Streptozotocin produces oxidative stress, inflammation and decreases BDNF concentrations to induce apoptosis of RIN5F cells and type 2 diabetes mellitus in Wistar rats

Siresha Bathina a, **, Nanduri Srinivas b, Undurti N. Das a, c, *

a BioScience Research Centre, Department of Medicine, Gayatri Vidya Parishad Hospital, GVP College of Engineering Campus, Visakhapatnam 530048, India
b National Institute of Pharmaceutical Education and Research, Hyderabad, India
c UND Life Sciences, 2221, NW 5th St, Battle Ground, WA 98604, USA

** Corresponding author. BioScience Research Centre, Department of Medicine, Gayatri Vidya Parishad Hospital, GVP College of Engineering Campus, Visakhapatnam 530048, India.
* Corresponding author.

Article history:
Received 12 March 2017
Accepted 13 March 2017
Available online 15 March 2017

Keywords:
Hyperglycemia
Oxidative stress
BDNF
Protein expression
Diabetes mellitus

1. Introduction

Type 2 diabetes mellitus (type 2 DM) is a major global health problem whose neurological consequences include: cognitive deficit, decline in memory and higher incidence of Alzheimer's disease possibly, due to alterations in neurotransmission and neuronal damage secondary to hyperglycemia and its associated metabolic abnormalities [1,2]. The cognitive impairment seen in diabetes mellitus are not only due to metabolic derangements seen in diabetes but also because of structural and functional changes that occur within the central nervous system [3,4].

Brain derived neurotrophic factor (BDNF), one of the neurotrophins, that was first identified in the brain [5,6] and subsequently found in several tissues in the body especially in the gut [6,7] promotes the optimum communication between neurons by enhancing “plasticity” at the synapse [7,8]. Since, brain is mainly a glucose-dependent organ, its dysfunction may occur due to...
disturbances of neuronal glucose transport and metabolism in hyperglycemia, increased production of free radicals and decreased production of BDNF [9–18]. BDNF plays an important role in the survival of neurons, their growth (axons and dendrites), and formation and function of synapse and ability to reduce hyperglycemia [18–21]. Thus, reduced concentrations of BDNF may contribute to reduced memory and cognitive impairment in diabetes.

It was reported that administration of BDNF to db/db mice enhances insulin-stimulated tyrosine phosphorylation of insulin receptors in liver and insulin-stimulated PI-3 kinase activity in liver, skeletal muscle and intercapsular brown adipose tissue. BDNF was reported not to affect either glucose uptake or gluconeogenesis in cultures hepatocytes, L6 muscle cells or 3T3-L1 adipocytes, leading to the suggestion that enhanced insulin signal transduction in liver produces its hypoglycaemic action in diabetic mice [22]. On the other hand, Yamanaka et al. [23] reported that repetitive administration of BDNF to db/db mice for 8 days ameliorates glucose metabolism by enhancing glucose utilization in muscle and brown adipose tissue by modulating the central and peripheral nervous systems [23].

But, it is not known whether BDNF concentrations are altered not only in the plasma but also in other tissues as well such as liver and brain. To verify this possibility, the present study was performed wherein we measured plasma, liver and brain BDNF concentrations in streptozotocin (STZ)-induced type 2 DM model. In addition, we also performed histological studies to know the neuronal cell number and their morphology in hippocampus and hypothalamic areas.

2. Materials and methods

2.1. Chemicals

All chemicals (including STZ) were purchased from Sigma Aldrich chemical company, USA.

2.2. Cell culture

RIN5F cells, insulin secreting rat pancreatic β cell line, were obtained from National Center for Cell Science (Pune, India). RIN5F cells were grown in RPMI1640 medium supplemented with bicarbonate (1.5 g/L), Glucose (4.5 g/L) 10% FBS, Penicillin (100 U/ml), Streptomycin (100 µg/ml), Amphotericin B (1.25 µg/ml), HEPES (10 mM) and l-Glutamine (2 mM) at 37 °C in humidified air with 5% CO₂. After 48 h of incubation time, cells reached confluent stage at which time they were harvested for further studies [24].

