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NEUROGENESIS AND ASTROGENESIS CONTRIBUTION TO RECOVERY OF VESTIBULAR FUNCTIONS IN THE ADULT CAT FOLLOWING UNILATERAL VESTIBULAR NEURECTOMY: CELLULAR AND BEHAVIORAL EVIDENCE

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Abstract—In physiological conditions, neurogenesis occurs in restricted regions of the adult mammalian brain, giving rise to integrated neurons into functional networks. In pathological or postlesional conditions neurogenesis and astrogenesis can also occur, as demonstrated in the deafferented vestibular nuclei after immediate unilateral vestibular neurectomy (UVN) in the adult cat. To determine whether the reactive cell proliferation and beyond neurogenesis and astrogenesis following UVN plays a functional role in the vestibular functions recovery, we examined the effects of an antimitotic drug: the cytosine-β-D arabinofuranoside (AraC), infused in the fourth ventricle after UVN. Plasticity mechanisms were evidenced at the immunohistochemical level with bromodeoxyuridine, GAD67 and glial fibrillary acidic protein (GFAP) stainings. Consequences of immediate or delayed AraC infusion on the behavioral recovery processes were evaluated with oculomotor and posturo-locomotor tests. We reported that after UVN, immediate AraC infusion blocked the cell proliferation and decreased the number of GFAP-immunoreactive cells and GABAergic neurons observed in the vestibular nuclei of neurectomized cats. At the behavioral level, after UVN and immediate AraC infusion the time course of posturo-locomotor function recovery was drastically delayed, and no alteration of the horizontal spontaneous nystagmus was observed. In contrast, an infusion of AraC beginning 3 weeks after UVN had no influence neither on the time course of the behavioral recovery, nor on the reactive cell proliferation and its differentiation. We conclude that the first 3 weeks after UVN represent a possible critical period in which important neuroplasticity mechanisms take place for promoting vestibular function recovery: reactive neurogenesis and astrogenesis might contribute highly to vestibular compensation in the adult cat.

Key words: adult neurogenesis, GABA, GFAP, vestibular compensation, vestibular nuclei complex, AraC.

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Abbreviations: ANOVA, variance analysis; AraC, cytosine-ß-D arabinofuranoside; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; GAD, glutamate decarboxylase; GFAP, glial fibrillary acidic protein; HSN, spontaneous nystagmus; Ir, immunoreactivity; IVN, inferior vestibular nucleus; LVN, lateral vestibular nucleus; Max P, maximal locomotor balance performance; MVN, medial vestibular nucleus; NaCl, sodium chloride; SVN, superior vestibular nucleus; SVZ, subventricular zone; UVN, unilateral vestibular neurectomy; VN, vestibular nuclei.

Adult neural stem progenitors reside in many areas of the adult mammalian CNS, but continuous neurogenesis occurs only in two restricted regions: in the subgranular zone of the dentate gyrus (DG) and in the subventricular zone (SVZ) of the lateral ventricles (Gross, 2000; Ming and Song, 2005). Among the questions raised by this new form of adult plasticity is the function of these new neurons (Zhao et al., 2008). *In vitro* experiments in rodents have shown that new DG or SVZ neurons develop electrophysiological and synaptic properties very similar to or even indistinguishable from mature neurons (Carlen et al., 2002; van Praag et al., 2002; Laplagne et al., 2006; Ge et al., 2007b). Spontaneous neurogenesis leads to functional integration into pre-existing neural networks of the cells newly generated in the DG (Schmidt-Hieber et al., 2004; Ge et al., 2008) and in the olfactory bulb (Carlen et al., 2002; Carleton et al., 2003). These cells become active and contribute to the transmission of information in the brain. Outside these two discrete areas, proliferating cells give rise to glia but not neurons in the intact adult CNS. However, in pathological or injured states of the brain, neurogenesis and gliogenesis have been reported both in known neurogenic zones and in other areas (de Waele et al., 1996; Campos Torres et al., 1999; Arlotta et al., 2003; Kokoeva et al., 2005; Moyse et al., 2006). Together, these results suggest that the adult mammalian CNS is able to generate the main characteristic cell types of nervous tissue (neurons, astrocytes and microglial cells) and to integrate them into pre-established networks. But the functional benefit of this form of structural plasticity remains poorly documented.

We have previously demonstrated in the adult cat that unilateral vestibular neurectomy (UVN) (Tighilet et al., 2007) causes a marked reactive cell proliferation in the deafferented vestibular nuclei (VN). Most of these new cells survive 1 month after injury and give rise to astrocytes, microglial cells, and neurons. The newly generated neurons express a GABAergic phenotype. The question raised in the present study is whether reactive astrogenesis and GABAergic neurogenesis contribute functionally to vestibular compensation, that is, the postlesional restoration of the impaired vestibular functions (Lacour, 2006). To determine whether reactive cell proliferation plays a functional role in the vestibular compensation process, the mitotic activity of the dividing new cells was blocked by a continuous infusion of cytosine-β-D arabinofuranoside (AraC, S-phase-specific antimitotic drug) in the fourth ven-

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tricle. In order to clarify whether delayed AraC infusion had an effect on vestibular compensation, the drug was administered to adult cats either immediately after being subjected to UVN, or 3 weeks after UVN when all the newly generated cells have been formed. Cell division blockade and its consequences were characterized at the cellular level with bromodeoxyuridine (BrdU), glial fibrillary acidic protein (GFAP), and glutamate decarboxylase (GAD)-67 immunostainings. Consequences of AraC infusion on the behavioral recovery processes were evaluated with oculomotor and posturo-locomotor tests.

