Dendritic cell nuclear protein-1, a novel depression-related protein, upregulates corticotropin-releasing hormone expression

Tian Zhou,1,2 Shanshan Wang,3 Haigang Ren,1 Xin-Rui Qi,1,2 Sabina Luchetti,2 Willem Kamphuis,2 Jiang-Ning Zhou,1 Guanghui Wang1 and Dick F. Swaab2

1 Key Laboratory of Brain Function and Diseases, School of Life Sciences, University of Science and Technology of China, Chinese Academy of Sciences Jinzhai Road 96, Hefei 230026, Anhui, People’s Republic of China
2 Netherlands Institute for Neuroscience, 1105 BA Amsterdam, The Netherlands
3 Chinese PLA General Hospital, Beijing 100853, People’s Republic of China

Correspondence to: Prof. Dick F. Swaab, MD, PhD, Netherlands Institute for Neuroscience, Meibergdreef 47, 1105 BA Amsterdam, The Netherlands
E-mail: d.f.swaab@nin.knaw.nl

Correspondence may also be addressed to: Dr. Guanghui Wang, Key Laboratory of Brain Function and Diseases, School of Life Sciences, University of Science and Technology of China, Chinese Academy of Sciences Jinzhai Road 96, Hefei 230026, Anhui, People’s Republic of China
E-mail: wghui@ustc.edu.cn

The recently discovered dendritic cell nuclear protein-1 is the product of a novel candidate gene for major depression. The A allele encodes full-length dendritic cell nuclear protein-1, while the T allele encodes a premature termination of translation at codon number 117 on chromosome 5. In the present study we investigate whether the two forms of dendritic cell nuclear protein-1 might act on corticotropin-releasing hormone, which plays a crucial role in the stress response and in the pathogenesis of depression. The messenger RNA expression of dendritic cell nuclear protein-1 appeared to be increased in the laser micro-dissected paraventricular nucleus of patients with depression compared with control subjects. Dendritic cell nuclear protein-1 was also found to be co-localized with corticotropin-releasing hormone in paraventricular nucleus neurons. Moreover, full-length dendritic cell nucleus protein-1 bound to and transactivated the promoter of corticotropin-releasing hormone in human embryonic kidney 293 cells. We propose that full-length dendritic cell nucleus protein-1 may play a role in the pathogenesis of depressive disorders by enhancing corticotropin-releasing hormone expression in the hypothalamic paraventricular nucleus.

Keywords: DCNP1; depression; CRH; PVN; hypothalamus

Abbreviations: cDNA = complementary DNA; CRH = corticotropin-releasing hormone; DCNP1 = dendritic cell nuclear protein-1; EGFP = enhanced green fluorescent protein; PCR = polymerase chain reaction
**Introduction**

Dendritic cell nuclear protein-1 (DCNP1) is officially designated chromosome 5 open reading frame 20. It was named after its localization in the perinucleus of mature and, to a lesser extent, immature dendritic cells. DCNP1 is a recently discovered protein that is expressed mainly in human brain and skeletal muscle (Masuda et al., 2002). No information has been available on the pattern of its localization in the human brain. Interestingly, DCNP1 has been proposed to be a novel candidate gene for major depression (Willis-Owen et al., 2006) and this finding has been recently replicated (Bosker et al., 2010). Homozygous carriers of the T allele of DCNP1, which encodes a premature termination of translation at codon number 117 on chromosome 5, were reported to have an increased risk for major depression, but the A/T heterozygotes were excluded from the analysis (Willis-Owen et al., 2006). A recent replication reported that not only the homozygous carriers of the T allele of DCNP1, but also the A/T heterozygotes had an increased risk for major depression (Bosker et al., 2010). We tested the functional effects of both forms of DCNP1: the A allele, which represents full-length DCNP1, and the T allele, which denotes the truncated form of DCNP1 (DCNP1^A/T^). We studied whether its relationship to depression might be explained by an effect of DCNP1 on corticotropin-releasing hormone (CRH).

CRH, produced in hypothalamic paraventricular nucleus neurons, plays a crucial role in the stress response and in the pathogenesis of depression (Raadsheer et al., 1994, 1995; Bao et al., 2008; Belmaker and Agam, 2008; Wang et al., 2008; Holsboer and Ising, 2010). In a subpopulation of patients the role of hyperactivated CRH expression in depression appears from (i) an increase in CRH messenger RNA (mRNA) in the paraventricular nucleus (Raadsheer et al., 1994; Wang et al. 2008); (ii) a 4-fold increase in the number of CRH expressing neurons in the paraventricular nucleus (Raadsheer et al., 1995; Bao et al., 2005); (iii) an increased number of CRH neurons co-expressing vasopressin (Raadsheer et al., 1994); and (iv) increased CRH levels in the cerebrospinal fluid (Banki et al., 1992). Additional evidence for the role of CRH in depression is that similar symptoms, such as decreased food intake, decreased sexual activity, disturbed sleep and anxiety, can all be induced in experimental animals by intracerebroventricular injection of CRH (Holsboer, 2001). An additional argument comes from a transgenic mouse model with over-expression of CRH that showed increased anxiety, a symptom related to major depression, which could be counteracted by injection of a CRH antagonist (Stenzel-Poore et al., 1994). Furthermore, depression is susceptible to treatment with CRH receptor antagonists (Keck and Holsboer, 2001; O’Brien et al., 2001; Grammatopoulos and Chrousos, 2002). Lastly, anti-depressants attenuate the synthesis of CRH, possibly by upregulation of corticosteroid receptor expression, and cause a decrease in CRH CSF levels (Heuser et al., 1998). These arguments have led to the CRH hypothesis of depression (Bao et al., 2008; Belmaker and Agam, 2008; Holsboer and Ising, 2010). A number of studies have described different genes that are involved in the regulation of activity of CRH neurons in the paraventricular nucleus and may take part in the pathogenesis of affective disorders (Bao et al., 2005; Wang et al., 2008; Chen et al., 2009).

