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Pharmacology & Toxicology
ISSN 0901-9928

Annotations & Reflections

Excitotoxic Mechanisms of Apoptosis in the Mammalian Visual System Following Monocular Visual Deprivation

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(Received February 28, 2002; Accepted March 20, 2002)

The visual system in new-born mammals is extremely susceptible to plastic adaptation in response to non-physiological visual experience. For instance, brief occlusion of the vision in one eye (monocular deprivation) during early postnatal life causes an almost total loss of visual responses from the deprived eye in cortical neurones. As a consequence, vision in the occluded eye is impaired and the eye becomes blind (Wiesel & Hubel 1963). The sensitivity to monocular deprivation is limited to a time period, also referred to as the critical period; at the end of this period, ocular preference of cortical neurones is stabilized and will no longer be influenced by manipulation of the visual environment.

Similar forms of deprivation-induced synaptic depression have been observed in many species, including monkeys (Hubel & Wiesel 1977), rats (Fagiolini *et al.* 1994) and mice (Gordon & Stryker 1996). It is well known that in human beings during post-natal life, altered visual experience, such as mono- or binocular visual deprivation (i.e. congenital cataract, ptosis) or abnormal visual stimulation (strabismus, anisometropia), induces a progressive decline of the visual performances of the affected eye causing a neurophthalmologic disease termed amblyopia. During the last three decades, many studies have been devoted to the understanding of the mechanisms underlying this disease but only recently new insights have been gained. Here we focus on the recent discovery of the mechanisms underlying neuronal cell death caused by monocular deprivation in the lateral geniculate nucleus that we believe open new venues for novel therapeutic approaches to the treatment of amblyopia in man.

Morphologic changes induced by monocular deprivation

Several studies show that the lateral geniculate nucleus is the relay station of the visual system most affected by monocular deprivation. Wiesel & Hubel (1963) were the first to discover that in the deprived laminae of the lateral geniculate nucleus of young cats (those receiving afferents from the closed eye), the diameter of the neuronal cell bodies is about two-thirds of that of neurones in the experienced layers. This observation has also been reported *post mortem* in the lateral geniculate nucleus of patients suffering of amblyopia (von Noorden & Crawford 1992), thus confirming the usefulness of the animal model to dissect the pathophysiology of the disease. Interestingly, of the two main classes of relay cells in the lateral geniculate nucleus, deprived Y cells seem particularly sensitive to physiological, metabolic and morphological effects elicited by deprivation (Sherman & Spear 1982; Guimaraes *et al.* 1990).

Our recent studies have shown that monocular deprivation causes cell death of the apoptotic type in the lateral geniculate nucleus of new-born rats (Nucci *et al.* 1998 & 2000a & b) (fig. 1). In fact, under these experimental conditions, high magnification light microscopy analysis of tissue sections from the brain of monocular deprivation rats stained with haematoxylin and eosin revealed the occurrence of marginalisation and condensation of the nuclear matrix (Nucci *et al.* 1998 & 2000a & b). Fragmentation of nuclear DNA has also been confirmed by the observation in adjacent tissue sections, obtained from rats monocularly deprived for 2, 7 and 14 days, of lateral geniculate nucleus cells positive to the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) technique (Gavrieli *et al.* 1992). Finally, cell death by monocular deprivation was accompanied by the appearance in the lateral geniculate nucleus of cells immunopositive for the tumour suppressor protein p53 (Nucci *et al.* 1999a&b), a gene product activated by cellular damage. Collectively, these three criteria support the hypothesis that cell death caused by monocular deprivation in the lateral geniculate nucleus of rat pups may be of the apoptotic type (Clarke *et al.* 1993; Kerr *et al.* 1987). As a consequence of cell death, we observed that the number of viable cells in the deprived

