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Full Length Article

Differential exposure to N-ethyl N-nitrosourea during pregnancy is relevant to the induction of glioma and PNSTs in the brain

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ABSTRACT

Exposure to N-nitroso compounds (NOCs) during pregnancy has been associated with an increase in brain tumors in the progeny. This study investigated the brain tumorigenic effect of N-ethyl N-nitrosourea (ENU) after differential exposure of rats during pregnancy.

Sprague Dawley rats were exposed to a single dose of ENU (80 mg/kg) in three different circumstances: 1) at first, second or third week of gestation; 2) at the 15th embryonic day (E15) in consecutive litters and 3) at E15 in three successive generations. Location and characterization of the offspring's brain tumors were performed by magnetic resonance imaging and histopathological studies. Finally, tumor incidence and latency and the animals' survival were recorded.

ENU-exposure in the last two weeks of pregnancy induced intracranial tumors in over 70% of the offspring rats, these being mainly gliomas with some peripheral nerve sheath tumors (PNSTs). Tumors appeared in young adults; glioma-like small multifocal neoplasias converged on large glioblastomas in senescence and PNSTs in the sheath of the trigeminal nerve, extending to cover the brain convexity. ENU-exposure at E15 in subsequent pregnancies lead to an increase in glioma and PNST incidence. However, consecutive generational ENU-exposure (E15) decreased the animals' survival due to an early onset of both types of tumors. Moreover, PNST presented an inheritable component because progeny, which were not themselves exposed to ENU but whose progenitors were, developed PNSTs.

Our results suggest that repeated exposure to ENU later in pregnancy and in successive generations favours the development of intracranial gliomas and PNSTs in the offspring.

1. Introduction

The incidence of primary tumors in the central nervous system (CNS) in the United States was 21.97/100,000 persons in the period 2008–2012 with a mortality rate of 4.31/100,000 (Ostrom et al., 2015). Gliomas are the most representative CNS tumors. They are associated with significant morbidity and mortality in adult populations. The etiology of primary brain tumors remains largely unknown, but their incidence increases with exposure to environmental pollutants or dietary intake. Among the factors governing disease susceptibility, there are some concerning habits, such as smoking or alcohol abuse; the environmental exposure to chemical carcinogens or radiation; biology, puberty, or ageing; or genetical inheritance of tumor suppressor mutations or susceptibility alleles of modifier genes (Reilly, 2009; Mackenzie

Abbreviations: A, adult; BBB, blood brain barrier; BT, basal tumor; CNS, central nervous system; CT, convexity tumor; E, embryonic; EB, Evans Blue; ENU, N-ethyl-N-nitrosourea; ETI, extra-axial tumor incidence; ETL, extra-axial tumor latency; EW1, embryonic week 1 (first prenatal week); EW2, embryonic week 2 (second prenatal week); EW3, embryonic week 3 (third prenatal week); F1, first generation; F2, second generation; F3, third generation; ITI, intra-axial tumor incidence; ITL, intra-axial tumor latency; L1, first litter; L2, second litter; MPNST, malignant peripheral nerve sheath tumors;; MRI, Magnetic resonance imaging; NF1, Type 1 Neurofibromatosis; NF2, Type 2 Neurofibromatosis; NOCs, N-nitroso compounds; NTDs, neural tube defects; OS, overall survival time; PN, postnatal; PNST, peripheral nerve sheath tumors; SVZ, subventricular zone; TI, tumor incidence; TL, tumor latency.

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and Harrison, 2016).

Multiple epidemiological studies point to the exposure to chemical agents as an important factor for glioma development (Michaud et al., 2009; Milne et al., 2013). More than 200 chemical compounds, acting as alkylating agents, have been described as neurotoxic substances for human beings. Some of them are associated with neurodevelopmental disabilities in children, including autism, dyslexia, and attention-deficit hyperactivity disorder (Grandjean and Landrigan, 2006, 2014). These include N-nitroso compounds (NOCs), which are among the simplest chemical carcinogens. NOCs are divided into two major groups: N-nitrosamides and N-nitrosamines, which are formed by the reaction of amides and amines, respectively, with nitrosating agents derived from nitrites. Moreover, NOCs can be endogenously formed by intake of nitrite and nitrate (Dubrow et al., 2010). In fact, some cancers such as colorectal, bladder and breast cancer have been associated with nitrate ingestion in drinking water (Ward et al., 2018). Food treated with nitrites for colouring, flavouring and preservation contain NOCs (Bielecka and Markiewicz-Żukowska, 2020). It is known that processed fish and meat products, such as bacon, sausages, and salami, have elevated levels of NOCs or precursors to their endogenous formation (Dietrich et al., 2005). In addition, NOCs have been detected in tobacco smoke, in the air near rubber manufacturing plants, and in some cleaners and pesticides (Haorah et al., 2001; Braganza et al., 2014). Exposure to pesticides early in life has been related with the risk of CNS tumor development in observational epidemiological studies (Patel et al., 2020). Animal research has also demonstrated that NOCs are potent neurocarcinogens, especially via transplacental or perinatal exposure (Pogoda et al., 2009). Many of the NOC-induced experimental tumors are similar to analogous human tumors, a fact that makes us wonder if some human tumors could be caused by exposure to NOCs (Lijinsky, 1999).

N-ethyl-N-nitrosourea (ENU) is a carcinogenic and teratogenic NOC that induces tumors and congenital abnormalities, especially in the CNS (Hallas and Das, 1978; Papathanasiou and Goodnow, 2005). This alkylating substance is a mutagenic compound widely used in the laboratory to generate murine tumor models. The first research trials with ENU showed that the highest incidence of CNS malignancies was obtained by intravenous injection of a dose between 40 and 80 mg/kg during the gestation period Martinez et al., 1974. Although administration of ENU to adult rodents did not induce tumors, transplacental administration in the last week of gestation (from E15 to E21) or during the first week (first 7 days, PN7, of postnatal development) induced tumors in more than half of the offspring (Jones et al., 1973; Slikker et al., 2004). On the other hand, intraperitoneal injection of a dose of 80 mg/kg of ENU to Sprague Dawley rats during pregnancy, specifically on the 15th day of gestation (embryonic day E15), induces the development of gliomas and schwannomas in offspring (Zook and Simmens, 2005; Koelsch et al., 2013; Bulnes et al., 2018). ENU-induced tumors mimic the evolution and development of human brain tumors following a sporadic exposure to carcinogens, and neoplasms appear after several months of latency (Bulnes et al., 2012, 2018). Gliomas induced by transplacental exposure to ENU show histopathological characteristics of oligodendrogliomas containing exclusively NG2-expressing slow dividing precursor cells, which express early markers of oligodendroglial lineage (Briançon-Marjollet et al., 2010; Bulnes and Lafuente, 2007). ENU exposure also induces malignant schwannomas that arise predominantly in the trigeminal nerves. A point mutation of the neu/erbB-2 gene characteristic of ENU-induced rat schwannomas is an early marker of Schwann precursor cells at high risk of subsequent malignant transformation (Gering et al., 2006). The mechanism by which NOCs induce tumors is well understood. It is known that the amount of the dose, the frequency, and the route of administration to a certain species is determinant for tumor induction. These factors can change the affected organ and even the target cell from which the tumor develops.

