. Our funding does not correspond to the results of previous studies which reported an increase in 5HT2A receptors measured by $^{3}$H-ketanserin binding in the same region of the suicide victims [7-9] . We found a significant negative correlation between TPH immunoreactivity and 5HT2A receptor density. We also found that the presence of the AA genotype of the A218C polymorphism of the TPH gene was associated with lower 5HT2A receptor density and higher TPH immunoreactivity. Therefore, the A218C polymorphism can cause up- or down-regulation of the 5HT2A receptor through producing changes in the TPH activity. We suggest that this discrepancy regarding 5HT2A receptor density in the prefrontal cortex of suicide victims may result from differences in genotype distribution of the A218C polymorphism in the samples examined. Moreover, the discrepancy between our results and those of other previous studies[6-9] might be due to differences in the ligands used to measure 5HT2A receptor density or to differences in the regions studied in postmortem brains. Another possible reason for this discrepancy may be found partly in the differences in the psychiatric status of the subjects.

In conclusion, our findings suggest that the A218C polymorphism of the TPH gene is likely to affect the regulation of TPH expression and may provide new insights not only for the biological studies of suicide but also for research into the behavioral characteristics that may be associated with serotonergic dysfunction.

## Methods

*Chemicals*

Anti-TPH polyclonal antibody was obtained from Chemicon International (Temecula, CA, USA). Peroxidase-linked anti-rabbit and the enhanced chemiluminescence Western blot detection system were obtained from Amersham (Buckinghamshire, UK), and the molecular weight standards from Bio-Rad Laboratories (Richmond, CA, USA). $^{3}$H-ketanserin (76.7 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). The DNA Extractor WB kit was obtained from Wako Chemicals (Tokyo, Japan). Restriction enzymes *Sau3A* I, *Nhe* I and *Hpa* II were obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of analytical grade and obtained from commercial sources.

*Subjects*

Table 1

Clinical characteristics of the subjects in the postmortem study are shown in Table 1. Information about the suicide victims and the controls was obtained from a wide range of sources, including autopsy, medical examiner's interviews with family, and police reports. None of the suicide victims or controls was consulting a psychiatrist or taking psychotropic medication at the time of death.

TPH immunoreactivity and $^{3}$H-ketanserin binding were studied in the prefrontal cortex (Brodmann's area 9) obtained from 10 suicide victims (six males and four females; age: mean ± S.D. = 49.5 ± 11.7 yr; postmortem delay: mean ± S.D. = 15.9 ± 8.8hr) and 12 deceased controls (nine males and three females; age: 56.6 ± 11.8yr; postmortem delay: 8.8 ± 6.5 hr). There was no significant difference in age between the suicide victims and the controls, but postmortem delay was significantly different ($z = -1.98$, $p = 0.048$). A tissue block was excised from each of the left hemispheres and kept at –80 °C until the assay.

The relationships between gene polymorphism and TPH immunoreactivity or $^{3}$H-

ketanserin binding were examined in 28 postmortem brain samples (nineteen males and nine females; age: 59.0 ± 15.4 yr; postmortem delay: 11.4 ± 7.5 hr) consisting of the above-mentioned 22 brain subjects and an additional 6 control subjects (four males and two female; age: 79.8 ± 2.9 yr; postmortem delay: 9.1 ± 2.9 hr,) which were excluded from the original control group because of age mismatch.

Postmortem brain samples of the suicide victims were obtained from the Division of Legal Medicine, Department of Environmental Health and Safety, Faculty of Medical Sciences, Kobe University Graduate School of Medicine. Control brain samples were obtained from the Department of Anatomy, Kobe University School of Medicine, with the consent of the relatives after the purpose and procedures of the study were fully explained. This study was approved by the Kobe University Graduate School of Medicine Ethical Committee for Genetic Studies.