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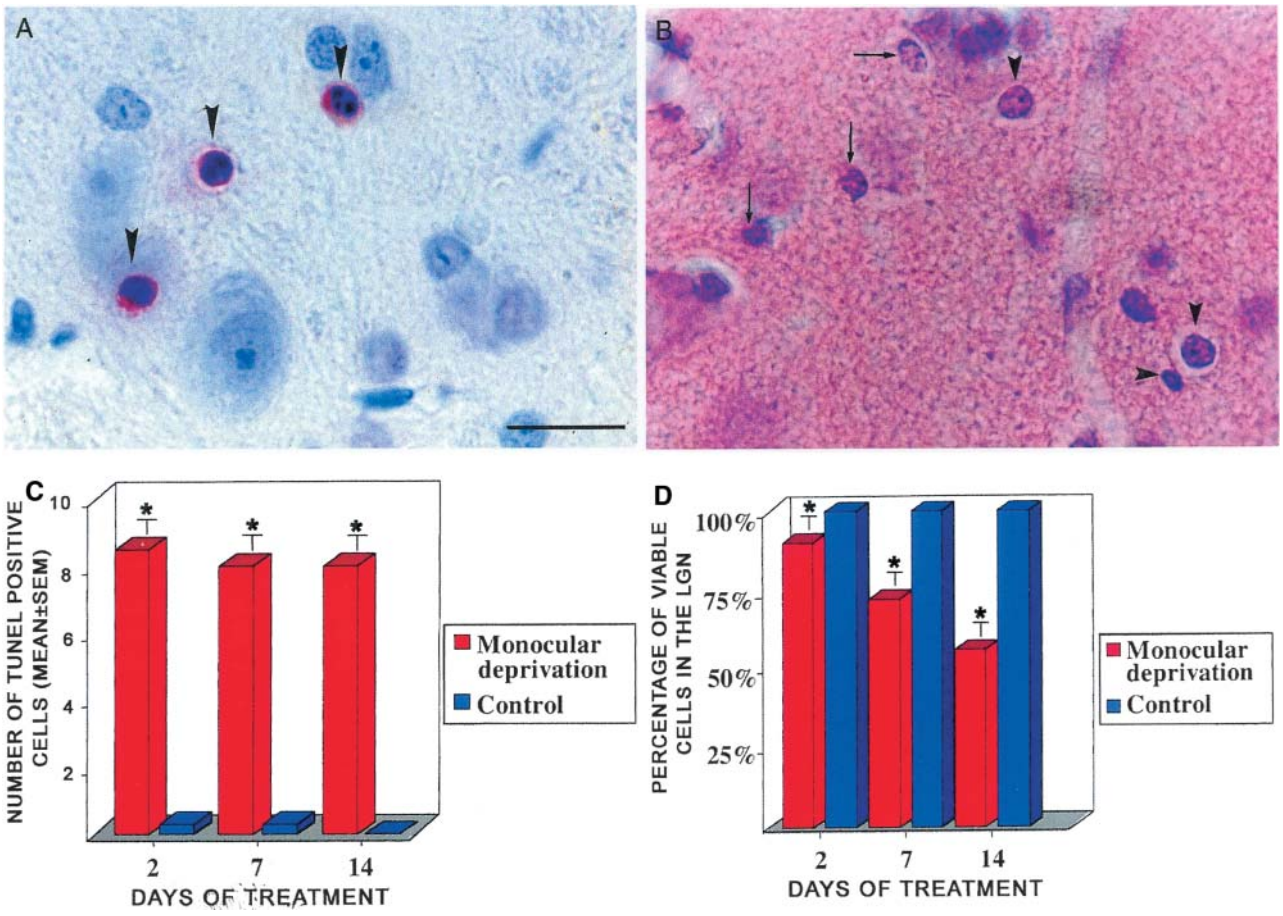

Fig. 1. Monocular deprivation (MD) induces apoptotic cell death in the lateral geniculate nucleus (LGN) of new-born rats. (A) *In situ* DNA fragmentation (TUNEL-positive cells) is evident in the LGN of a brain tissue section obtained from a rat deprived for 7 days. (B) Nuclei with condensed (arrow heads) and marginalized (arrows) chromatin are apparent in an haematoxylin and eosin-stained section from a newborn rat after 7 days of MD. (C) TUNEL-positive cells of the LGN were scored in coronal sections from newborn rats after MD for 2, 7 and 14 days or from age-matched controls. Statistical differences were evaluated by using Student's t-test. * $P<0.01$ versus control. (D) The percentage of viable cells was counted using the optical disector method in sections incorporating the LGN from rats deprived for 2, 7 and 14 days or from age-matched controls. Data were evaluated statistically using Student's t-test. * $P<0.01$ versus control.

lateral geniculate nucleus progressively decreases as the interval of deprivation is prolonged. In fact, in haematoxylin & eosin-stained sections the number of cells per mm^3 in the lateral geniculate nucleus contralateral to the deprived eye was reduced by approximately 10%, 27% and 44% after 2, 7, or 14 days of deprivation, respectively (fig. 1d).

