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NESTIN DOWN-REGULATION OF CORTICAL RADIAL GLIA IS DELAYED IN RATS SUBMITTED TO RECURRENT *STATUS EPILEPTICUS* DURING EARLY POSTNATAL LIFE

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Abstract – **Objective:** Nestin is temporarily expressed in several tissues during development and it is replaced by other protein types during cell differentiation process. This unique property allows distinguishing between undifferentiated and differentiated cells. This study was delineated to analyze the temporal pattern of nestin expression in cortical radial glial cells of rats during normal development and of rats submitted to recurrent *status epilepticus* (SE) in early postnatal life (P). **Method:** Experimental rats were submitted to pilocarpine-induced SE on P7–9. The cortical temporal profile of nestin was studied by immunohistochemistry at multiple time points (P9, P10, P12, P16, P30 and P90). **Results:** We observed delayed nestin down-regulation in experimental rats of P9, P10, P12 and P16 groups. In addition, few radial glial cells were still present only in P21 experimental rats. **Conclusion:** Our results suggested that SE during early postnatal life alters normal maturation during a critical period of brain development.

KEY WORDS: nestin, pilocarpine, *status epilepticus*, development, radial glia.

Atraso no desaparecimento da nestina na glia radial cortical de ratos submetidos a recorrentes *status epilepticus* durante o desenvolvimento pós-natal precoce

Resumo – **Objetivo:** A nestina, temporariamente expressa em diversos tecidos durante o desenvolvimento, é substituída no processo de diferenciação celular, o que permite a distinção entre células diferenciadas e indiferenciadas. O objetivo deste estudo foi verificar o padrão temporal da expressão da nestina nas células da glia radial cortical de ratos durante o desenvolvimento normal e nos ratos submetidos a sucessivos *status epilepticus* (SE) no periodo pós-natal precoce (P). **Método:** Os animais foram submetidos ao SE induzido pela pilocarpina em P7–9. O perfil temporal da nestina foi estudado por imuno-histoquímica em P9, P10, P12, P16, P30 e P90. **Resultados:** Nos ratos experimentais, observamos atraso no desaparecimento da nestina nos grupos P9, P10, P12 e P16. Ainda, encontramos algumas glias radiais corticais apenas em P21 experimental. **Conclusão:** Nossos resultados sugerem que o SE durante o desenvolvimento pós-natal precoce altera o processo de maturação durante um periodo crítico do desenvolvimento encefálico.

PALAVRAS-CHAVE: nestina, pilocarpina, *status epilepticus*, desenvolvimento, glia radial.

The intermediate filament protein denominated nestin is expressed by undifferentiated cells derived from nervous system, muscular and by cells of several other tissues[1]. Nestin is an acronym for neuroepithelial stem cell protein and it is generally considered a marker of neural stem cells[2]. Upon differentiation, nestin expression is down-regulated and replaced by other tissue-specific intermediate filament proteins, such as glial fibrillary acidic protein (GFAP) in astrocytes, neurofilaments in neurons, and desmin in the muscle[3]. Earlier reports had implied that radial glia cells of the mammalian cortex may be potential precursor cells since they were found to be immunopositive for nestin[4]. These cells do not just provide the scaffolding for migrating neurons, but they serve as precursors

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cells that give origin to all major class of neurons and astrocytes[4-6]. Nestin has been also associated with dividing and migrating cells and with cells which are rapidly changing their morphology. This protein also participates in the cytoskeleton of newly formed endothelial cells and represents a novel and reliable vascular marker[1]. Moreover, neural stem cells in the adult mammalian brain maintain radial glia features[7]. The maintenance of radial glial cells or their key aspects from development seems to be a crucial feature to allow neuronal repair[8]. Interestingly, nestin is re-expressed during various regenerative and degenerative conditions in the fully differentiated cells[9].

In humans with epilepsy, as well as in animals submitted to experimental models of epilepsy, the seizure susceptibility and associated neuronal damage are age-dependent[10-12]. While the immature brain appears to be less vulnerable to the adverse effects of prolonged seizures than the mature brain[13], seizures in the early life can be associated with later cognitive and behavioral disturbances, even in the absence of overt structural neuronal damage[14-16]. Previous study of our laboratory showed that 3 consecutive episodes of long-lasting pilocarpine-induced SE in developing rats (P7–9) is not able to promote neuronal loss but induces important electrographic and cognitive changes during adulthood[17]. Similarly, subtle impairments in intellectual performance have been reported in many individuals with a history of seizures in early life[18].