Due to the central role of CRH in the pathogenesis of depression and the risk of depression associated with DCNP1, we hypothesized that DCNP1 expression may be enhanced in depression, acting on CRH transcription in these neurons and thus increasing the risk for this mood disorder. To test this hypothesis, DCNP1 transcript levels were determined in the paraventricular nucleus and, as a control area, the supraoptic nucleus of seven patients with depression and seven matched controls by means of laser micro-dissection of cryostat sections followed by real-time quantitative polymerase chain reaction (PCR) (Wang et al., 2008). To examine the possible underlying mechanism by which DCNP1 influences CRH expression, we showed that DCNP1 is co-localized with CRH in hypothalamic paraventricular nucleus. We also performed chromatin immunoprecipitation and showed that DCNP1 interacts with the CRH promoter. Subsequently, we showed that the full-length DCNP1 could significantly transactivate CRH expression in cells.

The data from the present study indicate the presence of a molecular mechanism by which full-length DCNP1 could upregulate CRH activity and may thus be involved in depression. The widespread distribution of DCNP1 in the human brain suggests that DCNP1 may, in addition, be involved in a variety of other central processes.

**Materials and methods**

**Subjects**

Brain material was obtained from the Netherlands Brain Bank following permission from the patient or the next of kin for a brain autopsy and for the use of the brain material and clinical information for research purposes. We used brain material from a total of 49 patients for this study. Each patient with mood disorder and each control were confirmed by systematic neuropathological investigation (Van de Nes et al., 1998).

For the laser micro-dissection experiment, the hypothalamus was obtained by autopsy and snap frozen from 14 subjects, seven of whom were clinically diagnosed with depressive disorder and seven served as controls matched for sex, age, post-mortem delay, season and clock time at death (for clinicopathological information see Table 1, C8–C14 and D1–D7) (Wang et al., 2008). Patients suffering from depression were diagnosed during life and the diagnosis was checked retrospectively by a certified psychiatrist (Dr G. Meynen) using the medical records. The medical records did not reveal any alcohol or other drug abuse amongst subjects of either group (Wang et al., 2008).