Thus, our results indicate that monocular deprivation is associated with neuronal cell death that is responsible for a progressive decrease in the total number of viable cells in the lateral geniculate nucleus.

The neuropathological characteristics of the lateral geniculate nucleus of new born rats undergone monocular deprivation are in apparent contrast with the evidence that during the critical period of development the electrophysiological and functional changes induced by monocular light deprivation are mostly reversible if the previously occluded eye is reopened and the originally opened eye is closed for a proportional period of time (reverse lid suture). To rationalize these data, it may be assumed that visual recovery may reflect the capacity of residual neurones to compensate for the lost cells. This resource may also be influenced by a reduction in the total number of viable neurones in the contralateral lateral geniculate nucleus, that probably occurs during reverse lid suture. Thus, it is conceivable that the normal binocular vision after reverse lid suture may result from a novel equilibrium in the total number of viable neurones in the lateral geniculate nucleus of either side of the brain. This equilibrium is likely to be reached when monocular deprivation is maintained for a limited period of time during the critical period, with prolonged visual deprivation (in rats for more than 1 month) leading to very small number of residual, viable, cells in the lateral geniculate nucleus and irreversible damage of the vision.

Mechanisms involved in monocular deprivation-evoked neuronal death

The importance of retinal activity. Recent data obtained in kittens demonstrate that monocular deprivation for 48 hr

leaves sufficient residual activity in the deprived retina to induce homosynaptic long-term depression in the visual cortex (Bear & Rittenhouse 1999; Rittenhouse *et al.* 1999). In fact, it has been observed that elimination of retinal activity by intraocular injection of tetrodotoxin prevents the depression of closed-eye responses in the visual cortex of kitten (Bear & Rittenhouse 1999; Rittenhouse *et al.* 1999). The presynaptic activity is an important element in triggering synaptic depression. It has been proposed (Bienenstock *et al.* 1982) that activation of an excitatory input will lead to an increase or decrease in synaptic effectiveness depending on whether the coincident activity of the postsynaptic neurone falls above or below a critical value, called the modification threshold (Bienenstock *et al.* 1982). Therefore, it was hypothesized that the necessary condition for long-term synaptic depression may be presynaptic activity that consistently fails to evoke or correlate with a postsynaptic response large enough to trigger long-term synaptic depression. In agreement with the data reported of the visual cortex, we have observed that the residual pre-synaptic retinal activity (Bear & Rittenhouse 1999) is also necessary for the induction of neuronal death in the lateral geniculate nucleus of monocular deprivation rats. In fact, we have recently shown that optic nerve transection prevents cell death in the lateral geniculate nucleus (table 1) whereas in the deafferentated retina we observed a remarkable alteration of the tissue architecture associated with cell death (Nucci *et al.* 2000a & b). The importance of presynaptic activity in the induction of long-term synaptic depression and cell death during monocular deprivation suggests that these two phenomena could be related. Therefore, it is conceivable that apoptosis represents a structural change consequent to the induction of long-term synaptic depression in the lateral geniculate nucleus. The likely candidate for such a mechanism is glutamate, an excitatory neurotransmitter involved in synaptic plasticity which is released in the lateral geniculate nucleus by optic nerve endings (Sillito *et al.* 1990).

The role of excitotoxicity and strategies for neuroprotection

In the visual system, several studies have documented that N-methyl-D-aspartate (NMDA) and non-NMDA subtypes of glutamate receptors, beside their physiological function as mediators of excitatory synaptic transmission, are also involved in the mechanisms of synaptic plasticity in the visual cortex. In particular, it has been observed that infusion of the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (APV) into the visual cortex reduces the ocular dominance shift and prevents the neuronal shrinkage induced by monocular deprivation (Bear *et al.* 1990). A similar result was obtained with infusion of the NMDA channel blocker MK801 directly into the visual cortex (Rauschecker *et al.* 1990) or administered intramuscularly (Daw *et al.* 1999).

In accordance with these data we observed that in the lateral geniculate nucleus the glutamatergic pathway is also involved in the mechanisms of monocular deprivation-evoked cell death. In fact, intraperitoneal administration of selective antagonists of the NMDA (MK801 and CGP040116) or non-NMDA (GYKI52466) subtype of glutamate receptors protected against cell death in the lateral geniculate nucleus caused by seven days monocular deprivation. In particular, administration of MK801 or CGP040116 reduced the number of TUNEL-positive cells by 73% and 79%, respectively, whereas administration of GYKI52466 yielded a 91% reduction (Nucci *et al.* 2000a & b). Furthermore, treatment with MK801, CGP040116 or

Table 1.