In this context, the present study was delineated to verify nestin temporal expression in the cortical radial glial cells of rats, under physiological conditions of brain development and after three consecutive episodes of pilocarpine-induced SE (P7–P9). We studied animals from early postnatal life (P9) to adulthood (P90).

METHOD

Animals and *status epilepticus* induction

Wistar rats were housed under environmentally controlled conditions using a standard light/dark cycle of 12 hours (7 am–7 pm), with rat chow pellets, food and water *ad libitum*. The colony room had a temperature of 21°C. The rats were bred in our laboratory and the day of birth was considered as day 0 (P0). The pups were housed with their mothers in individual cages until weaning at day 21. Older animals were housed in groups of three to five per cage. We used only male rats randomly selected for SE induction and they were transiently separated from their mothers during the experimental procedures. The rats were submitted to SE induced by pilocarpine 2% (ip) (4 hours of SE) (Sigma, USA) on P7–P9 (380 mg/Kg), with no previous scopolamine treatment. Rats, with the same age, which received saline instead pilocarpine were used as control. The average weight was 17 gr (P7), 19 gr (P8) and 21 gr (P9) for the control rats and 17 gr (P7), 16 gr (P8) and 15 gr (P9) for the rats submitted to SE. The experiments were performed under strict institutional and ethical approval of the protocol (CEP – Comitê de Ética e Pesquisa, UNIFESP) and all efforts were made to minimize the suffering of the animals as well as reducing the number of animals used. The following groups were studied by immunohistochemistry: P9, P10, P12, P16, P21, P30 and P90, for both groups (n=4 per group).

Immunohistochemical staining

Anaesthetized (Ketamine 5 mg/Kg) animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brains were removed immediately after perfusion, post-fixed and soaked overnight in 30% sucrose in PBS. Brain coronal sections (40 μm) were made using a cryostat. Free-floating paired sections from experimental and control animals at matched levels were processed in the same vial in order to minimize the inter-group differences, during immunohistochemical procedure. In order to distinguish between slices, control ones were marked with a small cut in the surface. All sections were treated with 0.1% H_2O_2 in PBS and then incubated in PBS containing 0.3% Triton X-100. After that, the sections were incubated for 2 hours in 10% bovine serum albumin in PBS and then incubated overnight with the primary anti-nestin antibody (0.5μg/ml of monoclonal mouse anti-nestin, BD Pharmingen). The sections were incubated in the biotinylated secondary antibody (anti-mouse IgG peroxidase conjugate Sigma, 1:200), treated with the ABC reagent (Elite Kit Vector, CA, USA) and the tissue-bound peroxidase was developed using diaminobenzidine as chromogen (DAB 0,05% plus 0,01% hydrogen peroxidase solution). The reaction was initially monitored under the microscope in a few sections to determine the optimal duration of incubation with minimal background. Sections were mounted on gelatinized slides, air-dried, dehydrated, cleared and permanent mounted. Primary somatosensory cortical slices were studied by Sony digital camera coupled to a Nikon Eclipse E600 microscope using bright-field illumination. In order to test the antibody specificity, the primary antibody was omitted. Omission of the primary antibody in our immunhistochemical procedures did not detect any signal, revealing the fact that background and non-specific staining was kept to a minimum level.

RESULTS

Behavioral analysis

Few minutes after pilocarpine systemic administration, all animals presented continuous scratching, strong body tremor, masticatory automatisms, clonic movements of forelimbs and head bobbing culminating in SE. The rats presented long-lasting SE in each one of the three consecutive sessions (P7–P9). The mortality rate was low, less than ten percent. As reference of developmental aspects of pilocarpine model of epilepsy see Priel et al.[19].

