Altered functional brain networks in Prader–Willi syndrome

Yi Zhang, Heng Zhao, Siyou Qiu, Jie Tian, Xiaotong Wen, Jennifer L. Miller, Karen M. von Deneen, Zhenyu Zhou, Mark S. Gold and Yijun Liu

Prader–Willi syndrome (PWS) is a genetic imprinting disorder characterized mainly by hyperphagia and early childhood obesity. Previous functional neuroimaging studies used visual stimuli to examine abnormal activities in the eating-related neural circuitry of patients with PWS. It was found that patients with PWS exhibited both excessive hunger and hyperphagia consistently, even in situations without any food stimulation. In the present study, we employed resting-state functional MRI techniques to investigate abnormal brain networks related to eating disorders in children with PWS. First, we applied amplitude of low-frequency fluctuation analysis to define the regions of interest that showed significant alterations in resting-state brain activity levels in patients compared with their sibling control group. We then applied a functional connectivity (FC) analysis to these regions of interest in order to characterize interactions among the brain regions. Our results demonstrated that patients with PWS showed decreased FC strength in the medial prefrontal cortex (MPFC)/inferior parietal lobe (IPL), MPFC/precuneus, IPL/precuneus and IPL/hippocampus in the default mode network; decreased FC strength in the pre/postcentral gyrus and dorsolateral prefrontal cortex (DLPFC)/orbitofrontal cortex (OFC) in the motor sensory network and prefrontal cortex network, respectively; and increased FC strength in the anterior cingulate cortex/insula, ventrolateral prefrontal cortex (VLPFC)/OFC and DLPFC/VLPFC in the core network and prefrontal cortex network, respectively. These findings indicate that there are FC alterations among the brain regions implicated in eating as well as rewarding, even during the resting state, which may provide further evidence supporting the use of PWS as a model to study obesity and to provide information on potential neural targets for the medical treatment of overeating. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: Prader–Willi syndrome; eating disorder; obesity; amplitude of low-frequency fluctuation; resting-state network; functional MRI

INTRODUCTION

Approximately 90 million Americans are overweight or obese, and more than 400 000 deaths are related to obesity or its associated diseases per year in the USA (1). Although much attention has been given to obesity in the Western world (2), developing countries are not immune to the ‘globesity’ epidemic (3,4). As obesity is associated with an increased risk of morbidity and mortality, there is a sense of urgency to understand the processes contributing to this epidemic.

Studies utilizing both human and animal models have revealed different aspects of this complicated disease (5–12). A genetic imprinting disorder, Prader–Willi syndrome (PWS), results in profound hyperphagia and early-childhood-onset

Abbreviations used: ACC, anterior cingulate cortex; ALFF, amplitude of low-frequency fluctuation; AMY, amygdala; BA, Brodmann area; BMI, body mass index; DLPFC, dorsolateral prefrontal cortex; DMN, default mode network; EPI, echo planar imaging; FC, functional connectivity; HIPP, hippocampus; HPAL, hypothalamus; IPL, inferior parietal lobe; MPFC, medial prefrontal cortex; MPRAGE, magnetization prepared rapid acquisition gradient echo; NAC, nucleus accumbens; OFC, orbitofrontal cortex; PCC, posterior cingulate cortex; PWS, Prader–Willi syndrome; ROI, region of interest; RS-fMRI, resting-state functional MRI; RSN, resting state network; VLPFC, ventrolateral prefrontal cortex; VMPC, ventromedial prefrontal cortex.
obesity caused by excessive and pathological overeating and reinforcement of food (13–20). It is important to note that
PWS is one of very few human genetic models for the study of
obesity. PWS has been associated with substance dependence
and has been chosen as a well-defined genetic model as it
may help to explain certain neuropsychological mechanisms that
affect appetite and food addiction (20,21). Thus, this model can
be investigated more comprehensively and applied to obesity
research in a better way than using animal models of obesity,
especially as a neuroimaging model (12).

