

# Growth Hormone Modulates mRNA Expression of the GABA<sub>B1</sub> Receptor Subunit and GH/IGF Axis Genes in a Mouse Model of Prader-Willi Syndrome

JY LEE, JY KANG, SY LEE, SY CHO, DK JIN

## Abstract

**Objectives:** The aim of this study was to investigate the effects of recombinant human GH (rhGH, henceforth designated GH) on the gene expression of GABA<sub>B</sub> receptor subunits and GH/insulin-like growth factor (IGF) axis genes IGF-1, IGF-1R, IGF-2 and IGF-2R in the brain regions of Prader-Willi syndrome (*Snord116del*) mice, a dwarf strain exhibiting cognitive impairment. **Methods:** *Snord116del* mice were treated with GH (1.0 mg/kg) or saline for seven days before decapitation and tissue dissection. The collected brain tissues were analysed for mRNA content using quantitative PCR (qPCR) in the cerebellum, hippocampus and cerebral cortex. **Results:** In the cerebellum, GH restored the mRNA expression level of the GABA<sub>B1</sub> receptor subunit (GABA<sub>BR1</sub>) and IGF-1R. Furthermore, a significant positive correlation was found between the level of GABA<sub>BR1</sub> mRNA and the expression of the IGF-1R transcript. GH also induced an increase in the mRNA expression of IGF-2 and IGF-2R. **Conclusions:** These data suggest a modulatory effect of GH on the expression of GABA<sub>BR1</sub> and GH/IGF-1 axis genes in cerebellum may provide a mechanism for the GH-induced brain function in PWS patients.

## Key words

Cognitive impairment; GABA<sub>B</sub> receptor subunit; Growth hormone; Prader-Willi syndrome; *Snord116del* mice

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Received March 13, 2015

## Introduction

Prader-Willi syndrome (PWS) is a neurodevelopmental disorder caused by the lack of paternally expressed imprinted genes on human chromosome 15q11-q13.<sup>1</sup> PWS is characterised by hypothalamic dysfunction including growth hormone (GH) deficiency (GHD) with short stature, hyperphagia, obesity, neurobehavioural abnormalities, and cognitive impairment.<sup>2</sup> Clinical studies have shown that GH replacement therapy improves cognitive development in infants and adults with PWS,<sup>3-5</sup> prevents cognitive deterioration and improves cognitive skills in children with PWS.<sup>6</sup> Early GH therapy has been reported to increase the rate of language and neurodevelopment in infants with PWS.<sup>7</sup> However, the physiological and molecular mechanisms underlying the improvement in cognitive function after GH treatment

have not been investigated in PWS.

GH/insulin-like growth factor (IGF)-1 axis is involved in the growth, development and function of the central nervous system (CNS).<sup>8</sup> Individuals with GHD show cognitive impairment, which can be ameliorated by GH treatment.<sup>9</sup> GH administration attenuates cognitive deficits and improves memory in hypophysectomised rodents.<sup>10</sup> Another mediator of GH effects, IGF-2, has been proposed as a novel cognitive enhancer.<sup>11</sup> The presence of binding sites for GH and IGF-1 in the brain suggested that GH crosses the blood-brain barrier,<sup>12,13</sup> although the mechanisms behind the actions of GH on brain function remain unclear.

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the CNS, acting via GABA<sub>A</sub> and GABA<sub>B</sub> receptors. GABA<sub>B</sub> receptors are heterodimer and composed of GABA<sub>B</sub> receptor subunit 1 (GABA<sub>BR1</sub>) and GABA<sub>BR2</sub>,<sup>14</sup> which are responsible for the neuromodulatory effect of GABA.<sup>15,16</sup> Recent studies have reported that exogenous GH increases the abundance of the GABA<sub>B</sub> receptor in the area of the rat brain associated with cognition<sup>17</sup> and GABA<sub>BR1</sub> gene expression in hypophysectomised rat.<sup>18</sup> These findings indicate the possible correlation between GH-induced cognitive function and the GABA<sub>B</sub> receptor.