Hence, the aim of this study was to assess the tumorigenic effect of a NOC on the intracranial nervous tissue in the context of differential maternal exposure. For this purpose, we analyzed the incidence and latency of intracranial glioma and PNST development, as well as the survival time of offspring after exposing dams to ENU at different weeks of pregnancy, on the same day in consecutive pregnancies, and on the same day of pregnancy in successive generations.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats (300 g) were housed in groups of four animals under standard laboratory conditions (22 ± 1 °C, 55 ± 5% relative humidity, and 12:12 h light/dark cycle) with food and water provided ad libitum. Every effort was made to minimize animal suffering and to use the minimum number of animals per group and experiment. In this sense, following the principles of the 3Rs of animal wellness (Russell and Burch, 1959), in order to reduce the number of animals, we re-analyzed data from 55 animals belonging to previous assays, in which we applied the same methodology (Bulnes et al., 2009, 2010, 2012). From these three papers, we selected the material of 67 intra-axial gliomas induced in 55 rats. These rats were exposed to ENU at 15th prenatal day (E15) and sacrificed from 6th month to one year of age. The gliomas were segregated into 34 microtumors obtained from 27 rats and 33 macrotumors from 28 rats. In previous studies, in this same material we studied the microvascular network changes from low grade to high grade gliomas (Bulnes et al., 2009), we explored the immunoexpression of VEGF and eNOS, two molecules of vascular permeability (Bulnes et al., 2010) and identified a subpopulation of glioma stem like cells by two markers: nestin and CD133 (Bulnes et al., 2012).

Experimental protocols were approved by the Local Ethical Committee for Animal Research of the University of the Basque Country (UPV/EHU, CEEA, ref. CEBA/82b/2010/LAFUENTESANCHEZ). All the experiments were conducted according to the European Community Council Directive on “The Protection of Animals Used for Scientific Purposes” (2010/63/EU) and with Spanish Law (RD 53/2013) for the care and use of laboratory animals.

2.2. Experimental design

In recent years, we have conducted several assays looking for cellular and molecular features of ENU-induced gliomas in Sprague Dawley rats (Bulnes et al., 2009, 2018). Rats were prenatally exposed to ENU, and subsequently, epidemiological data about tumor incidence and latency were recorded. The current study reports chronological data of intracranial tumors development after ENU exposure protocols.

2.2.1. Chronological tumor screening

Rats were mated and the females checked daily for vaginal plugs to identify the embryonic day (E0). Ten dams were intraperitoneally injected with ENU (80 mg/kg, 10 mg/ml in 0.9% NaCl, Ref.: E2129, Sigma-Aldrich, Spain) at the 15th day of pregnancy (embryonic day E15). Their 108 offspring, 57 male and 51 female rats, were examined monthly using the “Health Status Assessment” (see section 2.3.) and MRI (see section 2.4), from 4th to 12th month of age to detect the presence of tumors. The animals were sacrificed monthly for the histopathological study. The number of rats sacrificed each month is reported in Table 2.

2.2.2. Carcinogenic effects of differential ENU exposure

Three groups were analyzed, depending on different ENU exposure protocols, as follows (Table 1):

2.2.2.1. Group 1: pregnancy time. 116 offspring from 12 dams, intraperitoneally injected with ENU (80 mg/kg), were included in this assay. The offspring were segregated as follows: 5 offspring (1 male and 4 females) from two litters of 2 dams that received ENU during their first prenatal week (EW1); 23 rats (14 males and 9 females) from two litters

Table 1
Groups of Sprague Dawley rats used to study the brain tumorigenic effects of differential exposures to ENU.

Group		Time of exposure (embryonic day)	Dams (n)	Rats studied		
				Total (n)	Male (n)	Female (n)
Pregnancy time	EW1	E3	2	5	1	4
	EW2	E8	2	23	14	9
	EW3	E15, E20	8 (4, 4)	88 (39, 49)	48(18/30)	40 (21/19)
Consecutive litter	L1	E15	2	29	18	11
	L2	E15		25	16	9
Successive generation	F1	E15	3	39	20	19
	F2	E15	2	22	12	10
	F3	E15	3	10	4	6
	F2*	Not exposed	2	23	11	12

E, embryonic day (prenatal day); EW, embryonic week (prenatal week); L1, first litter; L2, second litter; F1, first generation; F2, second generation; F2*, second generation rats not directly exposed to ENU, but are offspring of females prenatally exposed to ENU; F3, third generation.

of 2 dams injected on the second prenatal week (EW2); and 88 rats (48 males and 40 females) from eight litters of 8 dams that were exposed to ENU during their third prenatal week (EW3). The last 88 rats were further divided into two subgroups: 39 rats (18 males and 21 females) from four litters exposed to ENU at E15 and 49 rats (30 males and 19 females) from four litters exposed at E20.

2.2.2.2. Group 2: consecutive litters. Two dams were intraperitoneally injected in two consecutive pregnancies, both on the 15th embryonic day (E15). Finally, a total of 54 offspring rats from both litters: 29 rats (18 males and 11 females) from the first litter (L1) and 25 rats (16 males and 9 females) from the second litter (L2) were used for this study.

2.2.2.3. Group 3: successive generations. Inherited tumorigenic effects of ENU were analyzed in two studies:

A) ENU effects in offspring from three successive generations. Initially, three dams received ENU on the 15th day of their first pregnancy and 39 rats (20 males and 19 females) from F1 three litters were analyzed. Then, two males (F1) were mated with two female rats (F1). These females from F1 received ENU at E15. The resulting 22 rats (12 males and 10 females) from F2 two litters were analyzed. Since males from F2 presented infertility (see Johnson et al., 1993; Yin et al., 2015), Three dams from F2 were mated with male control animals (Fig. 1A) and received ENU at E15. 10 rats (4 males and 6 females) from F3 three litters were finally analyzed.

B) ENU effects in a second generation of offspring rats not exposed prenatally to ENU (F2*). Briefly, one control female, mated with a control male, was exposed to ENU on the 15th day of gestation. Then two females and two males from the first generation (F1), prenatally exposed to ENU, were mated but in this case the dams were not exposed to ENU during their gestation. Finally, the 23 offspring rats (11 males and 12 females) from F2 two litters not prenatally exposed to ENU were used in this study (Fig. 1B).

2.3. Health status assessment

Offspring were examined every two days from the 4th month to sacrifice in order to establish health status and to identify neurological

