ES cell derived neural progenitors improves visual functions in retinal ganglion cells - depleted mouse models

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Abstract

Background: Glaucoma is a chronic neurodegenerative disease, characterized by progressive, permanent visual loss resulting from irreversible retinal ganglion cells (RGC) degeneration. Replacement of degenerated RGCs is a promising strategy to restore visual function. We previously demonstrated FGF2 induced differentiation of mouse embryonic stem cells (ESC) to RGC lineage, capable of retinal Ganglion Cell Layer (GCL) integration upon transplantation in mice. Here, we evaluated possible improvement of visual function by transplantation of ES cell derived neural progenitors in RGC depleted glaucoma mice models.

Methods: ESC derived neural progenitors (ES-NP) was transplanted into NMDA (N-Methyl-D-Aspartate) injected, RGC-ablated mouse models and a pre-clinical glaucoma mouse model (DBA/2J) having sustained higher intra ocular pressure (IOP). Visual acuity and functional integration was evaluated by behavioural experiments and immunohistochemistry, respectively.

Results: Transplantation of GFP expressing ES-NPs in NMDA injected RGC depleted mice, differentiated to RGC lineage and got integrated into GCL. An improvement in visual acuity was observed after two months of transplantation, when compared to the pre-transplantation values. Expression of c-Fos in the transplanted cells, upon light induction, further suggests functional integration into the host retinal circuitry. However, transplantation in DBA/2J mouse showed no significant improvement in visual functions, due to the cell death by inherent high IOP.

Conclusions: We showed that, transplantation of ES-NPs into the retina of RGC-ablated mouse models could survive, differentiate to RGC lineage, and functionally integrate into GCL to improve visual function. However, for the survival of transplanted cells in glaucoma, strategies to control the IOP are warranted.

Keywords: Embryonic Stem cell derived Neural Progenitors, Retinal Ganglion Cells, Glaucoma, Transplantation, Functional integration

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Introduction

Glaucoma is a chronic neurodegenerative disease, characterized by progressive, permanent visual loss resulting from the degeneration of retinal ganglion cells (RGCs) [1]. An increased intra ocular pressure (IOP) resulting from the disrupted dynamics of aqueous humor flow is mainly attributed to the progressive damage of RGC and their axons [2]. Current treatment strategies focus mainly on pharmacological and surgical interventions to control the elevated IOP, which is the prime risk factor for glaucoma [3]. However, the irreversible vision loss as well as RGC degeneration often persists, even when IOP is controlled.

Thus selective replacement of degenerated RGCs to restore lost visual function forms an alternative strategy to regenerate the RGC layer [4]. However, vision restoration at translational level in clinics has been hindered by several challenges; such as source of the stem cells, differentiation into appropriate cell type, transplantation approaches, survival and functional integration of cells into the target retina [5]. Additionally, to establish functional neuronal connectivity, optimal microenvironment mediated by the trophic factors is crucial [6]. Moreover, survival of the transplanted cells also depends on the glaucomatous retinal microenvironment. Although several studies in animal models and isolated clinical trials for cell transplantations have been reported in ocular neurodegenerative diseases [4, 7, 8 9], results were not substantial to be translated as a therapeutic measure in clinics, owing to the safety concerns, elusive molecular mechanism in establishing the functional connectivity and survival of transplanted neurons [8]. Despite these advances less progress has been made in cell replacement strategies pertaining to RGC loss.

We have previously demonstrated the potential of embryonic stem (ES) cells to differentiate along RGC lineage induced by growth factors, which were capable of integrating into the Ganglion Cell Layer (GCL) of the retina upon transplantation [10]. In the current study, we evaluated restoration of visual function by transplantation of ES cell derived neural progenitors in (i) chemically-induced glaucoma model generated by intravitreal injection of NMDA in C57BL/6 mice and (ii) a pre-clinical model for glaucoma (DBA/2J mice), characterized by high IOP. From the visual behavioural and functional connectivity experiments in NMDA injected RGC-depleted mouse models; we showed that transplanted cells could survive, differentiate to RGC lineage, and integrate into GCL resulting in improved visual function. However, the DBA/2J animals did not show any significant improvement in visual acuity behaviour, owing to the degeneration of transplanted cells, resulting from inherent high IOP in the genetic model.

