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NMDAR1-like immunoreactive fibers appear in the ipsilateral optic tract during optic nerve regeneration in *Rana pipiens*

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Abstract

N-Methyl-D-aspartate receptor subunit 1-like immunoreactivity (NMDAR1-LI) was investigated in the brain of *Rana pipiens* during optic nerve regeneration. Following unilateral optic-nerve crush, frogs were tested for prey-catching and optokinetic nystagmus responses to assess return of visual function. At 1, 2, 3 and 5 months after the surgery, NMDAR1-LI was assessed in central visual pathways. At 3 and 5 months, conspicuous ipsilateral NMDAR1-LI fibers were detected in the thalamic and pretectal nuclei, and the time of their appearance coincided with the onset of behavioral recovery. Also, only ipsilateral retinorecipient layers in the optic tectum showed increased NMDAR1-LI during optic nerve regeneration. These results suggest that NMDA receptors may be present on retinal ganglion cell axons and terminals that have been misrouted during regeneration. © 1997 Elsevier Science Ireland Ltd.

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Several lines of evidence suggest that the regeneration of non-mammalian retinal ganglion cells (RGCs) and reformation of central nervous system retinotopic maps involve glutamate NMDA (*N*-methyl-D-aspartate) receptors. During optic nerve regeneration in goldfish, long-term potentiation (LTP) can be induced in the optic tectum; this LTP can be blocked by NMDA receptor antagonists, AP5 and AP7 [18]. If a third eye is implanted into *Rana pipiens* tadpoles, the RGC axons grow into the tectum, leading to formation of eye-specific stripes [15]. These eye-specific stripes can be desegregated with chronic application of AP5 to the tectum or sharpened with NMDA application to the tectum [3].

Involvement of NMDA receptors in optic-nerve regeneration is not restricted only to refinement of the retinotopic map. For example, expression of NMDA receptor-mRNA in regenerating goldfish RGCs increases only if functional, postsynaptic, tectal NMDA receptors are present and the axons have reached the tectum [7]. Regenerating RGCs also express a growth-associated protein, GAP-43 [17], and NMDA receptors may regulate this expression because NMDA receptor blockade prevents kainate induction of GAP-43 in sprouting mossy fibers in the rat hippocampus [14].

Although binding of NMDA has been studied in the frog brain [4,13], just how the density of NMDA receptors changes during regeneration is not known. To address this question, we analyzed NMDA receptor subunit 1-like immunoreactivity (NMDAR1-LI) in *Rana pipiens* central visual pathways after unilateral crush of one optic nerve. The NMDAR1 subunit is considered to be a necessary component of functional NMDA channels [1].

Adult frogs were anesthetized by immersion in a 0.2% MS-222 (3-aminobenzoic acid ethyl ester) solution (pH 7.0). An incision was made in the roof of the mouth, and the left optic nerve exposed, with care not to damage the ophthalmic artery. The optic nerve was tightly squeezed several times with fine forceps until two optic nerve stumps separated by a transparent area were visible within the optic nerve sheath. The incision was closed, and frogs were allowed to survive for 1, 2, 3 or 5 months. Two frogs were investigated at 1 month, and three frogs at every other survival time. In addition, two other frogs were sham-operated, and allowed to survive for 3 months.

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Each frog was tested for prey-catching behavior and optokinetic nystagmus (OKN) before surgery and each week thereafter. Prey-catching behavior was tested by randomly dropping a single mealworm into the right or left monocular field of view, as described previously by Fite and Hayden [5]. Each trial was recorded as a positive response (i.e. the frog turned at the correct angle towards the mealworm) or no response, and the percentage of responses were calculated for each frog, for each session. Optokinetic nystagmus was tested using a cylindrical optokinetic drum, 38 cm in diameter and 56 cm in height. The interior of the drum was covered with a repetitive pattern of alternating black (1.9 cm) and white (2.5 cm) stripes and was evenly illuminated from above. The drum was mounted on a platform, and the frog was placed in a clear 1000 ml glass beaker, suspended in the center of the drum. The drum was rotated at 6°/s, and the direction of movement was reversed several times during each session. Because frogs show a monocular response asymmetry during horizontal OKN response (the temporal-to-nasal direction is more effective in eliciting OKN than the nasal-to-temporal direction), frogs with the left optic nerve crushed failed to react to the clockwise movement of the drum. This response reappeared during optic nerve regeneration. The OKN responses were directly observed and graded as 'present', 'weak', or 'absent'. In order to determine whether changes in NMDAR1-LI might be induced during the OKN testing sessions, sham-lesioned frogs underwent the same OKN testing as frogs with a crushed optic nerve.