The Snord116 deletion (*Snord116del*) mouse, a genetic model of PWS, is a dwarf strain caused by the deletion of the Snord116 C/D box snoRNA cluster. This is characterised by a subset of PWS symptoms such as growth retardation,<sup>19,20</sup> elevated anxiety and deficiency in motor learning ability.<sup>20</sup> Although not obese,<sup>19,20</sup> *Snord116del* mice are hyperghrelinemic and moderately hyperphagic.<sup>20</sup> This mice have early-onset postnatal growth deficiency and exhibit the decrease in liver *Igf-1* mRNA and serum IGF-1,<sup>20</sup> suggesting that GH/IGF axis undergoes decreased GH concentrations in the brain and this decline of GH levels could be associated with changes of certain CNS functions such as impairment of cognitive function.<sup>8</sup>

Since GH is an important regulator of developmental and cognitive functions in the CNS, the aim of this study was to investigate the effects of GH on the expressions of GABA<sub>B</sub> receptor subunits as well as the GH/IGF axis gene in specific brain regions known to be affected by GH treatment<sup>21,22</sup> in *Snord116del* mice.

## Materials and Methods

### Animals and Drug Treatment

All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee, Laboratory Animal Research Center, Samsung Biomedical Research Institute (Seoul, Korea). Mice were housed under standard vivarium conditions and provided food and water ad libitum. *Snord116del* mice (B6 (Cg)-Snord116tm1.1Uta/J) were obtained from The Jackson Laboratory (Bar Harbor, 352 Maine, USA). Male *Snord116del* mice and their wild-type littermates aged six-twelve months with C57BL/6J background were used and genotyped as described.<sup>20</sup> At the start of the experiment, the mice were three weeks old (n=6-8 for each group), corresponding to adolescent age. Male *Snord116del* mice were injected subcutaneously with 1.0 mg/kg GH (Growthropin, provided from Dong-A Pharmaceutical Co., Yongin-si, Korea) or saline, once daily for seven days. Male C57BL/6J littermates were used as controls and given saline injections. All animals were weighed every day to monitor their biological response in weight gain. On day 8 of the experiment, the mice were sacrificed and the cerebellum, hippocampus and cerebral cortex were dissected using a brain matrix. The collected tissues were immediately placed in RNALater solution (Applied Biosystems, Foster City, CA, USA) and stored at 4°C prior to total RNA extraction.

### RNA Extraction and cDNA Synthesis

Brain tissues were prepared for RNA extraction using the RNeasy Lipid Tissue Mini Kit (QIAGEN, MD, USA), according to the protocol provided by the manufacturer. Briefly, the tissue samples were quickly homogenised in 1000 µl Qiazol tissue lyzer (Qiagen, Sollentuna, Sweden), and 200 µl chloroform was then added to each sample. The samples were centrifuged at 4°C (12,000 g, 15 minutes) and 70% ethanol was added to the supernatant. Mini Spin columns were then used to elute the samples. The quantification of total RNA was later assessed in a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA) to provide total RNA concentrations and a preliminary quality control. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio and analysed on Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, USA). The conversion of total

RNA to cDNA was performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 20  $\mu$ l. In total, 2  $\mu$ g RNA was used for the synthesis. The cycling parameters were as follows: 37°C for 60 minutes and 95°C for 5 minutes.

### Quantitative Polymerase Chain Reaction

The expression of six genes (*Gabbr1*, *Gabbr2*, *Igf-1*, *Igf-1r*, *Igf-2* and *Igf-2r*) was quantified using a TaqMan® Gene Expression Assay (Applied Biosystems), which included a TaqMan® real-time quantitative polymerase chain reaction (qPCR) in the cerebellum, hippocampus and cerebral cortex. qPCR analysis was performed using a PRISM 7900HT Sequence Detection System (Applied Biosystems) with SDS 2.3 software (Applied Biosystems) in 384-well plates containing cDNA template (10 ng), primers, probes and TaqMan® Universal PCR Master Mix to a final volume of 10  $\mu$ l/well. Each assay included individual samples for a specific gene in triplicate, with corresponding negative controls. Cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minutes. Predesigned gene-specific primers and probes were used to detect each gene (Applied Biosystems), presented in Table 1. For all primers, more details can be obtained from <http://www.appliedbiosystems.com>. The amount of each transcript was normalised to the amount of GAPDH expressed in the same sample.