2.3. Cell viability assay

Cell viability was performed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, as described previously [24]. For this test, 5 × 10⁴ cells/ml/well of RIN5F cells was cultured in 96-well plates for 48 h. After the end of various treatment schedules, the cells were harvested, spent media is removed and incubated with MTT prepared in phosphate buffered saline (PBS). After 4 h of incubation, the formazan crystals formed were dissolved in 10% SDS in 0.01 M HCL for 18 h. Quantification of was done by measuring the absorbance at 450 nm and background at 620 nm.

2.3.1. Dose and time optimization studies with STZ on RIN5F cells

RIN5F cells were seeded at a density of 5 × 10⁴ cells/100 µL of culture media in 96-well plates. STZ was dissolved in 100 mM citrate buffer (pH 4.5). After 44 h of attachment period, cells were treated with different doses of STZ (1 mM-30 mM) and incubated from 12 to 48 h. At the end of incubation, cell viability was determined by MTT assay.

2.3.2. Effect of BDNF on STZ-induced cytotoxicity to RIN5F

This study was performed with 5 × 10⁴ cells/100 µl of culture media in 96-well plates. The RIN5F cells were treated with optimal doses of STZ and BDNF (20 mM) and three types of experiments were performed to study the effect of BDNF on the cytotoxic action of STZ against RIN5F cells as described below and detailed previously [24].

2.3.2.1. In vitro study protocol. Three types of treatment protocol (pre-, simultaneous and post-treatment with STZ) were employed in this study. In the pre-treatment protocol, RIN5F cells were first incubated with BDNF for 5 h at the end of which excess BDNF was removed and cell were incubated with optimized dose of STZ. In the simultaneous treatment protocol, cells were incubated with medium for 5 h s and then BDNF and STZ were added simultaneously and incubated for optimized period. In the post-treatment protocol, RIN5F cells were supplemented first with STZ for 24 h at the end of which the spent medium was removed and supplemented with fresh medium containing BDNF and incubated for an additional 5 h. At the end of this 5 h of incubation, the cells were harvested and MTT assay and other tests were performed. In all these studies, appropriate controls were used.

2.4. Estimation of nitric oxide, lipid peroxides and antioxidant enzymes

For this study, RIN5F cells were seeded at a density of 1 × 10⁶ cells/ml/well in 6 well culture plates. After 48 h of attachment period, cells were treated with STZ (20 mM) for 24 h s. At the end of the treatment, cells and their supernatant were collected and used for analysis of various anti-oxidants as described previously [24].

2.5. Effect of STZ on the levels of BDNF in vitro

RIN5F cells were cultured in 24 well culture plates at a concentration of 0.5 × 10⁴/well in 500 µl of RPMI1640 and treated with different doses of STZ (20 and 40 mM) for 24 and 48 h. At the end of incubation period, the supernatant was collected and their content of BDNF was measured by Elisa (CYT306) per manufacturer instructions.

2.6. In vivo studies

Wistar rats of 3–4wk old, purchased from National Institute of Nutrition, (Hyderabad, India) were used for this study. The animals were housed at 25°C room temperature with 12-hr dark and 12-hr light cycle. Animals weighing around 180 gm were segregated into two groups of 10 animals: controls received PBS and citrate buffer; diabetic group received STZ dissolved in citrate buffer. Experiment was approved by Institutional Animal Ethical Committee.

2.6.1. Induction of type 2 diabetes mellitus in Wistar rats

Type 2 DM was induced by treatment with nicotinamide (NAD) and STZ. Freshly prepared nicotinamide in PBS at the rate of 175 mg/kg body weight was administered intraperitoneally. After 15 min s, freshly prepared STZ in 50 mM citrated buffer pH 4.5 was injected intraperitoneally at a dose of 65 mg/kg body weight as described previously [25]. Fasting blood glucose was measured by using Accu-Check blood glucose meter on 10th, 20th and 30th day from day 1 of the injection of STZ.
2.6.2. Estimation of blood glucose, food intake and body weight

The animals were confirmed to have developed type 2 diabetes when fasting blood glucose levels were >250 mg/dL measured using Accucheck glucometer. Body weight and food consumption was measured twice a week. The total duration of the study was 30 days from the day of injection of STZ (Fig. 2A). At the end of 30 days, animals were sacrificed to collect blood and various tissues for further studies. All samples were stored at −80 °C till further analysis.