The rest of the materials for the present study were formalin-fixed and paraffin-embedded. Two male subjects without mood disorder were used for in situ hybridization (Table 1, C1–C2). Ten different brain regions, i.e. the paraventricular nucleus, supraoptic nucleus, thalamus, nucleus basalis of Meynert, occipital cortex, caudate nucleus, cingulate cortex, frontal cortex, cerebellum and parietal cortex from five matched subjects without mood disorder were used for immunohistochemical analysis (Table 1, C3–C7). Three patients with depression were used for immunofluorescence (Table 1, D8–D10). From 14 patients with depression and 14 matched controls, the superior gyrus of prefrontal cortex was used, and from 12 patients with
<table>
<thead>
<tr>
<th>Code</th>
<th>NBB no.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PMD (h:min)</th>
<th>pH CSF</th>
<th>BW (g)</th>
<th>CTD (h:min)</th>
<th>Medication taken in the past</th>
<th>Medication in the last 3 months</th>
<th>Month of death</th>
<th>Cause of death</th>
<th>Neuropathological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>00-029</td>
<td>M</td>
<td>47</td>
<td>29:13</td>
<td>ND</td>
<td>1284</td>
<td>11:47</td>
<td>None</td>
<td>None</td>
<td>1</td>
<td>Acute myocardial infarction</td>
<td>Non-demented control</td>
</tr>
<tr>
<td>C2</td>
<td>97-161</td>
<td>M</td>
<td>53</td>
<td>24:00</td>
<td>ND</td>
<td>1587</td>
<td>10:55</td>
<td>None</td>
<td>None</td>
<td>5</td>
<td>Sepsis</td>
<td>Control with space-occupying process</td>
</tr>
<tr>
<td>C3</td>
<td>97-045</td>
<td>F</td>
<td>55</td>
<td>5:35</td>
<td>ND</td>
<td>1363</td>
<td>10:10</td>
<td>None</td>
<td>None</td>
<td>5</td>
<td>Intraventricular bleeding, rupture in ventricles</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C4</td>
<td>94-125</td>
<td>M</td>
<td>51</td>
<td>06:00</td>
<td>6.5</td>
<td>1518</td>
<td>17:15</td>
<td>Chlorpromazine, Primperan, Morphine Fraxipar, Fentanyl, Domicum</td>
<td>None</td>
<td>12</td>
<td>Progressive liposarcoma and ileus</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C5</td>
<td>98-006</td>
<td>M</td>
<td>50</td>
<td>08:30</td>
<td>6.7</td>
<td>1436</td>
<td>11:00</td>
<td>None</td>
<td>None</td>
<td>1</td>
<td>Cardiac arrest</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C6</td>
<td>01-011</td>
<td>F</td>
<td>46</td>
<td>10:25</td>
<td>5.8</td>
<td>1197</td>
<td>02:35</td>
<td>None</td>
<td>None</td>
<td>1</td>
<td>Multi organ failure</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C7</td>
<td>95-007</td>
<td>M</td>
<td>54</td>
<td>09:10</td>
<td>6.9</td>
<td>1335</td>
<td>09:15</td>
<td>None</td>
<td>None</td>
<td>1</td>
<td>Bleeding from right communicating carotid artery</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C8</td>
<td>05-068</td>
<td>M</td>
<td>56</td>
<td>9:15</td>
<td>6.54</td>
<td>1553</td>
<td>4:45</td>
<td>None</td>
<td>None</td>
<td>10</td>
<td>Myocardial infarction</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C9</td>
<td>99-067</td>
<td>F</td>
<td>59</td>
<td>6:20</td>
<td>6.67</td>
<td>1156</td>
<td>13:30</td>
<td>None</td>
<td>None</td>
<td>6</td>
<td>Ileus, larynx carcinoma</td>
<td>Non-demented control</td>
</tr>
<tr>
<td>C10</td>
<td>06-073</td>
<td>M</td>
<td>66</td>
<td>7:45</td>
<td>6.7</td>
<td>1590</td>
<td>17:45</td>
<td>Testosterone</td>
<td>Testosterone</td>
<td>5</td>
<td>Ruptured abdominal aorta aneurysm</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C11</td>
<td>05-084</td>
<td>M</td>
<td>56</td>
<td>14:00</td>
<td>7.03</td>
<td>1323</td>
<td>2:00</td>
<td>None</td>
<td>None</td>
<td>55</td>
<td>Congestive heart failure</td>
<td>Non-demented control; Braak stage 0</td>
</tr>
<tr>
<td>C12</td>
<td>05-019</td>
<td>M</td>
<td>74</td>
<td>5:00</td>
<td>6.7</td>
<td>1125</td>
<td>2:00</td>
<td>Digoxin</td>
<td>Mo, Hal</td>
<td>4</td>
<td>Bronchus carcinoma, cardiac decompensation</td>
<td>Non-demented control; Braak stage III</td>
</tr>
<tr>
<td>C13</td>
<td>01-033</td>
<td>M</td>
<td>75</td>
<td>6:20</td>
<td>6.18</td>
<td>1180</td>
<td>6:10</td>
<td>None</td>
<td>None</td>
<td>3</td>
<td>Dehydration/Pneumonia</td>
<td>Non-demented control; Braak stage I</td>
</tr>
<tr>
<td>C14</td>
<td>99-111</td>
<td>F</td>
<td>88</td>
<td>5:40</td>
<td>6.07</td>
<td>1054</td>
<td>3:05</td>
<td>Digoxin</td>
<td>None</td>
<td>1</td>
<td>Respiratory insufficiency</td>
<td>Non-demented control; Braak stage III</td>
</tr>
<tr>
<td>D1</td>
<td>01-074</td>
<td>M</td>
<td>45</td>
<td>7:00</td>
<td>6.55</td>
<td>1427</td>
<td>2:30</td>
<td>SSRI, BZD</td>
<td>SSRI</td>
<td>6</td>
<td>Haemorrhage in pons</td>
<td>Major Depression; Braak stage 0</td>
</tr>
<tr>
<td>D2</td>
<td>99-115</td>
<td>F</td>
<td>57</td>
<td>5:30</td>
<td>6.28</td>
<td>1345</td>
<td>20:45</td>
<td>TCA, BZD, ZUC</td>
<td>BZD</td>
<td>9</td>
<td>Legal euthanasia because of multiple acquired handicaps, intractable pain, shortness of breath</td>
<td>Major depression (melancholic subtype)</td>
</tr>
<tr>
<td>D3</td>
<td>02-014</td>
<td>M</td>
<td>68</td>
<td>16:46</td>
<td>6.64</td>
<td>1424</td>
<td>ND</td>
<td>Li, ZUC</td>
<td>None</td>
<td>2</td>
<td>Subdural haemorrhage</td>
<td>Bipolar disorder; Braak stage I0</td>
</tr>
<tr>
<td>D4</td>
<td>99-118</td>
<td>M</td>
<td>68</td>
<td>5:55</td>
<td>6.82</td>
<td>1204</td>
<td>23:15</td>
<td>Li, SSRI</td>
<td>None</td>
<td>10</td>
<td>Cardiac ischaemia</td>
<td>Major depression; Braak stage IA</td>
</tr>
<tr>
<td>D5</td>
<td>02-051</td>
<td>M</td>
<td>81</td>
<td>6:00</td>
<td>6.5</td>
<td>1345</td>
<td>15:30</td>
<td>TCA, Hal</td>
<td>Hal</td>
<td>6</td>
<td>Renal insufficiency</td>
<td>Major depression; Braak stage IIIC</td>
</tr>
<tr>
<td>D6</td>
<td>06-021</td>
<td>M</td>
<td>70</td>
<td>6:23</td>
<td>6.53</td>
<td>1488</td>
<td>13:07</td>
<td>Li</td>
<td>Li, Mo</td>
<td>3</td>
<td>Severe neck trauma and pneumonia</td>
<td>Bipolar disorder; Braak stage III</td>
</tr>
<tr>
<td>D7</td>
<td>06-011</td>
<td>F</td>
<td>60</td>
<td>4:20</td>
<td>ND</td>
<td>1080</td>
<td>16:10</td>
<td>SSRI, BZD, Hal, Mo, Tamoxifen Temesta, Mogamid Mianserin, Loxazepam Temazepam, Digoxine</td>
<td>Hal</td>
<td>1</td>
<td>Legal euthanasia because of metastasized mamma carcinoma</td>
<td>Major depression; Braak stage IA</td>
</tr>
<tr>
<td>D8</td>
<td>92-003</td>
<td>F</td>
<td>55</td>
<td>06:45</td>
<td>6.4</td>
<td>1320</td>
<td>07:45</td>
<td>Mogamid Mianserin, Loxazepam Temazepam, Digoxine</td>
<td>Fluoxetine</td>
<td>11</td>
<td>Suspected urosepsis</td>
<td>Depression and diabetes mellitus type II</td>
</tr>
<tr>
<td>D9</td>
<td>94-065</td>
<td>F</td>
<td>72</td>
<td>28:25</td>
<td>ND</td>
<td>116</td>
<td>04:20</td>
<td>Mogamid Mianserin, Loxazepam Temazepam, Digoxine</td>
<td>Brotizolam</td>
<td>4</td>
<td>Septic shock</td>
<td>Depression; Braak stage IA</td>
</tr>
<tr>
<td>D10</td>
<td>94-082</td>
<td>M</td>
<td>71</td>
<td>16:15</td>
<td>ND</td>
<td>975</td>
<td>16:15</td>
<td>Mogamid Mianserin, Loxazepam Temazepam, Digoxine</td>
<td>MAO-antagonists</td>
<td>2</td>
<td>Bronchopneumonia, cerebral ischaemia</td>
<td>Depression</td>
</tr>
</tbody>
</table>