**Table 1** List of genes and assays for real-time PCR

Gene name	Gene ID	ABI assay number
Insulin-like growth factor 1	<i>Igf-1</i>	Mm00439560_m1
Insulin-like growth factor 1 receptor	<i>Igf-1r</i>	Mm00802831_m1
Insulin-like growth factor 2	<i>Igf-2</i>	Mm00439564_m1
Insulin-like growth factor 2 receptor	<i>Igf-2r</i>	Mm00439576_m1
Gamma-aminobutyric acid B receptor 1	<i>Gabbr1</i>	Mm00444578_m1
Gamma-aminobutyric acid B receptor 2	<i>Gabbr2</i>	Mm01352554_m1
Glyceraldehyde-3-phosphate dehydrogenase	<i>Gapdh</i>	Mm99999915_g1

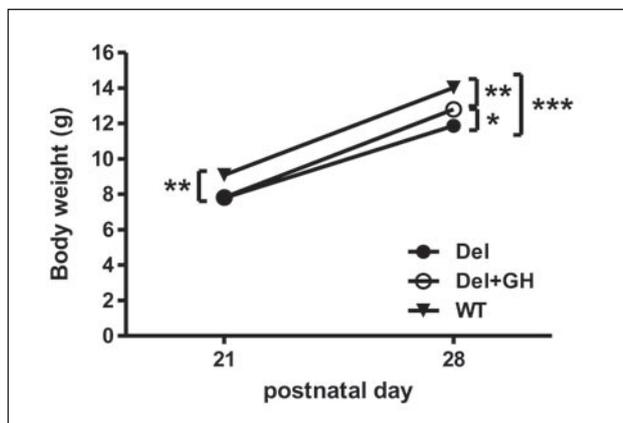
### Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5.0b (GraphPad Software, Inc., La Jolla, USA). The weight measurements were analysed using two-way repeated ANOVA. The results from the qPCR were analysed using one-way ANOVA with a *post hoc* Student-Newman-Keuls test for the statistical analysis of the differences between the groups. The correlation was tested by simple regression analysis. Values are presented as mean  $\pm$  SEM and *p*-value less than 0.05 were considered significant.

### Results

Compared to the WT mice, the *Snord116del* mice with GHD exhibited reduced body weight, and GH treatment significantly increased gains in body weight (Figure 1). This indicates that the administered GH was physiologically active and had an expected systemic effect on body growth.

The expression of six genes (*Gabbr1*, *Gabbr2*, *Igf-1*, *Igf-1r*, *Igf-2* and *Igf-2r*) in the cerebellum, hippocampus and cerebral cortex was analysed in *Snord116del* mice treated with GH (Del + GH) or saline (Del) and wild-type

