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Hippocampal neurogenesis in the prenatally stressed rat is enhanced by resveratrol treatment

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Abstract

An increasing number of reports have provided correlational evidence that prenatal stress caused a suppressed hippocampal neurogenesis. Resveratrol, a polyphenolic activator of sirtuin 1, is known to exert its neuroprotective potential by enhancing neurogenesis in hippocampus. But the efficacy of resveratrol against prenatal stress was not studied to the best of our knowledge. To address this issue we evaluated the neuroprotective action of resveratrol on prenatal stress-induced impaired neurogenesis. Pregnant rats were subjected to restraint stress during early or late gestational period. Another sets of rats received resveratrol during entire gestational period along with early or late gestational stress. Neurogenesis in the hippocampus was assessed by using a neuronal marker doublecortin on 21st postnatal day. Both early and late gestational stress resulted in significant decrease in neurogenesis and in hippocampus. The decrease of neurogenesis was more profound in the offspring who received late gestational stress compared to early gestational stress Resveratrol treatment has improved the neurogenesis. These data suggest the neuroprotective efficacy of resveratrol against prenatal stress induced impaired neurogenesis.

KEY WORDS: DOUBLECORTIN; HIPPOCAMPUS; NEUROGENESIS;PRENATAL STRESS; RESVERATROL

Introduction

Prenatal stress (PS) has been linked to abnormal cognitive, behavioural and psychosocial outcomes in both animals and humans (1). Prenatal stress during pregnancy induces neurobiological and behavioural defects in offspring, some of them involving the hippocampal formation (2). Indeed, prenatal stress results in an enhanced production of stress hormones by the mother during critical periods of fetal brain development and provokes a definitively longer corticosterone response to stress in the offspring associated with a reduction in the number of hippocampal corticosteroid receptors (2). Behaviourally, the progeny, from adulthood to senescence, exhibit memory deficits in a hippocampal-dependent task (2).

Recently, it has been hypothesized that hippocampal-mediated learning may be related to the generation of new neurons in the adult dentate gyrus (3).These new neurons are produced from progenitor cells located in restricted brain regions, including the subgranular zone of the dentate gyrus of the hippocampal formation. Daughter cells, generated locally at the border between hilus and granule cell layer, migrate into the granule cell layer where they develop morphological and biochemical characteristics of mature neurons (4). They receive synapses, extend axonal connections to CA_3 and become functionally integrated into existing neuronal circuitries (5) and it is well documented that prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus and this may be due to the altered hypothalamo-pituitary-adrenal (HPA) axis (6,7).

Resveratrol (3, 4', 5 trihydroxystilbene) is a naturally occurring phytoalexin present in high concentration in the skin and seeds of grapes (8). Recently several animals has focused on the neuroprotective effects of resveratrol, showing it to slow the neuropathology associated with Alzheimer's disease (9) and protect against injury from brain trauma and cerebral ischemia (10). Mokni et al. (11) reports that resveratrol is able to cross the blood brain barrier and exerts potent antioxidant features. Williams et al. (12) also reported that resveratrol is able to cross placental barrier and also did not induce any adverse reproductive effects and mortality in an embryo-fetal toxicity study in rats.

Resveratrol is recognized as a polyphenolic activator of sirtuin 1 (Sirt1), a protein deacetylase (13). Sirt1 can protect neurons from DNA damage-induced apoptosis by downregulating p53 acetylation, an apoptotic mediator (14). Resveratrol treatment of neural progenitor cells (NPCs) is capable of increasing differentiation and directing neurogenesis through a mechanism requiring Sirt1 (15). Thus resveratrol may be beneficial for prenatal stress induced neurotoxicity and even play a role in improving structural and/or functional defects of the hippocampus.

Although mounting evidence convincingly demonstrates the neuroprotective and antioxidant activity of resveratrol in adult animals, but the efficacy of resveratrol against prenatal stress was not studied to the best of our knowledge. These raised a critical question as to whether resveratrol can able to prevent prenatal stress-induced impaired neurogenesis.

To test this hypothesis, we first examined neuronal proliferation in progeny of stress and resveratrol treated mothers with doublecortin (DCX), a microtubule-associated protein specifically expressed in migrating and differentiating neurons. This neuronal specific marker (DCX) were used to phenotype the newly born neurons.

Materials & Methods

Animals

Animals and housing conditions: In-house bred male and female albino Wistar rats (3-4 months old) of weight 200-230gm were selected for the study. The rats were maintained in 12 hours light and dark cycle in temperature and humidity controlled environment. The rats were fed with standard food pellet and water *ad libitum*. Polypropylene cage with paddy husk as bedding materials was used for

housing the rats. Breeding and maintenance of the animals were done as per the guidelines of Government of India for use of Laboratory animals (Government of India notifies the rules for breeding and conducting animal experiments, proposed in the gazette of India Dec 15, 1998: which was reproduced in Ind. Journal of Pharmacol 31:92-95, 1999). Institutional Animal Ethics Committee (I.A.E.C) approval was obtained before the conduct of the study (IAEC/KMC/2010) and care was taken to handle the rats in humane manner.

Mating of rats and animal groups: Three female rats were allowed to mate with one fertile sexually active male rat for 4 hours per day (separate male rats for each group). At the end of 4 hours, female rats were separated and vaginal smears taken to detect the presence of sperm for the confirmation of pregnancy and the rats were designated as day 0 of pregnancy for further counting the days. The pregnant rats were housed individually in separate cages with proper label indicating the day of conception and randomly allocated into six groups of six each. One male and one female pups from each mother were considered for hippocampal neurogenesis (n=12; six male and six female pups) study on 21st postnatal day. All the mothers delivered at term (22-24th day of gestation). The offspring were raised by their biological mothers until weaning (21 days after birth). The number of offspring considered for neonatal parameters is in accordance with Holson & Pearce (16).

Stressing procedure: The pregnant rats were stressed (restraint stress) using a wire mesh restrainers (17), for three times daily for 45 minute (08:00 AM–11:00 AM, 12:00 AM–3:00 PM, and 4:00 PM–7:00 PM). The wire mesh restrainer will have a wooden base and stainless steel wire mesh restrainer hinged to the base. A pad lock and latch will help to secure the rat in the restrainer. The restrainer with dimension 11 cm (L) x 6cm (B) x 6 cm (H) was used for rats with gestation day 1 to 10. Restrainer of 11cm (L) x 8 cm (B) x 8 cm (H) was used for rats with gestation day 11 to till delivery. This type of restrainer will only restrict the animal movement without any pain, discomfort or suffocation.

Animal groups:

Group 1. (Control) The pups belonging to the pregnant rats who received only 0.5% carboxy methyl cellulose in a dose of 10ml/kg body weight (oral) throughout pregnancy.

Group 2. The pups belonging to the pregnant rats who received only resveratrol alone in a dose of 10mg/kg body weight (oral) throughout pregnancy.

Group 3. The pups belonging to the pregnant rats who received restrain stress from gestation day 1 to 10.

Group 4. The pups belonging to the pregnant rats who received restrain stress from gestation day 11 to till delivery.

Group 5. The pups belonging to the pregnant rats who received restrain stress from gestation day 1 to 10 and resveratrol (10mg/kg body weight, oral) throughout pregnancy.

Group 6. The pups belonging to the pregnant rats who received restrain stress from gestation day 11 to till delivery and resveratrol (10mg/kg body weight, oral) throughout pregnancy.

Resveratrol (Cat. no. 70675, Cayman chemicals, USA) was obtained from Pro Lab marketing, New Delhi, India. The dose of resveratrol considered in the present study is according to the earlier study by Kumar et al (18).

Immunohistochemical assay for Doublecortin (DCX) in the Hippocampus: On 21st postnatal day, female and male rats were used for immunohistochemical assay. Animals were sacrificed by cardiac perfusion with 4% paraformaldehyde under ether anaesthesia. The brains were removed, postfixed in

the same fixative 48 hours. Paraffin blocks were made in an embedding bath and blocked coronally and sectioned from the septal area where the two blades of the dentate are equal and formed a V-shape at post-Bregma. Sections of 7 ìm were cut in the dorsal hippocampus using a rotary microtome (Jung Biocutt 2035,Leica, Germany) and sections were mounted on poly-L-lysine-coated glass slides. Immunohistochemical procedures for doublecortin immunostaining (19) were carried out using a one-in-fifteen series of sections. Sections were washed in phosphate buffer solution, contained 3.0% H_2O_2 to remove the endogenous peroxidase activity. Sections were blocked in 10% Normal Horse serum, with 0.01% triton X-100 for 30 minutes. These sections were incubated with anti doublecortin antibody (1:250; Santa Cruz, CA, sc-8066) 4 hour at room temperature. These sections were washed and incubated in biotinylated anti goat IgG as secondary antibody (1:200; Vector Lab, BA-9500) for 1 hour, followed by avidin biotin complex (1:50;Vector Lab, SK-3600) for one hour. Sections were developed colour with diaminobenzedine / vector grey (Vector Lab, SK-5671) as chromogen. All sections were observed using light microscopy at 40X magnification. The numbers of newly born neurons, i.e. doublecortin positive neurons were counted (500µ) in subglanular zone (SGZ)/granular cell layer (GCL) of hilus of dentate gyrus using occulomicrometer.

Statistical analysis: Data are presented as the means±SE. Statistical analysis for multiple comparisons was performed by one way analysis of variance (ANOVA) with Bonferroni's corrections. Comparison of data between male and female group was assessed by unpaired "t" test. *p* values < 0.05 were considered as significant.

Results

There was no sexually dimorphic effect was observed in all the assessed parameters, hence mean values for both male and female were collapsed together. Restraint stress (both early and late) during pregnancy and resveratrol treatment do not have any significant effect on gestational length (p=0.077, F=2.231) and litter size (p=0.689, F=0.614). There was no mortality in any of the group till 40th postnatal day.

Quantification of DCX +vet neurons (marker of neurogenesis) in 21 days old rat brain: The DCX +ve neuron is a marker of neurogenesis. Both early and late gestational stress (group-3,p<0.01 and group-4, p<0.001) found to produce a significant decrease in DCX positive neurons in subglanular zone (SGZ)/granular cell layer (GCL) of hilus of DG in 21 days old rat brain. Offspring received parental stress during late gestation (group-4) showed a significant (p<0.001) decrease in DCX positive neurons in DG compared to the offspring received early gestational stress (group-3). Offspring who received both prenatal stress during early and late gestation and resveratrol treatment (group-5 and group-6) showed a significant increase in DCX positive neurons in SGZ/GCL of hilus of DG when compared to prenatally stressed offspring of group-3(p<0.05) and group-4(p<0.001) respectively in both 21st postnatal day rat brain. No significant change in DCX positive neurons in DG was observed in offspring whose mothers were treated with resveratrol alone (group-2). (Figure 1 and Figure 2)

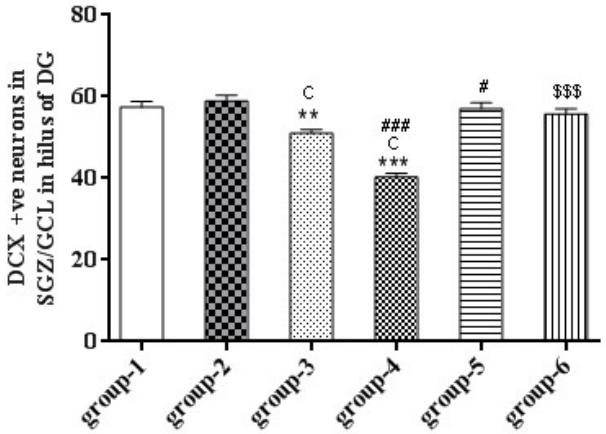
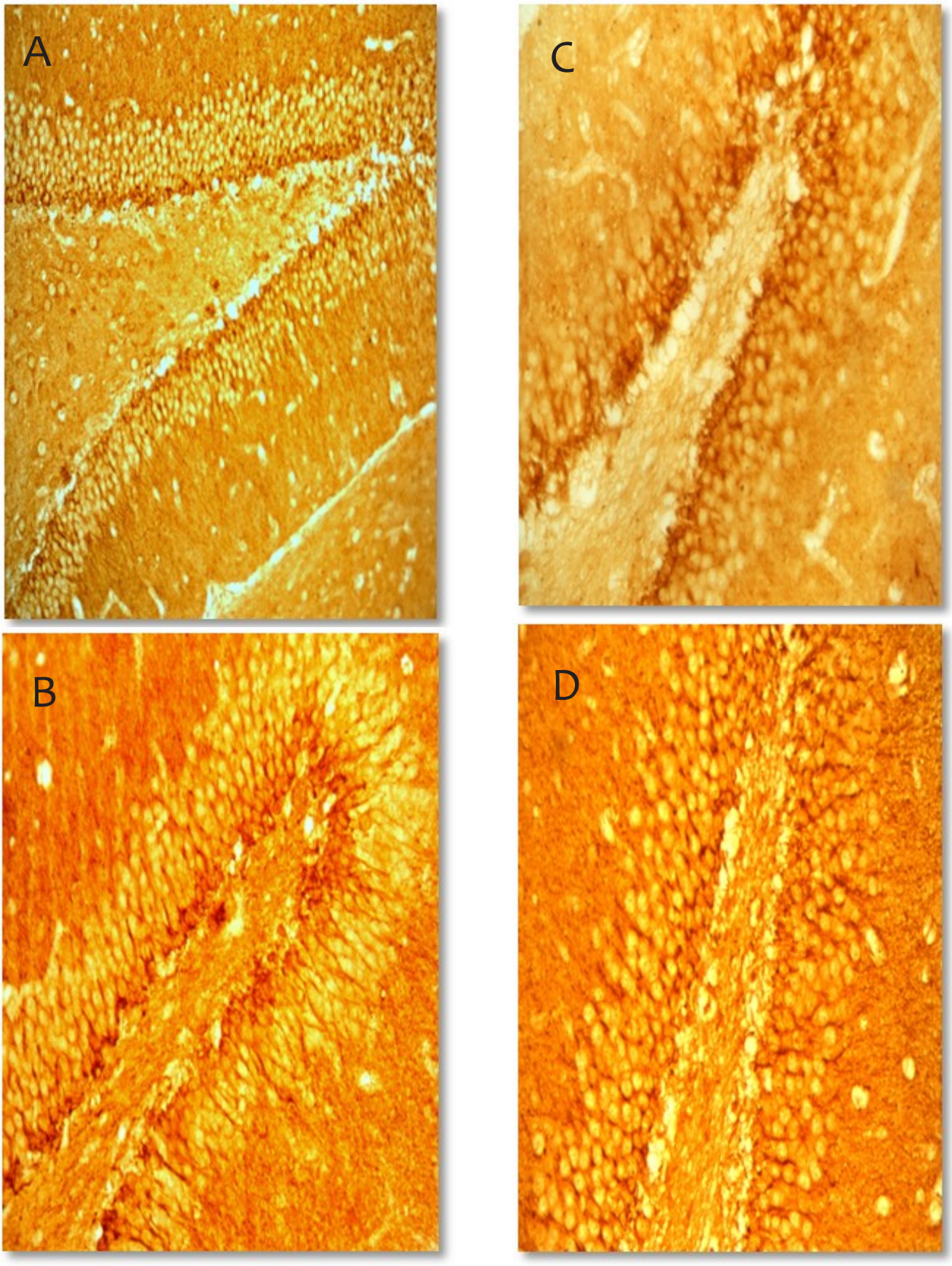


Figure 1. Effect of prenatal stress and resveratrol treatment on neurogenesis (21 days old rat brain). Values are expressed as Mean±SEM (n=66 slides per group). For comparison with Group-1, **p< 0.01; ***p < 0.001, for comparison with Group-2, ^{c}p< 0.001, for comparison with Group-3, $^{\#}$ p< 0.05; $^{\#\#\#}$ p< 0.001 and for comparison with Group-4, $^{\$\$\$}p$< 0.01. (One way ANOVA, Bonferroni's test).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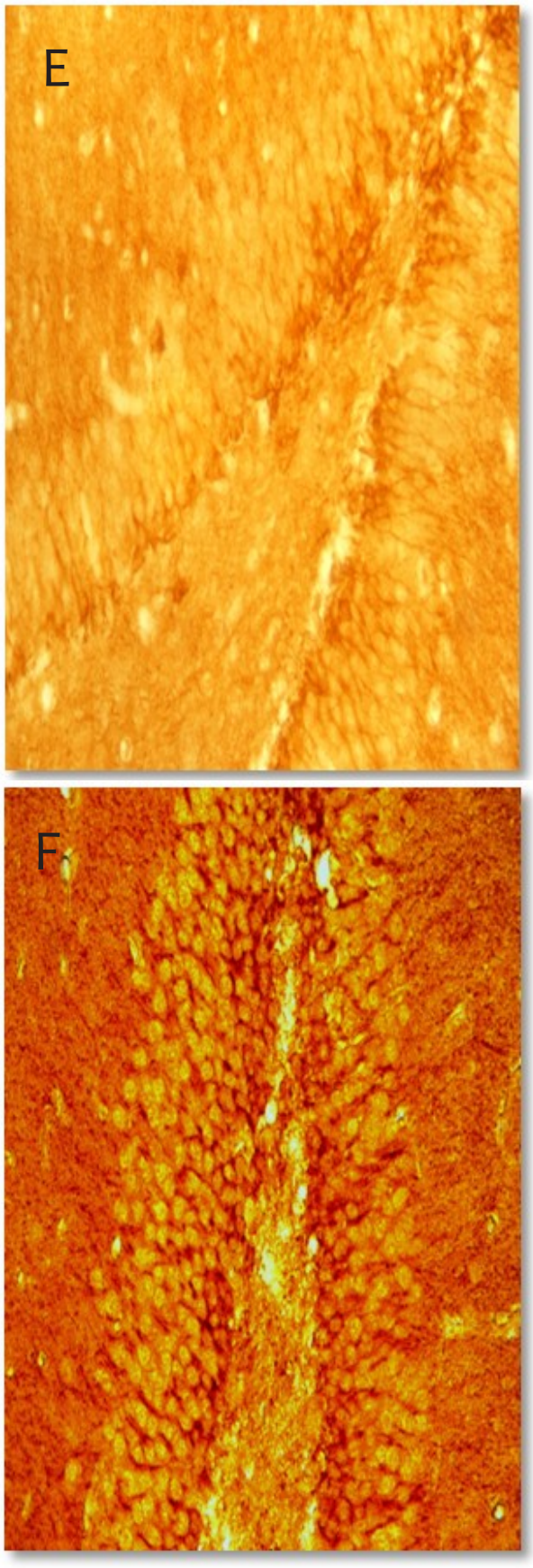

Figure 2. Photomicrographs of doublecortin positive neurons in hilus of DG in 21 days old rat brain under 40X: (a) control,group-1, (b) pups received prenatal resveratrol during entire gestation period, group-2; (c) pups received prenatal stress during day 1 to 10, group-3; (d) pups received stress during day 11 till delivery, group-4; (e) pups received prenatal stress during day 1 to 10 and resveratrol during entire gestation period, group-5; (f) pups received prenatal stress during day 11 to till delivery and resveratrol during entire gestation period, group-6.(from left to right)

Discussion

The results of the present study demonstrate that prenatal stress during early and late gestation caused a reduction of neurogenesis in dentate gyrus. The decrease of neurogenesis in dentate gyrus was more profound in the offspring who received late gestational stress compared to early gestational stress. Our primary findings show that resveratrol treatment enhances significantly the neurogenesis in dentate gyrus in prenatally stressed rat brain.

Earlier studies have demonstrated that prenatal stress decreases hippocampal neurogenesis (6). Prenatal stress and decreased neurogenesis are putatively associated with memory impairment (6). Exposure to prenatal stress is reported to decrease cell proliferation, survival and differentiation (20). Our results are in agreement with these reports that prenatal stress affects neurogenesis in the dentate gyrus. A number of studies suggest that prenatal stress is associated with alterations in the offspring HPA axis activity. In our previous study we confirmed that prenatal stress associated with higher basal glucocorticoid (GC) secretion in the off spring (21). A large body of evidence has been gathered, demonstrating that elevated levels of glucocorticoids, affects both hippocampal structure and function (22), as the hippocampus densely populated with receptors for GC hormones, stress and glucocorticoids strongly inhibit adult neurogenesis, leads to cognitive impairments and depressive illness (6).

It is well documented from various studies that prenatal stress during late gestation compared to early gestation results in an enhanced production of glucocorticoids by the mother, which crosses the placental barrier. This is due to reduced expression

of placental 11b-hydroxysteroid dehydrogenase type 2 (11b-HSD-2, barrier to maternal glucocorticoids) and provokes a longer corticosterone response to stress in the offspring associated with a reduction in the number of neuronal corticosteroid receptors (23) and caused a decreased neurogenesis and BDNF level in hippocampus of the offspring brain. While there is a plentiful 11b-HSD-2 in the CNS at early and mid-gestation (24), which presumably protects vulnerable developing brain from maternal glucocorticoid action.

As a strategy to reverse the prenatal stress-induced deficits, we used maternal resveratrol treatment during pregnancy, which has a major impact on neuroprotection. For the first time in our knowledge we report here that prenatal resveratrol treatment restores the restraint stress induced decrease in survival and differentiation of progenitor cells i.e. DCX positive neurons. Resveratrol has been shown to possess significant free-radical scavenging properties in a variety of cellular types and this antioxidant properties believed to protect the offspring hippocampus against prenatal stress induced neuronal loss due to oxidative stress (25). Resveratrol is recognized as a polyphenolic activator of sirtuin 1 (Sirt1). Sirt1 is a nicotinamide adenine dinucleotide-dependent histone deacetylase that downregulates acetylation levels of many regulatory proteins involved in cell survival, energy homeostasis, DNA repair, and life span extension (26). It is widely expressed in the CNS to promote its development (27). Sirt1 can protect cells from stress damage through deacetylating its targets (28,29). Sirt1 can deacetylate p53 at the Lys residues (Lys373 and/or Lys382) and protect various cells from DNA damage-induced apoptosis (30). Resveratrol treatment of neural progenitor cells (NPCs) is capable of increasing differentiation and directing neurogenesis through a mechanism requiring Sirt1. In another report (31), resveratrol induced neurogenesis by enhancing AMP-activated protein kinase (AMPK) in neurons and in Sirt1-deficient mice brain. AMPK activation stimulates neuronal differentiation as well as mitochondrial biogenesis in neurons. More cellular energy including ATP can be produced and neuronal activity is enhanced accordingly. More ATP produced by mitochondria can activate BDNF (32) and synthesis of brain derived neurotrophic factor stimulated in an activity-dependent manner will increase (33). It is important to confirm whether these findings are associated with the AMPK activation in the future.

In summary, we observed prenatal stress during early and late gestation caused a significant decrease of neurogenesis in offspring. Resveratrol treatment was effective in improving prenatal stress induced decrease in neurogenesis. Hence this study demonstrates that, neuroprotective action of resveratrol against prenatal stress induced decreased neurogenesis. Future studies aiming at precisely understanding the cellular mechanisms involved in the neuroprotective effects of resveratrol could thus open new avenues for the treatment of prenatal stress induced disorders.

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