2.6.3. Estimation of nitric oxide, lipid peroxides and antioxidant enzymes

Nitric oxide, lipid peroxides and antioxidant enzymes: super oxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione-S-transferase (GST) were estimated in the plasma and lysates of the collected pancreas and brain tissues samples as described previously [24–30].

2.6.4. Estimation of plasma BDNF, IL-6 and TNF-α

The levels of BDNF were measured from the plasma collected at the end of experiment (day 30) using Chemikine Sandwich ELISA kit, Millipore, USA (CYT306). Plasma TNF-α (by using Quantikine TNF-α Immunoassay ELISA kit (R&D Systems, MN, USA) and IL-6 were measured by using Abcam (Ab100772) Rat IL-6 ELISA kit, Kendall Square, Cambridge, MA USA, on day 30 of the study employing manufacturer’s protocol.

2.6.5. BDNF protein expression in brain and other tissues

The brain, pancreas and liver samples were kept in cold and all the steps were performed on ice. A small sample of fresh, unfixed tissue (brain, liver and pancreas) was finely chopped using a sharp scalpel. The chopped tissue was homogenized in a minimum volume of T-PER (tissue protein extraction lysis buffer with protease inhibitor mixture). The sample was centrifuged for 10,000 × g for 5 min to collect the debris. The supernatant (containing solubilized proteins) was obtained and the pellet (intact Cells/cell debris) was discarded. The supernatants were directly used for BDNF Elisa and done per manufacturer instructions (CYT306).

2.6.6. Collection of brain

At the end of 30 days of study, animals were sacrificed and transcardial perfusion with 1 x PBS was done. Later blood, pancreas and brain were collected for further studies.

2.6.6.1. Brain tissue preparation/Fixation

The whole brain was fixed in 4% paraformaldehyde and later dissected out and kept in 10% sucrose for 24 h and subsequently kept in 20% sucrose (day 2 after dissection) and then, finally transferred into 30% sucrose solution until equilibrated.

2.6.6.2. Sectioning of brains

The hippocampi were sectioned in the coronal plane on a sliding brain samples of animals that developed type 2 DM as described previously [31]. Neuronal proliferation was studied by Nissl staining and Ki67-immunohistochemistry. For this purpose, brains were sliced by cryostat (Leica CM 1510S) at 40 μm size and were transferred to 1 x PBS solution. Rat brain slides were placed onto gelatinized slides [31].

2.6.6.3. Nissl staining

Slides of hippocampal sections are immersed for 10 min each of the following solutions: 96% alcohol, 70% alcohol, distilled water and 0.5% cresyl violet prepared in distilled water. Later, slides were dipped in 10 ml of 10% glacial acetic acid with 90% of 70% alcohol and 10 ml of 10% glacial acetic acid with 90 ml of 96% alcohol. The slides were kept for 1–2 min in 96% alcohol, isopropyl alcohol and xylene for 2–3 min. DPX mounting was done and later kept for overnight drying and observed under microscope.

2.6.6.4. Immunohistochemistry (Ki67-proliferating cell marker staining)

To test the hypothesis that in diabetic animals there could be a change in the proliferation of neuronal cells, brain tissue sections taken from the control and diabetic animals were labelled for Ki67, a proliferating cell marker, to detect the extent of neurodegeneration and identify newly developed neurons in DG (dentate gyrus region). Assessing staining for Ki67 and newly developed neurons will give an insight into whether there is any association between cell proliferation (as detected by Ki67 staining) and neurogenesis. The cut sections of brain tissues were labelled with specific antibodies for: Ki-67, a nuclear protein easily detected in newly dividing cells. After staining, pictures of the stained tissue sections were taken by Microscope (Labomed LX400).

2.7. Statistical analysis

All studies were repeated thrice each time in triplicate. All results are expressed as mean ± SEM and all values obtained were analyzed employing paired t-test with equal variance in Microsoft Excel statistical analysis tool.

3. Results

3.1. Effect of STZ on viability of RIN5F cells

Studies with STZ showed that exposure of RIN5F cells to 1–30 mM for 12, 24, and 48 h, there was a significant decrease (P < 0.001) in their survival in a dose-dependent fashion as shown in Fig. 1A. STZ at 20 mM for 24 h reduced the viability of RIN5F cells to ~60%. Based on these results, all subsequent studies were performed using 20 mM and an incubation time of 24 h.