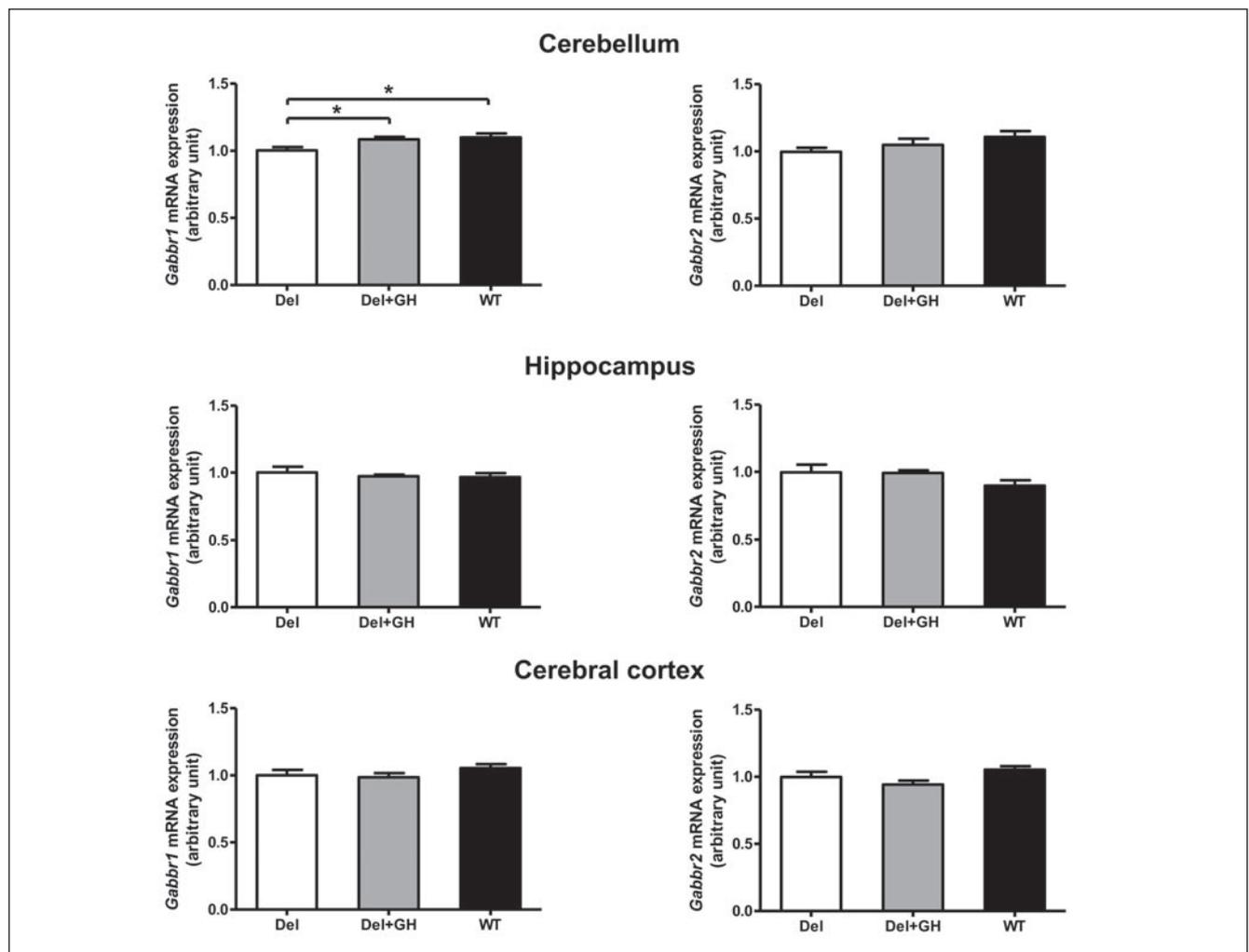


**Figure 1** Effects of GH treatment on body weight. Mice received daily subcutaneous injections of GH (1.0 mg/kg) or saline injection for 7 days. Body weights were measured over 8 days. Data represent mean  $\pm$  S.E.M.  $n=7-8$  for each group. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ . Abbreviations: GH, growth hormone; Del, *Snord116del* mice injected with saline; Del + GH, *Snord116del* mice injected with recombinant growth hormone; WT, wild-type mice injected with saline.

(WT) mice with saline. The results from the gene expression analysis of *Gabbr1* and *Gabbr2* in the cerebellum, hippocampus and cerebral cortex are displayed in Figure 2. In the cerebellum, there were significant differences between the treatment groups regarding the mRNA expression of *Gabbr1* ( $p < 0.05$ ) where both the Del + GH and WT groups showed increased *Gabbr1* mRNA expression compared with the Del group, but no effect on the *Gabbr2* expression was observed. However, the administration of GH did not alter the expression of *Gabbr1* or *Gabbr2* in the hippocampus and cerebral cortex.

The results from the gene expression analysis of *Igf-1*, *Igf-1r*, *Igf-2* and *Igf-2r* in the cerebellum, hippocampus and cerebral cortex are shown in Figures 3 and 4. There was a

significant difference between the treatment groups regarding the *Igf-1r*, *Igf-2* and *Igf-2r* expression in the cerebellum. A significant decrease of *Igf-1r* mRNA expression was found in the Del group compared to the WT group and GH administration induced an increase of *Igf-1r* expression ( $p < 0.05$ ). In addition, alterations of *Igf-2* and *Igf-2r* mRNA expression were found; the Del + GH group had increased the *Igf-2* and *Igf-2r* expression ( $p < 0.05$ ). However, GH administration did not alter the expression of *Igf-1*, *Igf-1r*, *Igf-2* and *Igf-2r* in the hippocampus and cerebral cortex (Figures 3 & 4). By comparing the expression of the gene transcripts for IGF-1R, IGF-2 and IGF-2R with those of the GABAB receptor subunits, a significant positive correlation was observed in cerebellum,