*TPH immunoreactivity*

The tissue blocks were homogenized in 10 volumes of ice-cold 50 mM Tris-HCI buffer (pH 7.4 at 25 °C) containing 1 mM EDTA, 5 mM EGTA, 20 units/ml aprotinin, 20 μg /ml antipain, 20 μg /ml leupeptin, 10 μM calpain inhibitor I, 1 mM phenylmethylsulfonyl fluoride and 25 mM 2-mercaptoethanol. The homogenates were centrifuged at 48,000 xg for 30 min at 4 °C, and the supernatant was used as the soluble fraction. The protein concentration was determined with Lowry's method[27] and using bovine serum albumin as the standard. The samples (10 μg protein/lane) were fractionated in a 10 % sodium dodecyl sulfate/polyacrylamide gel by electrophoresis according to the method of Laemmli[28] and transferred to a polyvinylidene difluoride membrane. The membrane was then incubated for 90 minutes at room temperature with rabbit anti-TPH polyclonal antibody diluted to 1:1000

and further incubated for 60 minutes with peroxidase-linked second antibody diluted to 1: 2000. Immunoreactive bands were visualized with an enhanced chemiluminescence Western blot detection system and scanned with a Scanjet 3c scanner (Hewlett-Packard Co., Greeley, CO). Densitometric readings were obtained with a computer program (NIH Image version 1.59). The densitized signals of the immunoreactive bands were confirmed on each membrane as being within the linear range by means of processing blots with four different concentrations (1,5,10 and 20 μg of protein) of stock preparations in a control brain sample. Densitometric reading of immunoblots over the membranes were normalized with this standard line. All measurements were made in duplicate. Data were calculated as percentages of the mean values in the control subjects or the subjects with the wild homozygotic genotype of each of the polymorphisms.

*5HT2A receptor binding assay*

The density of the 5HT2A receptor was determined by measuring $^{3}$H-ketanserin binding, in the following manner[29]. The tissue blocks were homogenized in 10 volumes of ice-cold 50 mM Tris-HCI buffer (pH 7.4 at 25 °C and then centrifuged at 48,000 xg for 10 min at 4 °C. The pellets were washed five times by repeated centrifugation and then re-suspended in the same buffer. The membrane preparations were incubated with 1 nM $^{3}$H-ketanserin (76.7 Ci/mmol) in a final volume of 0.6 ml Tris-HCl buffer for 60 min at 25 °C. The specific binding of $^{3}$H-ketanserin was defined in terms of the presence or absence of 1 μM pipamperone. After incubation, the mixture was rapidly passed through a Whatman GF/B glass fiber filter that was presoaked with 0.3 % v/v polyethyleneimine for at least 1 hr before use in order to prevent nonspecific binding of free ligands to the filter. The filters were then rinsed three times in a vacuum and the radioactivity on the dried filter was measured with a liquid scintillation counter.

*Identification of TPH gene and 5HT2A receptor gene polymorphisms*

DNA was extracted from whole blood or brain tissue with the sodium iodide method using a DNA Extractor WB kit. Genotyping for the A-6526G polymorphism of the TPH gene was based on the procedure by Rotondo et al. [22]. Target sequences were amplified in a polymerase chain reaction (PCR) with a Takara PCR Thermal Cycler MP (Takara Shuzo, Kyoto, Japan). The PCR products were then digested with *Sau3A* I, followed by electrophoresis on a 2 % agarose gel. DNA was visualized by ethidium bromide staining and UV transillumination. The A allele was cut into 194 bp and 86 bp fragments, while the G allele was not cut and kept at 280 bp. The A218C polymorphism of the TPH gene was genotyped as described by Bellivier et al.[20]. Target sequences were amplified by PCR and digested with *Nhe* I. After digestion, the A allele was cut into 860 bp and 58 bp fragments, while the C allele was cut into 615 bp, 245 bp and 58 bp fragments. The A-1438G polymorphism of the 5HT2A receptor gene was examined with the method of Collier et al. [30]. The PCR product for the G allele was digested with *Hpa* II into 244 bp and 224 bp fragments, whereas that for the A allele was left undigested.

*Statistical significance*

Mann-Whitney's test was used to estimate significance of the differences between the two groups. The significance of differences among the three groups was tested by Kruskal-Wallis ANOVA, and if significant, Mann-Whitney's test was applied. Frequencies of occurrence of the genotypes in suicides and controls were compared by using a chi-square test. Probability differences of $P < 0.05$ were considered statistically significant.