Patients with PWS can be diagnosed genetically. About 70% of
cases are caused by a paternal genetic deletion on chromosome
15 (15q11-13) and 25% are from a maternal uniparental disomy
of chromosome 15 (22). The remaining 1–5% of cases of PWS
result from certain imprinting defects, which have a 50% poten-
tial risk of reoccurring in future offspring (23–25). In patients
diagnosed with PWS, specific brain genes, such as MAGEL2, 
MKNR3, NDN, SNURF-SNRPN and sno-RNA, are misrouted or lost,
resulting in abnormal cortical development (26).

Individuals with PWS are characterized as having dolichoce-
phaly, almond-shaped eyes, small mouth, small appendages,
decreased muscle tone (27), infantile hypotonia, hypogonadism,
short stature and early onset of obesity as a result of central
dysfunction (around 18–36 months of age) (28). They also show
major disturbances in appetite, sleep, breathing and metabolism
regulation. The abnormal eating behavior of patients with PWS is
manifested by delayed satiety, premature return of hunger after
eating a meal, the seeking and hoarding of food and food-
related objects and the ingestion of inanimate items (17). This
irregular pattern of food consumption leads to symptoms
displayed as excessive daytime drowsiness, poor ventilation,
hypercapnia and dental cavities (29). In addition to the common
propensity of overeating, compulsive and ritualistic behaviors,
patients with PWS also show pronounced emotional volatility
and a striking inability to control their emotions, which results
in frequent temper outbursts (30).

Functional neuroimaging studies of obesity and/or eating
disorders have been performed in patients with PWS using visual
cues to investigate the abnormalities of eating-related neural
activity (13–17,19,31–33). In response to high-versus low-calorie
food stimulation, these studies mainly found that patients with
PWS exhibited a delayed signal reduction after glucose adminis-
tration which was located in the hypothalamus (HPAL), insula,
ventromedial prefrontal cortex (VMPFC) and nucleus accumbens
(NAc) (19); hyperactivity in the limbic and paralimbic regions
that drive eating behavior [e.g. the amygdala (AMY)] and in
regions that suppress food intake [e.g. the medial prefrontal
cortex (MPFC)] (15,31); and increased activation in HPAL and
the orbitofrontal cortex (OFC) (13,14,32), VMPFC (17) and bilat-
eral middle frontal, right inferior frontal, left superior frontal
and bilateral anterior cingulate cortex (ACC) (16,32).

Most of the aforementioned studies investigated the
abnormalities of eating-related neural circuitry using food cues.
Recently, Kullmann et al. (34) used resting-state functional MRI
(RS-fMRI) to investigate the functional connectivity (FC) integrity
of resting-state networks (RSNs) related to food intake in lean
and obese subjects; García-García et al. (35) found that the FC
strength of the putamen nucleus in the salience network was
increased in the obese group during the resting state. The
relationship between the abnormalities in patients with PWS
and intrinsic or resting brain function involved in the regulation
of food-related and reward processing has yet to be evaluated.

In this study, we applied RS-fMRI to assess the baseline brain
activity levels and to examine the FC of brain networks (36–38)
in patients with PWS compared with their sibling control group.
We focused on four RSNs that involve brain areas reported previ-
sely in food and reward processing. The default mode network
(DMN) includes the MPFC, precuneus, hippocampus (HIPP), pos-
terior cingulate cortex (PCC) and inferior parietal cortex (34–39).
The second network, termed the core network, includes the ACC
and bilateral insula, which are part of the primary gustatory
cortex (40). The third network is the prefrontal lobe network,
which includes the dorsolateral prefrontal cortex (DLPFC),
ventrolateral prefrontal cortex (vPFC) and OFC. The prefrontal
cortex is involved in inhibitory processes (13,17,19,31). The fourth network, termed the motor sensory network, includes
the precentral and postcentral gyri (36,41,42).

Based on previous findings, we hypothesized that there are
abnormalities which may contribute to overeating in patients
with PWS during the resting state. These abnormalities alter
the FC of the four RSNs (DMN, core, prefrontal and motor
sensory), especially in brain regions known to be important
in food and reward regulation.