3.1.1. Effect of STZ on BDNF secretion by RIN5F cells in vitro

The results of this study shown in Fig. 1B revealed that though BDNF is effective in preventing the cytotoxic action of STZ at all the three doses (10 ng, 50 ng and 100 ng/ml) tested, the most effective doses are 50 ng (P < 0.01) and 100 ng (P < 0.001) (100 ng > 50 ng). Of the three treatment schedules tested, pre-treatment and simultaneous treatment schedules are the most effective treatment schedule.

3.2. STZ-induced changes in antioxidants

To know whether cytotoxic action of STZ on RIN5F cells is due to changes in the concentrations of various anti-oxidants, we studied the activity of SOD, catalase, glutathione S-transferase, glutathione peroxidase and alterations in lipid peroxides and nitric oxide in these cells (Table 1). STZ produced significant changes (P < 0.05) in the concentrations of SOD, catalase, glutathione S-transferase, glutathione peroxidase, lipid peroxides and nitric oxide in RIN5F cells. STZ induced changes in the activity of various anti-oxidants in cell lysates and lipid peroxides and nitric oxide in the cell culture supernatants.

3.3. Effect of STZ on BDNF secretion by RIN5F cells in vitro

Results of this study given in Fig. 1C, it is evident that, under the conditions employed, STZ significantly decreased (P < 0.01, P < 0.001) the formation and secretion of BDNF by RIN5F cells in the cell supernatants in a dose dependent fashion.
3.4. Induction of type 2 DM by in vivo

To verify whether in vitro results are relevant to an in vivo situation, we tested the effect of STZ-induced type 2 DM in Wistar rats. The protocol of this study is shown in Fig. 2A.

3.4.1. Blood glucose/body weights/food intake

Fasting blood glucose levels were significantly (P < 0.05) increased from day 2 following the administration of STZ (Fig. 2B) that persisted when measured on 10th, 20th and 30th days of the study confirming the development of type 2 DM in these animals. Simultaneously, body weight was reduced and food consumption was increased in these STZ-induced type 2 DM Wistar rats (Data not shown).

3.4.2. Nitric oxide, lipid peroxides and antioxidant enzymes

Nitric oxide, lipid peroxides and antioxidant enzymes: super oxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione-S-transferase (GST) were found to be elevated both in the plasma and lysates of the collected pancreas and brain tissue samples (P < 0.05) (Table 1), suggesting that STZ induces significant oxidative stress.

3.4.3. Plasma BDNF, IL-6 and TNF-α levels

Plasma levels of BDNF were significantly reduced (P < 0.01), while IL-6 and TNF-α were significantly elevated in STZ-induced type 2 DM animals (Fig. 2C) compared to control, when measured on day 30th of the study. Since BDNF is a neurotrophic factor that is needed for the survival of neurons and IL-6 and TNF-α are pro-inflammatory and cytotoxic molecules, these results imply that these alterations could lead to an increase in neuronal apoptosis.

3.4.4. BDNF Elisa in liver, pancreas and brain tissues

The total BDNF protein levels in brain, pancreas and liver of diabetic rats are decreased significantly (P < 0.01, P < 0.001) compared to the control.