EXPERIMENTAL PROCEDURES

Animals and surgeries

Experiments were performed on 40 adult domestic cats (3–5 kg) obtained from the "Centre d'élevage du Contigné" (Contigné, France). All experiments were carried out in line with the Animals (scientific procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 8023, revised 1978). Every attempt was made to minimize both the number and the suffering of animals used in this experiment. Cats were housed in a large confined space with normal diurnal light variations and free access to water and food.

Animals were anesthetized with ketamine dihydrochoride (20 mg/kg, i.m., Rhône Poulenc, Mérieux, France), received analgesic (tolfenamic acid, 4 mg/kg, i.m.; Vetoquinol, Lure, France) and were kept at physiological body temperature using a blanket. The vestibular nerve was sectioned at a postganglion level after mastoïdectomy, partial destruction of the bony labyrinth, and surgical exposure of the internal auditory canal. The classical postural, locomotor, and oculomotor deficits displayed by the animals in the days following UVN were used as criteria indicating the effectiveness of the vestibular nerve lesion. Completeness of UVN had already been assessed by histological procedures in previous studies (Lacour et al., 1976).

For the implantation and use of osmotic minipumps containing AraC or sodium chloride (NaCl), a stainless steel cannula was implanted under anesthesia into the fourth ventricle of the brain and connected to a subcutaneous minipump (Alzet, Alza Corporation, Palo Alto, CA; flow rate 2.5 μl/h for 30 days). A midline incision was made through the skin and musculature in the back of the neck, and a cannula connected to plastic tubing was inserted between the dorsal wall of the brainstem and the ventral face of the cerebellum and then cemented with dental cement to the skull. The air in the system was removed by filling up with saline or AraC (diluted into a NaCl solution, 0.13 mM), after which the tubing was connected to an osmotic minipump, and the skin was incised. Cats (n=34) were infused continuously into the cerebrospinal fluid of the fourth ventricle with an antimitotic agent, AraC, known to block cell proliferation in brain structures, or with NaCl, using the osmotic minipump.

Study design

To determine the functional role of the reactive cell proliferation occurring in the VN after UVN and its possible functional critical period, five groups of cats were used for both cellular and behavioral investigations: (i) control that did not undergo UVN (n=4), (ii) UVN with early NaCl infusion (UVN/NaCl $D_{0\text{-}30}$, n=8), (iii) UVN with early AraC infusion (UVN/AraC $D_{0\text{-}30}$, n=8), (iv) UVN with delayed NaCl infusion (UVN/NaCl $D_{20\text{-}50}$, n=8), (v) UVN with late AraC infusion (UVN/AraC $D_{20\text{-}50}$, n=8). In addition, to determine if AraC provided behavioral changes (conditioning on rotating beam, postural function and global sensorimotor activity) and/or side effects (salivation, vomiting), a group of non-lesioned cats (n=2) underwent an infusion of AraC during the conditioning period (Non lesioned AraC group). Another group of nonlesioned cats (n=2) was used for determining if the conditioning on rotating beam affects cell proliferation in the VN (conditioning control group) (see Fig. 7). Immunostaining analyses of postlesional cell proliferation and cell differentiation were performed in the VN and the SVZ. The changes in behavioral recovery profile were analyzed with different behavioral tests (ocular horizontal nystagmus, support surface, and conditioning on rotating beam).

BrdU labeling and immunohistochemistry

Animals were injected with BrdU (200 mg/kg) and killed 27 (UVN $D_{0\text{-}30}$) or 47 (UVN $D_{20\text{-}50}$) days later (Fig. 7). The low doses administered to animals are not liable to generate side effects but are sufficient to mark the cells in S-phase synthesizing DNA (Tighilet et al., 2007).

The cats were deeply anesthetized with ketamine dihydrochoride (20 mg/kg, i.m., Rhône Poulenc, Mérieux, France) and killed by paraformaldehyde perfusion. See Tighilet et al. (2007) for details. Immunohistochemical labeling of BrdU-, GFAP-, and GAD67-immunoreative (Ir) cells was performed according to Tighilet et al. (2007). Double immunofluorescent stained sections were incubated with GAD67 or GFAP combined with BrdU-Ir. The optimal antibody dilutions and staining procedures are described in Table 1. Differentiation of the newly generated cells was analyzed with double labeling analysis performed using confocal imaging with a Leica TCS SP2 laser scanning microscope equipped with a 63×/1.32 NA oil immersion lens. The fields of view were then examined by confocal microscopy, and 1 μm-step Z series were obtained.

Spontaneous nystagmus recovery

The cat was placed on an apparatus with its head fixed and bent forward 23 deg, thus maintaining the horizontal semicircular canals in the horizontal plane. The frequency of the horizontal spontaneous nystagmus (HSN) was recorded in the light using a video camera (Sony HDV) as the number of quick phase beats toward

Table 1. Combination and sequential processing of primary and secondary antibodies used for immunohistochemical and immunofluorescent stainings of BrdU, GFAP, or GAD67

Marker	Primary antibody	Secondary antibody	Technique/coloration
BrdU	Mouse 1/100, Dako	Horse anti-mouse 1/200, Vector	DAB–brown
GFAP	Rabbit 1/200, DaKo	Goat anti-rabbit 1/200, Vector	DAB–brown
GAD67	Mouse 1/10000, Chemicon	Horse anti-mouse 1/200, Vector	DAB–brown
BrdU	Rat 1/100, Oxford Biot	Rabbit anti-rat 1/200, Interchim	Alexa 594–red
GFAP	Rabbit 1/200, Dako	Goat anti-rabbit 1/200, Interchim	Alexa 488–green
GAD67	Mouse 1/10000, Chemicon	Rabbit anti-mouse 1/200, Interchim	Alexa 488–green