METHODS

Subjects

Twenty-seven patients with PWS and 21 of their siblings partici-
pated in the study of both functional changes in the brain and
neuroanatomical variability across their lifespan (43,44). Among
them, 21 children with PWS (10 girls, 11 boys; mean age, 7.3 ± 9.3 years) and 18 healthy siblings of these patients with
PWS (control group; 10 girls, eight boys, p = 0.632; mean age,
11.1 ± 8.4 years, p = 0.184) were selected. Other participants
were removed from the current analysis because of either exces-
sive head motion or failure to perform functional scanning. The
subjects with PWS were significantly more obese than controls,
as assessed by the body mass index (BMI; mean BMI, 33.1 versus
24.8; p = 0.02). None of the patients with PWS were being treated
with growth hormone or estrogen/androgen replacement at the
time of MRI scanning. Molecular testing was performed on all
subjects with PWS. Fourteen had a deletion of the chromosomal
15q11-13 region, whereas the remainder had maternal uniparen-
tal disomy of chromosome 15 (17). The overall research protocol
was approved by the Institutional Review Board at the University
of Florida, and informed consent was obtained from each partic-
ipant or from his/her legal guardian.

Image acquisition

The experiments were carried out on a 3.0-T head-dedicated
Siemens Allegra MRI scanner (Siemens, Munich, Germany). A
set of T1-weighted, high-resolution structural images was
acquired using a magnetization prepared rapid acquisition gradi-
ent echo (MPRAGE) sequence with a matrix size of 512 × 512,
TR = 1500 ms, TE = 4.38 ms, field of view of 240 mm × 240 mm,
flip angle of 8° and 160 contiguous slices (1.1–1.4 mm thick).
Then, a gradient echo T2*-weighted echo planar imaging (EPI)
sequence was used to acquire resting-state functional images
with the following parameters: TR = 3 s; TE = 25 ms; flip
angle, 90°; matrix size, 64 × 64; field of view, 240 mm × 240 mm;
in-plane resolution, 3.75 mm × 3.75 mm; 36 axial slices
(3.8 mm thick with no gap). The scan for RS-fMRI lasted for 300 s (5 min), containing 100 brain volumes.

**Image processing and measurement**

All of the imaging data were preprocessed and analyzed using Statistical Parametric Mapping 5 (SPM5, http://www.fil.ion.ucl.ac.uk/spm). The functional images first underwent slice-timing correction for within-scan acquisition time differences between slices, and were then realigned to the first volume to correct for interscan head motion. Next, we spatially normalized the realigned images to the standard EPI template and resampled them to a voxel size of 3 mm × 3 mm × 3 mm. Finally, the functional images were spatially smoothed with a Gaussian kernel of 6 mm × 6 mm × 6 mm full width at half-maximum (FWHM) to decrease spatial noise. After the linear trends had been removed, a band-pass filter between 0.01 and 0.08 Hz was applied to the data to remove the effects of very low-frequency drift and high-frequency noise using REST software (http://resting-fmri.sourceforge.net). Finally, the nuisance covariates, including head motion parameters, global mean signals, white matter signals and cerebrospinal fluid signals, were regressed and removed from the blood oxygenation level-dependent signals.

**Amplitude of low-frequency fluctuation (ALFF) analysis and definition of regions of interest (ROIs)**

The ALFF analysis was carried out using REST software (http://resting-fmri.sourceforge.net) to define the ROIs. The calculation procedure was the same as that reported in a previous study (45). Specifically, a filtered time series was transformed to the frequency domain with a fast Fourier transform, and thus the power spectrum was obtained. Because the power of a given frequency is proportional to the square of the amplitude of this frequency component, the square root was calculated at each frequency of the power spectrum and the averaged square root was obtained across 0.01–0.08 Hz at each voxel. This averaged square root was taken as the ALFF measurement. For standardization, the ALFF of each voxel was further divided by the global mean of the ALFF values. Then, voxel-wise two-sample t-tests were employed to compare the differences in ALFF between the PWS and control groups using SPM5 software. The brain regions showing significant ALFF alterations were selected as a mask to define the ROIs (p < 0.05, false discovery rate corrected). The ALFF values of each ROI were then averaged across subjects in the PWS and control groups, respectively. The significant ALFF difference of each ROI between the two groups was detected by a two-sample t-test.

