*Neuroscience* 130 (2005) 697–712

# ONTOGENIC STUDY OF THE INFLUENCE OF TISSUE PLASMINOGEN ACTIVATOR (t-PA) IN NEONATAL EXCITOTOXIC BRAIN INSULT AND THE SUBSEQUENT MICROGLIA/MACROPHAGE ACTIVATION

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**Abstract —Intracerebral injections of ibotenic acid in neonatal mice produced white and gray matter lesions that mimic some aspects of the acquired cerebral injuries observed in human newborns (i.e. periventricular leukomalacias in preterm newborns and post-ischemic cortical necrosis in at term infants). We have evaluated the effects of tissue plasminogen activator inactivation (t-PA$^{-/-}$) on the effects of ibotenic acid (0.01–20 μg), and on F4/80 labeling of microglia/macrophages at different stages. Three ontogenic periods have been identified. In mice injected the day of birth, postnatal (P) day 0, ibotenic acid induced neuronal migration disorders together with low local microglial activation in wild-type and t-PA$^{-/-}$ mice. In P2 and P5 mice, ibotenic acid induced diffuse microglial activation in the whole cortex and subcortical areas; e.g. caudate nucleus and septum. In wild-type mice, cystic lesions of the white matter were consistently observed, surrounded by macrophages. In t-PA$^{-/-}$ mice, non-cystic lesions filled of macrophages were more frequent than cysts. Macrophages were virtually absent in the gray matter. White and gray matter lesions were reduced in t-PA$^{-/-}$ mice. The plasmin inhibitor aprotinin reduced white and gray matter lesions only in wild-type mice injected with high ibotenic acid doses (2.5–5 μg). During this period, a transient F4/80 immunoreactive cell population was detected in the cingulum. At P10, the salient lesion characteristic was a large gray matter lesion containing macrophage accumulation. Microglial activation was confined to the injection site in the white matter. t-PA$^{-/-}$ mice showed reduced lesion size under high doses (>5 μg) of ibotenic acid. Similarly, aprotinin diminished the lesion in wild-type animals exposed to 10 μg ibotenic acid. These data demonstrate that t-PA and microglia do not actively participate in the migration disorders induced in P0 mice. Conversely, t-PA was implicated in cyst formation in older (P2-P10) mice, and in their subsequent growth. t-PA was also involved in GM lesions, probably through an inflammatory process involving macrophages.** 

**Key words: brain development, ibotenic acid, inflammation, ischemia, neuronal migration disorders, periventricular leukomalacia.**

Cerebral palsy remains a common cause of disability in childhood. Most etiological factors of cerebral palsy and/or cognitive deficits in human infants have a prenatal or neonatal origin (Kuban and Leviton, 1994). Factors linked to the development of perinatal brain lesions include prematurity, multiple births, infection/inflammation status, hypoxic/anoxic, and ischemia/reperfusion events (Nelson and Willoughby, 2000; Evrard, 2001; Saliba and Marret, 2001). The patterns of anatomical injuries associated with neurological disorders depend on the morphogenetic stages of brain development. In the less mature preterm infants (aged 24–28 weeks of gestation) a particular prevalence of intraventricular/intra-parenchymal hemorrhage is observed, that could further evolve toward ventriculomegaly and hydrocephalus (Kuban et al., 1999; Whitelaw et al., 2002). Infants aged 24–33 weeks show a huge white matter (WM) vulnerability (Dammann and Leviton, 1999; Volpe, 2001) and may develop periventricular leukomalacia (PVL), a necrotic cystic lesion of the WM, associated with subsequent cortex and basal ganglia injury (Marin-Padilla, 1997). In contrast, in term neonates, brain lesions mainly consist of neuronal losses and consecutively impaired synaptogenesis in the cerebral cortex (Volpe, 1995).