Braak stages (neuropathological distribution of neurofibrillary Alzheimer's disease changes over the brain): stage 0: no neurofibrillary changes; stages I/II: mild/severe alterations in the entorhinal cortex; stage III: first involvement of the hippocampus. Clinically, stages 0–II are unaffected controls and in stage III mild cognitive impairment may start (Braak and Braak, 1991). BW = brain weight; BZD = benzodiazepine; C = control; CSF = cerebrospinal fluid; CTD = clock time at death; D = depression; F = female; Hal = haloperidol; Li = lithium; M = male; Mo = morphine; NBB no = Netherlands Brain Bank number; ND = no data; PMD = post-mortem delay; SSRI = selective serotonin reuptake inhibitor; TCA = tricyclic antidepressant; ZUC = zuclopenthixol.

Dendritic cell nuclear protein-1 and corticotropin-releasing hormone Brain 2010: 133; 3069–3079 | 3071
depression and 12 controls the anterior cingulate cortex was used to verify the specificity of the increased transcription of DCNP1 in the paraventricular nucleus.

**Human brain material**

Tissues were dissected and fixed in 0.1 M phosphate buffered 4% w/v formaldehyde (pH 7.2) for 1–2 months (Table 1). After dehydration in graded ethanol, they were embedded in paraffin and the hypothalami were serially cut in frontal sections (6 μm) on a microtome (Leitz). Every 100th section was stained with thionine (0.1% w/v thionine in acetate buffer, pH 4) in order to localize the structures before immunohistochemical staining.

**In situ hybridization**

Full-length human DCNP1 complementary DNA (cDNA) was amplified by real time PCR using the primers 5’-gaattcgaactatgacatgacgagca-3’ and 5’-ttgatcctggctatgcagttc-3’, with total RNA extracted from human embryonic kidney 293 cells and inserted in-frame into the expression vector EGFP-N1 (Clontech) at Sal I and BamH I sites. N-terminal 1-116 amino acid of DCNP1 was amplified by PCR from full-length DCNP1 using the primers 5’-gaattcgaactatgacatgacgagca-3’ and 5’-ctggtacctctgctgtaatcttgc-3’ and inserted into EGFP-N1 at Sal I and BamH I sites.

Digoxigenin-labelled RNA probes were generated from a completely linearized full-length DCNP1 using the appropriate RNA polymerases (T7 or T3). The sections were treated with 0.1 M HCl after deparaffinization and hydrated. The sections were then incubated in 0.1 M triethanolamine containing 0.25% acetic anhydride, dehydrated in an ascending ethanol series and air-dried. The digoxigenin-labelled full-length DCNP1 probe was prepared at a concentration of 1.5 ng/μl. Hybridization was performed overnight at 60°C. After hybridization, sections were washed in 0.2× standard saline citrate at 60°C, incubated with alkaline phosphatase-coupled antibodies to digoxigenin (Boehringer Mannheim) and developed in the dark with 4-nitroblue tetrazolium chloride (Sigma), 5-bromo-4-chloro-3-indolylphosphate (Sigma) and levamisole (Sigma). After development the sections were dried and mounted with glass cover slips. The anti-sense probe (Sigma) and levamisole (Sigma). After development the sections were then incubated with secondary horseradish peroxidase–conjugated antibody (1:100) (Dakocytomation). Sections were then incubated for 10 min in Tris buffered saline containing 0.05% 3,3’-diaminobenzidine (Sigma), 0.01% hydrogen peroxide and 0.3% nickel–ammonium sulphate. Having been developed, dehydrated and cleared, the sections were then cover slipped with entellan mounting medium (Merck).

**Laser micro-dissection of the paraventricular nucleus and supraoptic nucleus**

Freshly frozen hypothalami were serially sectioned at −15°C on a cryostat (Leica CM 1850 UV) at a thickness of 20 μm from rostral to caudal. The tissue sections were thaw-mounted on a slide coated with a plain film (Birkelbach Film). Every 10th section from rostral to caudal throughout the hypothalamus was stained by thionine and used to define the beginning and the end of the paraventricular nucleus or supraoptic nucleus. One out of three unstained paraventricular nucleus or supraoptic nucleus sections was collected for laser micro-dissection. The frozen sections were dissected by a photo-activated localization microscopy microlaser system (P.A.L.M Microlaser Technologies) and collected by hand using a tiny needle (Koning et al., 2007; Wang et al., 2008).