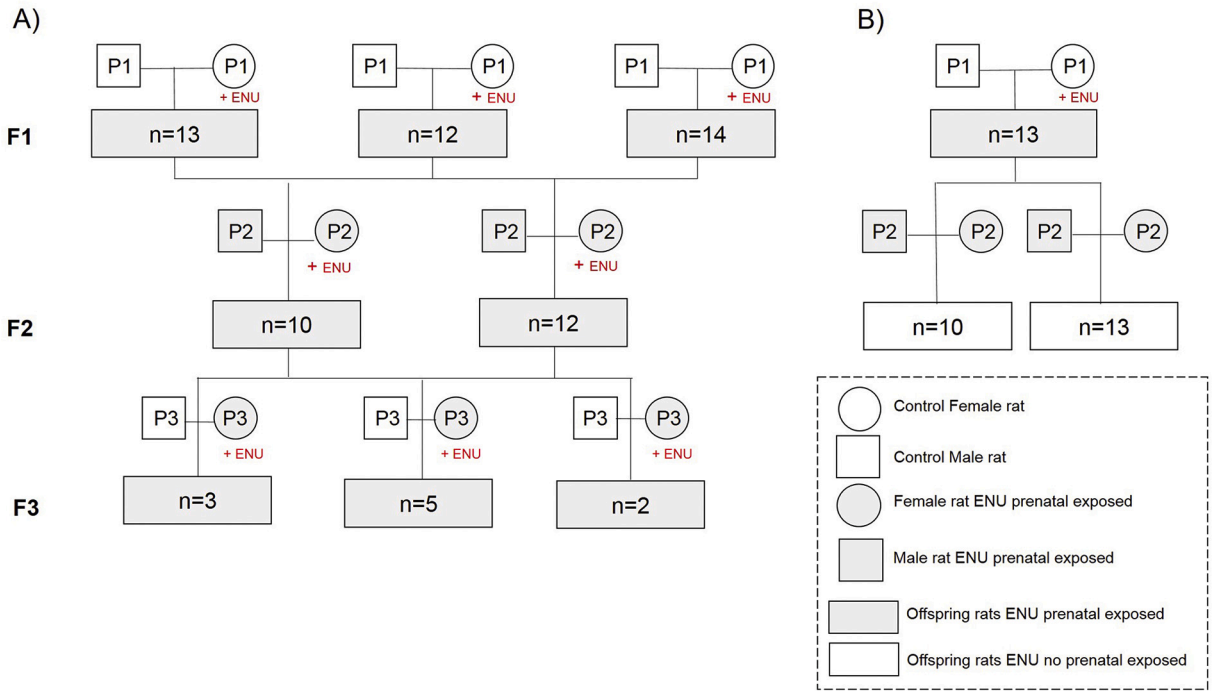

Fig. 1. Genealogical tree of Sprague Dawley rats used to study the inheritable tumorigenic effects of ENU. (A) Three generations of rats prenatally exposed to ENU. All dams were exposed to ENU on the 15th embryonic day (E15). (B) ENU inheritance effects studied in a second generation of offspring rats not directly exposed to ENU during pregnancy, but whose progenitors were prenatally exposed to ENU. *P1, progenitor of the first generation; P2, progenitor of second generation; P3, progenitor of the third generation; F1, first generation; F2, second generation; F3, third generation.*

deficits related to the presence of intracranial tumors. Health status was evaluated following the Morton-Griffiths inventory (Morton and Griffiths, 1985) and, in addition, abnormal postures or vocalizations, unusual weight loss, extreme immobility or swollen eyes were recorded. Rats presenting deficits incompatible with life or achieving 15 or more points in the adapted Morton-Griffiths scale were sacrificed (Bulnes-Sesma et al., 2006, 2007). The tumor incidence, latency and survival of animals was recorded.

2.4. ENU-induced intracranial tumor screening

Screening of intracranial tumors was carried out in vivo by magnetic resonance imaging (MRI, Biospec BMT 47/40, Bruker, Ettlingen, Germany, operating at 4.7 Tesla) using gadolinium contrast (1.5 ml/kg Gd-DTPA, Magnevist, Schering AG, Berlin, Germany), and by Evans Blue dye (EB, Sigma Aldrich, Spain, E2129, 28.5 mg/kg) at necropsy time. Intracranial tumors were segregated into intra and extra-axials tumors according to their location in the brain. After transcardial perfusion with 4% fresh paraformaldehyde in 0.1 M PBS (pH 7.4), brains were removed, post-fixed overnight in the same solution, embedded in paraffin and processed for histological studies. Finally, histopathological diagnoses of these tumors were carried out by hematoxylin-eosin stain. Tissue slices were examined to record the presence of histological features, such as cellular proliferation and anaplasia, atypical mitosis, microvascular proliferation, haemorrhages, necrosis, or cysts.

2.5. Data analysis

The effects of different exposures to ENU were analyzed by recording tumor incidence (TI), tumor latency (TL) and the overall survival time (OS) of rats. TI was calculated as the average number of rats (% of rats) that displayed tumors, and TL, the mean age (postnatal days, dpn) of tumor appearance. We performed a logistic regression analyses to assess whether the differential ENU exposure during the pregnancy was associated with intracranial tumor development, while controlling for the effect of litter. Comparisons of TL between groups were performed using one-way ANOVA using litter as covariate. Finally, Kaplan-Meier analysis with censoring was used to plot survival probability, and survival curve differences between groups were assessed with log-rank test. Statistical analysis was performed using IBM SPSS software (IBM Statistics 24). Data was presented as group means ± standard error of the mean. The level of statistical significance was set at $p < 0.05$.

3. Results

Offspring from dams exposed to ENU developed intra and extracranial tumors affecting central as well as peripheral nervous system. At six months of age, some rats showed a change in behavior, becoming hyperactive or confused, walking in circles, running compulsively into objects, and looking dishevelled. Clinical signs such as head lateralization, ataxic walk or anxiety were recorded. Symptomatology became severe with age. Four months later, they displayed numbness, apathy, seizures, and occasionally sudden death (Video 1). After necropsy, the rats showed intracranial tumors developed in the brain parenchyma or growth associated with the nerve sheath, mainly from the fifth cranial nerve. In addition, some animals showed paralysis of the lower extremities (Video 2) related to extra-cranial tumors growing inside the spinal cord or the nerve spinal sheath.

3.1. Intracranial tumors after prenatal exposure to ENU at E15

73.1% of offspring rats developed intracranial tumors. From a total of 57 male and 51 female rats, intracranial tumor incidence was similar between males (51%) and females (48%). Intracranial tumor prevalence increased from 40% at the 4th month and 90% at the 8th month to 100% after one year, affecting both the brain (intra-axial tumors) and the cranial nerve sheath (extra-axial tumors). Overall, 62% of rats developed intra-axial tumors and 26% extra-axial ones. The rate of intra-axial and extra-axial tumor development over time was similar between males and females (Table 2).

Intra-axial tumors mainly located in parietal and occipital areas (Fig. 2) while extra-axial ones outside the brain were associated with the sheath of the trigeminal nerve (Fig. 3). Both types of tumors could be present at the same time in the same animal (Table 2). Incidence of intra-axial tumors was three-fold higher than that of extra-axial tumors.

3.1.1. Lntra-axial tumors

Intra-axial tumors (Fig. 2) started their growth as a proliferation of cells around the 4th month. At this time, 43% of the rats displayed proliferation of cells detected by microscopic examination in subcortical white matter near the subventricular zone (SVZ). These groups of cells became microtumors at the 6th month. MRI showed T2-w homogeneous hyperintense signals that corresponded to microtumors denoting small nodular shapes distributed in the cerebral cortex (Fig. 2A). At the 8th month of age, 83% of the rats studied displayed intra-axial tumors. 60% of these tumors were large enough to be considered as macrotumors (Table 2, Fig. 2A). Finally, 100% of one-year-old rats displayed intra-axial tumors that could infiltrate a whole hemisphere. Thus, the size of intra-axial tumors increased with the age of the rats. Indeed, there was a predominance of a single focus of intra-axial tumors with respect to multifocal locations. More than the 60% of the rats displayed a single focus (Fig. 2B, C).

By histopathological study, microtumors were identified as classic oligodendrogliomas constituted by cells with rounded, homogeneous

Table 2
Chronological screening of intracranial tumors induced in Sprague Dawley rats after exposure of 80 mg/kg of ENU at E15.

Time (month)	Rats (n) (female/male)	Tumor Incidence (% of rats)			
		Total % (ratio female:male)	Intra-axial Tumor % (ratio female:male)	Extra-axial Tumor % (ratio female:male)	Both Tumors %(ratio female:male)
4	7 (1/6)	42.8 (–)	42.8 (–)	0 (–)	0 (–)
5	7 (1/6)	57.1 (1:3)	42.8 (–)	14.3 (–)	0 (–)
6	29 (15/14)	72.4 (1.25:1)	41.4 (2.8: 1)	41.4 (1:1.5)	10.3 (1.8:1)
7	15 (9/6)	73.3 (1:1.3)	66.7 (1.5:1)	46.7 (1:1.12)	40 (1:1.5)
8	12 (4/8))	91.7 (1.15:1)	83.3 (1.38:1)	33.3 (1:1.5)	25 (1:1)
9	18 (10/8)	77.8 (1:1.25)	77.8 (1: 1.25)	11.1 (1:1.25)	11.1 (1:1.25)
10	15 (9/6)	66.7 (1:1)	66.7 (1:1)	6,7 (–)	6.7 (–)
12	5 (2/3)	100(1:1)	100 (1:1)	40 (–)	40 (–)
Total	108 (51/57)	73.1 (1:1.03)	62 (1.08:1)	26 (1.12:1)	14.8 (1.4:1)

Proportion (%) of rats displaying newly developed intracranial tumors (intra-axial and/or extra-axial tumors and/or both type of tumors) each month, from 4 to 12 months of age. Tumor incidence is shown as the proportion of rats showing tumors each month, and the sex ratio is provided in parentheses. (–) no ratio is calculated due to low number of affected rats.