**Figure 2** Effects of GH treatment on mRNA expression of GABA<sub>BR1</sub> and GABA<sub>BR2</sub> in the cerebellum, hippocampus and cerebral cortex. Values are expressed as mean  $\pm$  SEM, n=7-8/group. \* $p < 0.05$ , \*\* $p < 0.01$ . Abbreviations: GH, growth hormone; *Gabbr1*, GABA<sub>B1</sub> receptor subunit; *Gabbr2*, GABA<sub>B2</sub> receptor subunit; Del, *Snord116del* mice injected with saline; Del + GH, *Snord116del* mice injected with recombinant growth hormone; WT, wild-type mice injected with saline.

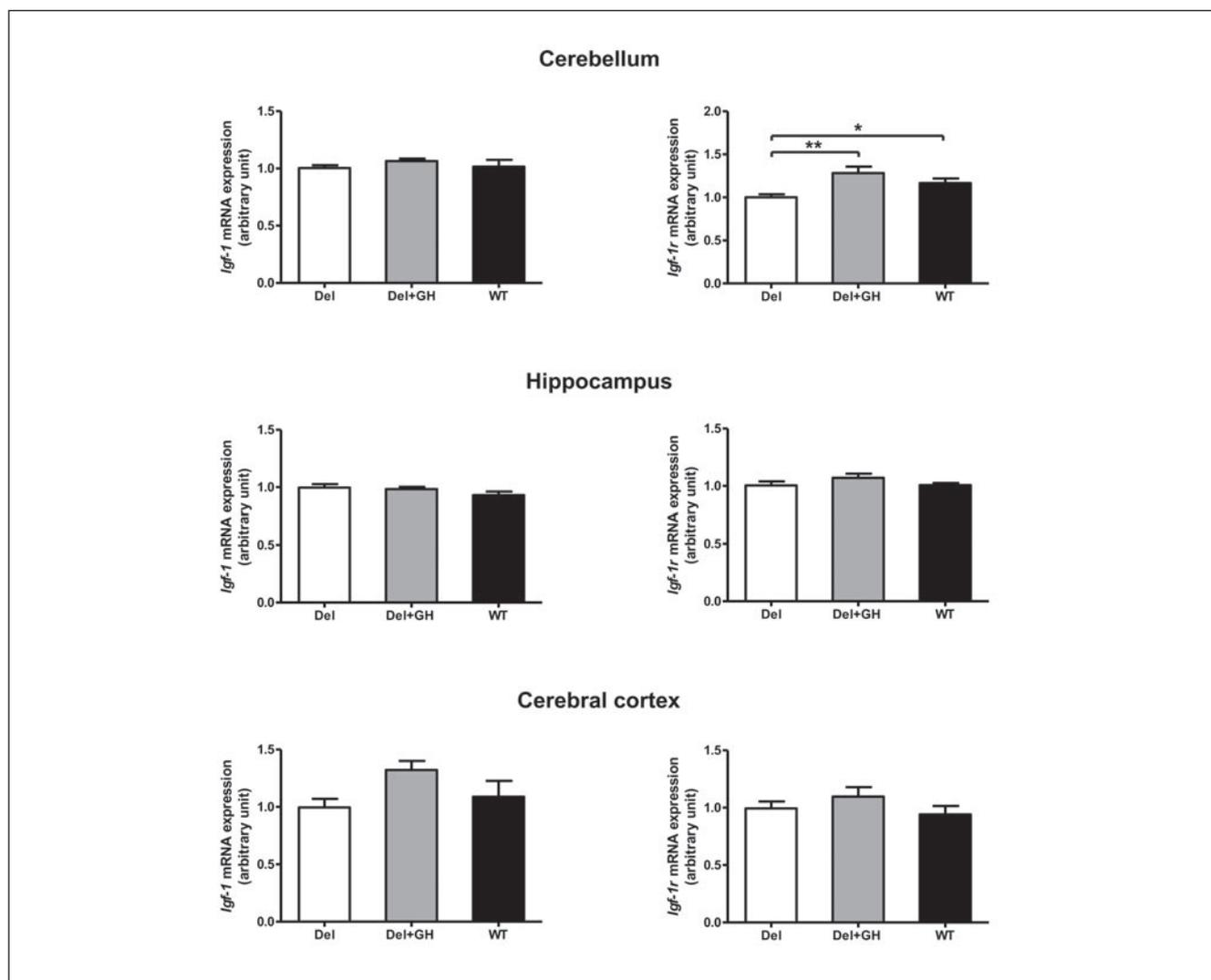
between the level of IGF-1R mRNA and the level of the transcript for the *Gabbr1* ( $r^2=0.62$ ,  $p<0.05$ ) (Figure 5). However, no significant correlation could be seen between the expression level of mRNA for IGF-2, IGF-2R and any other GABAB receptor subunits in any brain regions.

