Toxicology Letters 226 (2014) 20–27

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Maternal single injection of *N*-methyl-*N*-nitrosourea to cause microcephaly in offspring induces transient aberration of hippocampal neurogenesis in mice

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HIGHLIGHTS

- Maternal exposure effect of MNU on hippocampal neurogenesis was examined in mice.
- Single injection of MNU caused microcephaly and dentate gyrus hypoplasia.
- Doublecortin$^+$ cells transiently decreased reflecting aberration in differentiation.
- Reelin$^+$ and parvalbumin$^+$ cells increased reflecting disruption of neurogenesis.
- MNU mainly targets transient populations of highly proliferative progenitor cells.

ARTICLE INFO

Article history:
Received 26 November 2013
Received in revised form 7 January 2014
Accepted 8 January 2014
Available online 22 January 2014

Keywords:
N-Methyl-*N*-nitrosourea (MNU)
Microcephaly
Hippocampal dentate gyrus
Neurogenesis
γ-Aminobutyric acid (GABA) ergic interneuron

ABSTRACT

N-Methyl-*N*-nitrosourea (MNU) is an alkylating agent having antiproliferative cytotoxity targeting the neural stem/progenitor cells to cause microcephaly by maternal exposure. This study investigated the effect of transient exposure to MNU on the process of hippocampal neurogenesis in later life using mice. Pregnant mice received a single injection of MNU at 0, 5 and 10 mg/kg body weight, intraperitoneally on gestational day 14, and their offspring were examined on postnatal day (PND) 21 and PND 77. On PND 21, offspring displayed microcephaly and hippocampal formation hypoplasia at 10 mg/kg, decrease of doublecortin (Dcx)$^+$ cells in the dentate subgranular zone from 5 mg/kg, and decrease of TUNEL$^+$ apoptotic cells and increase of transcript expression of anti-apoptotic *Bcl-2* at 10 mg/kg in the dentate gyrus. In the dentate hilus, numbers of reelin$^+$ or parvalbumin (Pvalb)$^+$ interneurons or neuron-specific nuclear protein$^+$ neurons increased at 10 mg/kg. Microcephaly and hippocampal formation hypoplasia continued through PND 77 at 10 mg/kg. Thus, apart from the massive cell killing at the migratory stream causing microcephaly, MNU may decrease Dcx$^+$ cells reflecting disruption of the differentiation process of late-stage neuronal progenitors and immature granule cells through defective molecular functions by gene mutations. Increase of reelin$^+$ and Pvalb$^+$ cells may reflect the disruption of neurogenesis and following neuronal migration. All of the granule cell lineage and interneuron changes disappeared at the adult stage on PND 77 suggesting that MNU mainly targets transient populations of highly proliferative progenitor cells but hardly affects their stem cells having self-renewal ability.

Abbreviations: Bax, BCL2-associated X protein; Bcl2, B cell leukemia/lymphoma 2; BW, body weight; CA, cornu ammonis; Dcx, doublecortin; DG, dentate gyrus; GABA, γ-aminobutyric acid; GCL, granule cell layer; GD, gestational day; GFAP, glial fibrillary acidic protein; Hprt, hypoxanthine-guanine phosphoribosyltransferase; MNU, *N*-methyl-*N*-nitrosourea; NeuN, neuron-specific nuclear protein; Pax6, paired box 6; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PND, postnatal day; Pvalb, parvalbumin; Reln, reelin; RT-PCR, reverse-transcription polymerase chain reaction; SGZ, subgranular zone; Sox2, SRY (sex determining region Y)-box 2; Tbr2, T box brain 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; VZ, ventricular zone.