**RNA isolation**

An adaptation of the Qiagen RNeasy protocol (Qiagen) was used for RNA extraction of laser micro-dissected tissues. Trizol (Invitrogen Life Technologies) was added to the vial containing all dissected material of the paraventricular nucleus or supraoptic nucleus of one patient. After spinning down, the supernatant was transferred to a new vial and mixed with chloroform and centrifuged for 15 min. The upper aqueous phase was transferred into a clean vial and an equal volume of freshly prepared 70% ethanol was added, after which the sample was loaded onto an RNeasy column. The RNA quantity was measured by NanoDrop 1000 spectrophotometer (NanoDrop Technologies) and the quality of RNA was determined by means of a 2100 BioAnalyser (Agilent Technologies). RNA integrity number was used to assess the RNA quality (scale 1–10, with 1 being the lowest and 10 being the highest RNA quality).

**Antibodies**

A polyclonal antibody against DCNP1, recognizing both forms of DCNP1, was raised by immunizing a New Zealand white rabbit with glutathione-S-transferase conjugated purified DCNP1 1–116 fusion protein as an antigen. To confirm the specificity of our polyclonal antibody, we performed two independent experiments in human embryonic kidney 293 cells and inserted in-frame into the expression vector EGFP-N1 at Sal I and BamH I sites.

A polyclonal antibody against DCNP1, recognizing both forms of DCNP1, was raised by immuno-izing a New Zealand white rabbit with glutathione-S-transferase conjugated purified DCNP1 1–116 fusion protein as an antigen. To confirm the specificity of our polyclonal antibody, we performed two independent experiments in human embryonic kidney 293 cells and inserted in-frame into the expression vector EGFP-N1 at Sal I and BamH I sites.

**Immunohistochemical studies**

Localization of DCNP1 was performed after deparaffinization and hydration of the sections, antigen retrieval in Tris–HCl buffer (pH 9.0) for 10 min at 90°C, followed by incubation with anti-DCNP1 antibody (1:300) for 1 h at room temperature and overnight at 4°C. The following day, sections were incubated with secondary horseradish peroxidase–conjugated antibody (1:100) (Dakocytomation). Sections were then incubated for 10 min in Tris buffered saline containing 0.05% 3,3’-diaminobenzidine (Sigma), 0.01% hydrogen peroxide and 0.3% nickel–ammonium sulphate. Having been developed, dehydrated and cleared, the sections were then cover slipped with entellan mounting medium (Merck).

**cDNA synthesis**

For each sample, 300 ng RNA was used for cDNA synthesis. After mixing with oligo deoxynucleotide (100 μg/ml) and 10× hexanucleotide (Roche) the vial was heated and then quickly transferred to ice. A mixture of 5× first-strand buffer, 100 mM dithiothreitol, 10 mM deoxy-ribonucleoside triphosphate and RNase inhibitor was then added together with reverse transcriptase Superscript II RT (Invitrogen Life Technologies). The synthesis reaction was allowed to proceed at 42°C, after which cDNA was either stored at −20°C or used immediately.

**Real-time quantitative PCR**

The nucleotide and translated protein sequences of DCNP1 came from the National Centre for Biotechnology Information database at http://www.ncbi.nlm.nih.gov (AB074498). The primer pairs were 5’-aggcaaccccatacagatcg-3’ and 5’-ctcttcagtttgggtc-3’. The quantitative PCR reaction contained 10 μl of 2× SYBR Green Mastermix (Applied Biosystems), 1 μl of each primer pair (1 μM) and...
5 μl (equivalent to 2 ng/μl total RNA) of template cDNA in a 20 μl reaction volume. The quantitative PCR reaction was performed in a GeneAmp 7300 thermocycler. The specificity of the amplification and the size of the PCR products were checked. Sterile water, RNA samples without reverse transcriptase and DNA samples were used as controls. The linearity of the quantitative PCR assay with cDNA was tested by a series of dilutions of the same stock in multiple plates. The absolute amount of target gene was calculated by $10^{ \text{[E - 10^{ct}]}}$ (Wang et al., 2008).

**Normalization strategy**

In order to remove the sampling differences (RNA quality and RNA quantity) for the laser micro-dissection experiment, eight candidate reference genes were tested for cDNA samples. The normalization factor was the geometric mean of the following genes: glyceraldehyde-3-phosphate dehydrogenase, actin-β, 18S ribosomal RNA, hydroxymethylbilane synthase, hypoxanthine phosphoribosyltransferase 1, ubiquitin C, tubulin-α and tubulin-β. The geNorm analysis (Vandesompele et al., 2002) revealed that all eight reference genes determined in the paraventricular nucleus and supraoptic nucleus showed a good correlation in expression levels and could be included in the calculation of the normalization factor. The absolute amount of transcript thus determined was then divided by the normalization factor to obtain the normalized values (Wang et al., 2008).

**Immunofluorescence and confocal laser scanning microscopy**

Sections were incubated with rabbit polyclonal DCNP1 antibody (1:50) and rat monoclonal anti-CRH antibody ‘PFU 83’ (1:3000, IgG2a subclass) (Wu et al., 2006). Primary antibody incubation for 4 h at room temperature was followed by overnight incubation at 4°C. DCNP1 was visualized in red by SA-Alexa594 (1:200, Invitrogen) using a biotinylated anti-rabbit antibody (1:100, Vector Laboratories Inc.). CRH was detected in green by anti-Rat-Alexa488 (1:200, Invitrogen). Fluorochrome-conjugated antibody incubation was performed for 4 h at room temperature, followed by overnight incubation at 4°C. Sections were analysed using a Zeiss 510 confocal laser scanning microscope equipped with lasers emitting at 488 and 543 nm.