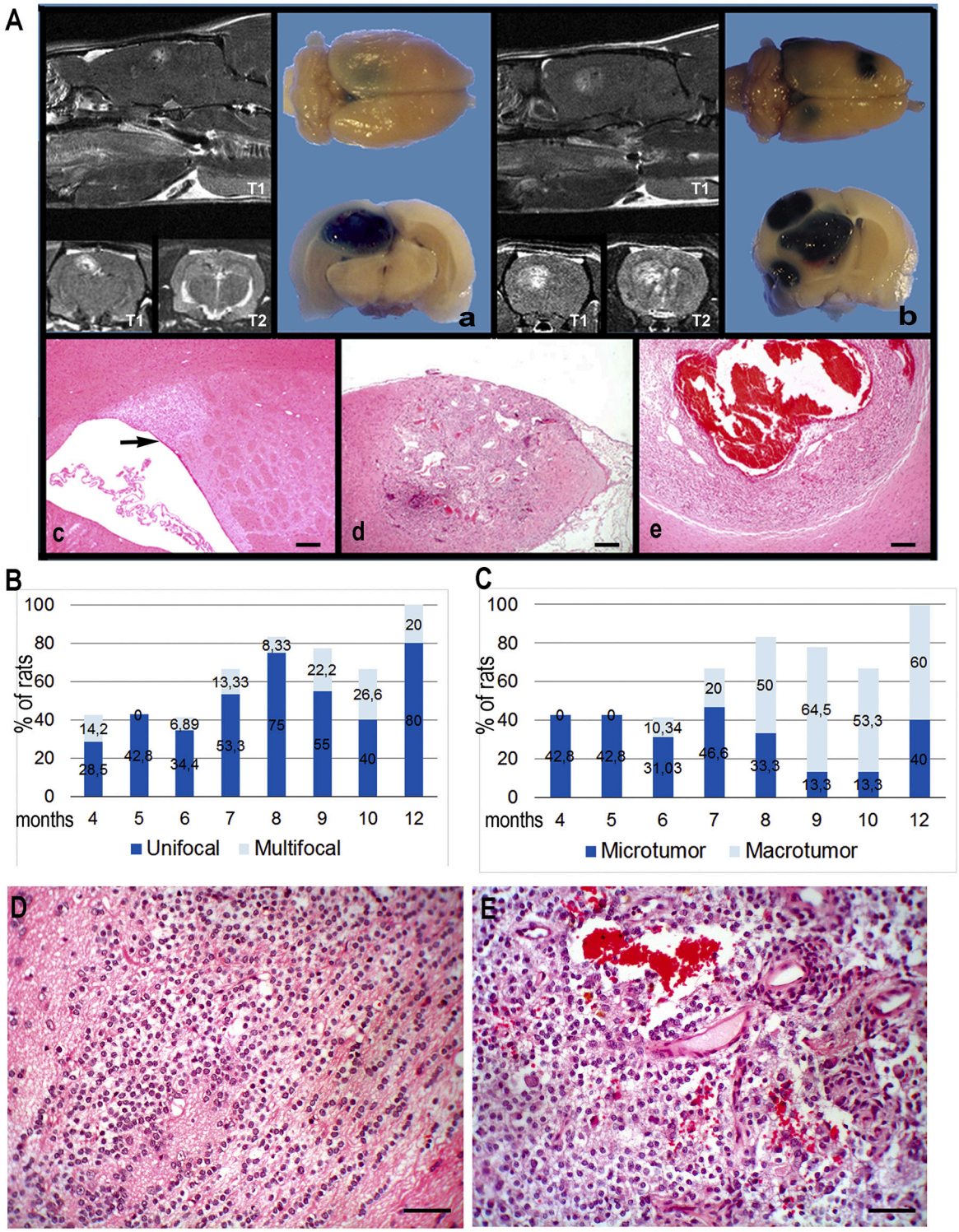

Fig. 2. Intra-axial tumors developed after prenatal exposure to ENU at E15. (A) Single (a) and multifocal (b) tumors located on parietal and occipital areas shown on MRI (T1 sagittal section showing the head of the rat and T1, T2, coronal sections of the brain) and Evans blue staining. HE staining reveals (c) a small proliferative mass of cells in the subventricular zone (SVZ, arrow), (d) a tumor with a nodular shape, and (e) a large tumor occupying a hemisphere. (B, C) Graphs representing tumor location and size during rat development. Single macrotumors predominated in rats of advanced age. (D) Homogeneous pattern of round fusiform cells distributed adjacent to the subcortical white substance characteristic of proliferative cell masses of cells. (E) Macrotumor displaying cellular anaplasia, hyperplastic vessels and haemorrhagic areas. Scale bar of 200 μm (Ac, Ad, Ae) and scale bar of 50 μm (D, E).

nuclei, and clear cytoplasm (honeycomb pattern) (Fig. 2D). On the other hand, macrotumors showed polymorphous, anaplastic areas with marked cellular anaplasia, atypical mitosis, haemorrhagic areas, and microvascular hyperplasia (Fig. 2E). They were mainly diagnosed as anaplastic gliomas. However, the macrotumors, that showed endothelial proliferations, macrocysts and necrosis with pseudopalisading, were diagnosed as glioblastoma multiforme. Therefore, low grade gliomas were observed in rats from 6 to 7 months old, anaplastic gliomas at the 8