3.4.5. Immunohistochemistry of hippocampus

There is evidence to suggest that type 2 DM may be due to hypothalamic dysfunction [7,8,11,12]. To know whether STZ-induce type 2 DM may also involve hypothalamic dysfunction, we performed immunohistochemical study of hippocampus and hypothalamus. We studied neuronal morphology in brain sections of STZ treated animals specifically the dorsal hippocampus region (Fig. 3A and B) with focus on the hippocampal pyramidal cell layers of cornu ammonis 1 (CA1) and CA3 and the granule cell layer of the DG (Fig. 3A–B). In addition, we also studied the hypothalamus region especially the PVN (paraventricular) regions as shown in Fig. 3C. These results (Fig. 3) revealed necrosis of neurons as well as dispersed nature of neurons in STZ treated brain samples of rats. A typical histopathological change seen following STZ administration is shown in Fig. 3A corresponding to CA1, CA2/3 and DG regions of the hippocampus. It was noted that neuronal loss in these regions is almost ~50% as visualized by Nissl staining. The number of neurons in CA1 region were counted and confirmed that STZ not only reduced number of neurons but also showed visible difference in their morphology. The number of neurons in the region of CA1 in STZ samples were reduced significantly (#P < 0.01) compared to control. In contrast, no signs of neurodegeneration were detected in the preoptic and lateral regions, while STZ induced neurotoxicity was observed in the PVN and ARC region of the hypothalamus (Fig. 3). This is a region-specific effect; neuronal damage could not be detected in other regions of the hypothalamus in STZ treated rats. A significant reduction (#P < 0.05) in neuronal proliferation was detected in hypothalamus in STZ treated animals. These results suggest that STZ can cause neurodegeneration that could have a role in the pathogenesis of type 2 DM. In addition, it was also noted that Ki67 antigen, which is a nuclear protein and proliferative cell marker expressed most often during the active phases of the cell cycle including G1, S, G2, and M phases, showed significantly decreased expression (#P < 0.05) in the hippocampus, dentate gyrus (DG) region and PVN region of the hypothalamus confirming that STZ reduces neurogenesis (Fig. 3).
Fig. 2. A) STZ-induced type 2 diabetes protocol. Animals were housed for 1 week for acclimatization after which single I.P injections of STZ was given and blood glucose levels were estimated once in 10 days until the end of the study. Body weight and food consumption of rats treated with STZ as well as control was measured weekly once. At the end of day 30, study was terminated and organs were collected for various molecular and biochemical analysis.

B) Blood glucose levels were measured once in 7 days until the end of the study.

C) Plasma BDNF/TNF-α/IL-6 levels. All values are expressed as mean ± SEM. *P<0.05 compared to respective control value §P<0.01 compared to control values. *P<0.05 compared to control (BDNF measurements).

D) Relative BDNF protein expression of brain, pancreas and liver of diabetic and control rats. Data are expressed as mean for the control brain, diabetic brain, liver and the pancreas. §P<0.01 compared to control values. *P<0.05 & §P<0.01 compared to control. All the above set of experiments were done in triplicate on two separate occasions (n = 10).

Table 1
Concentrations of various anti-oxidants, lipid peroxides and nitric oxide in RIN cells and pancreatic and brain tissues in vivo: Superoxide dismutase (SOD) is expressed as U SOD/mg of protein; Catalase (CAT) is expressed as µM of H₂O₂ consumed/minute/mg of protein. Glutathione-S-transferase (GST) is expressed as µM conjugate formed/minute/gm of protein; Glutathione peroxidase (GPX) is expressed as µg of glutathione consumed/minute/gm of protein. Lipid peroxides formed are expressed as µmoles of TMOP formed; Nitric oxide formed is expressed as µM of nitrite formed. *P<0.001, |P|<0.05 vs untreated control and **P<0.001 vs AL/STZ -treated group. All the above set of experiments were repeated on two separate occasions and each time in triplicate and n = 10 animal experiment in vivo. All values expressed as mean ± SEM. *P<0.05 vs untreated controls.

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>GROUP</th>
<th>SOD (Units/mg protein)</th>
<th>CAT (µM H₂O₂/min/mg protein)</th>
<th>GST (µM/min/gm protein)</th>
<th>GPX (µM/min/gm protein)</th>
<th>LPO (µM TMOP)</th>
<th>NITRIC OXIDE (µM Nitrite)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAIN</td>
<td>CONTROL</td>
<td>41.9 ± 1.1</td>
<td>511 ± 79.8</td>
<td>29.58 ± 0.6</td>
<td>121.5 ± 1.8</td>
<td>1.9 ± 0.05</td>
<td>0.78 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>STZ (65 mg/kg)</td>
<td>84.9 ± 2.9*</td>
<td>819 ± 28.6*</td>
<td>64.2 ± 0.76*</td>
<td>181.4 ± 4*</td>
<td>2.5 ± 0.03*</td>
<td>1.34 ± 1.2*</td>
</tr>
<tr>
<td>PANCREAS</td>
<td>CONTROL</td>
<td>36.9 ± 1.9</td>
<td>911 ± 62.8</td>
<td>19.58 ± 0.40</td>
<td>116.5 ± 2.6</td>
<td>1.3 ± 0.05</td>
<td>1.1 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>STZ (65 mg/kg)</td>
<td>74.4 ± 3.2*</td>
<td>1494 ± 41*</td>
<td>54.8 ± 0.36*</td>
<td>191 ± 3.9*</td>
<td>1.5 ± 0.1*</td>
<td>1.6 ± 0.8*</td>
</tr>
<tr>
<td>RIN5F cells</td>
<td>CONTROL</td>
<td>39.3 ± 2.4</td>
<td>677 ± 50.4</td>
<td>4.5 ± 0.156</td>
<td>129.5 ± 1.4</td>
<td>0.68 ± 0.7</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>STZ (20 mM)</td>
<td>25 ± 2.8*</td>
<td>1428 ± 67*</td>
<td>9.24 ± 0.80*</td>
<td>205.3 ± 7.4*</td>
<td>1.31 ± 0.3*</td>
<td>1.93 ± 0.05*</td>
</tr>
</tbody>
</table>
4. Discussion