For immunocytochemistry, frogs were anesthetized in 0.2% MS-222, and perfused transcardially with saline, followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB; pH 7.4). Brains were postfixed in the same fixative for 2 h at 4°C, transferred into 30% sucrose in PB and left overnight at 4°C. The next day brains were embedded in gelatin and serial, coronal sections were cut on a freezing microtome at 50 μm thickness. Sections were collected into cryoprotectant and stored at −20°C, rinsed in 0.05 M Tris-buffered saline (TBS; pH 7.6), and pretreated with 1% sodium borohydride (10 min) and 1% H_2O_2 (10 min). Following rinses, sections were incubated in 20% goat serum with 1% bovine serum albumin (BSA) in TBS for 30 min, transferred to the solution, containing 1 μg/ml rabbit-anti-NMDAR1 antibody (Chemicon), 1% normal goat serum and 0.5% Triton X-100 in modified TBS (mTBS; 0.1% gelatin and 0.02% NaN_3 added), and incubated for 2 days at 4°C. Sections were rinsed in mTBS, and incubated for 90 min in 2 μg/ml dilution of biotinylated goat-anti-rabbit antibody (Vector), containing 1.5% serum (in mTBS). Following rinses, sections were incubated in 1:100 dilution of ABC (Vector) in TBS for 90 min, rinsed, reacted with 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2, mounted on subbed slides, air-dried, and cover-slipped. No non-specific binding was observed in sections with the primary antibody omitted. Substitution of non-immune rabbit serum for the primary antibody resulted in non-specific staining in the nucleus of the basal optic root (nBOR), oculomotor nucleus, and the paraventricular cellular layers of the tectum. Therefore, these areas were excluded from further analysis.

In all frogs, prey-catching responses were completely abolished after optic nerve crush, and reappeared at 7.7 ± 1.7 weeks following surgery. The OKN response to the temporal-to-nasal direction of pattern motion (with respect to the operated eye) was completely abolished in all but one frog, and returned 9.1 ± 2.7 weeks postcrush. The time difference between appearance of the first prey-catching and the first OKN responses was non-significant across subjects ($F(1,13) = 1.20$, $P > 0.25$).

The distribution of NMDAR1-LI correlated well with the distribution of NMDA receptor binding in the *Rana pipiens* brain, as shown previously [4,13]. In particular, the telencephalon, pretectal area and cerebellum showed high levels of NMDAR1-LI. NMDAR1-LI was analyzed in thalamic visual nuclei (nucleus of Bellonci, corpus geniculatum, rostral visual nucleus), pretectal visual nuclei (posterior thalamic neuropil and nucleus lentiformis mesencephalis) and retinorecipient tectal layers. The posterior thalamic neuropil and the nucleus lentiformis mesencephalis were treated as one pretectal area (Pt) because of their close proximity.

In the thalamic and pretectal visual areas, no differences in NMDAR1-LI were observed between the ipsilateral and contralateral nuclei at 1 or 2 months after optic nerve crush. The only exception was one frog which survived for 1 month and showed NMDAR1-LI fibers exclusively in the ipsilateral Pt. However, this case also was unusual in that the optic nerve crush completely abolished prey-catching, but not OKN responses. At 3 months postcrush, conspicuous NMDAR1-LI fibers appeared in the ipsilateral Pt of one frog (Fig. 1A). These fibers were widely separated and closely followed the normal course of retinal axons in the marginal optic tract. In the same frog, NMDAR1-LI fibers were also detected in the ipsilateral corpus geniculatum. No NMDAR1-LI fibers were seen in any contralateral visual areas in the 3 month survival frogs. Neither normal nor sham-lesioned frogs showed NMDAR1-LI fibers in any of the retinorecipient areas.