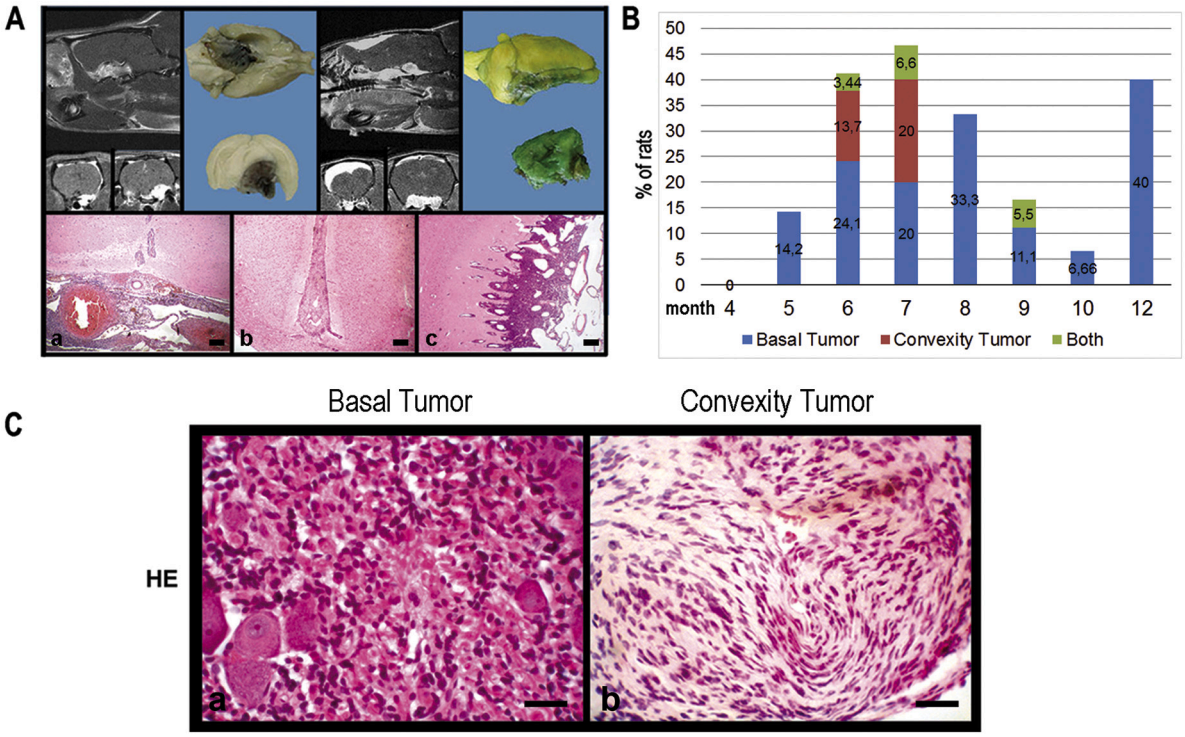

Fig. 3. ENU-induced extra-axial tumors associated with the Gasserian ganglion. A) Macroscopic images by MRI T1-w and Evans Blue dye reveal a growth pattern from the trigeminal nerve sheath covering the convexity throughout the meningeal sheaths. HE staining shows the two location of the tumors: (a) on the base of the brain and (b, c) on the convexity. Convexity tumor grows thorough the interhemispheric cleft (b), and Virchow Robin spaces (c). B) Bar chart showing the frequency of extra-axial tumor location by rat's age. Basal location of the tumor predominates over the expansion into the convexity area. At 6 and 7 months, the two locations are affected at the same time. C) Basal tumor shows cell bodies of sensory neurons of trigeminal ganglion and plenty of hyperchromatic neoplastic cells anarchically distributed around them. Convexity tumor displays fusiform cellular pattern with mitotic cells and hyperchromatic spindle cells with abundant, faintly eosinophilic cytoplasm. Scale bar of 200 μm (A) and scale bar of 20 μm (C).

month and glioblastomas at over one year of age.

Almost half of the brains showed two or more gliomas with different degrees of malignancy. Due to their natural growth, two or more microgliomas converged and, after one year, there was predominantly a single, extensive highly malignant glioma.

3.1.2. *Extra-axial tumors*

Extra-axial tumors (Fig. 3) developed from the sheath of the trigeminal nerve. Affected rats showed neurological signs such as numbness and seizures. MRI showed homogeneous strong gadolinium enhancement signals on the T1-w image. Due to their location, they could be segregated into basal tumors (BT) and convexity tumors (CT). Basal tumors grew out from the sheath of Gasserian ganglion, extending to invade and infiltrate both trigeminal nerves. Convexity tumors extended along the meninges covering the brain convexity, mainly in the frontal areas, occupying the subarachnoid space, and growing into the Virchow-Robin spaces (Fig. 3A).

The highest incidence of extra-axial tumors was found between the 6th and 7th months of age, corresponding with 40% and 50% of the rats studied. After the 7th month, their incidence decreased. This decrease might be related to basal tumor growth and subsequent pressure in the medulla oblongata, damaging the vital centers and resulting in animal death. Most of the animals that developed extra-axial tumors died at 7th month. This could be the cause of decreased incidence of intracranial tumors at 8th, 9th and 10th months (Fig. 3B).

Basal tumors predominated over convexity ones. Their incidence was three-fold higher than convexity tumors (Fig. 3B). This could be related to a later development of convexity tumors than basal ones.

According to the histopathological study, cellular pleomorphism, anaplasia and cellular atypia were characteristic of these tumors (Fig. 3Ca). Extra-axial tumors located on the convexity showed elongated nuclei with brisk mitotic activity and tightly packed fasciculate growth. Sometimes, cells were associated with fasciculus interweaving in all directions. This fasciculate growth acquired eddy-shaped structures, which are characteristic of schwannomas (Fig. 3Cb). Considering these histopathologic features, extra-axial tumors were diagnosed as peripheral sheath nerve tumors (PNSTs), concretely as schwannomas and malignant peripheral nerve sheath tumors (MPNSTs). Schwannomas appeared one month earlier than gliomas, at the 5th month, and MPNSTs two months later than schwannomas.

3.2. *Tumorigenic effects of ENU after differential exposure of dams*

3.2.1. *Pregnancy week of ENU-exposure: EW1, EW2 and EW3*

Regarding ENU-exposure at different time points, the data regarding the critical period for inducing intracranial results show that the exposure of dams to 80 mg/kg of ENU at the initiation of the gestational period, during the first week of prenatal development (EW1), lead to the development of severe congenital malformations in the offspring. Most of these were incompatible with life (Fig. 4). However, exposure to ENU after the initial week of gestation, during the second (EW2) and third (EW3) week of prenatal development induced intracranial tumors in the offspring. The results show no differences between intracranial tumor incidence (TI), tumor latency and overall survival between rats from EW2 and EW3 (Fig. 5A) (Table 3-4). Nearly 70% of the rats from both groups developed intracranial tumors: 60% of them inside the brain and 30% outside it (Table 3). Significant differences were observed for the incidence and the latency of the extra-axial tumors. A higher incidence of tumors was observed after ENU administration in EW3, but these tumors took one more month to develop (Table 3).

Moreover, it has been reported that from E15 to E18 is the critical period for the induction of intracranial tumors in offspring caused by

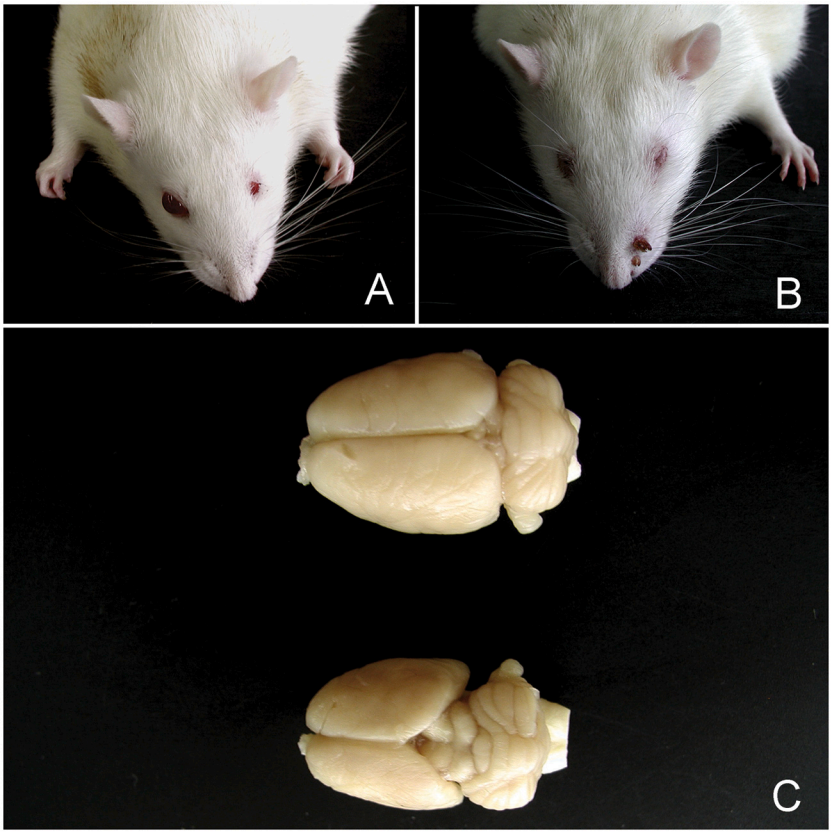

Fig. 4. Developmental defects induced by different exposure to ENU. Early prenatal exposure to ENU in the first week of pregnancy induced craniofacial malformations such as: unilateral anophthalmia in a female rat (A) and bilateral anophthalmia in a male (B). Advanced generation prenatal exposure to the chemical generates neural defects. (C) Macroscopic photograph of two brains of rats prenatally exposed to ENU from the first generation (above) and from the third one (below). Brain from the third generation shows hypoplasia of the hemispheres and elongated brain stem.