STZ is specifically toxic to pancreatic \( \beta \) cells due to the presence of a nitrosourea moiety in its structure. STZ decreases NAD levels in the pancreatic \( \beta \) cells that, in turn, leads to an increase in intracellular free radicals and thus, their apoptosis. Pancreatic \( \beta \) cells are known to have a relatively low NAD content that renders them particularly vulnerable to the toxic actions of STZ [32]. Both obesity and type2 diabetes could be due to altered expression of systemic and/or local BDNF. The results of the present study showed that in STZ-induced type 2 DM, plasma, hepatic and pancreatic levels of BDNF are decreased. This suggests that one mechanism by which STZ-induces type 2 DM is by decreasing BDNF levels. These results are in line with the previous observations that BDNF protects pancreatic \( \beta \) cells from various insults [24,33,34].

It was reported that ventromedial hypothalamic (VMH) lesions in non-obese non-insulin-dependent diabetic (Goto-Kakizaki [GK]) rats leads to the development of all features of typical type 2 DM seen in humans [35]. These results [11,12,23,35] suggest that hypothalamus has a critical role in the development of type 2 DM. The results of the Nissl stains performed in the present study indicated a decline in the number of neurons and alterations in their morphology in hippocampus and hypothalamic areas (Fig. 3) supporting the proposal that hypothalamus has a key role in the
pathogenesis of type 2 DM. Furthermore, low levels of BDNF noted in the liver of STZ-induced type 2 diabetic animals in the present study indicates that this may lead to a decrease in insulin signal transduction and contribute to hyperglycemia [22].

It is known that subjects with long-standing diabetes mellitus can develop defects in learning and memory. The demonstration that STZ-induced type 2 DM have a significant loss of hypothalamic and hippocampal neurons as seen in the present study lends support to this idea.

In summary, based on the present results and previous data it is reasonable to propose that STZ-induced type 2 DM is because of a significant increase in systemic and local (pancreas and brain tissue) inflammation and hypothalamic neuronal loss and decreased brain, hepatic and plasma concentrations of BDNF. These abnormalities are likely to lead to low-grade systemic inflammation, hypothalamic and cognitive dysfunction seen in type 2 DM.

Conflict of interest
Nil.

Disclosure statement
The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Author contributions
Conceived and designed the experiments along with idea proposal: UND.Performed the experiments: SB. Analyzed and interpreted the data: SB and UND. UND contributed reagents/materials/analysis tools. Drafting the paper: SB and UND. Read and approved the final manuscript: UND, SB and SN.

Acknowledgements and funding
SB is a Senior Research Fellow of Indian Council of Medical Research (ICMR), New Delhi and the work presented in this paper is part of her PhD work. This work was supported by ICMR (grant number: 3/1(2)/12-RCH) and in part, by grants from Defense Research and Development Organization, New Delhi (ITC/2519/INM - 03/2011/CARS) under R&D Project INM-311) to UND. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We wish to thank Dr. Monika Sadanandam of Mangalore University for extending immunohistochemical studies of brain.

References
[23] T. Nomoura, A. Tsuchida, K.M. Ono, et al., Brain-derived neurotrophic factor...