**Chromatin immunoprecipitation and immunoblot analysis**

Transfected human embryonic kidney 293 cells were cross-linked by 1% formaldehyde and neutralized with 0.125 M glycine. Solution with soluble chromatin was immunoprecipitated with anti-green fluorescent protein antibody (Roche) and Protein G agarose (Roche). DNA extraction, purification and amplification by PCR was then performed with the primers 5′-actggtcgctgtcgccttc-3′ and 5′-atgccgtgctgtcgccttc-3′.

Immunoblot analysis was performed to check the immunoprecipitation of enhanced green fluorescent protein (EGFP), DCNP1-EGFP and DCNP1-1–116-EGFP. Proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore) incubated overnight with a monoclonal anti-green fluorescent protein antibody (1:1000, Santa Cruz Biotechnology) and a sheep anti-mouse IgG-horseradish peroxidase antibody (1:5000, Amersham Pharmacia Biotech) for 1 h at room temperature. The proteins were visualized using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

**Dual-luciferase reporter gene assay**

Human embryonic kidney 293 cells were transfected with a CRH-luciferase construct and co-transfected with plasmids expressing DCNP1-EGFP, DCNP1-1–116-EGFP or EGFP. Renilla-expressing plasmid pRL-CMV was co-transfected to normalize the variations in transfection efficiency. The cell lysates were prepared via passive lysis buffer (Promega). Both firefly and renilla activities were measured with dual-luciferase reporter systems using a Veritas Microplate luminometer according to the manufacturer’s instructions (Promega). The absolute values of firefly luminescence were normalized to those of renilla and the ratios were presented as the median of three transfected experiments. For the three independent experiments, the conditions, including plasmids, cell lines, amount of cells and culture time, were kept the same.

**Statistical analysis**

Statistical analysis was conducted with Statistical Package for the Social Sciences (SPSS version 11.5). The differences in DCNP1 transcript level in the paraventricular nucleus and supraoptic nucleus between patients with depression and controls were statistically evaluated by a non-parametric Mann–Whitney U-test. Differences in clock time of death and month of death (circumstantial parameters) between patients with mood disorders and controls were tested with the Mardia-Watson-Wheeler test (Batschelet, 1981; Wang et al., 2008). We performed a Kruskal–Wallis test for differences in the three groups of the luciferase assay, after which we compared pairs by means of the Mann–Whitney U-test. All tests were two-tailed. Values of $P < 0.05$ were considered to be significant.

**Results**

**DCNP1 is expressed ubiquitously in the brain**

A strong in situ hybridization signal was present with a DCNP1 mRNA anti-sense probe in formalin-fixed, paraffin-embedded material of controls. In situ hybridization showed the presence of DCNP1 mRNA in the paraventricular nucleus, supraoptic nucleus and nucleus basalis of Meynert (Fig. 1D–I). The sense probe yielded a completely negative hybridization signal, supporting specificity of the procedure (Fig. 1A–C).

In addition, we performed immunohistochemistry to determine the expression of DCNP1 in a number of human brain regions. As shown in Fig. 2, DCNP1 immunostaining in the paraventricular nucleus, pulvinar of the thalamus and occipital cortex was found in the nucleus and cytoplasm of neurons, while some glial cells occasionally had a granular appearance. Nuclear staining was also found in the nucleus basalis of Meynert and caudate nucleus (Fig. 2D and F). In the supraoptic nucleus, cingulate cortex, frontal cortex, cerebellum and parietal cortex, DCNP1 was detected.
mainly in the cytoplasm, sometimes in a granular pattern (Fig. 2B, G–J). These results show that DCNP1 is expressed ubiquitously in the brain.

**DCNP1 expression is higher in the paraventricular nucleus of depressed patients**

We compared DCNP1 transcript levels both the paraventricular nucleus and, as a control area, the supraoptic nucleus in patients with depression and control subjects by laser micro-dissection and quantitative PCR. As shown in Fig. 3, the median level of DCNP1 mRNA in the paraventricular nucleus of patients with depression was 2.8 times higher than controls (P = 0.004). In contrast, no significant difference was found between patients with depression and controls in the supraoptic nucleus. When comparing transcript levels in the supraoptic nucleus and paraventricular nucleus, the level of DCNP1 was significantly higher in the supraoptic nucleus than in the paraventricular nucleus in controls (P = 0.048), while there was no such difference in patients with depression (P = 0.391). The RNA integrity value of these samples was 7.15 ± 0.63 in the paraventricular nucleus and 6.64 ± 0.66 in the supraoptic nucleus. There was no significant difference in RNA integrity value between patients with depression and controls (P > 0.13). There was no correlation of DCNP1 mRNA with possible confounders, such as autopsy delay, pH value or clock time of death. These data show that the transcription of DCNP1 was higher in the paraventricular nucleus in patients with depression than in controls.

**DCNP1 co-localizes with corticotropin-releasing hormone**

We performed immunofluorescence double staining and laser scanning microscopy to investigate possible co-localization of
Figure 2. Distribution of DCNP1 in different regions of the human brain. All sections from different brain areas were incubated with our DCNP1-antibody (1:300) and developed with nickel–ammonium sulphate. The material came from five patients without mood disorders. Arrows indicate the expression of DCNP1 in the nucleus. Arrowheads indicate DCNP1 localized in glial cells. Asterisks indicate stained cells with granular material. (A) paraventricular nucleus; (B) supraoptic nucleus; (C) thalamus (pulvinar); (D) nucleus basalis of Meynert; (E) occipital cortex; (F) caudate; (G) cingulate cortex; (H) frontal cortex; (I) cerebellum and (J) parietal cortex. Scale bar: 25 μm.
CRH and DCNP1 in the paraventricular nucleus. As shown in Fig. 4, cytoplasmic co-localization of DCNP1 and CRH was found in a subpopulation of the parvocellular neurons in the paraventricular nucleus. In all sections there were cells present that expressed only DCNP1 or CRH (Fig. 4).