chemical exposure (Thomas et al., 1994). We evaluated the tumor incidence in E15 and E20-exposed rats and observed that the time of prenatal exposure affected the incidence of intra-axial and extra-axial tumors. The intra-axial incidence was higher in rats exposed at E20 than at E15 ($p < 0.05$). However, the opposite was observed regarding the incidence of extra-axial tumor: the incidence was higher for rats exposed at E15 ($p < 0.001$). In addition, the data showed that exposure at E15 induced an earlier development of intracranial tumors compared to exposure at E20 ($p < 0.01$), being two months earlier for intra-axial and one month earlier for extra-axial tumors in E15 injected animals ($p < 0.01$). This time point also affected the survival of rats. Rats exposed to ENU at E15 showed a shorter survival time than E20 injected animals (two months less than the other groups, $p < 0.01$) (Table 5, Fig. 5A).

3.2.2. Consecutive Litters ENU-exposure at E15

Exposure to ENU during consecutive pregnancies of the same dam increased the intracranial tumor incidence and latency in the offspring. Briefly, the incidence of intra-axial as well as extra-axial tumors increased with the litter exposure. A significant difference was only found for extra-axial tumors (ETI = 34% from L1, and ETI = 64% from L2, $p < 0.05$).

In addition, intra-axial tumors developed later after exposure to ENU in two consecutive litters. They were detected one month later in L2 than L1 ($p < 0.05$) (Table 3). The survival time of rats from both litters was not affected by the successive exposure of the dams to ENU. Rats displaying intra- or extra-axial tumors survived the same time, around 7 months (Table 4). Moreover, half of the rats affected by any tumor were able to survive for 200 days (Fig. 5B).

3.2.3. Successive generation exposure to ENU at E15

The major inherited effect due to successive generation prenatal exposure to ENU was sterility. Since most of the male rats from the second generation were sterile, new progenitors were used for mating, while the females' reproductive capacity was drastically reduced. The number of offspring decreased from 11 to 13 in F1 to 6–8 in F3. In addition, teratogenic effects such as smaller brain size were observed (Fig. 4C).

An increase of intracranial tumor incidence and a decrease of tumor latency were appreciated with generational exposure to ENU (F1-TI = 76% and F2-TI = 90% of rats, F1-TL = 8 months and F2-TL = 7 months). Statistical analyses showed that generational exposure to ENU favoured an earlier development of intracranial tumors, concretely, intra-axial tumors (TL, $p < 0.001$, Table 3). Extra-axial tumor development was favoured by generational exposure to ENU. Tumor incidence was eight times greater in the second generation, compared to the first one (F1-ETI = 7% to F2-ETI = 61%) and they appeared two months earlier in F2 than in rats from F1 (F1-ETL = 9 months and F2-ETL = 7 months). However, we did not find statistically significant differences for extra-axial tumor incidence and latency after controlling for the effect of litters.

Regarding survival of animals, F2 rats displaying extra-axial tumors survived three months less than F1 rats ($p < 0.001$, Table 4). Half of the animals from F2 only reached 7 months of age, while those from F1 reached 9 months (Fig. 5C).

It must be noted that the tumor latency and survival of the offspring belonging to a second generation not exposed prenatally to ENU (F2*) were not measured because these rats did not develop any neurological symptom or sign suggestive of intracranial tumors. However, the animals were sacrificed at month 12 (mean age of 382.2 ± 26.7 dpn), and we observed that 21.7% of rats showed proliferative cell masses in the cerebral cortex and in intraventricular area (mean age of 389.4 ± 24.5 dpn). Small schwannomas associated with the Gasserian ganglion were also observed in the 30% of these rats (mean age of 383.4. ± 29.01 dpn).

These offspring were not exposed prenatally to ENU, but their progenitors were exposed during their prenatal time. This means that current tumors were not induced by direct exposure to ENU during the