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http://dx.doi.org/10.1016/j.toxlet.2014.01.014

1. Introduction

In the mammalian brain, the ability to generate new neurons continues into adulthood in a few selected regions, including the subventricular zone and subgranular zone (SGZ) of the hippocampal dentate gyrus (DG; Supplementary Fig. 1; Cameron and McKay, 1998). In the SGZ, postnatal neurogenesis, so called "adult neurogenesis", starts from type-1 stem cells and produces intermediate generations in the order of type-2a, type-2b, and type-3 progenitor cells. Type-3 cells undergo final mitosis to differentiate into immature granule cell, and then to mature granule cells in the granule cell layer (GCL; Hodge et al., 2008). Adult neurogenesis can be affected by various intrinsic and extrinsic stimuli, such as circulating hormones, brain neurotransmitter levels, enriched environments, running and induction of seizures (Lee and Son, 2009).

In the hilus of the DG, γ-aminobutyric acid (GABA) ergic interneurons control neurogenesis in the SGZ, providing projections to growing granule cells to help their differentiation (Masiulis et al., 2011). A subpopulation of GABAergic interneurons produces reelin, which is a secreted glycoprotein, and regulates the migration and correct positioning of granule cell progenitors (D'Arcangelo et al., 1997; Gong et al., 2007). It may be reasonable to evaluate the neurotoxic effects on neurogenesis by analyzing the distribution of neuronal progenitor cell populations in combination with interneuron subpopulations in the DG during the developmental period as we have previously done for neurotoxicants such as acrylamide (AA), manganese, chlorpyrifos, nicotine and glycidol (Akane et al., 2013; Ogawa et al., 2012; Ohishi et al., 2013a,b; Wang et al., 2012).

N-Methyl-*N*-nitrosourea (MNU) is an alkylating agent having antiproliferative cytotoxity and causes genotoxicity (Okada et al., 2008). MNU causes an O^6-methylguanine-induced point mutation that may contribute to oncogenesis targeting a wide range of organs/tissues (Becker et al., 1996). A single dose of MNU given to experimental animals at the perinatal age causes lethal damage to proliferating cells, targeting neural stem/progenitor cells in the neuroepithelium of the dorsal telencephalon, resulting in microcephaly when prenatally administered to rats and mice during gestational day (GD) 13.0–15.5 (Komada et al., 2010; Ohta et al., 1997). In mice, stem cells expressing glial fibrillary acidic protein (GFAP), i.e., radial glias, first appear in the ventricular zone (VZ) on GD 13.5 to form a migratory stream from the VZ to the developing DG, and establish germinal zones in the migratory stream finally to form the SGZ (Seki et al., 2014; Sugiyama et al., 2013). MNU exposure at this stage may target the migratory stream. MNU kills proliferating cells by DNA damage; however, MNU also causes disruption of gene regulation in surviving cells (Kokkinakis et al., 2004; Uehara et al., 2006). Stem cells and progenitor cell populations in the SGZ can be the target of MNU, and thus, MNU may affect neurogenesis in later life.

The present study was performed to investigate the impact of transient maternal exposure to MNU at the stage starting formation of the anlage of the DG on GD 14 on neurogenesis in later life in mice. For this purpose, we examined the subpopulations, proliferation, and apoptosis of the granule cell lineages in the SGZ and GABAergic interneuron subpopulations in the hilus of the DG at immature and mature stages.

2. Materials and methods

2.1. Chemicals and animals

MNU (purity: >98%) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). In all experiments, pregnant Slc:ICR mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) at GD 1 (the appearance of vaginal plugs was designated as GD 0). Pregnant mice were housed individually with their offspring in plastic cages with wood chip bedding until postnatal day (PND) 21 (where PND 0 is the day of delivery). Animals were maintained in an air-conditioned animal room (temperature: 23 ± 2 °C, relative humidity: 55 ± 15%) with a 12-h light/dark cycle. Dams were kept under free access to a pelleted basal diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water throughout the experimental period. Offspring were housed with three or four animals per cage and provided *ad libitum* with the MF basal diet and tap water from PND 21 onwards.