Glutamatergic excitotoxicity has been pointed out as a common final pathway of most deleterious processes in brain diseases (McDonald and Johnston, 1990; Lipton and Rosenberg, 1994). When injected in immature rodents, ibotenic acid, an excitotoxin that produces cerebral cortex lesions mainly through the activation of the glutamate *N*-methyl-D-aspartate (NMDA) receptors, mimics some aspects of the developmental lesions observed in human infants (Marret et al., 1995, 1996). Ibotenic acid injected the day of birth, postnatal day (P) 0 in hamsters, was shown to induce neuronal migration disorders (Marret et al., 1996). When administered at P0 or at P2 in mice, it induced neuronal death in the deeper cortical layers, resulting in an abnormal, microgyria-like sulcation of the cortex. At P5 or P10 neuronal loss affects all cortical layers, a characteristic which is observed in full term human neonates (Marret et al., 1995). In the WM, when delivered within a defined ontogenic window (P2–P10),

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*Abbreviations:* CP, cortical plate; GM, gray matter (in P2-10 mice); i.c., intracerebral; Neu-N, nuclear neuronal antigen; NMDA, *N*-methyl-D-aspartate; P, postnatal day; PBS, phosphate-buffered saline; PVL, periventricular leukomalacia; t-PA, tissue plasminogen activator; WM, white matter.

0306-4522/05$30.00+0.00 
doi:10.1016/j.neuroscience.2004.09.049

ibotenic acid produces cystic lesions that corresponds to retraction of radial glia, neogliogenesis of astrocytes and pre-oligodendrocytes, and microglia invasion of the parenchyma. This model has provided experimental evidence for a synergy between excitatory amino acids and pro-inflammatory cytokines (Dommergues et al., 2000; Patkai et al., 2001). The activation of microglia, the local inflammatory cells, occurs soon after ibotenic acid injection (Tahraoui et al., 2001; Dommergues et al., 2003). In fact, anti-inflammatory drugs have been reported to produce neuroprotective effects in P5 mice subjected to ibotenic acid (Arquié et al., 2002).

Furthermore, deleterious effects of microglial activation have been demonstrated in adult rodent brain, after excitotoxic, ischemic or hemorrhagic insults (Rogove and Tsirka, 1997; Rogove et al., 2002; Wang et al., 2003). Activated microglia elicit neuronal death through the release of pro-inflammatory cytokines (Perry et al., 1999) and enzymes, including the tissue plasminogen activator (t-PA; Tsirka et al., 1995; Smith et al., 1998; Flavin et al., 2000). t-PA is a serine protease that plays a critical role in fibrinolysis by converting plasminogen into plasmin. t-PA was also described as a mediator in a variety of tissue remodeling functions (Collen and Lijnen, 1991; Vassalli et al., 1991). In the adult CNS neurons, astrocytes, and microglia are able to release t-PA (Tsirka et al., 1995; Buisson et al., 1998), that promotes extracellular plasmin mediated proteolysis (Sappino et al., 1993). Under physiological conditions, the t-PA/plasmin system was involved in a number of developmental events in the CNS, including neuronal migration (Seeds et al., 1999), neurite outgrowth (Jacovina et al., 2001) and synaptic plasticity (Calabresi et al., 2000). During the inflammation process, neuronal t-PA activates microglia through a non proteolytic action on membrane targets (Rogove et al., 1999; Siao and Tsirka, 2002). This cytokine like effect of t-PA subsequently amplifies inflammation by promoting microglial cell recruitment and proliferation at the injured site (Siao et al., 2003). The t-PA of microglial origin may lead to neuronal degeneration via plasmin activation (Chen and Strickland, 1997; Nagai et al., 1999b; Siao et al., 2003).