## Discussion

This is the first study, to our knowledge, to examine the effects of GH administration on the expression of GABA<sub>B</sub> receptor subunits and GH/IGF-1 axis genes in specific

regions of the PWS (*Snord116del*) mice brain.

The present study demonstrated that in comparison to wild-type mice, both the expression of GABA<sub>BR1</sub> and IGF-1R transcripts are markedly decreased in the cerebellum of *Snord116del* mice and GH increases the expression of GABA<sub>BR1</sub> and IGF-1R transcripts. GABA<sub>B</sub> receptor has been shown to be important for neuronal excitability and plasticity and is suggested to be involved in the regulation of long-term potentiation, which is the cellular mechanism for learning and memory.<sup>16,23</sup> GH treatment has been reported to affect the functionality and density of GABA<sub>B</sub> receptors in the area of the brain associated with cognition.<sup>17</sup> Other

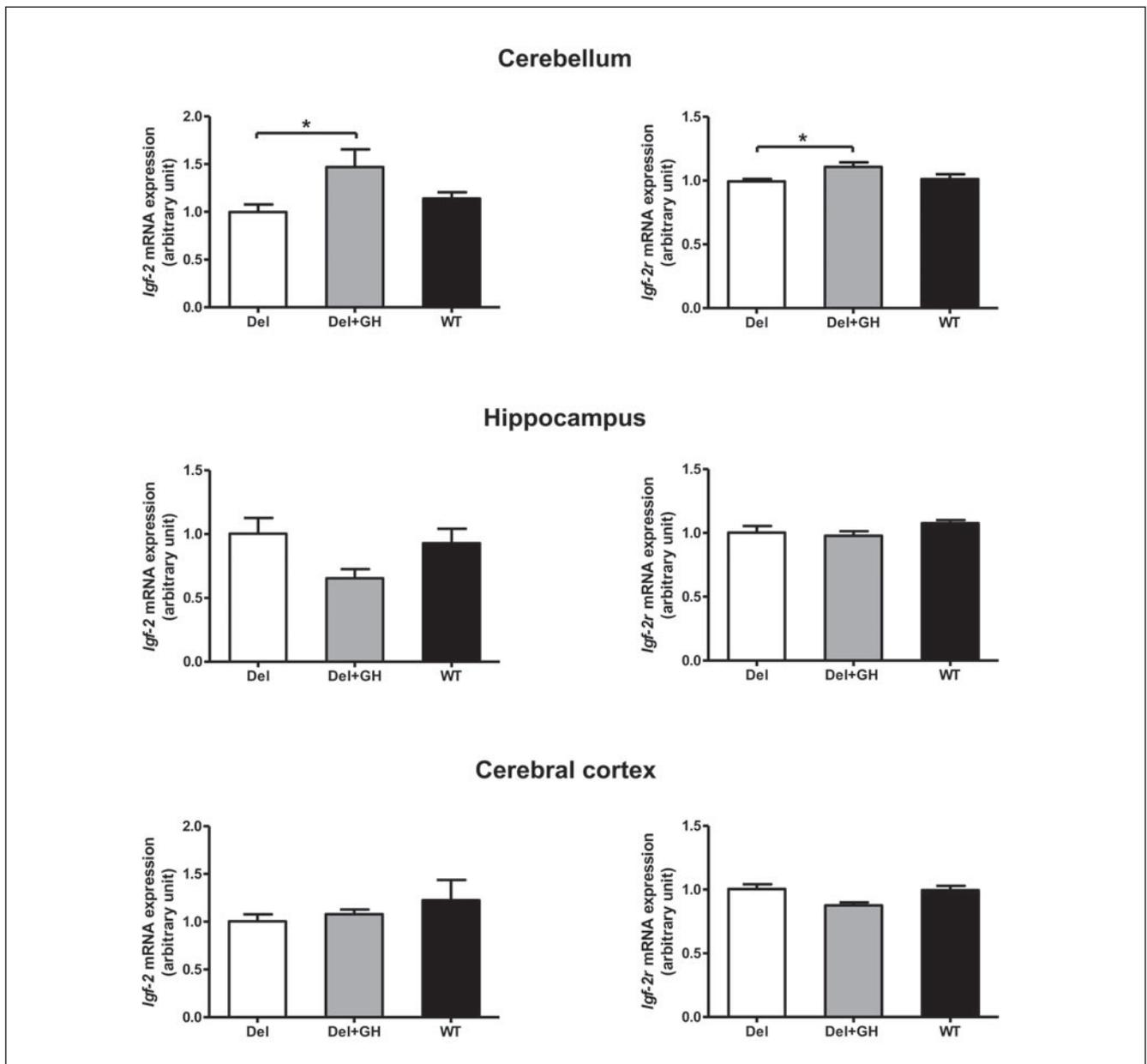


**Figure 3** Effects of GH treatment on mRNA expression of IGF-1 and IGF-1R in the cerebellum, hippocampus and cerebral cortex. Values are expressed as mean  $\pm$  SEM,  $n=7-8$ /group.  $*p<0.05$ . Abbreviations: GH, growth hormone; *Igf-1*, insulin-like growth factor 1; *Igf-1r*, insulin-like growth factor 1 receptor; Del, *Snord116del* mice injected with saline; Del + GH, *Snord116del* mice injected with recombinant growth hormone; WT, wild-type mice injected with saline.

studies revealing that GH administration up-regulated the expression of GABA<sub>BR1</sub> transcript in rat brain further have validated the connection between GH and GABA<sub>B</sub> system.<sup>18,24</sup>

We also detected a significant positive correlation between the mRNA level of IGF-1R and GABA<sub>BR1</sub> in the cerebellum. This finding, indicative of an IGF-1R-mediated