**DCNP1 interacts with and transactivates the corticotropin-releasing hormone promoter**

The transcription of DCNP1 was higher in the paraventricular nucleus in patients with depression and co-localized with CRH neurons, which play a crucial role in depression. Therefore we explored the relationship between DCNP1 and CRH.

We performed a chromatin immunoprecipitation assay and found that the CRH promoter could be immunoprecipitated by DCNP1-EGFP but not by DCNP11–116-EGFP or EGFP. Immunoblot analysis confirmed that EGFP, DCNP1-EGFP and DCNP11–116-EGFP were indeed immunoprecipitated by anti-green fluorescent protein antibody (Fig. 5).

Having found that full-length DCNP1 interacted with the CRH promoter, we performed a luciferase assay to explore if DCNP1 could regulate CRH expression and whether there was a difference between DCNP1 and DCNP1 1-116. Human embryonic kidney 293 cells were co-transfected with a CRH-luciferase construct and EGFP, DCNP1-EGFP or DCNP11–116-EGFP expression vectors. Cells were harvested 48 h after transfection and the extracts...
Dendritic cell nuclear protein-1 and corticotropin-releasing hormone

Brain 2010: 133; 3069–3079 | 3077

Discussion

Our data show for the first time that the novel candidate gene for depression, DCNP1, may play a role in the pathogenesis of this disorder by upregulating the CRH promoter in the paraventricular nucleus. CRH neurons are the driving force for the hypothalamic-pituitary-adrenal axis, which is the final common pathway for the stress response and also a crucial system in the pathogenesis of depression.

In situ hybridization and immunohistochemical data showed a widespread distribution of DCNP1 expression in the human brain at both the mRNA (Fig. 1) and protein levels (Fig. 2). In addition, we determined the transcription level of DCNP1 by quantitative PCR in nine different brain areas in three patients without mood disorder (data not shown). DCNP1 was detectable in all nine brain areas, but at a low and variable level.

A previous publication mentioned that a small fraction of microglial cells was stained with the DCNP1-antibody, but no neurons (Masuda et al., 2002). However, we observed that our DCNP1-antibody could stain both neurons and glial cells (Fig. 2). In order to see whether this discrepancy might be due to different antibodies, we compared our polyclonal antibody with the original antibody from Masuda’s group (Masuda et al., 2002). With the Masuda group’s antibody we obtained a similar staining pattern as with our own antibody, albeit with a lower background. The similar distribution and the fact that the same cells were stained by the two different DCNP1 antibodies (Supplementary Fig. 2) support the specificity of the immunohistochemical data. Our DCNP1 staining in neurons, in contrast to the absence of neuronal staining reported in Masuda’s original publication (2002), might be due to the more sensitive staining procedure in the antigen retrieval method we used.

DCNP1 mRNA was present in the paraventricular nucleus and supraoptic nucleus in patients with depression and controls (Fig. 3). The localization of DCNP1 in neurons of the paraventricular nucleus and supraoptic nucleus by two completely independent techniques, i.e. in situ hybridization and immunohistochemical analyses (Figs 1 and 2), is an additional strong indication in favour of the specificity of the localization of DCNP1 in these neurons.

Although the global distribution of this novel protein indicates the possibility of different central functions, the protein sequence of DCNP1 does not give any indication as to what these functions may be, as DCNP1 has no crucial sequence similarity to known genes of human or other species.

DCNP1 contains an open reading frame with an in-frame stop codon in the 5’ untranslated region, encoding a protein composed of 244 amino acids (Masuda et al., 2002) and it is a novel candidate gene for major depression (Willis-Owen et al., 2006; Bosker et al., 2010). We hypothesized that DCNP1 would be increased in depression and act by stimulating CRH release and in this way stimulate the hypothalamic-pituitary-adrenal axis, which is considered to be a crucial system in the pathogenesis of a major part of depressive symptomatology (Bao et al., 2008). To support our hypothesis, we first showed that DCNP1 mRNA was detectable in both the paraventricular nucleus and the

Figure 5 DCNP1 interacts with the CRH promoter in cells. Human embryonic kidney 293 cells were transfected with plasmids expressing EGFP, DCNP1-EGFP or DCNP11-116-EGFP and collected for chromatin immunoprecipitation assays. The inputs and chromatin immunoprecipitation products were amplified by PCR reactions. A response element at −434 to −109 bp of the CRH promoter was amplified. The amplification product was 300 bp. The input was 10% of the cell lysate.

Figure 6 CRH is transactivated by DCNP1 in cells. Human embryonic kidney 293 cells were co-transfected with equal amounts (400 ng) of a CRH promoter construct of luciferase reporter along with plasmids expressing DCNP1-EGFP, DCNP11-116-EGFP or EGFP. Renilla luciferase plasmid pRL-CMV was also introduced into each transfected sample as an internal control. The results represent the median from three independent transfection experiments. Asterisk indicates that CRH is significantly upregulated by full-length DCNP1 (P < 0.001).

were used for luciferase activity detection. CRH was significantly activated by DCNP1-EGFP (P < 0.001). No significant activation of CRH regulation was found with DCNP11-116-EGFP (P = 1.00) (Fig. 6). These data show that full-length DCNP1 may interact with the CRH promoter and transactivate CRH expression.
supraoptic nucleus dissected from snap-frozen human hypothalamus. In agreement with our hypothesis, the transcription of DCNP1 in the paraventricular nucleus was significantly (2.8-fold) increased in patients with depression, while the level in the supraoptic nucleus, as a control area that does not contain CRH (Wang et al., 2008), was unaltered (Fig. 3). As an additional control for the specificity of increased DCNP1 expression in the paraventricular nucleus, we compared DCNP1 transcription in two cortical brain areas in patients with depression and matched controls. Neither the superior prefrontal cortex (in 14 patients with depressive disorder and 14 controls) nor the anterior cingulate cortex (in 12 patients with depressive disorder and 12 controls) showed a difference in transcription of DCNP1 between patients with depression and controls ($P = 0.304$ and $P = 0.245$, respectively). The levels of DCNP1 transcription in these two areas were almost eight times lower (data not shown) than in the paraventricular nucleus.