We have recently demonstrated the involvement of t-PA in excitotoxic WM lesions elicited by ibotenic acid. t-PA knock-out mice (t-PA$^{-/-}$) have shown limited formation and growth of cystic lesions in the WM (Hennebert et al., 2004). However, the precise role played by t-PA, as well as its interaction with microglia in excitotoxic brain lesion occurring during mouse development, remains only partially described. The present study was designed to investigate the involvement of t-PA/plasmin system and microglia in the ontogeny of the various lesions that occur after an excitotoxic challenge during mouse brain development. Therefore, we have carried out F4/80 immunolabeling of microglia/macrophages over a wide dose range of ibotenic acid, in wild-type and t-PA$^{-/-}$ mice.

## EXPERIMENTAL PROCEDURES

### Animals

All experimental protocols and procedures were in agreement with the directive 86/609/CEE of the European Community, for the use of laboratory animals and were performed under the control of an authorized expert (P.L. authorization number: 76.A.16). All efforts were made to minimize the number of animals used and their suffering.

The study was performed in t-PA$^{-/-}$ mice (Carmeliet et al., 1994) and in wild-type control animals with the same genetic background (C57-Bl6/129; 75/25). The animals were permanently housed by pairs in a room controlled at a temperature of 21±2 °C on a 12-h light/dark cycle. They were fed rodent laboratory chow and were given water *ad libitum*. The first 6 h of life were considered P0, and then termed P1. For lesion identification and size measurements, several sex-matched litters (usually three or four), aged P0, P2, P5 or P10 were used. Immunohistochemistry was carried out in nontreated animals and ibotenic acid-injected animals (42 and 119 per group, respectively).

### Drugs administration

Intracerebral (i.c.) injections of ibotenic acid (0.01 μg to 20 μg; Sigma, St. Quentin Fallavier, France), diluted in 0.02% acetic acid–0.1 M phosphate-buffered saline (PBS), pH 7.4, were performed at P0, P2, P5 and P10 under light isoflurane anesthesia with a 26 gauge needle on a 50 μl Hamilton syringe (VWR, Fontenay-aux-Roses, France) mounted on a calibrated dispenser (VWR). The needle was inserted 2 mm under the external surface of scalp skin in the frontoparietal neopallium of the right hemisphere, 2 mm right from the coronal suture in the lateral-medial plane and 3 mm anterior from the sagittal suture in the rostrocaudal plane. Under these conditions, the tip of the needle reached the periventricular WM (Gressens et al., 1997). Two 1 μl boluses were injected 30 s apart.

The plasmin inhibitor aprotinin (20 kallikrein inhibiting units/pup; Trasylol; Bayer Pharma, Puteaux, France) was administered i.p. to pups of P0, P2, P5 or P10, 10 min before injection of ibotenic acid. In mice injected the day of birth, postnatal (P) day 0, aprotinin was administered before injection of a medial dose (1 μg) of ibotenic acid. At other developmental stages, aprotinin was administered in animals injected with either a low dose (0.1 μg) or a high dose of ibotenic acid. The high dose was defined as the lowest dose producing the maximum lesion size, depending on the age of animal; 2.5 μg, 5 μg and 10 μg in P2, P5 and P10 mice, respectively (see below).

### Histology of the lesions

Five days after i.c. ibotenic acid injection, animals were killed by decapitation. The brain was removed, fixed in formalin 4% for 7 days, and embedded in paraffin. Coronal serial sections, 10 μm thick, were cut and every other section was stained with Cresyl Violet (Sigma), for histological analysis of lesions. All sections between the occipital lobes (caudal) and the frontal lobe (rostral) were analyzed. The nature of lesions depends upon the stage of development.

Neuronal migration disorders induced by different doses of ibotenic acid at P0 were analyzed using neuronal labeling (see below). The disorders that affected the cortical plate (CP) at this stage were defined by their location: i) intracortical and less frequent focal periventricular neuronal heterotopia; ii) neuronal ectopia in the molecular layer; and iii) polymicrogyria, as previously described (Rorke, 1994; Marret et al., 1995, 1996). The occurrence rates of different types of neuronal migration disorders were scored as followed: 1 present versus 0 not present. The ratio of occurrence for each lesion type was expressed as percentage in