effect on the function of the GABA<sub>B</sub> receptor, is in agreement with a recent observation that the activation of the GABA<sub>B</sub> receptor induces IGF-1R transactivation leading to survival signaling in the cerebellum.<sup>25</sup> Thus, several studies have suggested that the GABA<sub>B</sub> receptor protects the brain from ischaemic damage and improves memory,<sup>26-28</sup> providing evidence that stimulation of the GABA<sub>B</sub> receptor may be



**Figure 4** Effects of GH treatment on mRNA expression of IGF-2 and IGF-2R in the cerebellum, hippocampus and cerebral cortex. Values are expressed as mean  $\pm$  SEM,  $n=7-8$ /group. \* $p<0.05$ . Abbreviations: GH, growth hormone; *Igf-2*, insulin-like growth factor 2; *Igf-2r*, insulin-like growth factor 2 receptor; Del, *Snord116del* mice injected with saline; Del + GH, *Snord116del* mice injected with recombinant growth hormone; WT, wild-type mice injected with saline.

involved in a mechanism by which GH regulates brain function, including a cognitive and neuroprotective effect.

Of particular interest from the present study is the GH-induced increase in the gene expression for IGF-2 and IGF-2R in the cerebellum. IGF-2, another mediator of GH action, is known to be important for brain development and to have neurotrophic or neuroprotective properties.<sup>29,30</sup> IGF-2 signaling has been implicated in cognitive function and it is suggested that the effect of IGF-2 as a memory enhancer is selectively mediated by IGF-2R. It was shown that IGF-2 promoted IGF-2R-dependent, persistent long-term potentiation, demonstrated by memory improvement.<sup>11</sup> While the precise mechanisms by which IGF-2 and IGF-2R are regulated remain to be investigated, our data suggest the possibility that IGF-2/IGF-2R signaling could have an important role in GH-induced cognitive function in *Snord116del* mice.