At 5 months postcrush, all three frogs showed densely labeled NMDAR1-LI ipsilateral fibers in all thalamic and pretectal retinorecipient nuclei. These were especially prominent in the ipsilateral Pt, with the same appearance as observed in the 3 month survival case. These fibers followed the normal course of optic tract axons, did not branch, and often had a darkly staining apical enlargement (Fig. 1B). Again, no NMDAR1-LI fibers were observed contralaterally.

In the optic tectum, differences between contralateral and ipsilateral sides were observed earlier than in the thalamus and pretectum. As early as 1 month after optic-nerve crush, NMDAR1-LI was higher in ipsilateral tectal layers 8 and 9. Since in normal frogs these layers show weak NMDAR1-LI, whether this difference was due to a decrease in immuno-

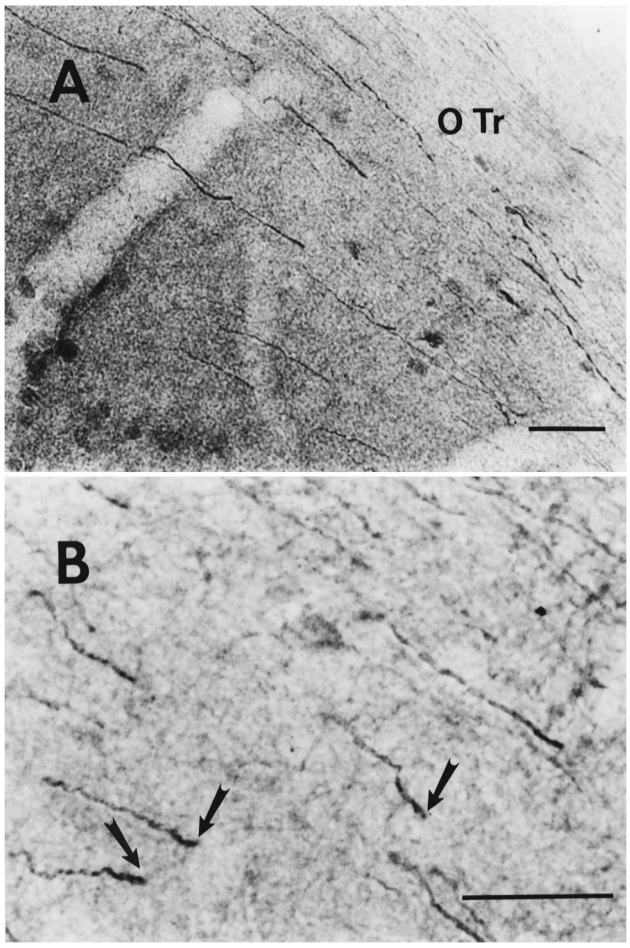

Fig. 1. NMDAR1-LI fibers in the ipsilateral pretectum (A) 3 and (B) 5 months after optic-nerve crush. Note apical enlargements (arrows). OTr, optic tract. Scale bar, 30 μm.

reactivity on the contralateral (deafferented) side or to an increase on the ipsilateral (intact) side was unclear. This contralateral-ipsilateral difference increased with longer postoperative survival times; and, at 5 months postcrush, ipsilateral tectal layers 8 and 9 were stained much stronger when compared with the contralateral side (Fig. 2). At that time, these ipsilateral tectal layers were stained more intensely than in normal frogs, with the strongest staining being restricted to the medial, posterior portion of the ipsilateral tectal lobe.

The consistent increase in NMDAR1-LI in the ipsilateral retinorecipient areas during optic-nerve regeneration was unexpected because, in normal frogs, most RGCs project contralaterally. However, these results are in general agreement with data from a variety of other studies. Chalmers and McCulloch [2] enucleated rats and found that NMDA-sensitive [^{3}H]glutamate binding was unaltered in the visually deprived hemisphere for up to 20 days after enucleation. On the other hand, Liu and Debski [11] reported recently that unilateral optic nerve transections in *Rana pipiens* decreased substance P (SP)-immunoreactivity on the ipsilateral side of the tectum 6 weeks after the operation, but did not affect SP levels on the contralateral side. They suggested that this change may involve pathways from the nuclei isthmi. Since NMDA receptors and SP can interact [6,8], the changes observed in Liu and Debski's experiments and in the present study may represent different aspects of such interaction.