Immunohistochemical analysis

Cortical radial glia cells were immunopositive for nestin at P9, P10, P12, P16 of experimental and control ani-

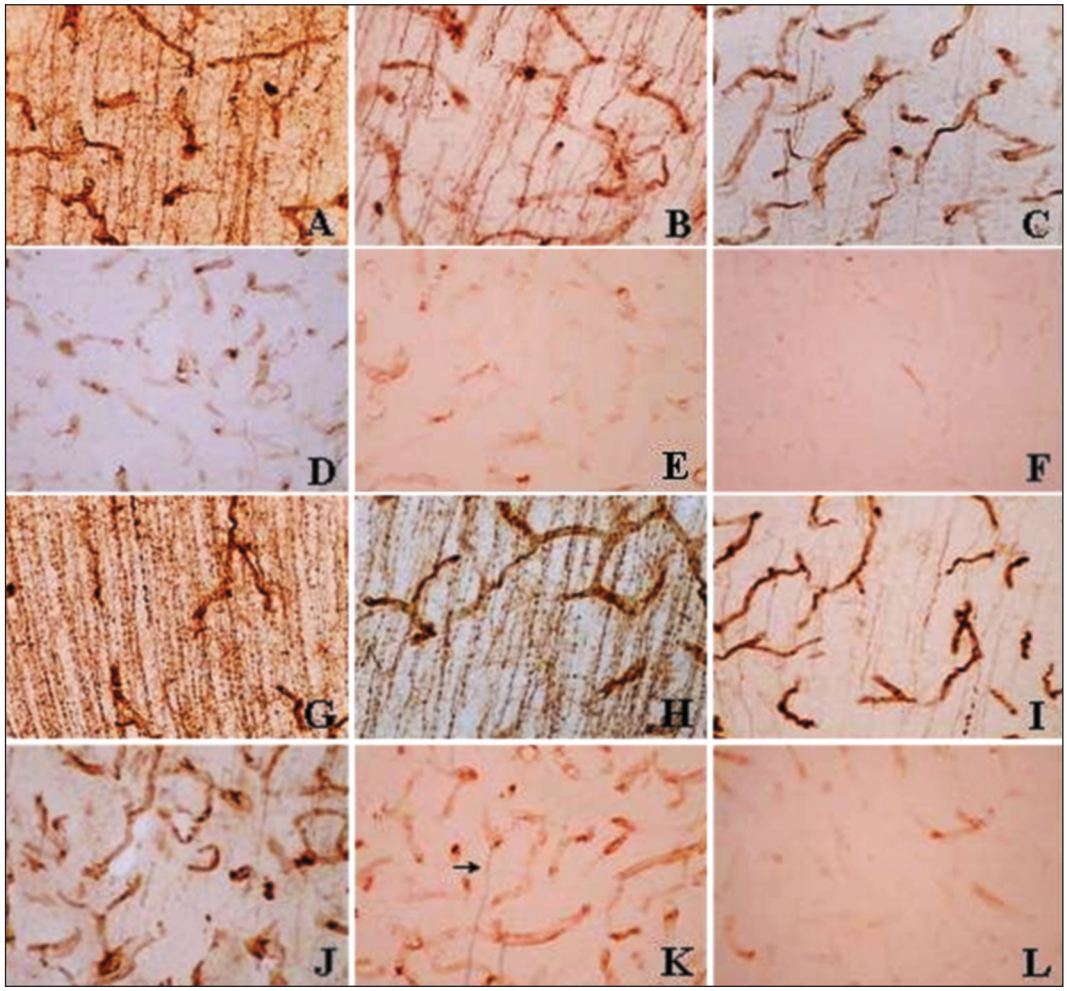

Fig 1. Photomicrographs of the primary somatosensory cortex showing nestin staining. [A–F] control rats; [G–L] experimental rats submitted to SE (P7-9). [A–G] P9; [B–H] P10; [C–I] P12; [D–J] P16; [E–K] P21; [F–L] P30. Arrow: radial glia; Open arrow: blood vessels. Magnification 400x.

mals. However, few radial glia cells were still present only in P21 experimental rats while no nestin immunoreactivity was observed by P21 in the control group. Moreover, cortical blood vessels were also labeled against nestin, gradually decreasing from P9 to P30 and almost inexistent by P90 (not showed), both in experimental and control groups (Fig 1).

Small varicosities were observed along the cortical radial fibers (Figs 2A,2B, P10 taken as representative Figure). In the cortical radial glia of control rats, nestin immunoreactivity (IR) was intense in P9 and P10 groups, decreasing strongly in P12 and P16 (Figs 1A,1B,1C,1D). In P21 control group, the nestin IR completely disappeared (Fig 1E), being inexistent in P30 (Fig 1F) and P90. In contrast, nestin IR was increased in the experimental groups from P9 to P16, when compared to their respective control animals (Figs 1G,1H,1I,1J). In addition, few radial glia cells were still present in the P21 experimental rats (Fig 1K), disappearing in P30 (Fig 1L) and P90. These data suggested that animals submitted to three consecutive SE presented delayed nestin down-regulation in cortical radial glia.