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**Table 1 Characteristics of the subjects in the postmortem study**

| Subject | Age | Gender[a] | Cause of Death | Postmortem delay (Hours) |
|---|---|---|---|---|
| Suicide | | | | |
| S-1 | 32 | F | Burning | 9 |
| S-2 | 33 | F | Hypothermia (by starvatic | 28.5 |
| S-3 | 41 | M | Jumping from a high plac | 9 |
| S-4 | 47 | F | Hanging | 16 |
| S-5 | 48 | M | Hypothermia (by alcohol) | 14 |
| S-6 | 50 | M | Jumping from a high plac | 28.5 |
| S-7 | 57 | M | Drowning | 22 |
| S-8 | 60 | M | Jumping from a high plac | 20 |
| S-9 | 62 | M | Cutting | 2 |
| S-10 | 65 | F | Drowning | 10 |
| Control | | | | |
| C-1 | 35 | M | Bleeding | 17 |
| C-2 | 42 | M | Bleeding | 4 |
| C-3 | 46 | M | Bleeding | 12 |
| C-4 | 49 | M | Falling | 2 |
| C-5 | 56 | M | Heart failure | 12 |
| C-6 | 57 | F | Heart failure | 2 |
| C-7 | 59 | M | Pulmonary tuberculosis | 9 |
| C-8 | 62 | M | Bleeding | 24 |
| C-9 | 62 | F | Myocardial infarction | 8 |
| C-10 | 65 | M | Pulmonary infarction | 6 |
| C-11 | 70 | M | Gastric cancer | 5 |
| C-12 | 76 | F | Retroperitoneal malignan | 5 |

[a] M: Male; F: Female.

# <Figure legends>

**Figure 1.** Representative immunoblots of TPH in soluble fractions from the prefrontal cortex

**Figure 2.** Correlation between TPH immunoreactivity and $^{3}$H-ketanserin binding in the prefrontal cortex of suicide victims and controls

Linear regression analysis of TPH immunoreactive and $^{3}$H-ketanserin binding in suicide victims (●) and controls (○).

**Figure 3.** Relationship between genotypes of the A218C polymorphism of the TPH gene and TPH immunoreactivity or $^{3}$H-ketanserin binding

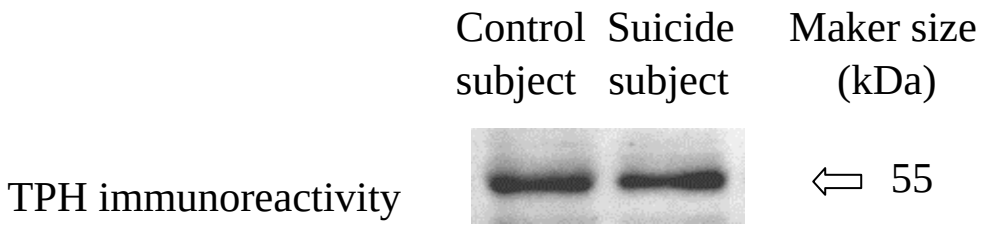
The results of TPH immunoreactivity are expressed as a percentage of the mean value of the AA genotype.

Age (mean ± S.D.), AA: 65.5 ± 18.2 yr; AC: 53.4 ± 13.5yr; CC: 59.5 ± 14.0 yr

Postmortem delay (mean ± S.D.), AA: 11.9 ± 7.5 hr; AC: 9.5 ± 7.0 hr; CC: 13.0 ± 8.4 hr