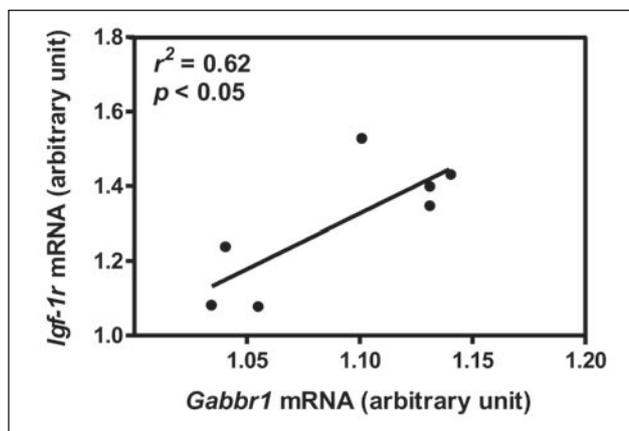
On the contrary to the effects seen in the cerebellum, the expression of GABA<sub>BR1</sub>, GABA<sub>BR2</sub>, IGF-1, IGF-1R, IGF-2 and IGF-2R in the hippocampus and cerebral cortex was unaffected by GH administration. The GH activity may be different regionally, because the brain is highly heterogeneously functional. Several potential mechanisms, such as differences in blood-brain barrier permeability and the distribution of GH receptor (GHR), may account for differences in the effects of GH on GABA<sub>B</sub> receptor subunits and GH/IGF axis expression in specific brain regions. When analysing the results in the present study we did not find any significant difference in cerebral GHR mRNA expression between the treatment groups (data not shown). Additionally, the amount of GH binding protein

may affect the response to GH by modulating the bound fraction of GH in *Snord116del* mice.

The clinical phenotype of individuals with PWS, including mental retardation, hypotonia, motor delay, and poor fine motor skills, support the idea that cerebellar development may be abnormal in these individuals. Several autopsy studies of individuals with PWS have shown abnormalities in the white matter of the cerebellum and one found partial hypoplasia of the right cerebellar hemisphere.<sup>31,32</sup> Additionally, quantitative structural magnetic resonance imaging (MRI) studies have been performed in patients with PWS that reported abnormalities in the cerebellum.<sup>33-36</sup> Many genetic disorders are associated with compromised cerebellar development.<sup>37</sup> In addition to being important for motor control, the cerebellum has connections to areas in the cerebrum which are relevant to cognition and behaviour.<sup>38</sup> A large structural imaging study found that there was a significant relationship between general cognitive ability (IQ) and the volume of the cerebellum.<sup>39</sup> Importantly, individuals with PWS have decreased cerebellar volumes and lower general cognitive ability (GIA) compared to controls.<sup>36</sup> Moreover, the neurobehavioural test of *Snord116del* mice has reported impaired motor learning in the rotarod test<sup>20</sup> that is a suitable test for evaluation of cerebellar deficits in rodents.<sup>40,41</sup> The rotarod task measures motor coordination and can also measure motor learning.<sup>42</sup> The cerebellum is highly implicated in the functioning of this task, and it is a well documented site of action for other learning paradigms.<sup>43</sup>

The local expression of GH and the presence of its receptor GHR in the cerebellum indicate that cerebellum is an autocrine and/or paracrine site of GH action.<sup>44</sup> As it is known that GH and IGF-I increase brain growth, myelination, and has neuroprotective properties<sup>45-47</sup> we could speculate that if the GH treatment had any effect on the brain, it would have a positive effect in terms of brain normalisation.

In conclusion, this study demonstrates that GH restores the gene expression of GABA<sub>BR1</sub> and IGF-1R and increases IGF-2 and IGF-2R in the cerebellum of *Snord116del* mice. The alterations of GABA<sub>BR1</sub> and IGF-1R observed in *Snord116del* mice could, at least partly, account for cognitive impairment. Because GHD during early life could impair proper brain development, thereby leading to cognitive deficits, it is suggested from the present study that a modulatory effect of GH on the expression of GABA<sub>BR1</sub> and GH/IGF-1 axis genes in brain may provide a mechanism for the GH-induced brain function in *Snord116del* mice, genetic models of PWS.



**Figure 5** Simple-regression analysis of the correlation between the GABA<sub>BR1</sub> and IGF-1R mRNA in the cerebellum of *Snord116del* mice (n=7) treated with GH (1.0 mg/kg).

## Acknowledgments

This paper was supported by Sungkyun Research Fund, Sungkyunkwan University, 2014 (# 2013-0716-000).

## Declaration of Interest

The authors declare that there is no conflict of interest.

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