**FC analysis**

After obtaining the ROIs that showed significant ALFF differences between the two groups, an FC analysis was carried out. We registered the ROIs backward to the original space of each subject, and then the time series of each ROI from the resting-state scan was extracted. For a given pair of ROIs, the zero-lag temporal correlation between them was calculated on the voxel time series for all pair-wise combinations between X and Y, and then averaged (46). After averaging the strength across the subjects of the two groups, we obtained the FC value between ROIs within the four RSNs, respectively. The significant FC alteration of each pair-wise ROI between the two groups was detected by a two-sample t-test (p < 0.0001).

**RESULTS**

**Altered resting-state ALFF in patients with PWS**

Compared with the controls, the patients with PWS exhibited a significant difference in ALFF in the MPFC (Brodmann area, BA 9/10), ACC (BA 32/24), parahippocampus (BA 28/34), HIPP, AMY, insula, thalamus, caudate, HPAL, DLPFC, VLPFC, OFC, midbrain, pre- and postcentral gyr, the temporal gyr, inferior and superior parietal lobule, and pre- and postcentral gyri. The temporal gyr, inferior and superior parietal lobule, and pre- and postcentral gyri. The temporal gyr, inferior and superior parietal lobule, and pre- and postcentral gyri.
parietal gyri, precuneus, cuneus and fusiform gyrus ($p < 0.05$, false discovery rate corrected). No significant difference was found in the PCC. Among these brain areas, the MPFC, ACC, insula, Hipp, DLPFC, VLPFC, OFC, IPL, precuneus, and pre- and postcentral gyri (Fig. 1 and Table 1) were chosen as the ROIs to be used in further FC analyses.

Further quantitative analyses of the mean ALFF values were calculated to compare the baseline brain activity level changes in these ROIs using a two-sample t-test (Fig. 2). Our results showed a trend of higher ALFF values in the IPL, ACC, insula and VLPFC, and lower ALFF values in the MPFC, Hipp, precuneus, pre- and postcentral gyri, DLPFC and OFC, in the PWS group relative to the controls. All of the regions showed statistically significant ALFF alterations between the two groups, except in the insula and DLPFC ($p < 0.001$).

### Altered FC in patients with PWS

Correlation values of the pair-wise ROIs from the two groups were obtained using an FC analysis. Compared with the control group, the PWS group showed decreased FC strength in the MPFC/IPL, MPFC/precuneus, IPL/precuneus and IPL/Hipp in the DMN; decreased FC strength in the pre-/postcentral gyri and DLPFC/OFC in the motor sensory network and prefrontal cortex network, respectively; and increased FC strength in the ACC/insula, VLPFC/OFC and DLPFC/VLPFC in the core network and prefrontal cortex network, respectively ($p < 0.001$) (Fig. 3). No significant FC alterations were observed in the MPFC/Hipp and Hipp/precuneus.

### DISCUSSION

The aim of this study was to explore the effects of PWS on the integrity of FC within the DMN, core, prefrontal lobe and motor sensory networks. In the present study, we used RS-fMRI to investigate the FC alterations of RSNs in children with PWS. We first used ALFF analysis to define the ROIs that showed significant alterations of resting-state brain activity levels in the patients. Then, based on the selected ROIs, we applied the FC method to characterize interactions among these brain regions. The evidence indicated that there were substantial differences during the resting state in the brain networks related to reward and food regulation compared with sibling controls.

In the DMN, patients with PWS showed decreased FC strength in the MPFC/IPL, MPFC/precuneus, IPL/precuneus and IPL/Hipp. The MPFC is closely connected to the OFC, which is involved in stimulus reward and evaluation in sync with direct multisensory input (47). Early reports on the neural mechanisms of hyperphagia noted strong MPFC activation in individuals with PWS (14,17,31,48). Hinton et al. (48) reported a lack of association between activation in the MPFC and ratings of the reward value of various foods in a group of individuals with PWS with deletion, suggesting that, although these individuals did not have higher ratings on food desirability compared with the control group, they displayed hyperactivity in regions associated with behavioral disinhibition and lack of self-control. The MPFC is also involved in the inhibition of emotion and regulation of appetitive drives. Results indicate that food motivation in obese individuals is associated with increased self-reported disinhibition and hunger, and increased activation in the ACC and MPFC.