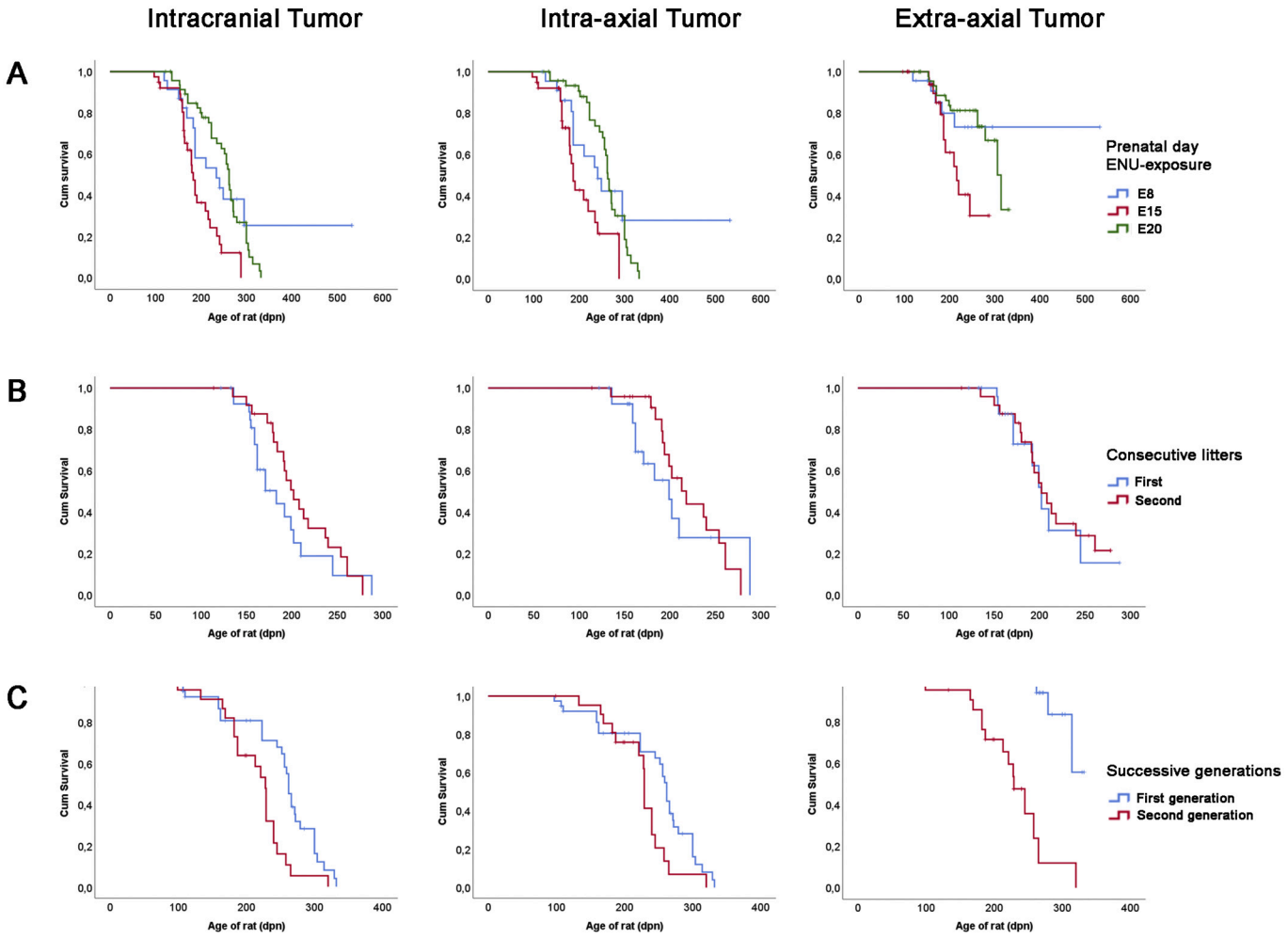

Fig. 5. Kaplan Meier survival curves of rats exposed to a single dose of ENU at different timings: (A) in the last two weeks of gestation at E8, E15 and E20; (B) in two consecutive litters: *L1, first litter; L2, second litter; and* (C) in successive generations: *F1, first generation; F2, second generation.*

Table 3
Study of intracranial tumor incidence and latency of rats subjected to different exposures to ENU.

Groups		Total				Intra-axial tumors				Extra-axial tumors			
		TI % rats	p	TL (mean ± SEM)	p	TI % rats	p	TL (mean ± SEM)	p	TI % rats	p	TL (mean ± SEM)	p
Prenatal Time	EW2	65.2	ns	199.5 ± 14.02	ns	56.5	ns	208.1 ± 14.5	ns	21.7	0.027	168.2 ± 15.1	0.025
	EW3	73.8		216.8 ± 7.2		61.3		225.1 ± 8.1		28.4		204.4 ± 9.5	
Consecutive Litters	L1	65.5	ns	178.9 ± 8.7	0.02	48.8	ns	179.1 ± 11.1	0.009	34.4	0.02	185.2 ± 9.4	ns
	L2	88		208.3 ± 8.7		68		218.5 ± 9.6		64		193.2 ± 8.04	
Successive Generation	F1	76.9	ns	240.4 ± 12.3	<0.001	76.9	ns	240.4 ± 11.7	<0.001	7.6	ns	285.0 ± 28.5	ns
	F2	90.9		211.1 ± 14.9		72.7		221.2 ± 16.1		61.6		211.6 ± 13.2	

Intracranial tumor incidence of rats according to several experimental procedures. Differences of tumor incidence between groups were examined by logistic regression to test whether ENU-exposure was associated with tumor development, while controlling for the effect of litter. Tumor latency was analyzed with one-way ANOVA using litter as covariate. *p*-values <0.05 are considered significant; ns: non-significant. TI, tumor incidence, proportion of rats developing tumors; TL, tumor latency, mean postnatal days until the development of tumors. EW, embryonic week; L1, first litter; L2, second litter; F1, first generation; F2, second generation.

Table 4
Study of survival time of rats subject to different exposures to ENU.

	Intracranial tumors			Intra-axial tumor			Extra-axial tumor		
Prenatal time	EW2	EW3	p	EW2	EW3	p	EW2	EW3	p
	288.4 ± 33.9	227.8 ± 6.8	ns	304.2 ± 35.7	239.47 ± 7.05	ns	435 ± 37.2	272.0 ± 8.8	ns
Consecutive litters	L1	L2	p	L1	L2	p	L1	L2	p
	190.6 ± 9.9	209.2 ± 8.5	ns	208.4 ± 14.2	221.6 ± 8.9	ns	211.1 ± 12.4	214.6 ± 9.4	ns
Successive Generation	F1	F2	p	F1	F2	p	F1	F2	p
	248.1 ± 10.6	213.9 ± 10.5	0.004	248.1 ± 10.6	226.9 ± 10.1	0.034	317.3 ± 7.0	229.7 ± 13.1	<0.001

Overall survival of animals separated by experimental groups. Data are reported as mean ± SEM. Group comparisons were performed using Log Rank test. Significant comparisons (p-value <0.05) are indicated with the corresponding *p*-value. ns, non-significant. EW, embryonic week; L1, first litter; L2, second litter; F1, first generation; F2, second generation.

prenatal time, but by induced mutations transferred by the ENU-exposed progenitors.

4. Discussion

In this study, we have documented the relevance of successive exposure and generational prenatal exposure to the neurotoxic compound ENU as two relevant circumstances in the development of intracranial tumors. Late or successive exposure to this alkylating agent during the gestational period leads to development of gliomas and PNSTs in the progeny. Glioma incidence was favoured by later prenatal exposure. Repeated generational exposure to ENU decreased the survival of rats due to an increased rate of PNSTs and early onset glioma development.

4.1. Gestational time is relevant to the induction of gliomas and PNST after exposure to ENU

There is little consensus about the tumorigenic capacity of some NOCs in primary CNS tumors. Maternal dietary exposure to NOCs during pregnancy has been associated with a risk of childhood brain tumors (Dietrich et al., 2005). Some animal studies have indicated that NOCs cross the placenta and affect the fetus in utero (Bruning-Fann and Kaneene, 1993). Indeed, the blood brain barrier (BBB) is incompletely developed in the prenatal period, and toxic agents can enter into the CNS, which is much more vulnerable to toxic agents than in the case of adults (Thomas et al., 1994).

ENU is a carcinogenic and teratogenic substance that freely crosses the placental barrier and does not require any metabolic activation. Our results demonstrate that a single dose of ENU to dams in the first week of pregnancy, corresponding with the beginning of the first trimester of human development (Chen et al., 2017), when organogenesis begins, induces severe congenital malformations (microcephaly, hydrocephaly) in the progeny. Several epidemiologic studies have shown an association between maternal exposure to nitrite and nitrate substances in food and drinks and birth defects, including neural tube defects (NTDs) such as anencephaly and spina bifida (Andersen et al., 2000; Massa et al., 2020). Results also corroborate the teratogenic effects of ENU in the early murine craniofacial development (Thomas et al., 1994; Sandell et al., 2011).

On the other hand, a key aspect for evaluating the tumorigenic effect of exposure to any toxic substance is the age of the animal (Rice, 2006). This work demonstrates the carcinogenic potential of ENU-exposure during the last two weeks of the gestational period. Prenatal exposure to ENU (80 mg/kg) induces intracranial tumors, diagnosed as gliomas, schwannomas and MPNSTs, in nearly 70% of the progeny. It is known that ENU acts by alkylating O6-guanine, O2-thymine and O4-thymine (Justice et al., 1999). These mutations affect the expression of the oncogenes neu/erbB, Ha-ras, p53, and genes coding for caspase-9, platelet-derived growth factor receptor alpha (PDGFRa), cyclin-dependent kinase inhibitor 2A (CDKN2A) and epidermal growth factor receptor (EGFR). The expression of neu/erbB2 is associated with development of schwannomas, and p53 with glial tumors (Thomas et al., 1994; Zook et al., 2000; Katayama et al., 2005; Mukherjee et al., 2006). Gil-Perotin et al. (2011) reported that after administering ENU late in the gestational period, the cell cycle was arrested in neuroepithelial cells of the subventricular zone (SVZ) and the ventricular zone (VZ) in a p53-dependent manner. These altered neuroepithelial cells generate glial lineage tumors such as oligodendroglioma, astrocytomas, mixed gliomas, and ependymomas (Mennel et al., 2004). In the current study, we determined that maternal exposure to ENU significantly favours the induction of gliomas comparable to schwannomas. These ENU-induced gliomas mimic the evolution and development of human gliomas after sporadic exposure to carcinogens, and neoplasm appears after several months of latency (Bulnes-Sesma et al., 2006; Bulnes et al., 2010; Bulnes et al., 2012; García-Blanco et al., 2016). It is common to find collections of isomorphic cells near the subventricular zone (SVZ). Then, some months later, the same cells form a small nodular low-grade glioma, typically diagnosed as oligodendroglioma, showing mild features of anaplasia. They usually involve subcortical white matter and the cortex. Tumors grow and converge into large tumors, becoming malignant and evolving into glioblastomas in older rats (around one year of age). Making a translational comparison between rat and human ages, the exposure to ENU at the end of the gestational period implies the development of primary brain tumors in young human adults (Quinn, 2005). As proposed by Recht et al. (2009), offspring rats prenatally

Table 5
Study of tumorigenic effect of ENU-exposure at E15 and E20.