Methods

Animals

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal experiments were approved and carried out according to the Johns Hopkins University, Animal Care and Use Committee (No: MO16A212), NIH guidelines, and Institutional Animal Ethics Committee (No: IAEC/215/JAC/2013) of Rajiv Gandhi Center for Biotechnology. All mice were housed in standard animal facility cages with access to food and water *ad libitum* with 12h light/dark cycle.

Generation of NMDA injected glaucoma model

C57BL/6 mice strain aged 6-8 weeks was used for generating RGC ablated model by injecting NMDA intravitreally. Animals (n = 20) were anesthetized with 0.3 - 0.5ml of Avertin (20mg/mL) and 2µl of 100µM NMDA was injected using graded glass micropipettes with a fine tip aperture into both eyes. The mice were analyzed for visual function behavioral tests (n =10), immunohistochemical analysis for RGC loss (n = 5), and anterograde tracing of RGC axonal projections (n =5) after 1 week, in comparison to the non-injection controls (n =10).

DBA/2J glaucoma model

The DBA/2J mice, pre-clinical model of glaucoma (8 months old, n = 6) exhibiting elevated IOP resulting from point mutations in two genes, *Gpnmb* and *Tyrp1* [11, 12] was also used for transplantation studies. DBA/2J-*Gpnmb*$^{+}$ genetic control mice strain (8 months old, n =3) with normal IOP was used as corresponding experimental control (Both mice strains were a kind gift from Nick Marsh Armstrong's Laboratory, Johns Hopkins University, School of Medicine, Baltimore, MD).

Transplantation

We used stable GFP expressing CE3 ES cells, (ATCC SCRC-1039) for our differentiation and transplantation experiments. The transplantation experiments were performed as per our previous report [10]. Briefly embryoid body (EB) induced from CE3 ES cells were partially differentiated on poly-D-Lysine (150 µg/ml) and Laminin (1 µg/ml) substrate for 2 days in neuron differentiation medium [DMEM/F12 supplemented with 1% N2 supplement (Invitrogen), 0.5% FBS, Heparin (2µg/ml) and FGF2 (10 ng/ml) (Chemicon)]. The partially differentiated EBs were trypsinized and plated on to uncoated

plates in medium consisting of DMEM/F12 supplemented with 1% N2 supplement, Heparin (2 µg/ml) and FGF2 (10 ng/ml) for 6 days to generate ES cell derived neural progenitor (ES-NP) cells. These ES-NPs were dissociated into single cell suspension (1×10^6 cells/µl in 1X PBS and 10 ng/ml FGF2) and were used for transplantation experiments. Approximately ~1million (ES-NP) cells were transplanted intravitreally in NMDA injected-RGC depleted mice models (n = 10) and DBA/2J mice, pre-clinical glaucoma mice model (n = 6). The behavior experiments for visual function were tested after two months of transplantation and were compared with prior to that of transplantation. Further the transplanted animals were sacrificed for immunohistochemical characterization of retina.

Behavioral Analysis

Optokinetic tracking experiments

We used an optokinetic tracking system (OptoMotry, Cerebral Mechanics Inc.) to evaluate the visual acuity by measuring the image-tracking reflex of mice [13]. Visual stimuli are projected on to the monitors so that a virtual cylinder with rotating gratings (sine wave grating) is produced. The mouse was allowed to track the grating and the tracking response was recorded. All acuity thresholds were determined using the staircase method with 100% contrast [14].

Light Avoidance Experiment

The innate aversion of mice to the brightly illuminated area was utilized here to evaluate the visual responses. The light avoidance test was conducted using a light-dark box consisting two equal sized compartments (20cm × 20cm). The light compartment was kept eliminated at a luminosity of 600 lux. An opening (7.5 × 7.5 cm) was located in the wall between the two chambers allowing free access between the light and dark compartments. The mice was placed in the light chamber to explore the box for five minutes and their movement across the chambers was tracked by computerized video tracking system (Anymaze, Stoelting, USA). The fraction of time spent by the mice in the open illuminated space is noted for calculating the percentage of time spent in light zone.