TPH immunoreactivity: H=7.32, df=2, p=0.026

$^{3}$H-ketanserin binding: H=10.78, df=2, p=0.005

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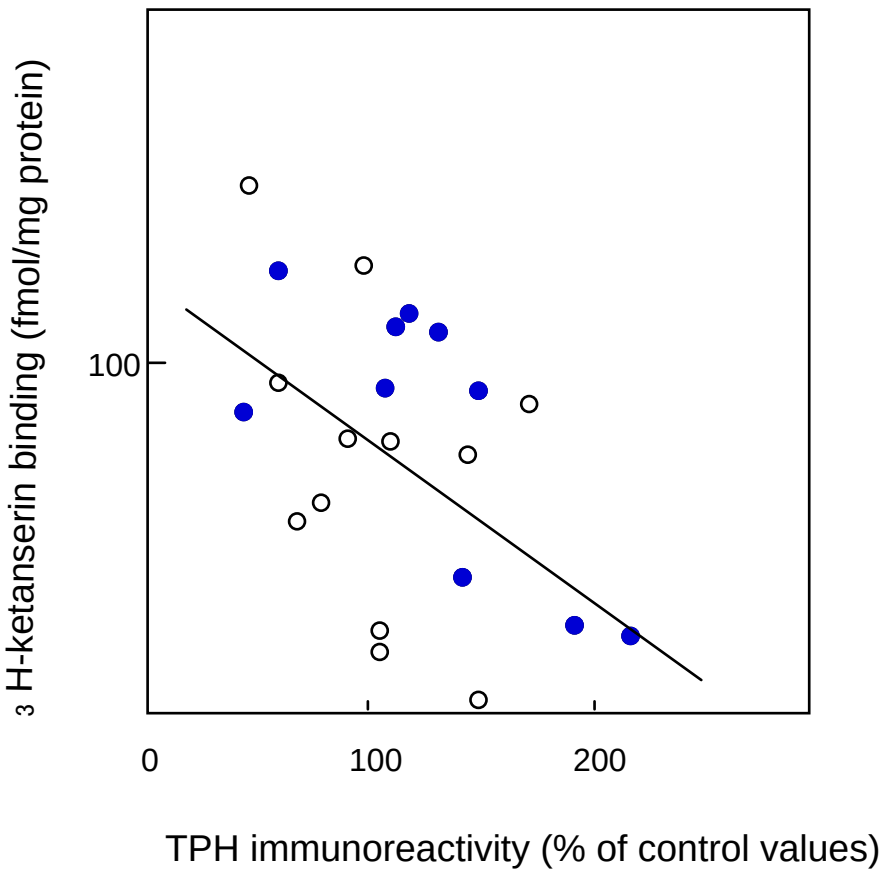


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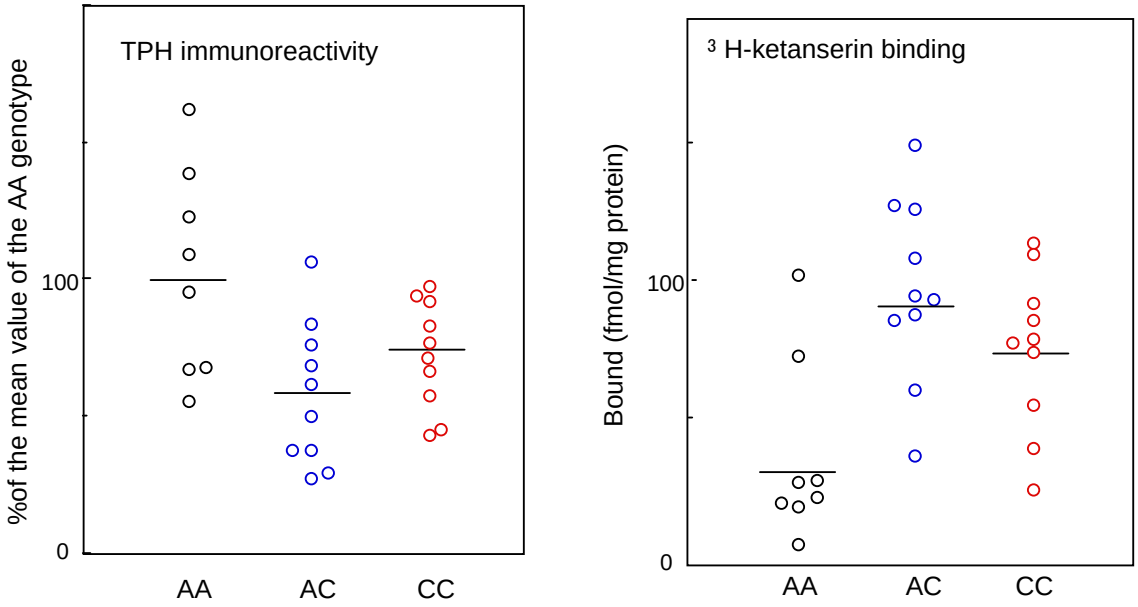


**Figure 3.** Relationship between genotypes of the A218C polymorphism of the TPH gene and TPH immunoreactivity or $^{3}$H-ketanserin binding

The results of TPH immunoreactivity are expressed as a percentage of the mean value of the AA genotype. Age (mean ± S.D.), AA: 65.5 ± 18.2 yr; AC: 53.4 ± 13.5yr; CC: 59.5 ± 14.0 yr. Postmortem delay (mean ± S.D.), AA: 11.9 ± 7.5 hr; AC: 9.5 ± 7.0 hr; CC: 13.0 ± 8.4 hr. TPH immunoreactivity: H=7.32, df=2, p=0.026. $^{3}$H-ketanserin binding: H=10.78, df=2, p=0.005