The morphology of postcrush NMDAR1-LI fibers indicates that they are likely to be axons coursing towards their targets, and their enlarged tips may be growth cones. So far, presynaptic NMDA receptors have not yet been reported in frogs. Cline et al. [4] have argued that presynaptic NMDA receptors are unlikely in *Rana pipiens* RGCs, because NMDA binding did not decline until 3 days after enuclea-

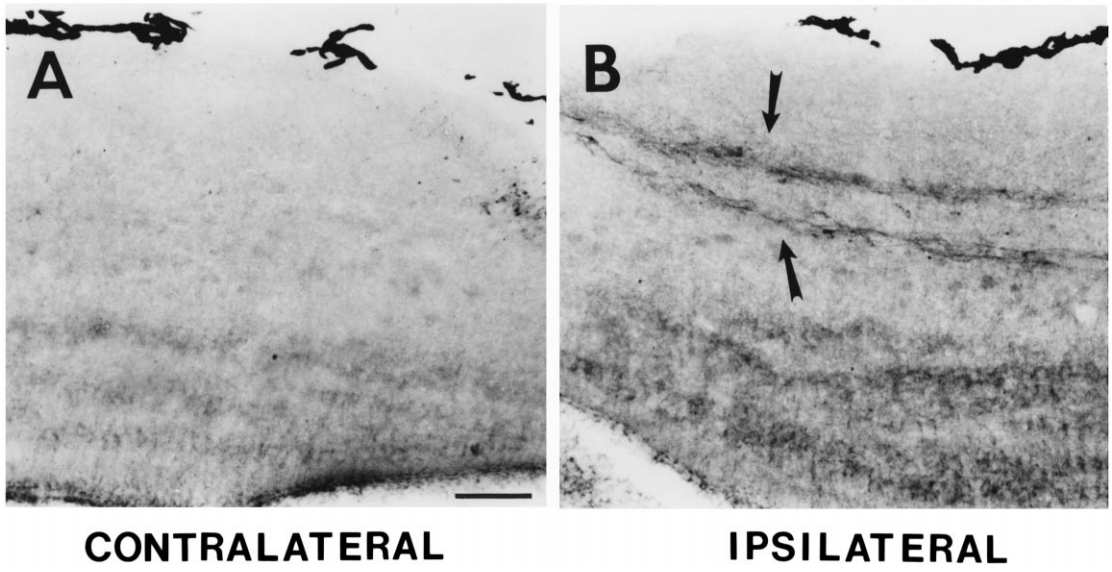

Fig. 2. Two of the retinorecipient layers (arrows) in the ipsilateral optic tectum show increased NMDAR1-LI 5 months after optic-nerve crush. (A) Contralateral tectum, (B) ipsilateral tectum of the same frog. Scale bar, 50 μm.

tion. They assumed that, after 3 days, RGC axons had degenerated, and the decrease in NMDA binding was due to loss of postsynaptic NMDA receptors. However, it has been shown that *Rana pipiens* RGC axons can survive for at least 2 months following enucleation [12].

During optic nerve regeneration, the ipsilateral RGC projection becomes abnormally strong in frogs [19], and the expression of presynaptic NMDA receptors may be involved in elimination of these misrouted RGCs. This hypothesis is consistent with the fact that injections of NMDA into the eye causes a significant loss of RGCs [16], and that a wave of RGC death generally occurs during optic-nerve regeneration [20]. Prevention of regeneration transiently delays the death of some RGCs [10], but RGC axons do not have to reach the tectum for RGC death to occur [9,20]. Therefore, the factor triggering death of RGCs may be expressed on the regenerating axons themselves. It is not clear, however, how the expression of NMDA receptors can be regulated by the route axons follow. It is conceivable that deafferentation may unilaterally alter the levels of a substance that modulates NMDA receptors. Since SP can potentiate NMDA responses [8], and SP-immunoreactivity decreases in the ipsilateral tectum after deafferentation [11], the decrease in SP-levels on the ipsilateral side might reduce the activity of presynaptic NMDA receptors and thus trigger their upregulation. Further research should help to clarify these questions.

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[1] Brose, N., Gasic, G.P., Vetter, D.E., Sullivan, J.M. and Heinemann, S.F., Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1, J. Biol. Chem., 268 (1993) 22663–22671.

[2] Chalmers, D.T. and McCulloch, J., Selective alterations in glutamate receptor subtypes after unilateral orbital enucleation, Brain Res., 540 (1991) 225–265.

[3] Cline, H.T., Debski, E. and Constantine-Paton, M., NMDA receptor antagonist desegregates eye specific stripes, Proc. Natl. Acad. Sci. USA, 84 (1987) 4342–4345.

[4] Cline, H.T., McDonald, J.W. and Constantine-Paton, M., Glutamate receptor binding in juvenile and adult *Rana pipiens* CNS, J. Neurobiol., 25 (1994) 488–502.

[5] Fite, K.V. and Hayden, D., The accessory optic system and temporal correlates of visuomotor orientation, Brain Behav. Evol., 27 (1985) 48–56.

[6] Hajji, M.D., Salin, P. and Kerkerianlegoff, L., Chronic dizocilpine maleate (MK-801) treatment supresses the effects of nigrostiatal dopamine deafferentation of enkephalin but not on substance P expression in the striatum, Eur. J. Neurosci., 8 (1996) 917–926.

[7] Hieber, V.C. and Goldman, D., Trans-synaptic regulation of NMDA receptor RNAs during optic nerve regeneration, J. Neurosci., 15 (1995) 5286–5296.

[8] Hornfeldt, C.S., Kitto, K.F. and Larson, A.A., Evidence that the NH_2-terminus of substance P modulates *N*-methyl-D-aspartate-induced activity by an action involving sigma receptors, Eur. J. Pharmacol, 306 (1996) 15–22.

[9] Humphrey, M.F., Effect of different optic nerve lesions on retinal ganglion cell death in the frog *Rana pipiens*, J. Comp. Neurol., 266 (1987) 209–219.

[10] Humphrey, M.F., Darby, J.E. and Beazley, L.D., Prevention of optic nerve regeneration in the frog *Hyla moorei* transiently delays the death of some ganglion cells, J. Comp. Neurol., 279 (1989) 187–198.

[11] Liu, Q. and Debski, E.A., Optic-nerve dependent changes in adult frog tectal cell phenotypes, J. Neurobiol., 29 (1996) 517–534.

[12] Matsumoto, D.E. and Scalia, F., Long-term survival of centrally projecting axons in the optic nerve of the frog following destruction of the retina, J. Comp. Neurol., 202 (1981) 135–155.

[13] McDonald, J.W., Cline, H.T., Constantine-Paton, M., Maragos, W.F., Johnston, M.V. and Young, A.B., Quantitative autoradiographic localization of NMDA, quisqualate and PCP receptors in the frog tectum, Brain Res., 482 (1989) 155–158.

[14] McNamara, R.K. and Routtenberg, A., NMDA receptor blockade prevents kainate induction of protein F1/GAP-43 mRNA in hippocampal granule cells and subsequent mossy fiber sprouting in the rat, Mol. Brain Res., 33 (1995) 22–28.

[15] Reh, T.A. and Constantine-Paton, M., Eye specific segregation requires neural activity in three-eyed *Rana pipiens*, J. Neurosci., 5 (1985) 1132–1143.

[16] Sabel, B.A., Sautter, J., Stoehr, T. and Siliprandi, R., A behavioral model of excitotoxicity: retinal degeneration, loss of vision, and subsequent recovery after intraocular NMDA administration in adult rats, Exp. Brain Res., 106 (1995) 93–105.

[17] Schaden, H., Stuermer, C.A.O. and Bahr, M., GAP-43 immunoreactivity and axon regeneration in retinal ganglion cells of the rat, J. Neurobiol., 25 (1994) 1570–1578.

[18] Schmidt, J.T., Long-term potentiation and activity-dependent retinotopic sharpening in the regenerating retinotectal projection of goldfish: common sensitive period and sensitivity to NMDA blockers, J. Neurosci., 10 (1990) 233–246.

[19] Stelzner, D.J., Bohn, R.C. and Strauss, J.A., Expansion of the ipsilateral retinal projection in the frog brain during optic nerve regeneration: sequence of reinnervation and retinotopic organization, J. Comp. Neurol., 201 (1981) 299–317.

[20] Stelzner, D.J. and Strauss, J.A., A quantitative analysis of frog optic nerve regeneration: is retrograde ganglion cell death or collateral axonal loss related to selective reinnervation?, J. Comp. Neurol., 245 (1986) 83–106.