2.2. Experimental design

All procedures for animal experiment of this study were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan (June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Dams were randomly divided into three groups of 12 animals per group and received a single injection of MNU intraperitoneally at GD 14. The high dose level was set at 10 mg/kg body weight (BW) according to the report by Komada et al. (2010), causing mild microcephaly by administration once at GD 13.5. The low dose level was set at 5 mg/kg BW. BW and food consumption of dams were measured at 3-day intervals during the lactation period. After delivery, the litters were culled randomly on PND 4 leaving 10 male offspring per dam thereafter. In cases where dams had fewer than 10 male offspring, more female pups were included to maintain a total of 10 pups per litter. The offspring were weighed once a 3-day until PND 21. On PND 21, 11 male animals per group (1 male offspring per dam) were anesthetized deeply with CO_2/O_2 and perfused through the left cardiac ventricle with cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). For perfusion, a Masterflex peristaltic pump (EW-7553-70/71; Cole-Parmer, Vernon Hills, IL, USA) was used applying a flow rate of 10 ml/min. Samples from 27 to 31 male offspring per group (1–4 males per dam) were prepared for further gene analyses including real-time reverse-transcription polymerase chain reaction (RT-PCR). All female offspring and dams were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anesthesia and subjected to necropsy. Female samples were preserved without further analysis because neurogenesis is influenced by circulating levels of steroid hormones during the estrous cycle (Pawluski et al., 2009). The remaining animals were maintained until PND 77, and BW and food consumption were measured weekly. On PND 77, 11 male offspring per group (1 male offspring per dam) were perfused for immunohistochemistry with the same flow rate applied to pups on PND 21. A total of 19–27 male offspring per group (1–4 males per dam) were euthanized, and brain tissues were prepared for further gene analyses.

2.3. Immunohistochemistry and apoptotic cell detection

For immunohistochemical analysis, perfused brains in the subgroups of male offspring euthanized at PND 21 and 77 were further fixed in PFA solution at 4 °C overnight. Coronal slices were prepared at −2.2 mm from the bregma and further fixed with 4% PFA overnight at 4 °C. Brain slices were routinely processed for paraffin embedding and prepared for immunohistochemical staining (3 μm in thickness).

Sections were subjected to immunohistochemistry for granule cell lineage markers using primary antibodies against glial fibrillary acidic protein (GFAP), expressed in not only astrocytes but also type-1 stem cells (Kempermann et al., 2004b), SRY (sex determining region Y)-box 2 (Sox2), paired box 6 (Pax6), expressed in type-1 stem cells and type-2a progenitor cells (Roybon et al., 2009; Matsuda et al., 2012), T box brain 2 (Tbr2), expressed in type-2b and subset of type-3 progenitor cells (Hodge et al., 2008), and doublecortin (Dcx), expressed in type-2b and type-3 progenitor cells and immature granule cells (Kempermann et al., 2004b) (Supplementary Table 1). For evaluation of cell proliferation activity in the SGZ, immunohistochemistry for proliferating cell nuclear antigen (PCNA) was carried out on brain sections.

With regard to GABAergic interneurons and mature neurons in the dentate hilus, immunohistochemistry was performed using primary antibodies against reelin, which is secreted extracellular matrix glycoprotein playing a role in neuronal migration and positioning during neuronal development (Gong et al., 2007), parvalbumin (Pvalb), a calcium-binding protein expressed in the most active interneurons showing high activity for GABAergic transmission (Gulyás et al., 2006), and neuron-specific nuclear protein (NeuN), which specifically detects postmitotic neurons (Mullen et al., 1992) (Supplementary Table 1). Incubation with the respective primary antibodies was performed overnight at 4 °C.

Deparaffinized sections were incubated in 0.3% hydrogen peroxide solution in absolute methanol for 30 min to quench endogenous peroxidase. Antigen retrieval was performed for Pax6, Tbr2, Pvalb and GFAP antibodies with the conditions shown in Supplementary Table 1, and no antigen retrieval was performed for the other antibodies. Immunodetection was carried out using a Vectastain® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine (DAB)/H_2O_2 as the chromogen. Immunostained sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

Apoptosis was evaluated in the SGZ of the DG of offspring by applying the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay to brain sections according to the method described previously (Akane et al., 2013). Detection of apoptotic cells was performed using an ApopTag *in situ* apoptosis detection kit (Millipore Corporation) with DAB/H_2O_2 as the chromogen.