Anterograde tracing of RGC axonal projections in NMDA model

NMDA injected mice (n = 5) and non-injection controls (n = 5) were anesthetized as mentioned previously and 2µl of cholera toxin B subunit conjugated with Alexa Fluor 488 (CTB-488, Invitrogen, CA) was injected intravitreally to track the RGC axonal projections.

Three to four days after injection, the retina and brain were fixed by perfusing with 4% Paraformaldehyde (PFA). The brain samples were dissected and coronal sections were taken for further analysis. RGC axonal targets in the brain such as Suprachiasmatic Nuclei (SCN), Lateral Geniculate Nuclei (LGN) and Superior Colliculus (SC) were analyzed for RGC axonal projection innervations. The levels of fluorescence of maximum RGC projections in brain regions and total fluorescence levels were measured with Image-J software (http://rsbweb.nih.gov/ij/) as described previously [15].

Whole mount Retina Immunostaining

To evaluate the RGC loss, NMDA injected animals (n = 5) and non-injection control animals (n = 5) were sacrificed and the eyes were fixed in 4% PFA for 30 minutes followed by dissection of retina. The retina was again fixed in 4% PFA for one hour followed by blocking in 0.6% blocking solution. The whole mount retina was incubated with rabbit anti-Brn3a primary antibody (1:500, Chemicon) at 4°C for 2-3 days followed by washing with 1X PBS three times for 30 minutes each. Goat anti-Rabbit IgG (H+L), Alexa Fluor 594 (1:400, Molecular Probes) was used as secondary antibody and incubated for two days at 4°C.

In order to assess functional integration of transplanted cells, we analyzed the expression of an immediate early gene c-Fos in transplanted NMDA injected mice housed in normal 12:12 light-dark cycle. Mice were given a light pulse of 500 lux intensity for 30 minutes after which mice were placed back in dark for 90 minutes. Animals were subsequently sacrificed, enucleated and whole mount retinal preparation were made as described earlier. The whole mount retinas were then incubated with rabbit anti-c-Fos antibody (1:10,000, Calbiochem) at 4°C for 2 days followed by secondary staining with Goat anti-Rabbit IgG (H+L), Alexa Fluor 594 (1:400, Molecular Probes). To confirm the RGC lineage of transplanted cells, immunostaining with the RGC marker, Calretinin [16, 17] (Rabbit anti-Calretinin, 1:500, Chemicon) was done. The sections were mounted on microscopic slides in mounting medium (Vectashield, Burlingame, CA) with DAPI for nuclear staining. The slides were viewed and imaged in an upright fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

All data were obtained by at least three independent experiments and represented as Mean ± SD. Statistical significance between two groups was calculated by unpaired *t-tests*

assuming two-tailed distribution and equal variances. *P* value <0.05 was considered as statistically significant.

Results

Visual acuity of the NMDA injected mice is significantly reduced along with RGC loss

The RGC ablated mice model was generated by intravitreal injection of NMDA (100µM) to induce excitotoxic damage to RGCs. After one week of NMDA injection, there was ~50% reduction in the number of Brn3a positive RGCs in the whole mount retina (33.85 ± 4.71) when compared to control animals with no NMDA injection (57.74 ± 4.66, p<0.001, **Fig.1 A-F&G**). In order to understand the possibility of whether the NMDA injected retina could regenerate the lost RGC's induced due to excitotoxicity, we also analysed the retina after 40 days of NMDA injection and observed that the loss is irreversible **(Fig. S1A, B)**. Visual acuity of the NMDA injected mice was tested by its ability to track black and white gratings on a virtual rotating cylinder using an optokinetic tracking system (Fig.1 H). Our results showed that there was a significant reduction in visual acuity (cycles/degree) of NMDA injected animals (0.209 ± 0.053, p < 0.001, **Fig.1J)**, compared to the pre-injection controls 0.427 ± 0.08. A light avoidance test **(Fig.1 I)** also supported the above observation, where the pre-injection controls avoided bright light and spent only ~17% of the time in light zone. The NMDA injected animals showed comparatively less light avoidance behaviour and spent ~43%, (p < 0.001) of time in the light zone, **(Fig.1 K)**. Similar results were also observed after 40 days of NMDA injection indicating that the animals have lost visual functions permanently with no recovery **(Fig. S1C)**. These results clearly suggested that ablation of RGCs by NMDA injection, leads to impairment in visual functions.