	Intracranial tumor			Intra-axial tumors			Extra-axial tumors		
	E15	E20	p	E15	E20	p	E15	E20	p
Tumor incidence	69.2	77.5	ns	53.8	67.3	0.024	30.8	26.5	<0.001
Tumor latency	180.2 ± 8.2	242.8 ± 8.7	<0.01	179.1 ± 9.8	254.3 ± 8.37	0.001	189.9 ± 8.12	217.0 ± 16.2	<0.01
Overall survival time	194.1 ± 8.9.	249.9 ± 8.1	<0.001	204.9 ± 10.6	260.9 ± 7.44	<0.001	225.5 ± 11.3	288.6 ± 9.8	0.003

Overall survival time and tumor latency are expressed by mean postnatal day ± SEM. Tumor incidence is expressed as the proportion (%) of rats that developed intracranial tumors. Differences in tumor incidence were examined using logistic regression to test whether the timing of ENU exposure was associated with tumor development, while controlling for the effect of litter. Tumor latency was analyzed with one-way ANOVA using litter as covariate. Significant comparisons (p-value <0.05) are indicated with the corresponding p-value. ns, non-significant. E, embryonic day (prenatal day).

exposed to ENU show a clinical situation analogous to that of young patients who develop brain tumors several years after receiving chemotherapy.

Many metabolism-dependent carcinogens are toxic to the fetal rat only when given to the dam at the very end of gestation, when fetal metabolic competence has presumably evolved to the stage that the carcinogen can be biotransformed by fetal tissues (Rice et al. 2006). In this work, we compare the ENU neurocarcinogenic effect in fetal rats from the last two weeks of gestation (E7-E14 and E15-E21, respectively), finding similar tumor incidence and latency. Rice et al. (2006) described how the rat fetus at E15 is at least 50 times more susceptible than adults and that well-tolerated single doses of ENU can cause multiple primary brain tumors in 100% of exposed offspring. In this study, we investigated the effect of this chemical in rat fetuses at E15 compared to E20. Our results show that E15 rats survived significantly less time than E20 rats, due to an earlier onset of gliomas. Moreover, comparing E15 fetal rats and early live rats (7 postnatal day, PN7) exposed to ENU, a significantly later development of gliomas in neonatal rats compared to prenatal ones was observed (data not published). Some experiments have demonstrated that fetus brains are less able to efficiently excise or repair the DNA alkylation induced by mutagenic agents than postnatal brains (Shrivastav et al., 2010). Mullerd and Rajewsky, in 1983, reported the relationship between prenatal day 15 and the expression and subsequent switch-off of the 06-EthylGuanine-eliminating enzyme(s) in the embryonic brain. This finding shows the importance of the 15th day of prenatal development of murine animals in the induction of gliomas. This time corresponds with the beginning of the third trimester of human gestation (Workman et al., 2013; Bolon and Ward, 2015).

4.2. Glioma and PNST development favoured by successive exposure of dams to ENU

Because the tumorigenesis process requires the accumulation of multiple mutations in cells, another purpose of this study was to assess the effect in the offspring of successive maternal exposure to ENU. Several ENU-exposures of the same dam during consecutive pregnancies favour the development of glioma and PNST. The glioma-induction increases in the consecutive litters, but their time of appearance in the cerebral parenchyma is delayed. It has been reported that repetitive administration of *N*-methyl-N nitrosourea implies an accumulation of O6-methylguanine in the rat brain (Margison and Kleihues, 1975).

4.3. Generational exposure to ENU during gestation favours PNST induction

Several tumors of the nervous system are associated with familial cancer predisposition syndromes. These include Li- Fraumeni syndrome, tuberous sclerosis and Turcot syndrome and neurofibromatosis (Frebourg et al., 2020; Malbari and Lindsay, 2020; Strowd, 2020). It has been reported that mutations of the type 1 Neurofibromatosis (NF1) gen and the type 2 Neurofibromatosis (NF2) gen are associated with sheath tumors (Jenkins et al., 2010; Chapman et al., 2015). Our results showed the inherited tumorigenic effect of exposure to ENU of the peripheral nervous system of prenatal animals. We found PNSTs in young rats not prenatally exposed directly to ENU but born from mothers exposed to it. Moreover, prenatal ENU exposure in consecutive generations mainly favoured the incidence and latency of schwannomas/MPNSTs. In line with this, a finding that must be noted is that offspring rats develop tumors without having been exposed to the toxin while their progenitors have. This could be explained because ENU is one of the most potent mutagens in murine germ cells, especially in stem-cell spermatogonia, by Stx2/Epim gene mutation and haemoglobin loci mutation (Murota, 1995; Akiyama et al., 2008). Thus, ENU prenatal exposure induces mutations in the germ cells that are transferred to the offspring.

The development of MPNSTs may be influenced by NF1, an illness caused by the inheritance of a defective allele of the NF1 gene. On the other hand, there is a close association of NF2 mutations in schwannoma with loss of merlin expression (the NF2 gene product) (Louis et al., 2016). NF1 is among other hereditary syndromes associated with a genetic predisposition to nervous system tumors (Wrensc et al., 2002; Bondy et al., 2008). The NebB2 gene, altered by prenatal exposure to ENU, may be an inherited gene with a strong influence on the risk of developing MPNSTs, and a person who was prenatally exposed to ENU might have a genetic predisposition to developing neurofibromatosis. Therefore, children of parents who are carriers of defective alleles of tumor-associated genes would be at risk for the familial cancer syndrome.

5. Conclusion

In conclusion, the fetal CNS is highly susceptible to the genotoxicity of ENU, resulting in brain abnormalities and tumors. Our data confirm that maternal exposure to ENU during successive generations and subsequent pregnancies could contribute to the development of glioma and PNSTs in the offspring. Moreover, progenitors exposed to ENU transmit to their germ cells the mutation necessary for the development of PNSTs in their offspring.

Therefore, one of the causes of the increase in brain tumor incidence in the last few decades might be the generational exposure to N-nitroso compounds during the third trimester of gestation. Moreover, not only direct prenatal exposure but also prenatal progenitor's exposure could be considered as a risk of brain tumors development.

One of the routes of exposure to these chemicals in pregnant women is their diet. Dietary factors that could significantly increase brain tumor development are still not clearly identified and described (Bielecka and Markiewicz-Żukowska, 2020). It is known that processed meat products, such as bacon, sausages, and salami, have elevated levels of NOCs or precursors for their endogenous formation (Dietrich et al., 2005). Thus, as diet is a potentially modifiable behavior, studying the association between NOC intake from foods and drinks and brain tumor development might result in an important disease prevention strategy.

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Author contributions

SB designed the study, performed the experiments, analyzed the data, prepared the figures and wrote the manuscript. AM reviewed the statistical analyses and the manuscript, JVL supervised, designed the study, and reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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