Monocular deprivation (MD)-evoked cell death in the lateral geniculate nucleus (LGN) is prevented by optic nerve transection, treatment with glutamate receptor antagonists or by inhibition of nitric oxide synthase.

Experimental conditions	TUNEL-positive cells	Viable cells (per mm^3)
Control	0.0±0.0	$14.5\times10^4\pm0.2\times10^4$
MD for 7 days	8.0±0.3	$11.1\times10^4\pm0.5\times10^{4}$**
Optic nerve transection	0.9±0.2*	ND
MD for 7 days + L-NAME	0.2±0.1*	$13.6\times10^4\pm0.7\times10^{4}$***
MD for 7 days + D-NAME	8.1±0.3	$10.2\times10^4\pm0.4\times10^{4\$}$
MD for 7 days + CGP040116	1.7±0.2*	$14.5\times10^4\pm0.8\times0^{4}$***
MD for 7 days + MK801	2.2±0.2*	$13.6\times10^4\pm0.6\times10^{4}$***
MD for 7 days + GYKI52466	0.8±0.1*	$15.3\times10^4\pm0.8\times10^{4}$***

Monocular deprivation was performed for 7 days in rats starting at 14 days of age. Age-matched non-deprived animals were used as controls. Different groups of MD animals were additionally treated with CGP040116 (7 $mgkg^{-1}$), MK801 (0.3 $mgkg^{-1}$), GYKI52466 (3.3 $mgkg^{-1}$), with L-NAME (3 $mgkg^{-1}$, twice daily), with D-NAME (3 $mgkg^{-1}$, twice daily) or by optic nerve transection (at the day of MD start). Adjacent brain tissue sections across the LGN were processed for *in situ* detection of DNA fragmentation (TUNEL technique) or stained with haematoxylin & eosin. The number of TUNEL-positive cells in the dLGN of either side of the brain and the number of viable cells per mm^3 in the LGN contralateral to the deprived eye in identical animals (n=6 per group) was scored. The results are expressed as mean±S.E.M. Data were evaluated statistically for differences using ANOVA followed by Tukey-Kramer multiple comparisons test. *Denotes $P<0.001$ versus MD. **Denote $P<0.01$ versus control. ***Denote $P<0.01$ versus MD and $P>0.05$ versus control; $^{4\$}$ denotes $P<0.01$ versus control and $P>0.05$ versus MD. ND=Not determined. Adapted from Nucci *et al.* (2000a).

GYKI52466 prevented the loss of viable neurones elicited by 7 days of monocular deprivation. Thus, glutamatergic stimulation seems to be essential for cell death triggered by monocular deprivation (table 1).

Excessive stimulation of glutamate receptors increases intracellular [Ca^{2+}] and this triggers downstream processes leading the cell to die (Meldrum & Garthwaite 1990). Among these, activation of nitric oxide synthase generates nitric oxide, a highly reactive radical species which plays important physiological roles in the CNS (Moncada *et al.* 1993) and has also been involved as a downstream mediator in various neurodegenerative processes. Interestingly, it has been reported that nitric oxide is necessary for the transmission of the visual input under normal visual stimulation and it is directly involved in visual information processing at the level of the lateral geniculate nucleus (Cudeiro *et al.* 1994). Interestingly, several evidence indicate that nitric oxide acts together with NMDA receptors in the activity-dependent refinement of the visual connections during development. Using a high performance liquid chromatography based technique, we have observed that citrulline, the coproduct of nitric oxide synthesis (Moncada *et al.* 1993), increases significantly in new born rats after 24 hr of monocular deprivation but not after 7 days when its levels were again similar to those of age-matched non-deprived control (Nucci *et al.* 1999a & b, 2000a & b). Thus, it seems conceivable that increased nitric oxide synthase activity is involved in the mechanisms which trigger cell death. This hypothesis is strengthened by the observation that treatment of monocular deprivation rats with L-NAME, an inhibitor of nitric oxide synthase, but not with D-NAME, a less active isomer, abolished the observed increase of citrulline levels and prevented neuronal cell death and loss in the lateral geniculate nucleus (Nucci *et al.* 1999a&b; 2000a&b) (table 1). In addition, elevation of citrulline was also prevented by treating the animals during monocular deprivation with antagonists of NMDA (MK801and CGP040116) or non-NMDA (GYKI52466) subtypes of glutamate receptors (Nucci *et al.* 2000a & b), indicating that during monocular deprivation there is an excessive stimulation of glutamate receptors, with consequent activation of nitric oxide synthase and abnormal production of nitric oxide which in turn participates in the mechanisms of neuronal cell death.

