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LENTIVIRAL VECTOR-MEDIATED GENE TRANSFER IN ADULT MOUSE PHOTORECEPTORS IS IMPAIRED BY THE PRESENCE OF A PHYSICAL BARRIER

THESE

préparée sous la supervision du Dr Yvan Arsenijevic, MER, PD

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RESUME

L'utilisation de la thérapie génique dans l'approche d'un traitement des maladies oculaires dégénératives, plus particulièrement de la rétinite pigmentaire, semble être très prometteuse (Acland *et al.* 2001). Parmi les vecteurs développés, les vecteurs lentiviraux (dérivé du virus humain HIV-1), permettent la transduction des photorécepteurs après injection sous-rétinienne chez la souris durant les premiers jours de vie. Cependant l'efficacité du transfert de gène est nettement plus limitée dans ce type cellulaire après injection chez l'adulte (Kostic *et al.* 2003). L'objet de notre étude est de déterminer si la présence d'une barrière physique produite au cours du développement, située entre les photorécepteurs et l'épithélium pigmentaire ainsi qu'entre les photorécepteurs eux-mêmes, est responsable de la diminution de l'entrée en masse du virus dans les photorécepteurs, minimisant ainsi son efficacité chez la souris adulte.

De précédentes recherches, chez le lapin, ont décrit la capacité d'enzymes spécifiques comme la Chondroïtinase ABC et la Neuraminidase X de modifier la structure de la matrice entourant les photorécepteurs (Inter Photoreceptor Matrix, IPM) par digestion de certains de ses constituants suite à leur injection dans l'espace sous-rétinien (Yao *et al.* 1990). Considérant l'IPM comme une barrière physique, capable de réduire l'efficacité de transduction des photorécepteurs chez la souris adulte, nous avons associé différentes enzymes simultanément à l'injection sous-rétinienne de vecteurs lentiviraux afin d'améliorer la transduction virale en fragilisant l'IPM, la rendant ainsi plus perméable à la diffusion du virus.

L'injection sous-rétinienne de Neuraminidase X et de Chondroïtinase ABC chez la souris induit des modifications structurales de l'IPM qui se manifestent respectivement par la révélation ou la disparition de sites de liaison de la peanut agglutinin sur les photorécepteurs. L'injection simultanée de Neuraminidase X avec le vecteur viral contenant le transgène thérapeutique augmente significativement le nombre de photorécepteurs transduits (environ cinq fois). Nous avons en fait démontré que le traitement enzymatique augmente principalement la diffusion du lentivirus dans l'espace situé entre l'épithélium pigmentaire et les photorécepteurs. Le traitement à la Chondroïtinase ABC n'entraîne quant à elle qu'une légère amélioration non significative de la transduction.

Cette étude montre qu'une meilleure connaissance de l'IPM ainsi que des substances capables de la modifier (enzymes, drogues etc.) pourrait aider à élaborer de nouvelles stratégies afin d'améliorer la distribution de vecteurs viraux dans la rétine adulte.

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BRIEF COMMUNICATION

Lentiviral vector-mediated gene transfer in adult mouse photoreceptors is impaired by the presence of a physical barrier

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Gene transfer offers a substantial promise for the therapy of degenerative ocular diseases. Lentiviral vectors have the ability to efficiently transduce murine photoreceptors during the first days of life, but they are poorly effective on photoreceptors during adulthood. Here, we studied whether a physical barrier was responsible for this impairment. Previous studies have described the capacity of enzymes, such as chondroitinase ABC and neuraminidase X, to modify the structure of the interphotoreceptor matrix (IPM) when subretinally injected. Considering the IPM as a physical barrier that may decrease photoreceptor transduction, we injected different enzymes into the subretinal space of the adult mouse simultaneously with the lentiviral vector preparation, to increase viral transduction by fragilizing the IPM. Subretinal injection of neuraminidase X and chondroitinase ABC induces modifications in the IPM by, respectively, revealing or decreasing peanut agglutinin sites on photoreceptors. The simultaneous subretinal injection of neuraminidase X with a lentiviral vector driving the expression of a reporter gene in the photoreceptors increases the number of transduced cells significantly (around five-fold). After the enzyme treatment, the diffusion of the vector between the pigmented epithelium and the photoreceptors appears to facilitate the lentiviral vector transduction. Such approach targeting the IPM may help to design new strategies to improve gene delivery into the adult photoreceptors.

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Retinitis pigmentosa, characterized by a loss of photoreceptors, appears to be a target of choice for gene therapy, because of the accessibility of the retina and because several types of retinitis pigmentosa are monogenic diseases in which a single mutation leads to a loss of function. The consecutive retinal degeneration is indeed often due to mutations of genes expressed in photoreceptor cells,[1] but also due to the dysfunction of the pigmented epithelium (RPE).[2] As several retinal degenerative diseases are a consequence of a genome alteration affecting specifically photoreceptors, it is of prime importance to efficiently and specifically target these cells. Several studies have shown that gene therapy is a promising tool to correct gene defects affecting the retina or to rescue retinal cells.[3–12] By transferring therapeutic genes into RPE cells with viral vectors, studies have also demonstrated vision recovery in large animal models suffering from retinitis pigmentosa, such as dogs.[6,10,13]

The lentiviral vector ability to transduce photoreceptors has been demonstrated,[3,4,14] but its therapeutic effect was documented only for postnatally-treated mice.[4] No data show whether a lentiviral vector can have a beneficial action on adult photoreceptors, whereas in adult-treated rats, retinal ganglion cells can be preserved by transgene delivery mediated by a lentiviral vector.[12] In the adult rat, transduction of the photoreceptors is confined to the injection site,[3] and in the mouse the percentage of transduced photoreceptors is low.[14] Moreover lentiviral transduction of photoreceptors seems more efficient in newborn and nonmature retinas than in adult mice after either intravitreous or subretinal injection.[3,4,14] Therefore, obstacles that prevent an efficient transduction of photoreceptors by the lentiviral vector may exist. One possibility is that the interphotoreceptor matrix (IPM), which occupies the space between the neural retina and the RPE, could play a role as a physical barrier. IPM is composed of domains associated with cone photoreceptors, termed 'cone matrix sheaths', composed of proteoglycan containing chondroïtin sulfate and oligosaccharides. They are interconnected with oligosaccharides and proteoglycans, which have terminal sialic acid residues associated with rod photoreceptors. The cone matrix sheaths form a structural and molecular bond between the photoreceptors and the RPE microvilli.[15]

Yao *et al*[16] have demonstrated that, in the rabbit, subretinal injections of specific enzymes (neuraminidase X and chondroitinase ABC) significantly decrease retina adherence to the RPE by changing the IPM structure. Indeed, the neuraminidase X removes the sialic acid residues, whereas the chondroitinase ABC digests proteoglycans. The use of the peanut agglutinin (PNA) binding affinity in the retina enabled the observation of certain properties of those enzymes. PNA binds with high affinity to galactose–galactosamin disaccharides present in the IPM domains associated with cones. For rods, sialyl residues mask these carbohydrate chains and prevent the binding of PNA. Neuraminidase X digests the syalic acid residues and so reveals the galactose–galactosamin disaccharides, allowing new accessible binding sites for the PNA. By contrast, chondroitinase ABC treatment of the retina leads to a decrease in PNA-binding site number by removing the proteoglycans.

In the present study, we have tested whether the diffusion of the lentiviral vector is impaired by a physical barrier and whether this barrier is determined by the presence of proteoglycans and sialic acid residues.

To evaluate the efficiency of gene transfer by lentiviral vectors into the photoreceptor layer of the adult mouse, we performed lentiviral vector injections into the vitreous and subretinal space in different groups of animals. When adult mice were injected intravitreously with a lentiviral vector encoding the GFP transgene under the control of the rhodopsin promoter (LV-Rho-GFP), none or rare cells expressed GFP 7 days after the injection as reported previously.[14] In contrast, the subretinal approach allowed to transduce more photoreceptors, but only in the vicinity of the injection site. Nonetheless, despite the fact that the subretinal injection improved photoreceptor transduction efficiency, only a low percentage (1–3%) of the total photoreceptor population at the site of injection expressed the transgene. We hypothesized that a physical barrier may prevent the viral vector diffusion, this barrier being between the photoreceptor and the RPE layers, as well as between photoreceptors themselves. Supporting this hypothesis, the best transduction efficiency was observed in areas where the photoreceptor layer was disturbed by the injection procedure, as was already observed in our precedent work.[14] Interestingly, when LV-PGK-LacZ, LV-EFs-GFP (previously shown to transduce RPE cells[14]), or LV-Ubi-GFP vectors were injected subretinally ($n=24$), a large portion of the RPE cells were positive for the transgene over a distance of 1.37 ± 0.15 mm, whereas rhodopsin-GFP-expressing cells were observed covering a length of 0.9 ± 0.19 mm ($n=9$), the total length of the retina being around 3.5 mm. These results suggest that the virus has a certain accessibility to the subretinal space, but has difficulty in targeting photoreceptors.

To determine whether a physical barrier prevents the subretinally injected vectors from reaching the photoreceptors in massive amounts, we investigated how proteoglycans maintain the rigidity of the outer nuclear layer (ONL) in the mouse by testing whether the co-injection of neuraminidase X or chondroitinase ABC with LV-Rho-GFP increases the lentiviral transduction of photoreceptors.

To monitor the changes in the photoreceptor cell layers, we used PNA coupled to a dye (Texas red) or to biotin (for revelation using DAB procedure). In the enzyme-untreated tissue, PNA binds preferentially to the terminal sugar of the cones (Figure 1a). However, the injection procedure itself has an impact on PNA binding to the outer segments (perhaps due to the detachment of the retina from the RPE) because injection of the vehicle

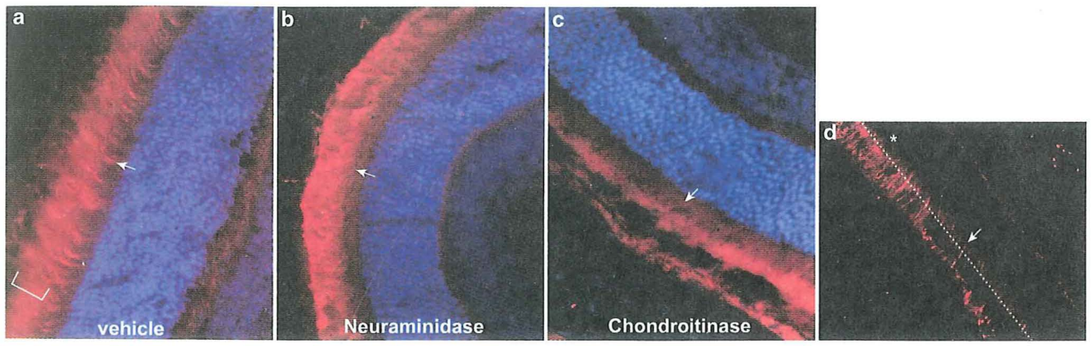

Figure 1 *PNA-binding sites after subretinal injection of neuraminidase X and chondroitinase ABC in mouse eyes. Groups of mice ($n=7$–9 per group) received subretinal injections of 1.5 µl of neuraminidase X (50 U/ml, Roche, Switzerland), or of chondroitinase ABC (0.2 U/ml, Seigakachu, Japan) or of vehicle (PBS containing 0.5% BSA), as described previously.[14] Mice were killed 1 or 3 days post-injection, their eyes fixed by immersion in 4% paraformaldehyde in PBS, cryoprotected in sucrose and cut in 15 µm slices with a cryostat. After blocking the nonspecific binding sites of eye sections, PNA labeling was performed overnight at 4°C using PNA coupled with Texas red (1:5000, Sigma) or with biotin (1:100, Sigma) and then washed three times with PBS. PNA affinity in the control eyes injected with the vehicle only is directed towards cone outer segments (a, arrow), as was described by Yao et al[16,21] in the rabbit. However, the most external part of these segments (a, brackets) shows an increase in PNA binding compared to uninjected eyes (not shown), which may be a consequence of the retinal detachment induced by the subretinal injection. At 1 day (b) or 3 days (not shown) after neuraminidase X injection, the PNA-binding affinity is modified and there is an increase in PNA binding in the outer segment layer (b, arrow) at the site of injection or in the vicinity of the injection site where the neural retina is still attached to the RPE. On the other hand, 1 day (c) or 3 days (not shown) after chondroitinase ABC injection, the PNA affinity for cone outer segments is lost at the injection site (c, arrow showing a remaining PNA-binding site), but only decreased in the neighborhood of the injection site (not shown). The boundary of the region where there are PNA-binding site modifications following chondroitinase ABC treatment is shown in (d). The arrow shows the typical PNA binding in the untreated region and the asterisk defines the region where the PNA labeling is decreased in the inner segment. The dotted line helps to separate the most external part of the outer segments, which shows an increase in PNA labeling independently of enzyme treatment (see a). In conclusion, the subretinal injection of neuraminidase X and chondroitinase ABC in the mouse retina alters the integrity of the IPM. (a–c) PNA labeling in red and Dapi staining in blue. (a–c) ×400 magnification; (d) ×200 magnification.*

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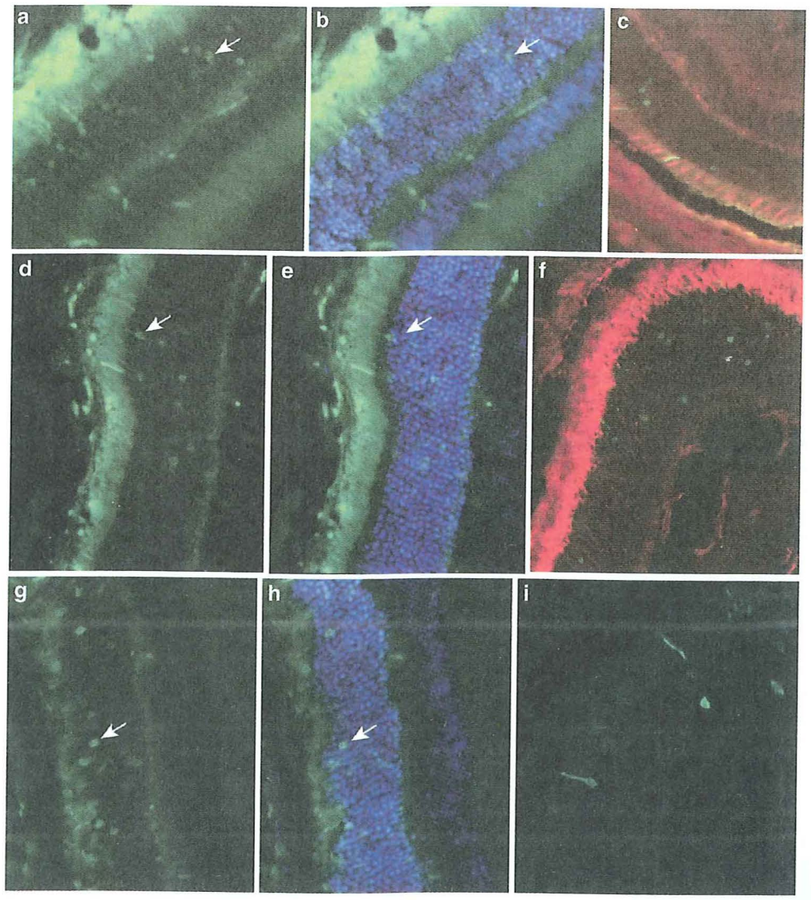

Figure 2 *Neuraminidase X and chondroitinase ABC co-injection with lentiviral vectors does not impair photoreceptor transduction. The Hlox-Rho-GFP (LV-Rho-GFP) vector construction, production and quantification were previously described in Kostic et al.[14] In the first group of animals (n = 9), we injected the equivalent of 150 ng of p24 per eye (1.5 µl), which was estimated previously[14] to correspond to around 2.6 × 10⁶ transduction units for this vector. In the two other groups, the same amount of LV-Rho-GFP was co-injected with the same amount of either neuraminidase X (n = 8) or chondroitinase ABC (n = 7) used in the preceding experiment (Figure 1) with the same injection volume per eye (1.5 µl). At 7 days post-injection of LV-Rho-GFP (a, b, c, i) or of a LV-Rho-GFP solution with neuraminidase X (d, e, f) or chondroitinase ABC (g, h), we observed cell transduction (GFP-positive cells, green) in the ONL (a, b, d, e, g, h arrow) at the injection site. (c) and (f) show co-labeling of PNA with GFP fluorescence in eyes injected, respectively, with LV-Rho-GFP only or LV-Rho-GFP+neuraminidase X. At 7 days after injection of neuraminidase X, the PNA-binding site pattern is not yet recovered. (i) shows that GFP-expressing cells have a photoreceptor morphology. Thus, co-injection of these enzymes does not hinder lentiviral transduction. Interestingly, LV-Rho-GFP co-injected with neuraminidase X appears to enhance the size of the region surrounding the injection site bearing GFP-positive cells. (a, d, i) GFP fluorescence; (b, e, h) GFP fluorescence in green and Dapi staining in blue. Magnification: (a, b, d, e, g, f, i) ×400; (c, f) ×200.*

only (Figure 1a) leads to an increase of labeling in the most external part of these segments in comparison to uninjected eyes (not shown). According to Yao *et al*,[16] subretinal injection of neuraminidase X in the rabbit leads to an increase in the number of PNA-binding sites, whereas chondroitinase ABC injection decreases the binding of PNA. We first injected the enzymes alone into the subretinal space of adult mice (neuraminidase X or chondroitinase ABC) and killed the animals 1 or 3 days post-injection. The eyes were cryosectioned, labeled with Texas red-linked PNA and analyzed with fluorescent microscopy or labeled with biotin-linked PNA and analyzed under direct light microscopy. Following neuraminidase X treatment, the PNA-binding sites available increased markedly. The labeling was observed throughout the outer segment layer (Figure 1b) and was mostly visible at the level of the inner segments of the photoreceptors (Figure 1b, arrow). The binding sites were so dense that it was impossible to count individual outer segment labeling. By contrast, after chondroitinase ABC injection, we observed a loss of PNA binding to the cone matrix sheaths, mostly to the inner segments, at the injection site (Figure 1c). The number of PNA-labeled cones was significantly decreased at the injection site (Figure 1c) in comparison to regions far away from the injection site (Figure 1d).

The present results show that neuraminidase X and chondroitinase ABC lead to changes in the IPM structure

of mouse retina, as was observed in the rabbit[16] (1992). We then tested whether subretinally injected enzymes lead to IPM changes that could facilitate lentiviral transduction of photoreceptors. LV-Rho-GFP and either neuraminidase X or chondroitinase ABC were mixed and injected into the adult mouse subretinal space. The mice were killed 7 days post-injection and the number of transduced photoreceptors was analyzed under fluorescent microscopy to detect GFP-expressing cells. Importantly, we observed that both neuraminidase X and chondroitinase ABC did not interfere with lentiviral vector infection, showing that this enzyme has no inhibitory effect on the use of this type of viral vector (Figure 2). Moreover, neuraminidase X significantly improved photoreceptor transduction. It increased by about five-fold the number of GFP-positive photoreceptors (Figure 3a). Chondroitinase ABC had a tendency to improve photoreceptor transduction (Figure 3a). However, this effect was not significant. Thus, it appears that the disruption of the IPM by specific enzymes leads to the improvement of lentiviral diffusion and infection. These experiments also reveal that the sialic acid residue is an essential component for the integrity of the interconnections of the outer segments and their adhesion to RPE.

Neuraminidase X has two different effects on the rabbit retina. It increases its detachment from the RPE and modifies the IPM structure.[16] To determine the extent of neuraminidase X action, we compared the number of sections per eye bearing GFP-positive photoreceptors with and without simultaneous injection of neuraminidase X (Figure 3b). This number reflects the diffusion of the virus under the subretinal space. When neuraminidase X and LV-Rho-GFP were co-injected, the number of GFP-positive photoreceptors increased in correlation with the number of GFP-positive sections (Figure 3). The enzyme increased the retinal detachment by forming a bleb which spread over the ONL evenly, separating the retina from the RPE. The density of GFP-positive photoreceptors was not increased at a precise site of the retina: there is no statistically significant difference between the eyes injected with LV-Rho-GFP only, which have on average 2.5% of the photoreceptors at the region of the ONL bearing the GFP signal that was GFP-positive (67 GFP-positive cells out of 2860 photoreceptor cells), and the eyes treated with LV-Rho-GFP+ neuraminidase X which have 4% of GFP-positive cells in similar regions (three microscopic fields containing GFP-positive cells for four eyes were counted: 305 GFP-positive cells over 7660 photoreceptors). Nevertheless, the total surface of transduction increased under the co-incubation with the enzyme (Figure 3c). This shows that neuraminidase X improves lentiviral vector transduction in the subretinal plan.

Previous studies have demonstrated that the integration of a therapeutic transgene into a degenerating retina can delay its degeneration and apoptosis of its cells.[4–7,9–12] Use of specific promoters enables the transgene to target a precise cell type, thus avoiding its expression in the neighboring different cell phenotypes.[4,14,17] To observe the transduction of photoreceptors by lentiviral vectors, we used the promoter controlling the rhodopsin gene.[14,18] Our and other groups have shown that the lentiviral vector is poorly efficient to transduce photoreceptors in the adult rodent retina,[14,19] whereas photoreceptors of young animals are more easily transduced.[4,14] This difference could be due to several parameters. In particular, a change in gene expression might occur after the full development of the retina. In the present case, the rhodopsin promoter is active all along adult life and the rhodopsin gene is expressed already at birth. The change of expression of a vector inserted in the genome can be due to a change of chromatin folding, but such a change rarely occurs in post-mitotic adult cells. This suggests that the reduced lentiviral transduction in the adult photoreceptor is probably due to a decrease of virus accessibility to its target. In the adult eye, we can envisage that RPE cells have a greater activity in comparison to the newborn eye

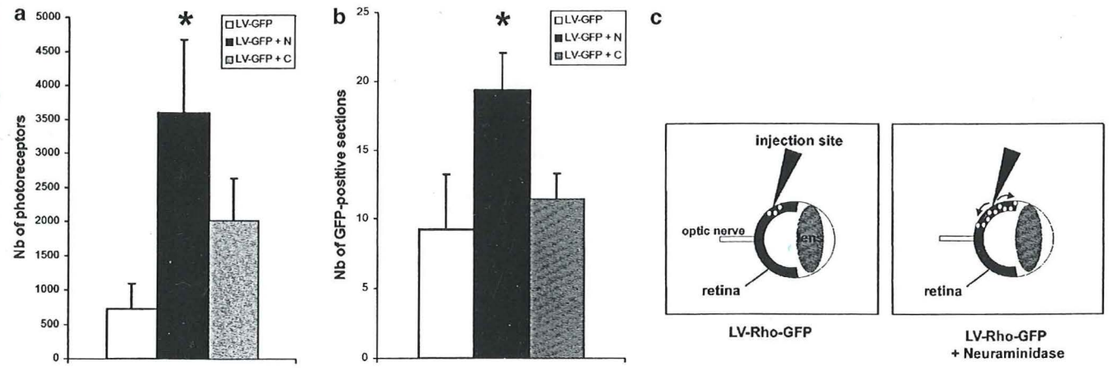

Figure 3 *Neuraminidase X treatment of mouse subretinal space significantly increases photoreceptor transduction by LV-Rho-GFP. (a) Neuraminidase X treatment (n = 8) significantly increases the number of photoreceptors transduced by LV-Rho-GFP ($P < 0.05$), whereas chondroitinase ABC (n = 7) has only a tendency to increase photoreceptor transduction. (b) We counted the number of GFP-positive sections per eye to evaluate the diffusion of the lentiviral transduction. Statistical analysis reveals that neuraminidase X treatment increases the diffusion of the lentiviral vectors in the subretinal space (in comparison to LV-Rho-GFP only injection, $P < 0.05$) in correlation with the increase of the number of transduced photoreceptors. (c) Diagram showing that the increase of GFP-positive photoreceptor number using neuraminidase X treatment is due to an increase in the diffusion of the lentiviral vectors from the injection site into the subretinal plan and not due to an increase of transduction rate at the injection site. LV-GFP: lentivirus Rho-GFP injection, LV-GFP+N: lentivirus Rho-GFP and neuraminidase X injection, LV-GFP+C: lentivirus Rho-GFP and chondroitinase ABC injection, *$P < 0.05$ versus LV-GFP.*

and that the majority of the viral vectors injected subretinally is phagocyted by the RPE cells. The fact that adult RPE cells are well transduced in a large area shows that the lentiviral vector can in part disperse in the subretinal space. Thus, a certain amount of vector should be available also for photoreceptors. The mean coverage of RPE transduction by vectors targeting the expression in the RPE is about 1.37 ± 0.15 mm in length, whereas photoreceptor transduction occurred on only 0.9 ± 0.19 mm. Moreover, the photoreceptors were transduced only sparsely and not side by side as observed in the RPE, showing that the ONL is reached with difficulty by the lentiviral vector. However, the neuraminidase X treatment increased the number of transduced photoreceptors (five-fold) and the distance to which cells were infected (1.7 mm). These results clearly show that a physical barrier impairs lentiviral diffusion into the subretinal space and the ONL when the retina is intact. The removal of the sialyl residues by neuraminidase X allows to disrupt the cell attachment between photoreceptor outer segments, as revealed by PNA staining. This release of photoreceptor connection allows the lentiviral vector to penetrate the ONL, but not evenly in all photoreceptor layers. Digestion of proteoglycans by chondroitinase ABC was less effective, probably because it mainly disrupts the matrix secreted by the cones, which represent only 3% of the photoreceptor population and thus contribute only weakly to the physical barrier that the IPM seems to represent for viral particles. This demonstrates that neuraminidase X has a major action on adherence between photoreceptors and reveals that lentiviral vector diffusion into the adult retina, and more specifically into the ONL, is impaired by components of the IPM. As neuraminidase X has no effect in deeper layers of photoreceptors, our results suggest that other components of the IPM or cellular structures like glial extension forming the outer limiting membrane[20] may prevent the lentiviral vector to diffuse between photoreceptors.

These observations have important consequences on the potential use of the lentiviral vector to replace genes in the adult photoreceptors. Indeed, Yao *et al*[21] showed that these retinal enzymatic treatments do not impair the functional retinal recovery of rabbit retina after the surgical act. Similarly, we observed no differences in ERG recordings between mouse eyes injected with lentiviral vector only or in the presence of such enzymes (Figure 4), which render this approach compatible with potential gene transfer therapies. However, even with the partial digestion of the IPM, the efficiency of photoreceptor transduction remains quite low. In this case, only around 4% of the photoreceptors are transduced in the regions reached by the vector, hiding a potential effective benefit due to transgene integration. Nonetheless, our experiments show that manipulation of the IPM barrier may improve lentiviral vector cell transduction and that the prospect of other enzymes or substance targeting the IPM should help to enhance lentiviral vector-mediated gene transfer into photoreceptors. So, identifying more precisely the constituents of the IPM and the structure of the ONL may give new strategies for increasing lentiviral vector-mediated gene transfer into photoreceptors.

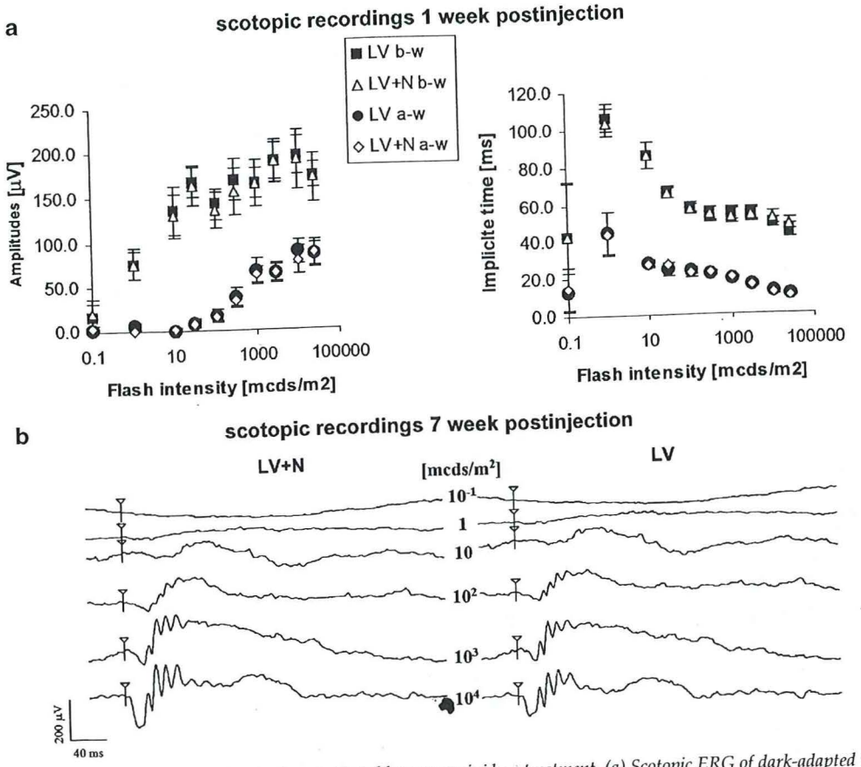

Figure 4 Retinal function of subretinally injected mice is not affected by neuraminidase treatment. (a) Scotopic ERG of dark-adapted mice (n = 4) injected in one eye with LV and in the contralateral eye with LV and neuraminidase X was measured 1 week post-injection using single flashes of increasing intensities (from 10^{-4} to 25 cds/m²). The amplitudes and the implicit times of a- and b-wave responses are reported in the two upper graphs showing the similarity of the responses between both groups (LV or LV+N). Similar responses between both groups were also obtained for scotopic flicker responses using repeated 3 cds/m² flashes of increasing frequencies (from 0.5 to 30 Hz) or for photopic responses (data not shown). (b) Representative scotopic single-flash responses 7 weeks post-injection of a mouse injected in one eye with LV only and in the contralateral eye with LV+N. LV: lentivirus Rho-GFP injection. LV+N: lentivirus Rho-GFP and neuraminidase X injection.

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