### Table 1. Brain regions of interest (ROIs) showing significant amplitude of low-frequency fluctuation (ALFF) alterations between patients with Prader–Willi syndrome (PWS) and controls in the resting state. The results were obtained using two-sample t-tests ($p < 0.05$, false discovery rate corrected)

<table>
<thead>
<tr>
<th>ROI</th>
<th>Hemisphere</th>
<th>Brodmann area</th>
<th>Montreal Neurological Institute atlas</th>
<th>t value (max)</th>
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<tbody>
<tr>
<td>Medial prefrontal cortex</td>
<td>L</td>
<td>9</td>
<td>x: -3, y: -24, z: 63</td>
<td>-3.07</td>
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<tr>
<td></td>
<td>R</td>
<td>9/10</td>
<td>x: 6, y: 54, z: 42</td>
<td>-2.91</td>
</tr>
<tr>
<td>Inferior parietal lobe</td>
<td>L</td>
<td>40</td>
<td>x: -51, y: -42, z: 48</td>
<td>2.31</td>
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<tr>
<td></td>
<td>R</td>
<td>40</td>
<td>x: 51, y: -39, z: 54</td>
<td>2.63</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>L</td>
<td>40</td>
<td>x: -30, y: -36, z: -9</td>
<td>-2.08</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>27</td>
<td>x: -14, y: -42, z: 3</td>
<td>-1.77</td>
</tr>
<tr>
<td>Precuneus</td>
<td>L</td>
<td>7</td>
<td>x: -3, y: -54, z: 51</td>
<td>-1.96</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>7</td>
<td>x: 15, y: -45, z: 54</td>
<td>-1.92</td>
</tr>
<tr>
<td>Anterior cingulate cortex</td>
<td>L</td>
<td>32/24</td>
<td>x: -9, y: 39, z: 15</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>32</td>
<td>x: 6, y: 9, z: 24</td>
<td>3.11</td>
</tr>
<tr>
<td>Insula</td>
<td>L</td>
<td>9/6</td>
<td>x: -36, y: -15, z: 6</td>
<td>1.93</td>
</tr>
<tr>
<td>Dorsolateral prefrontal cortex</td>
<td>L</td>
<td>9/46</td>
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<tr>
<td></td>
<td>R</td>
<td>9/46</td>
<td>x: 37, y: 50, z: 33</td>
<td>-2.89</td>
</tr>
<tr>
<td>Ventrolateral prefrontal cortex</td>
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<td>45</td>
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<td>2.85</td>
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<tr>
<td></td>
<td>R</td>
<td>45</td>
<td>x: 46, y: 42, z: 32</td>
<td>2.60</td>
</tr>
<tr>
<td>Orbitofrontal cortex</td>
<td>L</td>
<td>10</td>
<td>x: -34, y: 62, z: 5</td>
<td>-1.76</td>
</tr>
<tr>
<td>Precentral</td>
<td>L</td>
<td>4/6</td>
<td>x: -21, y: -24, z: 61</td>
<td>-2.39</td>
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<tr>
<td></td>
<td>R</td>
<td>4/6</td>
<td>x: 38, y: -11, z: 65</td>
<td>-1.80</td>
</tr>
<tr>
<td>Postcentral</td>
<td>L</td>
<td>10</td>
<td>x: -47, y: -31, z: 51</td>
<td>-1.92</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>46</td>
<td>x: 46, y: -28, z: 56</td>
<td>-2.28</td>
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autonomic and endocrine effects, such as gastric secretion (56) and increased food consumption (55). HIPP activation also occurs in a number of studies involving food-related stimuli, hunger and food craving (57–61).

In the core network, theinsula and ACC showed increased FC strength in patients with PWS. The insula cortex is considered to be the primary gustatory cortex (62,63) and is also involved in motivational processes (64,65), representing an important relay of the neural circuitry connecting HPAL, OFC and the limbic system (66). It is possible that the delayed satiety response is the result of disturbance in the perception of inner physiological states. This would relate to the suggestion that people with PWS may have a problem with interoception (insula), or the perception of internal sensation (67). The insula is also known to receive gustatory, olfactory and visceral afferents involved in taste memory (68), and has been implicated in the experience of emotion (69). Activation in the insula has also been reported in studies using other methodologies (e.g., hunger) to manipulate the desire for food (70–72). Insular activation in response to food cues has also been found to be correlated with the desire to eat, as well as to hunger and prospective food intake. These findings indicate that the insula is a brain region that is important in the processing of food-related cues, both internal and external, and appears to be important in processing the motivational value of food and feeding. The ACC is involved in the executive control of internal and external stimuli regulating context-dependent behaviors (73–75). The ACC has both cognitive (e.g., working memory or attention) and affective functions. The affective subdivision is involved in the assessment of the salience of emotional information and in the regulation of emotional responses, and is connected to a number of other areas that show liking- or craving-related activation, including the AMY, insula and HIPP (76). The ACC can contribute to an increased risk for overeating through an imbalance between cognitive and emotional processing.