We further investigated RGC innervations of brain targets, both in image forming visual function centres (LGN and SC) and non-image forming visual function centres (SCN). Anterograde tracing experiments with intravitreal injection of Cholera toxin B-488 was performed to track the RGC axonal projections. RGC innervations into the dorsal LGN of NMDA injected animals (218.00 ± 7.86) was significantly reduced compared to that of control animals (682.00 ± 42.41, p < 0.001, **Fig.2 A-D&E**). Similarly, SC also had significantly less RGC innervations in NMDA injected animals (274.24 ± 97.32) compared to that of controls (2502.85 ± 6.77, p < 0.001, **Fig.2 F-I&J**). However, the RGC innervations to the SCN, the brain region responsible for regulating the circadian rhythm were intact (Control = 634.47 ± 53.53, NMDA=404.28 ± 80.65, p < 0.21, **Fig.2 K-N&O**. These observations were in agreement with our results where the NMDA injected animals showed drastically reduced

visual acuity which might be due to the lesser RGC innervation in the image forming centres of the brain as of the RGC loss. The intact innervation in the SCN was in agreement with the reports that the NMDA injection does not affect ipRGCs [18]. We thus used animals, after 7 days of NMDA injection, as RGC ablated model for the transplantation studies.

ES-NPs transplantation in RGC depleted mice improved visual function

We have previously reported that FGF2-induced ES-NPs were able to differentiate along RGC lineage evidenced by the expression of RGC regulators and markers [10] **(Fig. S2 A-N)**. Further we have also shown that ES-NPs, after exposure to FGF2, were capable of integrating and differentiating into RGCs in vivo upon transplantation [10]. In the present study we adopted the same strategy. The FGF2 treated, differentiating ES-NPs (GFP expressing) were transplanted intravitreally into the NMDA injected-RGC depleted mice model. The animals were subjected to behavior tests two months after transplantation. Significant improvement in visual acuity was observed in transplanted animals as evidenced from optokinetic response, compared to NMDA injected animals, before transplantation (Transplanted = 0.233 ± 0.07, NMDA injected, prior to transplantation = 0.138 ± 0.07, $p < 0.05$ **Fig.3 K**). Though, we observed an improved visual function in NMDA model, we could not observe integration of transplanted cells into the optic nerve.

In the light avoidance test, animals after transplantation spent less time (90.7 ± 24.8 s, 30%) in the light zone compared to those prior to transplantation (129.7 ± 22.2 s, 43%), indicating a significant improvement ($p < 0.05$) in light responsiveness. However, the non-injected control animals spent only 17% (51.5 ± 23.2 s) of the time in light zone, exhibiting highest light avoidance behavior **(Fig.3 L)**. Thus the results indicate ES-NPs transplantation has shown improvement in visual behavioral tests suggestive of partial restoration of vision.

To confirm the integration of the transplanted cells into the GCL of host retina, the animals were sacrificed after behavioral analysis and whole mount retinal staining was done. Extensive integration of GFP expressing cells throughout the surface of retina with distinct RGC morphology was observed **(Fig.3 A-C)**. The RGC lineage was also confirmed by immunostaining with the RGC marker, calretinin **(Fig.3 D-G)**. Retinal sections also showed extensive integration of GFP expressing cells in the RGC specific GCL **(Fig.3 H-J)**.