The mechanism underlying nitric oxide-mediated, monocular deprivation-induced, neuronal death in the lateral geniculate nucleus of newborn rats is not known. However, using mice lacking the PARP gene (PARP -/-), (Wang *et al.* 1995), we observed that monocular deprivation failed to induce neuronal cell death in the lateral geniculate nucleus which, by contrast, was evoked in the heterozygous variant (Nucci *et al.* 2000b). This suggests that during monocular deprivation the observed glutamate-induced increase in nitric oxide production causes DNA fragmentation and activates PARP which in turn may take part in the mechanism of neuronal death in the lateral geniculate nucleus. PARP is a repair enzyme which catalyses the attachment of ADP-ribose units from NAD to nuclear proteins such as histone

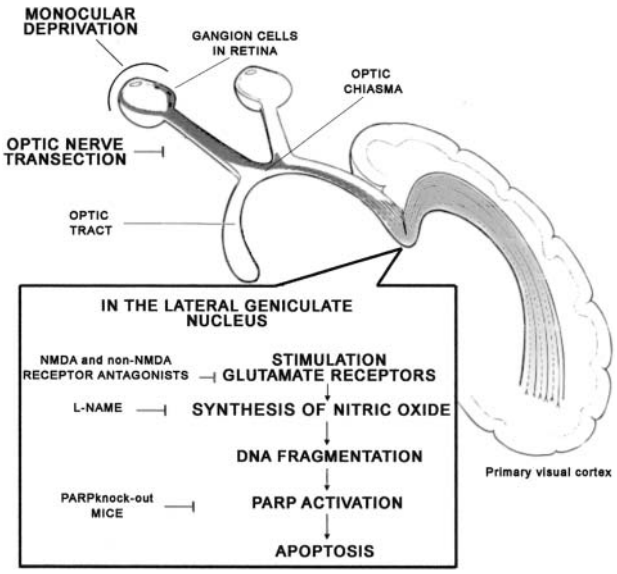

Fig. 2. During early postnatal development of the rat visual system, monocular deprivation causes excitotoxic, nitric oxide-mediated, cell death in the LGN that appears to be apoptotic and also requires activation of PARP. Interruption of retinal input signals by optic nerve transection or pharmacological treatment with either glutamate receptor antagonists or inhibitors of nitric oxide synthesis abolished MD-evoked cell death in the LGN (as indicated by the T bars). Also the absence of the PARP gene seems to confer neuroprotective properties.

and PARP itself. For every mole of ADP-ribose transferred from NAD, one mole of NAD is consumed and four free energy equivalents of ATP are required to regenerate NAD to normal cellular levels. Therefore, activation of PARP can result in a rapid drop in energy stores (Pieper *et al.* 1999). If this drop is severe and sustained it can lead to impaired cellular metabolism and ultimately death. In accordance, pharmacological inhibition of PARP has been shown to prevent cortical neuronal death induced by addition of some nitric oxide donors or due to stimulation of endogenous nitric oxide synthase by NMDA (Pieper *et al.* 1999). However, the same inhibitors are not effective when apoptosis is induced in cerebellar granule cell primary cultures by excitotoxic mechanisms (Pieper *et al.* 1999). A similar difference of effects was seen when primary neuronal cultures from PARP -/- mice were examined: apoptotic death of cerebellar granule cells was not affected by the absence of PARP, whereas cortical neuronal death was prevented (Pieper *et al.* 1999).

Conclusions and future prospects

Our studies demonstrate that visual deprivation in one eye during early post-natal development induces a reduction in the total number of viable cells in the lateral geniculate nucleus via the induction of an apoptotic programme which appears to be triggered by an excitotoxic, nitric oxide mediated, mechanism. The exact mechanism responsible for monocular deprivation evoked activation of the death cas-

cade in the lateral geniculate nucleus remains to be elucidated; however, the results obtained so far offer novel experimental venues for the development of a pharmacologic approach to the treatment of amblyopia (see fig. 2 for a schematic representation of possible targets for therapeutic intervention).

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