That DCNP1 expression increased in depression in the paraventricular nucleus but not in the supraoptic nucleus or in the prefrontal cortex may be related to the receptor imbalance we found earlier in the paraventricular nucleus in this disorder (Wang et al., 2008).

In a previous study, our group investigated gene expression in the human hypothalamus in depression (Wang et al., 2008). We found that the transcript levels of CRH were significantly higher in the paraventricular nucleus in patients with depression. Since these patients were the same as the patients with depression in the present study, we used non-parametric Spearman correlation to compare the data from the two studies. We found that DCNP1 mRNA levels were correlated with CRH mRNA levels ($P = 0.015$, $\rho = 0.635$ in all 14 subjects).

Subsequently we showed that DCNP1 immunostaining was localized in the nucleus and cytoplasm of paraventricular nucleus neurons (Fig. 2A). Double labelling experiments showed that DCNP1 was indeed co-localized in CRH neurons (Fig. 4).

Since DCNP1 was present in CRH neurons and transcript levels were increased in the paraventricular nucleus in patients with depression, we investigated whether it might act on the CRH promoter in cells. Full-length DCNP1 was shown to be able to bind to the CRH promoter (Fig. 5). Moreover, by means of the luciferase assay we found that full-length DCNP1 can significantly transactivate CRH promoter activity. In contrast, truncated DCNP1 (DCNP1$^{1-116}$) was unable to interact with or significantly regulate the CRH promoter (Figs 5 and 6), meaning that only full-length DCNP1 can act on CRH neurons.

Although the findings confirmed our hypothesis that DCNP1 is increased in depression and can stimulate CRH expression by acting on the CRH promoter, there are some limitations to this study. First, the group size of the patients with depression and controls studied is relatively small. However, quantitative PCR assays on laser micro-dissection material (Fig. 3) require well-documented patients with depression, well-matched controls and a high quality of RNA. These criteria are extremely difficult to fulfill, which limits the number of suitable donors. Secondly, the epidemiological study that showed the increased risk for major depression in relation to DCNP1 (Wills-Owen et al., 2006; Bosker et al., 2010) should be confirmed in a large study, including bipolar depression. In our study the two patients with bipolar depression disorder also had high DCNP1 values, well within the range of patients with major depression (Fig. 3). The two different types of depression, i.e. five patients with major depression and two patients with bipolar depression disorder, are in some aspects considered to be pathophysiologically non-identical entities (Videbech and Ravnkilde, 2004). Indeed, for each patient with major depression there are probably different epigenetic factors and a genetic background that may influence the pathogenesis of depression. On the other hand, previous studies have shown that both major depression and bipolar disorders are accompanied by a similar activation of the hypothalamic-pituitary-adrenal axis (Raadsheer et al., 1994, 1995; Bao et al., 2005). In light of our data it would be interesting to study the epidemiological risk for bipolar disorder depression in relation to the two forms of DCNP1. Lastly, due to the absence of a similar sequence to DCNP1 in known genes of other species (Masuda et al., 2002), experimental confirmation of the increased expression of DCNP1 in the paraventricular nucleus of a rodent model for depression is hampered. The same holds true for possible effects of antidepressants on DCNP1 expression.

**Conclusion**

By combining a number of techniques, i.e. in situ hybridization, immunohistochemistry, laser micro-dissection and quantitative PCR, we provided evidence for the presence of DCNP1 in a number of human brain areas at both the mRNA and protein level. CRH neurons of the hypothalamic paraventricular nucleus also express DCNP1 as shown by immunofluorescence and confocal laser scanning microscopy. Moreover, by using laser micro-dissection and quantitative PCR we found an increase of DCNP1 mRNA in the paraventricular nucleus in patients with depression. By means of chromatin immunoprecipitation and a luciferase assay, we showed that full-length DCNP1, but not DCNP1$^{1-116}$, can interact with the CRH promoter and upregulate the CRH promoter significantly. This study not only provides new data indicating that the activity of CRH may also be regulated by DCNP1 in depression, but it shows evidence for a cross-talk between CRH and DCNP1 as well. The question of whether DCNP1 may be a target for new therapeutic strategies should be a topic for further studies.

**Acknowledgements**

We are indebted to the Netherlands Brain Bank at the Netherlands Institute for Neuroscience (Director Dr I. Huizinga) for providing us with the brain material and patient information. We thank Prof. Satoru Senju for the DCNP1-antibody, Prof. Fraijese for the cDNA samples, Dr Michel A. Hofman and Prof. Ai-min Bao for their statistical assistance, Ronald Verwer, Joop Van Heerikhuize, Erkang Fei, Rawien A. Balesar, Arja A. Sluiter, Unga A. Unmehopa, Ling Shan, Ton Put and Bart Fisser for their technical advice and Wilma Verweij for correcting the English.
Funding
The China Exchange Programme of the Royal Netherlands Academy of Arts and Sciences (KNAW) (project 09CDP011) and the Nature Science Foundation of China (30530310).

Supplementary material
Supplementary material is available at Brain online.

References