DISCUSSION

Gliogenic activities remain intense during the postnatal period in the developing rat cortex. These include in-

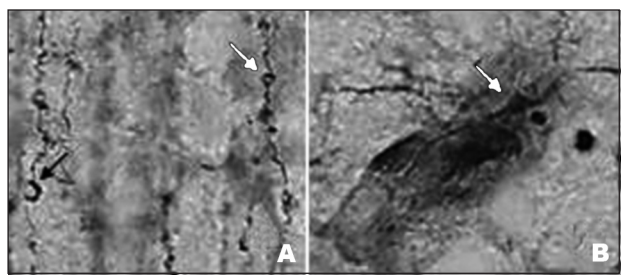

Fig 2. Details of cortical radial glia and blood vessels. [A] Varicosities (asterisk); characteristic association of radial fiber with blood vessel (arrow) 100x. [B] astrocytic end-feet encircles blood vessel (arrow). Magnification 1000x.

volution of radial glia, proliferation of astrocytes and oligodendrocytes and myelin formation. We addressed the question of how recurrent long-lasting SE in early postnatal brain development (P7–9) could affect temporal nestin expression in cortical radial glial cells. Nestin expression is transient and we found delayed nestin down-regulation in the rats submitted to SE. As expected, we observed small varicosities along cortical radial fibers. In 1888, Magini[20] had already detected these varicosities along the radial glial filaments (Fig 2A). Nowadays, some authors have suggested that they could be associated to a transitory cell form between radial glia and astrocytes[21,22].

Nestin is re-expressed during various regenerative and degenerative conditions in the fully differentiated cells, including lesions induced by kainic acid and pilocarpine in adult rats[9,23,24]. However, when considering developmental aspects and astrocytic response after injury the literature is still poor. In the present study, we did not observe such cortical reactive astrocytic response after injury, as largely found in adult rats. In order to support our findings, Rizzi et al.[25] reported that at P9 there are just a little activation of microglia and astrocytes after kainic acid-induced SE. Conversely, Sizonenko et al.[26] showed that the hypoxic-ischemic injury resulted in disruption of the normal radial glia architecture, which was paralleled by an increase in GFAP immunopositive reactive astrocytes. In addition, the morphology of these latter cells and the fact that they were immunolabelled for both nestin and GFAP suggested an accelerated transformation of radial glia into astrocytes. Anyway, the findings imply that glial responses are central to cortical tissue remodelling following neonatal injury.

We observed that experimental rats from P9 to P16 presented increased nestin IR labeling cortical radial glia cells, when compared to their respective controls. Kálmán and Ajtai showed that most radial glial cells disappear by P14, showing no reactivity at P18[22]. As observed in our study, normally, radial glial cells were already inexistent by P21 in the control rats. However, few radial glial cells were still present in the cortex of P21 experimental rats, showing a delay in cortical down-regulation of nestin protein and, therefore, suggesting that the appearance of these fibers in the P21 rats was abnormal. It could be that the SE injury simply acts to delay the transformation of radial glia cells to astrocytes but, eventually, all radial glial cells would transform. This result suggests that SE-induced injury could interfere in the release of factors responsible for the transformation of the radial glia cells.

In humans, SE is a common pediatric emergency and occurs mainly in children younger than 2 years, supporting the idea that during the development the brain is more vulnerable to long-lasting seizures[27,28]. Furthermore, retrospective studies indicate that adults with mesial temporal lobe epilepsy report a high incidence of childhood status epilepticus[29]. Previously, our laboratory showed that although most of the animals submitted to SE (P7–9) did not develop spontaneous seizures in adulthood, they presented learning impairment and significant changes in cortical recordings, with episodes of complex spiking activity[17].

Here we demonstrate increased temporal radial glia after injury induced by SE and delayed in radial glia disappearance. In conclusion, our study suggests that SE during early postnatal life can alters normal maturation during a critical period of brain development. The mechanism for the maintenance of the immature form of cells expressing nestin is unkown and studies of nestin are of relevance in neurobiology.

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