To test whether the transplanted cells form functional neural circuits with the photoreceptor expressing cells, expression of c-Fos, an immediate early gene, was probed in the transplanted cells, in response to light induction. The transplanted GFP-expressing cells

showed c-Fos expression, suggestive of getting activated by the light stimulus **(Fig.3 M-P)**. Thus the results are suggestive of transplanted cells capable of being functionally integrated into the host retinal circuitry by forming connections with the photoreceptor expressing host cells. Although the number of cells co-expressing c-Fos was less in number, this was a very promising result, which substantiates our results of improved visual acuity evidenced from behavioural experiments **(Fig.3 K, L)**.

ES-NPs transplantation in DBA/2J mice did not show significant improvement in visual functions

We further extended our transplantation protocol to the pre-clinical glaucoma model, DBA/2J mice of 8 months of age. The DBA/2J mice had deficits in visual functions and could hardly track the gratings in optokinetic tracking system when compared to the corresponding DBA/2J-*Gpnmb*$^+$ genetic control (DBA/2J-*Gpnmb*$^+$ genetic control = 0.276 ± 0.03, DBA/2J = 0.137 ± 0.02, $p < 0.001$ **Fig.4 A**). Further DBA/2J mice also showed less light avoidance behaviour compared to the DBA/2J-*Gpnmb*$^+$ genetic control animals confirming their visual deficits (Control 45.57 ± 11.64 s, 15%, DBA/2J 174.08 ± 23.32 s, 58 %, $p < 0.001$, **Fig.4 B**).

FGF2-induced GFP-expressing ES-NPs was transplanted into the visually challenged DBA/2J animal. Visual functions by behavioural experiments were tested after two months of transplantation. As evidenced from optokinetic response and light avoidance behaviour, visual acuity in DBA/2J animals did not improve after transplantation **(Fig.4 A, B)**. Moreover DBA/2J mice showed highly disorganized GCL with very low number of RGCs which are dispersed irregularly throughout **(Fig.4 E)** compared to that of controls **(Fig.4 C)**. We speculate that the elevated IOP inherent to the DBA/2J mice might induce oxidative stress [19] on the transplanted cells, preventing them from proper differentiation and innervation, limiting the improvement in visual acuity. Therefore, by comparing the NMDA and DBA/2J models, it appears that control of IOP is much more crucial for the transplanted cells to integrate efficiently for functional recovery.

Discussion

Large number of studies has been reported, where photoreceptor transplantation in appropriate disease models has been done with partial restoration of vision[4, 20-23]. However, data is limiting with RGC transplantation in glaucoma models. Here, we have evaluated the functional visual restoration upon transplantation of ES-NPs in two glaucoma animal models; NMDA-induced RGC-depleted model and DBA/2J mice, a standard pre-clinical genetic glaucoma model [24].

The transplantation results in the NMDA model demonstrated intact integration of ES cell-derived RGC like cells into GCL layer with proper RGC morphology and RGC marker expression. Further co-expression of c-Fos with GFP-expressing cells in transplanted animals upon light induction clearly demonstrated an improved functional integration into the GCL. In addition, behaviour experiments to evaluate visual acuity also augmented the functional vision restoration, though partial, in the transplanted animals. Similar observations have been reported previously, which showed an improved retinal scotopic wave function in NMDA-injected rats after transplantation of human Müller glial derived RGCs [25]. Improvement of visual functions evidenced by electroretinogram (ERG) and visual evoked potential (VEP) following the transplantation of retinal progenitor cells in rat models of retinal degeneration was also reported recently[26, 27].