In the prefrontal lobe network, the VLPFC/OFC and DLPFC/VLPFC exhibited increased FC strength, whereas the DLPFC/OFC showed decreased FC strength. The DLPFC is linked to numerous aspects of top-down control, especially executive functions; goal selection, planning, manipulation of information and response inhibition (77–79). Top-down processing in the lateral PFC allows regions such as the DLPFC to influence processing in other regions to obtain a common goal. This role in cognitive control might extend to decision-making related to...
food intake, as suggested by findings of increased DLPFC activity during satiation in a group of successful dieters (80). The DLPFC is also involved in working memory, including holding the reward value in mind whilst judging potential outcomes of a given set of behavioral responses (81). The VLPFC is involved in more cognitive aspects of emotional material. The findings of a positive correlation between increased heart rate deceleration and increasing activity in the VLPFC indicated that the more intense the orienting response, especially to food cues, the greater the activation of the VLPFC. The OFC is believed to play a role in stimulus–reinforcement learning (82), with extensive connections to AMY that have been implicated in affect-driven motivational behavior (31). In addition to the integration of information from multiple sensory inputs, the OFC serves a role in reward learning. The FC strength alteration of the OFC indicated dysfunction in the rewarding–learning circuit in the PWS group.

In addition, in the motor sensory network, the pre- and post-central gyri showed decreased FC strength. These two regions drive the control of motor activity and are involved in the execution of gestural or graphic sequences in motor program elaboration, and therefore are involved in visually guided movements (83). Abnormal FC strength between the regions results in perceptive agnosia, particularly in incorrect perceptions of the consistency, size, weight and shape of objects. Patients with PWS showed a significant delay in the ‘block design scale’ of the Wechsler test, which targets the ability to combine visual and motor processes (16).

The present study has several limitations. First, PWS is a rare disease and the small sample size limits the generalization of our findings. As a result of the low prevalence of the disease, it is difficult to find large numbers of participants, particularly when recruiting for an fMRI study which requires strict inclusion criteria. In addition, although we balanced the gender, the age range among the patients with PWS and the control samples is still relatively large.

CONCLUSIONS

In the present study, we sought to investigate the abnormal brain networks in children with PWS using rs-fMRI. Our results demonstrated that patients with PWS showed decreased FC strength in the MPFC/IPL, MPFC/precentral, IPL/precentral and IPL/HIP in the DMN; decreased FC strength in the pre/post-central gyri and DLPFC/OFC in the motor sensory network and prefrontal cortex network, respectively; and increased FC strength in the ACC/insula, VLPCF/OFC and DLPFC/VLPFC in the core network and prefrontal cortex network, respectively. These findings indicated that there were FC alterations among those brain regions implicated in eating as well as rewarding in patients with PWS during the resting state, which may provide further evidence supporting the use of PWS as a model to study food addiction and to provide information on potential neural targets for use in medical treatment for overeating.

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We employed resting-state functional MRI to investigate abnormal brain networks related to eating disorders in children with Prader–Willi syndrome (PWS). Our results demonstrate that patients with PWS show abnormal functional connectivity in the default mode network, core network, motor sensory network and prefrontal cortex network. These findings may provide information for potential neural targets for use in the medical treatment of overeating.
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- Click on the Strikethrough (Del) icon in the Annotations section.

### 3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

- Add sticky note Tool – for making notes at specific points in the text.

**How to use it**

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.
5. **Attach File** Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

**How to use it**
- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you’d like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

6. **Add stamp** Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

**How to use it**
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. **Drawing Markups** Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.

**How to use it**
- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: