Inhibition of the transforming growth factor-β/SMAD cascade mitigates the anti-neurogenic effects of the carbamate pesticide carbofuran

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The widely used carbamate pesticide carbofuran causes neurophysiological and neurobehavioral deficits in rodents and humans and therefore poses serious health hazards around the world. Previously, we reported that gestational carbofuran exposure has detrimental effects on hippocampal neurogenesis, the generation of new neurons from neural stem cells (NSC), in offspring. However, the underlying cellular and molecular mechanisms for carbofuran-impaired neurogenesis remain unknown. Herein, we observed that chronic carbofuran exposure from gestational day 7 to postnatal day 21 altered expression of genes and transcription factors and levels of proteins involved in neurogenesis and the TGF-β pathway (i.e., TGF-β; SMAD-2, -3, and -7; and SMURF-2) in the rat hippocampus. We found that carbofuran increases TGF-β signaling (i.e., increased phosphorylated SMAD-2/3 and reduced SMAD-7 expression) in the hippocampus, which reduced NSC proliferation because of increased p21 levels and reduced cyclin D1 levels. Moreover, the carbofuran-altered TGF-β signaling impaired neuronal differentiation (BrdU/DCX+ and BrdU/NeuN+ cells) and increased apoptosis and neurodegeneration in the hippocampus. Blockade of the TGF-β pathway with the specific inhibitor SB431542 and via SMAD-3 siRNA prevented carbofuran-mediated inhibition of neurogenesis in both hippocampal NSC cultures and the hippocampus, suggesting the specific involvement of this pathway. Of note, both in vitro and in vivo studies indicated that TGF-β pathway attenuation reverses carbofuran’s inhibitory effects on neurogenesis and associated learning and memory deficits. These results suggest that carbofuran inhibits NSC proliferation and neuronal differentiation by altering TGF-β signaling. Therefore, we conclude that TGF-β may represent a potential therapeutic target against carbofuran-mediated neurotoxicity and neurogenesis disruption.

Neurogenesis is a process of generation of new neurons throughout the life, from a population of dividing and precisely located neural stem cells (NSC) 5 (1, 2). NSC mainly reside in the dentate gyrus region of the hippocampus and subventricular zone of the brain (3, 4). The newly generated neurons functionally integrate into the existing neuronal circuitry to regulate normal functions of the brain, such as learning, memory, and response to injury (5, 6). The process of neurogenesis is known to be reduced with the advancement of age, chronic exposure to certain drugs, sleep deprivation, mental stress, and steroids, whereas it is increased by physical activity and voluntary exercise (7–12). Exposure to certain toxicants, such as methyl-mercury, lead, Bisphenol-A, and ethanol, leads to an alteration in the process of neurogenesis, resulting in motor and cognitive function deficits (13–18). Long-term exposure to these environmental contaminants together with bioaccumulation over an individual’s lifetime are speculated to induce neuroinflammation and neuropathology, causing development of certain neurological disorders.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methyl carbamate) is a commonly used carbamate insecticide, nematocide, and acaricide, to which humans can be exposed by contaminated food, vegetables, and drinking water (19). Carbofuran has a relatively good solubility in water and can contaminate surface and ground water; therefore, it carries a great risk of human exposure. Carbofuran is nonspecific in action and also affects non-target species, including humans (20). It has been reported to be highly toxic to the mammals, which can induce chromosomal aberrations, micronucleus formation, and sperm abnormalities (21). Carbofuran causes oxi-
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dative stress with increased generation of reactive oxygen species and reactive nitrogen species in the skeletal muscle and in the hippocampus of the brain (22, 23). Acute exposure of carbofuran can even cause death by inhibition of acetylcholinesterase at synaptic junctions (24). Carbofuran impairs the energy state of neurons by inhibition of glycolysis and decreases ATP levels along with reduced dendritic length and spine density of pyramidal neurons in the hippocampus. It induces DNA fragmentation, resulting in apoptosis and loss of hippocampal neurons (23, 25). It has also been reported that low-dose exposure of carbofuran causes changes in lipid composition and membrane-bound enzyme activity and also disturbs synapticominal calcium homeostasis along with neurophysiological and neurobehavioral alterations in rodents (26–28). Evidence has also shown that carbofuran causes Tau hyperphosphorylation with activation of glycogen synthase kinase-3β and inhibition of protein phosphatase-2A, raising the risk of early onset of Alzheimer’s disease (29).

The TGF-β family of proteins comprises the TGF-βs, bone morphogenetic proteins (BMPs), activins, nodal, and growth and differentiation factors. TGF-β1 is a cell cycle regulator and acts as a potential inhibitor for the proliferation of the hippocampal neural progenitor cells in mice, and it plays a key role in adult neurogenesis (30, 31). Overproduction of TGF-β1 from astrocytes creates inflammatory conditions in the central nervous system in mice (32). The level of TGF-β increases in the brain during Alzheimer’s disease and is responsible for the increase in vascular production of pro-inflammatory cytokines, IL-1β, and tumor necrosis factor-α (33). Elevated TGF-β signaling also induces stem cell quiescence in the subgranular zone of the hippocampus (34). The levels of TGF-β were also reported to be increased in the striatum and cerebrospinal fluid of ventricles in Parkinson’s disease (35).

TGF-β1 acts as an anti-proliferating agent and induces cell cycle exit by up-regulation of cyclin-dependent kinase inhibitor p21 in the developing cortex and in the hippocampus during aging (31, 36). A study showed increased levels of TGF-β in the hippocampus following prenatal exposure of ethanol (37). Another study reported that TGF-β1 induces cell death in NSC treated with ethanol via the FasL-mediated apoptotic pathway (38). TGF-β1 promotes differentiation of radial glia into astrocyte in the cerebral cortex, followed by a decreased number of neurons in the cortical plate (39). Inhibition of TGF-β and BMP signaling pathways induces neuronal differentiation of human adipose-derived stem cells (40). The inhibition of SMAD signaling greatly increases neural conversion of human embryonic stem cells and induced pluripotent stem cells (41, 42). The inhibition of TGF-β signaling also restores cell cycle progression defects in NSC of aged mice (43). A recent study showed that systemic attenuation of the TGF-β pathway restores hippocampal neurogenesis in aged mice (31).

In our previous study, we observed that prenatal carbofuran exposure causes reduced neurogenesis in the brain of offspring (44). Therefore, in the present study, we investigated the underlying molecular mechanism for reduced neurogenesis due to prenatal carbofuran exposure. We found a robust increase in the levels of TGF-β1 in the hippocampus of carbofuran-exposed rats. Attenuation of TGF-β signaling suggested that up-regulation of this pathway is responsible for reduced proliferation and neuronal differentiation of NSC as well as apoptosis of newly proliferating cells in carbofuran-treated animals. This study also demonstrated that inhibition of TGF-β signaling restores cognitive and memory deficits caused by prenatal carbofuran exposure in rats.

Results

Carbofuran decreases proliferation and neuronal differentiation of the hippocampus-derived NSC in vitro

We assessed the effects of different concentrations of carbofuran (1, 10, 20, 50, 100, 200, and 400 μM) on viability and proliferation of the NSC derived from the hippocampus by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the Alamar blue assay (Fig. 1, a and b). Carbofuran significantly decreased proliferation and viability of NSC after 24-h treatment above 50 μM (Fig. 1, a and b). Here, we found that a 50 μM concentration of carbofuran was a noncytotoxic concentration, which was further used to assess the effects of carbofuran in all in vitro experiments.

Next, we performed a neurosphere (clonal aggregates of NSC) growth kinetics assay to evaluate the effects of carbofuran on NSC proliferation. Dissociated NSC isolated from the hippocampus were cultured and allowed to grow as neurospheres in the presence of carbofuran (50 μM) and analyzed for gross morphology and number and size (diameter) of neurospheres. We observed a significant decrease in both the number and size of neurospheres after carbofuran treatment as compared with control (p < 0.05; Fig. 1 (c–e)). These results show that carbofuran treatment significantly decreases NSC proliferation, as suggested by the reduced number and size of neurospheres.

Next, we studied the effects of carbofuran on NSC proliferation by double immuno-co-labeling of BrdU with nestin (a marker for progenitor cells). We found that carbofuran treatment significantly reduced the number of BrdU/Nestin+ co-labeled cells in culture (Fig. 1g). Further, to assess the effect of carbofuran on the neuronal and glial differentiation of NSC, we performed co-labeling of BrdU with β-tubulin-III (neuronal marker), CNPase (oligodendrocyte marker), and glial fibrillary acidic protein (GFAP) (glial markers). To confirm the specificity of GFAP labeling, cells were also labeled with S100β (astrocyte marker) to show that these cells were indeed astrocytes (45–47). We observed a significantly increased astrocyte population (BrdU/GFAP/S100β+) and reduced number of mature neurons (BrdU/β-tubulin-III+ cells) and oligodendrocytes (BrdU/CNPase− cells and BrdU/MBP− cells) after carbofuran treatment as compared with the control group (p < 0.05; Fig. 1 (h and i) and supplemental Fig. S1 (a and b)).

Carbofuran impairs progenitor cell proliferation and differentiation in the hippocampus

To investigate the effects of prenatal carbofuran exposure on the proliferation of NSC in coronal hippocampal sections, we performed co-labeling of BrdU with Sox-2 (a marker of progenitor cells). We found that Sox-2/BrdU+ proliferating hippocampal neural stem and progenitor cells significantly (*, p < 0.05) declined in the dentate gyrus region due to prenatal carbofuran exposure (Fig. 2, a and b). These results imply that
Figure 1. Carbofuran reduces proliferation and neuronal differentiation of NSC derived from the hippocampus of rat embryo. (a) and (b), cultured primary hippocampal NSC in 96-well plates were treated with different concentrations of carbofuran for 24 h. Effects of carbofuran on cell viability of NSC were assessed by MTT and Alamar blue assay. The graph shows percentage cell viability as compared with control. *, $p < 0.05$ versus control. Values are expressed as mean $\pm$ S.E. (error bars) ($n = 3$ independent experiments). (c), representative phase-contrast photographic images of hippocampus-derived neurospheres treated with a non-cytotoxic concentration (50 $\mu$M) of carbofuran. (d) and (e), quantitative analysis shows that carbofuran significantly reduces the number and size (diameter) of neurospheres as compared with control. Scale bar, 100 $\mu$m. (f) and (g), immunofluorescence images of BrdU/Nestin– co-labeled cells counterstained with nuclear stain DAPI in NSC culture treated with carbofuran for 24 h. The bar diagram shows that the number of BrdU/Nestin– co-labeled cells significantly decreases in the presence of carbofuran as compared with control. (h) and (i), immunofluorescence photomicrographs showing the effects of carbofuran on the differentiation of NSC. Immunocytochemistry was performed for co-labeling of BrdU with neuronal marker $\beta$-tubulin-III, astrocyte markers GFAP and S100$\beta$, and oligodendrocyte marker CNPase. Quantitative analysis shows a significant reduction in the number of $\beta$-tubulin-III/BrdU and CNPase/BrdU– co-labeled cells, whereas there was a robust increase in the number of GFAP/ S100$\beta$/BrdU– triple-labeled cells following carbofuran treatment as compared with the control group. Scale bar, 20 $\mu$m.
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Figure 2. Carbofuran altered the proliferation and differentiation of NSC in the hippocampus of rat brain. a and b, immunofluorescence was performed for NSC marker Sox-2 (red) and BrdU (green). The representative graph shows a significant decrease in the number of Sox-2/BrdU–co-labeled cells in the hippocampus due to the treatment of carbofuran as compared with the control group. Arrows indicate Sox-2/BrdU–co-labeled cells. ML, molecular layer; GCL, granular cell layer; DG, dentate gyrus. Values are expressed as mean ± S.E. (error bars) (n = 6 rats). *, p < 0.05 versus control group. Scale bar, 20 μm. c and d, representative photomicrographs showing BrdU (red)-positive cells co-labeled with astrocyte markers GFAP (blue) and S100β (green) in the hippocampus of the rat brain. Arrows, GFAP/S100β/BrdU–triple-labeled cells. Quantitative analysis suggested a significantly increased number of GFAP/S100β-positive cells co-labeled with BrdU in the hippocampus of carbofuran-treated rats. e and f, representative images show that carbofuran treatment alters neuronal differentiation of NSC in the hippocampus, as assessed by co-labeling of BrdU with the mature neuronal marker (NeuN). Quantitative analysis shows that the number of NeuN/BrdU–co-labeled cells in the hippocampus of carbofuran-treated rats was significantly decreased as compared with the control group. Arrows, NeuN/BrdU–co-labeled cells. g and h, representative photomicrographs showing BrdU (green)-positive cells co-labeled with oligodendrocyte markers CNPase (red) in the hippocampus of the rat brain. Arrows, CNPase/BrdU–co-labeled cells. Quantitative analysis suggested a significantly reduced number of CNPase/BrdU–co-labeled cells in the hippocampus of carbofuran-treated rats as compared with control. i, quantitative real-time PCR mRNA expression analysis of neurogenic and gliogenic genes (Nestin, GFAP, S100β, β-tubulin-III, DCX, neurogenin, neuregulin-1, neuro-D1, Pax-6, Stat-3, Olig-1, CNPase, and MBP) in the hippocampus of the rat brain.
Carbofuran treatment decreases the NSC pool and their proliferation in the hippocampus of the rat brain.

The newly born BrdU+ proliferating cells in the hippocampus may have a different fate, where they may differentiate into either neuron or glia and some cells also die through apoptosis. Therefore, we studied the effects of prenatal carbofuran exposure on cell fate of BrdU+ cells in the hippocampus. We carried out co-labeling of cells with BrdU/GFAP/S100β (astrocyte marker), BrdU/NeuN (neuronal marker), and BrdU/CNPase (oligodendrocyte marker) to determine the effects of carbofuran exposure on cell fate and phenotype of newly born cells in the dentate gyrus. Quantitative analysis suggested that carbofuran treatment significantly increased the number of BrdU/GFAP/S100β+ triple-labeled cells in the DG with respect to the control group (Fig. 2, c and d). In contrast, we found a significantly reduced number of BrdU/NeuN+, BrdU/CNPase+, and BrdU/MBP+ co-labeled cells in the hippocampus as compared with control (Fig. 2 e–h) and supplemental Fig. S2 (a and b)). From these results we infer that carbofuran treatment causes a significant increase in astrocyte differentiation and reduction in neuronal and oligodendroglial differentiation of newborn cells in the rat hippocampus.

Next, we found that carbofuran caused significant down-regulation in the expression of nestin, β-tubulin-III, neurogenin, neuregulin-1, neuroD1, DCX, Pax-6, Olig-1, CNPase, and MBP genes, whereas the expression of GFAP, S100β, and Stat-3 genes was significantly increased (Fig. 2i). These results suggested that prenatal carbofuran exposure reduced the expression of neurogenic genes that ultimately leads to impaired hippocampal neurogenesis. The genes related to oligodendrocyte lineage (Olig-1, CNPase, and MBP) were also found to be down-regulated, whereas those of astroglial lineage (GFAP, S100β, and Stat-3) were increased significantly due to carbofuran exposure.

**Carbofuran increases TGF-β/SMAD signaling in the hippocampus**

TGF-β expression has been reported to increase with age in the subventricular zone and hippocampus of the brain, contributing to a decline in neurogenesis (31, 48). We studied whether the impaired neurogenesis due to carbofuran exposure correlates with levels of TGF-β in the hippocampus. Quantitative real-time PCR analysis showed that the mRNA expression of TGF-β1, SMAD-2, and SMAD-3 was significantly increased in the carbofuran-treated group (Fig. 3a). In contrast, the genes, such as SMAD-7 and SMURF-2, that negatively regulate the TGF-β pathway were down-regulated in the hippocampus following carbofuran treatment. The mRNA expression of TGFBR1 and TGFBR2 receptors, as well as SMAD-4, remained unaltered after carbofuran exposure. The mRNA expression of p21, a cell cycle inhibitor, was significantly increased, whereas the expression of cyclin D1 was reduced after carbofuran exposure in rats (Fig. 3a).

Immunoblot analysis revealed that carbofuran significantly enhanced the protein levels of TGF-β1 in the hippocampus as compared with control (Fig. 3, b and c). Interestingly, densitometry analysis suggested that carbofuran significantly increased the ratio of p-SMAD-2/3 to SMAD-2/3, which is a hallmark of the up-regulation of TGF-β pathway (Fig. 3, b and d). Along with this, the protein levels of SMAD-7 were significantly reduced by carbofuran treatment (p < 0.05), whereas there was no significant change in SMAD-4 protein levels. The p21 arrests progression of the cell cycle by inhibiting the activity of cyclin-dependent kinases (49). Cyclin-D1 acts as a regulatory subunit of CDK4 and -6 and plays a critical role in the regulation of G1 progression during the cell cycle (50). Interestingly, the levels of p21 were remarkably increased, whereas the levels of cyclin D1 were significantly decreased in carbofuran-treated groups as compared with control (p < 0.05; Fig. 3, b and c).

These results suggest that carbofuran significantly alters the expression of the TGF-β pathway genes in the hippocampus, which leads to reduced proliferation of NSC.

To assess the cellular source responsible for elevated levels of TGF-β1 in the hippocampus, immunostaining of TGF-β1 with GFAP/S100β (astrocyte markers) and Iba-1 (microglial marker) was carried out in the hippocampus. We found that in the hippocampus, both astrocytes and microglia expressed TGF-β1. The number of GFAP/S100β/TGF-β1 and Iba-1/TGF-β1 co-labeled cells was significantly increased in carbofuran-treated rats as compared with control, suggesting that they are the source of the carbofuran-mediated increase in levels of TGF-β (Fig. 3, e–h). To further confirm these results, we analyzed downstream pSMAD signaling in NSC in the rat hippocampus. We found that the levels of pSMAD-3 were up-regulated in Sox-2+ neural stem and progenitor cells in the hippocampus of carbofuran-exposed rats as compared with control (Fig. 3, i and j). All of these results confirmed an elevation of TGF-β signaling in the hippocampal stem cell niche due to carbofuran exposure.

**Effects of carbofuran on NSC proliferation and differentiation in vitro in the presence of TGF-β1 pathway activator (TGF-β1 protein) and inhibitor (SB431542)**

We studied whether carbofuran impairs NSC proliferation and differentiation via the TGF-β pathway. For this study, the hippocampus-derived NSC were treated with TGF-β pathway activator (TGF-β1 protein) and pharmacological inhibitor of the TGF-β receptor 1 kinase (SB431542) before carbofuran treatment. SB431542 is a small-molecule drug and selective pharmacological inhibitor of activin receptor-like kinase, receptors involved in TGF-β/SMAD signaling (51). We observed that carbofuran treatment significantly reduced proliferation (Sox-2/BrdU+ cells) and neuronal differentiation (β-tubulin-III+ cells) of NSC in culture (Fig. 4, a–d). Treatment of NSC cultures with TGF-β1 protein also significantly decreased NSC proliferation and neuronal differentiation (Fig. 4, a–d). In contrast, SB431542 significantly increased NSC proliferation and neuronal differentiation in vitro. Interestingly, carbofuran-mediated reduced proliferation and neuronal differentiation of NSC was significantly attenuated in the presence of SB431542, suggesting a role of the TGF-β pathway in carbofuran-mediated effects on neurogenesis. Moreover, co-treatment of carbofuran with TGF-β1 protein further reduced proliferation and neuronal differentiation of NSC (Fig. 4, a–d). These findings suggest that the TGF-β pathway activation is involved in carbofuran-mediated reduced proliferation and neuronal differentiation of NSC.
Genetic inhibition of the TGF-β pathway attenuates the effects of carbofuran on NSC neuronal differentiation

We found that carbofuran increased TGF-β1 mRNA expression and protein levels, leading to impaired neurogenesis. To further confirm the specific involvement of this signaling pathway and build upon these results, we attenuated the TGF-β pathway by genetic inhibition of the SMAD-3 gene in NSC culture. Genetic inhibition involved transfection of NSC culture with SMAD-3 siRNA before carbofuran treatment. Using immunoblotting, we found that the transfection of SMAD-3 siRNA efficiently knocked down the protein levels of SMAD-3 in NSC culture, whereas transfection with scrambled siRNA showed no significant changes in protein levels of SMAD-3 (Fig. 5, a and b). Neurospheres growth kinetics and neuronal dif-
differentiation were studied after the respective treatments. Knockdown of SMAD-3 reversed the effects of carbofuran-mediated reduced size and number of neurospheres (Fig. 5, c–e). Similarly, knockdown of SMAD-3 caused enhanced neuronal differentiation, as was evident from the increased number of β-tubulin-III+ neurons (Fig. 5, f and g). Transfection of NSC with scrambled siRNA showed no significant effects on proliferation and neuronal differentiation. These results indicate that carbofuran was involved in the up-regulation of the TGF-β pathway, leading to decreased neurogenesis.

Systemic attenuation of the TGF-β pathway exerts neuroprotection in carbofuran-treated rats

For this study, we used another set of animals with two additional groups (TGF-β pathway inhibitor SB431542 and carbofuran + SB431542–treated). The SB431542 (5 mg/kg body weight) was administered systemically via intraperitoneal injection into offspring once daily for 3 consecutive days/week from postnatal day 1 (PND1) to PND21. The dose for SB431542 was selected on the basis of other earlier studies (52, 53).

We quantified the numbers of BrdU/Sox-2+–co-labeled cells in the hippocampal sections. Interestingly, neurogenesis was significantly enhanced in rats treated with SB431542 as compared with the control. We observed that SB431542 treatment significantly increased the number of BrdU/Sox-2+–co-labeled cells in the hippocampus as compared with control (Fig. 6, a and b). Treatment of SB431542 in carbofuran-exposed rats provided protection against carbofuran-mediated impaired neurogenesis, as evident from the increased number of BrdU/Sox-2+–proliferating cells in the hippocampus as compared with carbofuran-exposed rats (Fig. 6, a and b).

Next, we performed double immunolabeling of BrdU with DCX (a marker of immature neurons) to analyze the effects of TGF-β pathway inhibitor on neuronal differentiation following carbofuran exposure. We observed that carbofuran treatment caused a reduction in the number of BrdU/DCX+ cells as compared with control (Fig. 6, c and d). Interestingly, there was a significant increase in the number of BrdU/DCX+ cells in the carbofuran + SB431542 group as compared with the carbofuran-treated group (Fig. 6, c and d). Thus, SB431542 amelio-

Figure 4. Effect of in vitro carbofuran treatment on proliferation and differentiation of the hippocampus-derived NSC culture in the presence of activator and inhibitor of the TGF-β pathway. a–d, hippocampus-derived NSC culture was treated with the TGF-β pathway activator (TGF-β1 protein) and inhibitor (SB431542) in the presence and absence of carbofuran to demonstrate their effects on proliferation and differentiation. Immunolabeling for Sox-2 (red) and BrdU (green) was performed and counterstained with DAPI (blue). Representative immunofluorescent images and quantitative analysis suggested that carbofuran and TGF-β protein significantly decreased Sox-2/BrdU–co-labeled cells in culture, but SB431542 ameliorated the inhibitory action of carbofuran on proliferation of NSC. c and d, representative immunofluorescent images and quantitative analysis suggested that carbofuran and TGF-β protein significantly decreased neuron-specific β-tubulin-III–labeled cells (red), whereas SB431542 ameliorated the inhibitory action of carbofuran on neuronal differentiation. Values are expressed as mean ± S.E. (error bars) (n = 3). *, p < 0.05 versus control; #, p < 0.05 versus carbofuran. Scale bar, 20 μm.
rates the effects of carbofuran by enhancing the number of neuroblasts in the subgranular zone of the hippocampus.

We also carried out co-labeling of NeuN and BrdU to study the effects on maturation of neurons. We found that a significantly increased number of newborn BrdU-positive cells were co-labeled with NeuN after treatment of SB431542, suggesting increased maturation of newly born cells into postmitotic neurons as compared with the carbofuran-treated group (Fig. 6, e and f). Further, densitometry analysis of Western blots confirmed the decrease in pSMAD signaling in the hippocampus of SB431542-treated rats. The ratio of pSMAD-2/3 to SMAD-2/3 was significantly reduced in SB431542-treated rats as compared with carbofuran-treated rats and SB431542–treated animals (Fig. 6, g and h). Along with this, the protein levels of DCX and NeuN were also significantly increased in the carbofuran + SB431542 group as compared with rats treated with carbofuran. Interestingly, the lev-
els of cell cycle inhibitor p21 were significantly reduced, whereas levels of cyclin D1 were significantly increased after treatment with SB431542 (Fig. 6, i and j).

In our previous study, we showed that carbofuran exerts neurotoxicity and neurodegeneration in the hippocampus through induction of apoptosis in NSC. Herein, we performed immune co-labeling of cleaved caspase-3 with BrdU to evaluate the effects of SB431542 on carbofuran-induced apoptosis in the hippocampal NSC (Fig. 7, a and b). Carbofuran treatment significantly enhanced BrdU/cleaved caspase-3 co-labeled cells, which were

Figure 6. Administration of pharmacological inhibitor (SB431542) of TGFBR1 reverses the inhibitory effects of carbofuran on NSC proliferation and neuronal differentiation in the hippocampus. a and b, co-labeling was performed for Sox-2 (red) and BrdU (green) counterstained with nuclear stain DAPI. Quantitative analysis of immunofluorescence images shows that treatment of SB431542 significantly ameliorated the inhibitory effects of carbofuran on NSC proliferation, as depicted by an increased number of BrdU/Sox-2–co-labeled cells followed by treatment with inhibitor. Values are expressed as mean ± S.E. (error bars) (n = 6). Scale bar, 20 μm. *, p < 0.05 versus control; #, p < 0.05 versus carbofuran. c and d, immunofluorescence staining was conducted for immature neuron marker DCX (red) and BrdU (green) to study the effects on neuronal differentiation of NSC in the hippocampus. The graphical representation suggested an increased number of BrdU/DCX–co-labeled cells in the presence of SB431542 followed by carbofuran treatment. e and f, immuno-co-labeling for NeuN (red) and BrdU (green) was carried out to study the effects on maturation of neurons in the hippocampus. Quantitative analysis suggested that the number of NeuN/BrdU–co-labeled cells significantly increased in the hippocampus of SB431542-treated rats following carbofuran exposure. g–j, Western blot analysis of the proteins in hippocampal tissue. *, p < 0.05 versus control; #, p < 0.05 versus carbofuran.
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markedly diminished by SB431542 in the hippocampus region (Fig. 7, a and b). Carbofuran significantly increased the levels of Bax (pro-apoptotic protein), which was reversed by SB431542 (Fig. 7, c and d). The levels of cleaved caspase-3 in the hippocampus were enhanced due to carbofuran treatment. However, SB431542 treatment remarkably reduced the protein levels of cleaved caspase-3 following carbofuran exposure (Fig. 7, c and d).

Next, we performed Fluoro-jade-B staining to observe the effects of SB431542 on neurodegeneration in the hippocampus following carbofuran exposure. Qualitative analysis shows that the number of degenerating neurons (Fluoro-jade B+ cells) was significantly decreased in the hippocampus region of the rat brain by administration of SB431542 following carbofuran exposure. Quantitative analysis of degenerating neurons shows a significant decrease in the number of degenerating neurons (Fluoro-jade B+ cells) in the hippocampus region of the rat brain by administration of SB431542 following carbofuran exposure (Fig. 7, e and f).

Figure 7. Inhibition of TGF-β pathway by administration of SB431542 reduces apoptosis and neurodegeneration in the hippocampus of the rat brain and restores learning and memory in adolescent rats following carbofuran exposure. a and b, immunofluorescence co-labeling was performed for cleaved caspase-3 (red) and BrdU (green). The bar graph shows that the number of BrdU/cleaved caspase-3–co-labeled cells was significantly decreased by treatment with SB431542 following carbofuran exposure in the hippocampus of the rat brain. Values are expressed as mean ± S.E. (error bars) (n = 6). Scale bar, 20 μm. *, p < 0.05 versus control; #, p < 0.05 versus carbofuran. c and d, Western blot analysis suggests significantly reduced levels of pro-apoptotic protein Bax and cleaved caspase-3 in the hippocampus tissue dissected from rats administered with SB431542 following carbofuran exposure. e and f, Fluoro-jade-B staining was performed to study the effects of SB431542 on neurodegeneration in the hippocampus following carbofuran exposure. Quantitative analysis shows that the number of degenerating neurons (Fluoro-jade B+ cells) was significantly decreased in the hippocampus region of the rat brain by administration of SB431542 following carbofuran exposure. Scale bar, 100 μm. g, cognitive ability (learning and memory) was measured following assessment of two-way conditioned avoidance behavior using a shuttle box apparatus. SB431542 significantly restored cognitive ability in the rats treated with carbofuran. Values are expressed as mean ± S.E. (n = 6). *, p < 0.05 versus control; #, p < 0.05 versus carbofuran.
of TGF-β pathway inhibition on carbofuran-mediated neurodegeneration in the hippocampus. The number of Fluoro-jade-B+ neurons significantly decreased in the hippocampus of the carbofuran + SB431542 group as compared with carbofuran-treated rats (Fig. 7, e and f). We performed a neurobehavioral assay to study effects on cognitive functions using conditioned avoidance in different groups. Administration of SB431542 in carbofuran-treated rats significantly restored learning and memory deficits as compared with the carbofuran-treated group (Fig. 7g).

On the basis of all of these experiments, we proposed a schematic mechanistic representation illustrating the possible mechanism(s) of carbofuran-mediated inhibition of hippocampal neurogenesis and involvement of the TGF-β/SMAD pathway (Fig. 8).

**Discussion**

Carbofuran is one of the most widely used carbamate pesticides applied on farms against enormous insect species. Due to its vast applications, it poses serious health risks to humans. The common routes of its exposure involve inhalation, ingestion, or absorption through the skin (54). Carbofuran disrupts various normal biological functions in humans when exposed during the gestational as well as the postnatal period, which includes endocrine disruption, neurobehavioral alternations, and reproductive abnormalities (55–57). Carbofuran causes several developmental abnormalities in the fetus due to its transfer through the placenta as well as the blood–brain barrier during gestation and through mother milk during the lactation period (58, 59). Our previous study showed that gestational carbofuran exposure causes learning and memory deficits in rat offspring due to inhibition of hippocampal neurogenesis (44).

But the underlying cellular and molecular mechanisms for carbofuran-mediated reduced neurogenesis were still unknown. The TGF-β pathway is known to be involved in cell cycle regulation, NSC maintenance, neuronal/glial differentiation, and apoptosis and negatively regulates the process of neurogenesis (30, 60, 61). In the present study, we investigated the role of TGF-β signaling in carbofuran-mediated altered neurogenesis in the rat brain.

In the current study, we demonstrated that oral exposure of carbofuran to the rats from gestational day 6 (GD6) to PND21 decreases hippocampal neurogenesis due to elevated TGF-β signaling in the brain. Carbofuran exposure up-regulates the TGF-β pathway, which leads to decreased proliferation and neuronal differentiation of NSC both in vitro and in vivo. Further, we found that the rise in TGF-β levels is responsible for apoptotic cell death of NSC and neurodegeneration in the hippocampus following carbofuran exposure, and all of these effects were reversed by inhibition of TGF-β in vitro and in vivo.

We observed that the proliferation and viability of NSC derived from the hippocampus decrease dose-dependently with increasing concentration of carbofuran (>50 μM) in vitro. These findings suggested that carbofuran has cytotoxic potential that inhibits the proliferation and viability of NSC in culture. Further, carbofuran treatment caused a significant decrease in size and number of free-floating primary neurospheres, suggesting a decrease in the self-renewing ability of NSC. Nestin, an intermediate filament protein,
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plays an important role in proper survival and self-renewal of NSC (62). Carbofuran decreased the pool of NSC, as depicted by the lesser number of nestin/Brdu immune-co-labeled cells in culture and reduced relative mRNA expression of nestin in the hippocampus. All of these results suggested that carbofuran treatment decreases proliferation and the self-renewable ability of multipotent NSC. We next studied the effects of carbofuran exposure during gestational periods on proliferation and differentiation of NSC in the hippocampus of the brain. Sox-2 is a transcriptional regulator, which plays an important role in directing the differentiation of pluripotent stem cells to neural progenitors and is responsible for maintaining the properties of stem cells (63). Carbofuran significantly decreased Sox-2/Brdu–co-labeled cells in the GCL of the hippocampus compared with control rats. Further, we evaluated the effects of carbofuran on the destiny and phenotype of proliferating cells. Carbofuran reduced the number of postmitotic neurons (Brdu/NeuN–co-labeled cells) and oligodendrocytes (Brdu/CNPase–co-labeled cells) while, interestingly, increasing the population of astrocytes (Brdu/GFAP/S100–triple-positive cells) in the hippocampus. These results suggest that carbofuran decreases neuronal and oligodendrocytes differentiation of newborn cells in the hippocampus, whereas it increases astrocyte differentiation. A plethora of cytotoxic cytokines secreted from activated glial cells in the neurogenic niches tightly regulate the inflammatory responses and the process of neurogenesis (64–66).

Several neurogenic and gliogenic genes, such as nestin, doublecortin, β-tubulin-III, GFAP, and transcription factor neurogenin and neuregulin 1, neuroD1, pax-6, and Sox-2, regulate neurogenesis (15, 70). Chlorpyrifos, another cholinesterase-inhibiting insecticide, shows similar effects, which also increases TGF-1 expression by astrocytes in transgenic mice promotes hallmarks of Alzheimer’s disease like amyloid formation and microvascular degeneration (79). During aging, vascular-derived TGF-1 increases in the stem cell niche and decreases neurogenesis in the adult mouse brain (80). TGF-1 acts as a negative modulator of adult neurogenesis (60). Chronic overexpression of TGF-1 also alters dentate gyrus microstructure and macrostructure in the hippocampus and causes spatial learning deficits (81).

Our study demonstrated that carbofuran reduced the hippocampal neurogenesis through up-regulation of the TGF-β signaling pathway. In our study, we found that the number of GFAP/TGF-β1 and Iba1/TGF-β1–co-labeled cells significantly increased following carbofuran exposure. These findings suggested that astrocytes and microglia are responsible for the elevated levels of TGF-β in the stem cell niche of the hippocampus in carbofuran-treated rats. We also found increased mRNA expression and protein levels of TGF-β1 and associated proteins of this pathway, such as SMAD-2 and SMAD-3, in carbofuran-treated rats. Interestingly, carbofuran reduced the expression of inhibitory SMAD-7 and SMURF-2, which negatively regulate the TGF-β pathway. SMAD-7 blocks the phosphorylation of regulatory Smads (SMAD-2 and SMAD-3) by forming stable complexes with activated TGFβR1 receptors (82, 83). SMURF-2 recruits ubiquitin E3 ligases, which results in ubiquitination and degradation of activated type I receptors (84). This up-regulation of the TGF-β pathway could be correlated with increased neurodegeneration and learning and memory deficit in carbofuran-treated rats in our study.

TGF-β1 specifically arrests neural stem and progenitor cells in the G0/G1 phase of the cell cycle and acts as a potent inhibitor of the hippocampal neural progenitor cell proliferation in adult mice (30, 60). The reduced proliferation is strongly correlated with an increased accumulation of phospho-SMAD-2, an effector of the TGF-β signaling in TGF-β1–infused brains (60). TGF-β1 promotes cell cycle exit mediated by up-regulation of cyclin-dependent kinase inhibitor p21 and a decrease in the levels of cyclin D1 (36, 85). In our study, we also observed a robust increase in the number of Sox-2/pSMAD-3–co-labeled cells along with a significant increase in p21 mRNA and protein levels and reduced cyclin D1 levels in the hippocampus of the carbofuran-treated group. Interestingly, all of these carbofuran-mediated effects were reversed by inhibition of the TGF-β pathway using SB431542 both in vitro and in vivo. A study showed that TGF-β greatly reduces the number and self-renewal ability of primary and secondary neurospheres derived from midbrain cells (86). These findings are in support of our study in which we observed the number and size of neurospheres is significantly increased in the presence of SMAD-3 siRNA in the carbofuran-treated NSC cells. Collectively, all of these findings reflect that up-regulation of TGF-β signaling could be the possible cause of reduced proliferation of NSC in carbofuran-exposed rats.

TGF-β modulates inflammatory responses in the CNS and controls the proliferation of microglia and astrocytes. TGF-β promotes the growth of glioma cells via activating SMAD and ERK-1/2 pathways (87). TGF-β1 signaling is associated with
SMAD-2/3 nuclear translocation; a hallmark of TGF-β1 pathway activation that induces astrocyte fate commitment of radial glia cells in vitro (88). Overexpression of TGF-β resulted in a reduction of DCX-expressing immature neuron population in the hippocampus of transgenic mice (30). Treatment of astrocytes in vitro with TGF-β1 resulted in a significant increase in GFAP mRNA and protein (89). Infusion of TGF-β1 lowered the number of DCX-expressing neuronal precursors in the neurogenic niches (subventricular zone and hippocampus) of the brain (60). All of these earlier reports again support our findings that up-regulation of TGF-β pathway is responsible for increased astrocyte differentiation (GFAP/S100β/BrduU+ cells) and reduced neuronal differentiation (DCX/BrduU+ and NeuN/BrduU+ cells) in the hippocampus of carbofuran-treated rats. In contrast, treatment with SB431542 or knockdown of SMAD-3 increased the neuronal differentiation of newly proliferating BrduU+ cells both in vitro and in vivo. TGF-β1 potentiates apoptotic cell death by up-regulating extrinsic pathway transcripts and proteins in NSC. TGF-β1 increases Fas expression at the transcript and protein levels (38). 

Inhibition of the TGF-β1 pathway rejuvenates hippocampal neurogenesis in aged mice (31). A recent study showed that systemic attenuation of the TGF-β1 pathway SB431542–treated group, which received a daily single oral gavage of carbofuran (1 mg/kg body weight) in corn oil as vehicle from GD7 to postnatal day 21 (PND21); 2) the carbofuran–treated group, which received a daily single oral gavage of carbofuran suspended in corn oil (1 mg/kg body weight) during GD7–PND21; 3) the inhibitor of TGF-β1 pathway SB431542–treated group, which received a daily single oral gavage of corn oil during GD7–PND21 and intraperitoneal injection of SB431542 (5 mg/kg body weight) for 3 consecutive days/week during PND1–PND21; and 4) the carbofuran + SB431542–treated group, which received a daily single oral gavage of carbofuran (1 mg/kg body weight) in corn oil during GD7–PND21 and intraperitoneal injection of SB431542 (5 mg/kg body weight) for 3 consecutive days/week during PND1–PND21.

The dose of carbofuran (1 mg/kg body weight) was selected on the basis of our previous study, in which carbofuran reduced hippocampal neurogenesis in the rat brain (44). Similarly,

**Materials and methods**

Carbofuran, BrduU, SB431542, BSA, MTT, Tris-base, poly-L-lysine, basic fibroblast growth factor (bFGF), EGF, rabbit anti-GFAP (G9269, lot 025M4843V), mouse anti-β-tubulin-III (T8578, lot 054M4819V), rabbit anti-SMAD-7 (AV32008, lot QC1577), rabbit anti-caspase-3 active (C8487, lot 104M4842V), mouse anti-β-actin (A5441, lot 123M4887V), rabbit anti-CNPase (C9743, lot 118K0441), and mouse anti-S100β (S2532, lot 02K4879) primary antibodies were procured from Sigma-Aldrich. Neurobasal medium, Hanks’ balanced salt solution, N-2 supplement, B-27 supplement, Alamar blue, normal goat serum (NGS), anti-oxidant-antimycotic solution, and TRIzol reagent were obtained from Gibco. Lipofectamine was procured from Invitrogen. A set of three Stealth SMAD-3 siRNAs (catalog no. 1330001, assay ID RSS332753, RSS352298, and RSS352299) and Stealth siRNA negative control (catalog no. 12935110) was procured from Invitrogen. Alexa Fluor 488-, 594-, and 350–conjugated secondary antibodies were procured from Molecular Probes (Invitrogen). A protease and phosphatase inhibitor mixture was purchased from Thermo Scientific. Monoclonal rabbit anti-neuronal nuclei (NeuN) (ABN78, lot 2748625), and chemiluminescence substrate and PVDF membrane were obtained from Chemicon (Millipore). Mouse anti-BrdU (sc-32323, lot H2813), rabbit anti-SMAD-2/3 (sc-8332, lot A2513), goat anti-pSMAD-2/3 (sc-11769, lot C2913), and rabbit anti-SMAD-4 (sc-7154, lot J2312) primary antibodies were obtained from Santa Cruz Biotechnology, Inc. Rabbit anti-TGF-β1 (ab66043, lot GR276420-2), goat anti-Iba1 (ab5076, lot GR153106-5), mouse anti-Sox-2 (ab79351, lot GR168366-5), rabbit anti-Sox-2 (ab97959, lot GR128998-1), rabbit anti-BrduU (ab152095, lot GR256486-11), rabbit anti-cyclin D1 (ab134175, lot GR192733-1), rabbit anti-SMAD-3 (ab28379, lot GR155884-7), rabbit anti-pSMAD3 (ab51177, lot GR123828-1), rabbit anti-MBP (ab40390, lot GR225584-1), and rabbit anti-p21 (ab109199, lot GR296632-7) primary antibodies were obtained from Abcam. Rabbit anti-DCX (4604S, lot 01/2016), rabbit anti-Bax (2772, lot 02/2016), and mouse anti-nestin (4760S, lot 02/2016) were purchased from Cell Signaling Technology (Danvers, MA). Primers were procured from Integrated DNA Technologies, and SYBR Green was obtained from Applied Biosystems. Anti-fade mounting medium with DAPI was obtained from Vector Laboratories (Vectorshield). Cultureware was obtained from Nunc (Denmark).

**Animals and carbofuran treatment**

Adult female Wistar rats (180–200 g) were obtained from the Animal Breeding Colony of the CSIR-Indian Institute of Toxicology Research. All of the experimental animals were handled following the guidelines of institutional animal ethical committee. A water and pellet diet was provided ad libitum, and the animals were maintained under standard temperature and humidity conditions with a 12-h light/dark cycle. The female rats were paired with male rats for breeding, and a vaginal smear test was used to confirm their pregnancy. Females with a vaginal smear test positive for the presence of sperm were considered as pregnant, and the day was designated as GD0. Pregnant rats were randomly segregated into the following groups: 1) the control group, which received a daily single oral gavage of corn oil as vehicle from GD7 to postnatal day 21 (PND21); 2) the carbofuran–treated group, which received a daily single oral gavage of carbofuran suspended in corn oil (1 mg/kg body weight) during GD7–PND21; 3) the inhibitor of TGF-β1 pathway SB431542–treated group, which received a daily single oral gavage of corn oil during GD7–PND21 and intraperitoneal injection of SB431542 (5 mg/kg body weight) for 3 consecutive days/week during PND1–PND21; and 4) the carbofuran + SB431542–treated group, which received a daily single oral gavage of carbofuran (1 mg/kg body weight) in corn oil during GD7–PND21 and intraperitoneal injection of SB431542 (5 mg/kg body weight) for 3 consecutive days/week during PND1–PND21.
the dose for SB431542 was selected on the basis of an earlier study showing loss of TGF-β signaling during oligodendrogenesis (52).

**BrdU administration for labeling proliferating cells and cell fate/survival analysis**

A set of pups from all of the groups received a single intraperitoneal injection of BrdU (50 mg/kg body weight) for 3 consecutive days during PND19–PND21 for detection of proliferating and newborn cells as described in our previous studies (15, 44). BrdU-injected rats from all of the groups were divided into two subgroups at PND21, for NSC proliferation and differentiation studies. A set of pups from each group was sacrificed 4 h after the last BrdU injection at PND21 for the NSC proliferation study. Further, to determine the effects of carbofuran on long-term survival of NSC and neuronal differentiation, pups of another set were allowed to survive for an additional 3 weeks after the last BrdU injection and sacrificed at PND42 for NSC survival and cell fate analysis.

**Immunohistochemistry**

After respective treatments, animals were deeply anesthetized with ketamine and xylazine (3:1) and were sacrificed by transcardial perfusion with normal saline followed by ice-cold 4% paraformaldehyde (pH 7.2). The brains were placed in 4% paraformaldehyde for 30 min, washed three times with 0.1% Triton X-100 in PBS. Sections were then incubated in blocking buffer containing 3% NGS, 0.5% BSA, then kept in citrate buffer (pH 6.0) at 60 °C for 30 min. Sections were washed three times for 15 min with PBS followed by a 2-h incubation in blocking buffer containing 3% NGS, 0.5% BSA, and 0.1% Triton X-100 in PBS. Sections were then incubated with primary antibodies overnight at 4 °C with denature DNA followed by neutralization with borate buffer (0.1 M, pH 8.5) for 10 min at room temperature. Sections were then washed in citrate buffer (pH 6.0) at 60 °C for 30 min. Sections were then incubated with secondary antibodies (1:200) linked with Alexa Fluor 488, 594, 647, or 555 for 2 h at room temperature. Finally, sections were mounted on a glass slide with anti-fade mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). The images were captured and analyzed under a Nikon Eclipse Ti-S inverted fluorescent microscope. The labeled cells were analyzed in a total of six sections per rat of each group. The quantitation of immunolabeled cells in the hippocampus region for proliferation and differentiation studies was carried out following our earlier studies (15, 44, 91).

**Immunocytochemistry**

To evaluate the effects of carbofuran on proliferation and differentiation of NSC in the presence and absence of TGF-β pathway inhibitor, immunocytochemistry was performed. In brief, primary neurospheres were harvested by centrifugation at 1000 rpm and dissociated into single-cell suspension using 0.25% trypsin. Dissociated NSC were plated on a poly-l-lysine–coated 4-well chamber slide at a density of 10,000 cells/well in proliferation (neurobasal medium containing 20 ng/ml bFGF and EGF, antibiotic antimycotic, l-glutamine, B-27, and N-2 supplement) and differentiation media (neurobasal medium containing antibiotic, antimycotic, l-glutamine, B-27, and N-2 supplement). Cells were treated with carbofuran (50 μM) in the presence and absence of TGF-β1 protein (10 ng/ml) and SB431542 (10 μM) for 24 h (30, 41, 92). Cells were then fixed with 4% paraformaldehyde for 30 min, washed three times with PBS, and incubated in blocking buffer (2% NGS, 0.5% BSA, and 0.1% Tween 20) for 1 h at room temperature. Further, cells were incubated overnight at 4 °C with monoclonal primary antibodies mouse anti-nestin (1:200), rabbit anti-Sox-2 (1:250), and mouse anti-BrdU (1:200) for proliferation studies and mouse anti-β-tubulin-III (1:200), rabbit anti-GFAP (1:200), mouse anti-S100β, and rabbit anti-CPNPase (1:250) for differentiation studies. After washing with PBS, cells were then incubated with Alexa Fluor 488 or 594–conjugated secondary antibodies for 2 h at room temperature. Slides were coverslipped with DAPI containing antifade mounting medium, and fluorescent images were acquired using an inverted fluorescent microscope.

**Gene expression analysis using quantitative real-time PCR**

To assess the effects of carbofuran on the expression of genes involved in neurogenesis and the TGF-β pathway, quantitative real-time PCR analysis was carried out following our earlier published studies (44, 93). In brief, total RNA was isolated using TRIzol reagent from the hippocampus region of control and carbofuran-treated rats according to the manufacturer’s instructions. An equal amount of RNA was reverse-transcribed using the Superscript first-strand cDNA synthesis kit and diluted in nuclease-free water to a final concentration of 10 ng/μl. The expression of the cellular housekeeping gene β-actin was used as internal control. Real-time PCR was performed using SYBR Green and an ABI PRISM 7900 sequence detection system (Applied Biosystems). Reactions were run in triplicate, and the relative expression was calculated using the ∆∆Ct method.

**Protein level analysis by Western blotting**

To determine the levels of proteins involved in neurogenesis and the TGF-β pathway, Western blotting was performed as described earlier (44). The hippocampus region was dissected from the brain, and tissue was lysed with cell lytic MT mammalian tissue lysis reagent (Sigma) with a protease and phosphatase inhibitor mixture. Equal amounts of protein (60 μg) were loaded on the Tris-glycine gel and transferred onto PVDF membrane. Membranes were blocked in Western Blocker solution (Sigma-Aldrich) for 2 h at room temperature. Blots were incubated overnight at 4 °C with primary antibodies TGF-β1 (1:1000), Iba-1 (1:250), pSMAD-3 (1:200), DCX (1:250), and cleaved caspase-3 (1:250) followed by incubation in secondary antibodies (1:200) linked with Alexa Fluor 488, 594, or 350 or 2 h at room temperature. Finally, sections were mounted on a glass slide with anti-fade mounting medium containing DAPI. The images were captured and analyzed under a Nikon Eclipse Ti-S inverted fluorescent microscope. The labeled cells were analyzed in a total of six sections per rat of each group. The quantification of immunolabeled cells in the hippocampus region for proliferation and differentiation studies was carried out following our earlier studies (15, 44, 91).
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Primary culture of the hippocampal NSC

NSC were isolated from the hippocampus of rat embryos (embryonic day 12) and cultured as in our earlier published studies (94). Briefly, hippocampal tissues were collected in cold Hanks’ balanced salt solution and chopped into small pieces followed by incubation in 0.25% trypsin for 30 min at 37 °C. Trypsin was neutralized with 0.5 mg/ml soybean trypsin inhibitor followed by gentle trituration and centrifugation. Cells were resuspended in neurobasal medium supplemented with 2 mm l-glutamine, 1% antibiotic antimycotic, 2% B-27, 1% N-2 supplement, EGF (20 ng/ml), and bFGF (20 ng/ml). The cells were transferred in 25-cm² flasks and allowed to grow in a CO₂ incubator at 37 °C, where the neurospheres gradually formed after 4–5 days of culture.

Cell viability assessment by MTT assay

To examine the effects of carbofuran on NSC proliferation in culture, an MTT assay was carried out (93). NSC were seeded in a 96-well plate at a density of 1 × 10⁴ cells/well. Cells were treated with different concentrations of carbofuran in DMSO (1, 10, 20, 50, 100, 200, and 400 μM) for 24 h. Following treatment, MTT (0.5% (w/v) in PBS) was added to each well and incubated for another 4 h at 37 °C. The formazan crystals were dissolved in 200 μl of DMSO, and the absorbance was measured at 570 nm using a multidetection microplate reader. We found that carbofuran was nontoxic for NSC at concentrations up to 50 μM.

Neurosphere growth kinetics assay

To assess the effects of carbofuran on proliferation potential of NSC in the presence and absence of TGF-β1 protein (10 ng/ml) and TGF-β pathway inhibitor SB431542 (10 μM), we performed a neurosphere growth kinetics assay as described earlier (15, 30, 41, 92, 93). Single-cell suspension of primary NSC isolated from the hippocampus was seeded in a 12-well plate at a density of 5 × 10⁴ cells/well and treated with carbofuran (50 μM), TGF-β1 (10 ng/ml), and SB431542 (10 μM). The number and size of neurospheres were analyzed in all of the groups using an inverted phase-contrast microscope (Nikon).

Knockdown of SMAD-3 in NSC culture

SMAD-3 knockdown in NSC culture was performed at nearly 70% cell confluence. The primary NSC cultures derived from the hippocampus were transiently transfected with a pool of three SMAD-3 siRNA (10 nm each) and scrambled siRNA (non-targeting negative control) using Lipofectamine transfection reagent as per the manufacturer’s protocol. We performed Western blotting and densitometry analysis to determine the transfection efficiency of siRNA in our experiment. We found ~70% knockdown of SMAD-3 protein levels in the NSC culture after 24 h of SMAD-3 siRNA transfection. The cells were harvested 24 h post-transfection. The neurosphere growth kinetic assay and immunostaining were performed to assess the effects on NSC proliferation and differentiation.

Conditioned avoidance response

We performed two-way conditioned avoidance behavior to assess the learning and memory ability of control–, carbofuran–, SB431542–, and carbofuran + SB431542–treated rats using a shuttle box apparatus (Columbus Instruments) as described in our earlier studies (44, 91). The percentage of conditioned avoidance response was considered as a measure of cognitive ability among all of the groups for analysis of learning and memory. Learning and memory in treated rats were calculated as compared with the percentage of control.

Statistical analysis

All of the data were statistically analyzed using InStat software (GraphPad Software, La Jolla, CA). All of the values are expressed as mean ± S.E. The mean significant difference among multiple treatment groups was determined using one-way analysis of variance followed by the Tukey–Kramer post hoc multiple-comparison test, whereas the individual treatment between the two groups was assessed by Student’s t test using t values by keeping degrees of freedom at the 5% level of significance and a 95% confidence interval. p values of <0.05 were considered to be statistically significant.

Author contributions—B. S. and R. K. C. conceived and coordinated the study, performed experiments, analyzed data, and wrote the paper. B. S., S. A., S. K. T., and A. Y. designed, performed, and analyzed the experiments shown in Figs. 1–7. All authors reviewed the results and approved the final version of the manuscript.

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