Nevertheless, in our study integration of transplanted cells into optic nerve was not observed, which was in line with reports where transplanted cells were seen to extend processes into the RGC layer, but not towards the optic nerve [25, 28]. We speculate that the transplanted cells integrated into the GCL layer may establish local interneuron connectivity and also secrete neurotrophic factors exerting a protective effect on optic nerve preventing the further neuronal loss and, thus resulting in improved visual functions [25, 29]. Moreover, NMDA-induced model showed only ~50% RGC depletion which suggested possible contribution of the transplanted cells in survival of remaining RGCs in the retina and their axons providing a repairing effect on optic nerve that innervate the visual centres in the brain. However, present study was limited by the neuronal tracking to show the restored neuro-anatomy in the visual centres in brain. Further electrophysiological evidences were also lacking to support the recovery, but the improved visual functions evidenced from behavioural studies along with the observations from c-Fos expression results clearly showed functional integration of the transplanted cells proving intact functional anatomy. Though this integration was promising the number of c-Fos co-expressing cells was less, which could be

attributed to the lesser RGC yield after FGF2 induced differentiation [10]. Integration of transplanted primary mice RGCs in uninjured rat retina was recently reported with axons extending to visual centres in brain with electrical excitability and light response [30]. This integration might have been benefitted from the factors derived from donor RGCs as it was isolated from developing retina [28, 31]. Therefore future studies are warranted for precise RGC fate acquisition from pluripotent stem cell source with good yield for testing long range arborisation to visual centres after transplantation.

Meanwhile, the DBA/2J animals did not show any significant improvement in vision after transplantation. This could be attributed to the inherent high IOP in DBA/2J animals resulting in the degeneration of the transplanted cells owing to the oxidative stress [19]. Since the GCL of retina is highly dispersed in these mice model, it can be speculated that the transplanted cells were not able to integrate. Thus the dispersed nature of the GCL may limit the scaffolding support to the transplanted cells. Moreover, the transplantation was done at the age of 8 months, where RGC degeneration occur maximum because of high IOP implying the requirement of pharmacological IOP alleviation for effective transplantation. However, our study was limited by transplantation experiments in DBA/2J controlled for IOP. Therefore, for the survival of transplanted cells in glaucoma, strategies to control the IOP are warranted.

Although, there was a recovery of visual function in the NMDA model, it can be considered as the recovery from the pathological state of glaucoma by replenishment of cells. Since elevated IOP forms a critical characteristic feature of glaucoma and the NMDA model represents only a static glaucomatous stage of degenerated RGC population. Thus, the ocular disease phenotype modelled in the animal plays a significant role in evaluating the efficacy of the cell transplantation. Altogether, our results showed; the prospect of cell replacement therapy in glaucomatous condition, requirement of appropriate animal models mimicking the pathological human conditions, and advanced transplantation strategies to promote the survival of the transplanted cells.

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List of abbreviations

RGCs: Retinal Ganglion Cells; ES cell: Embryonic Stem Cells; ES-NP: Embryonic stem cell derived Neural Progenitors; IOP: Intra Ocular Pressure; NMDA: N-Methyl-D-Aspartate; GCL: Ganglion Cell Layer; SCN: Suprachiasmatic Nuclei; LGN: Lateral Geniculate Nuclei; SC: Superior Colliculus; ERG: Electro Retinogram; VEP: Visual Evoked Potential

Authors' contributions

MSD, JJ and SH conceived and designed the experiments; MSD, performed the experiments; MSD, VAR and TS collected, analyzed and interpreted data. SL generated supplementary figure S1. The manuscript was written through contributions of all the authors. All authors have given approval to the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *British Journal of Ophthalmology* 2006;90:262-267.

2. Sherwood MB, Crowston JG, Shaarawy T. *Glaucoma: Medical Diagnosis and Therapy*: Elsevier Health Sciences; 2009.

3. Lee DA, Higginbotham EJ. Glaucoma and its treatment: a review. *American journal of health-system pharmacy* 2005;62:691-699.

4. Chamling X, Sluch VM, Zack DJ. The Potential of Human Stem Cells for the Study and Treatment of GlaucomaHuman Stem Cell for Treatment of Glaucoma. *Investigative ophthalmology & visual science* 2016;57:ORSFi1-ORSFi6.

5. Hertz J, Goldberg JL. Stem Cells and Glaucoma. *Stem Cell Biology and Regenerative Medicine in Ophthalmology*: Springer; 2013:75-97.

6. Johnson TV, Martin KR. Cell transplantation approaches to retinal ganglion cell neuroprotection in glaucoma. *Current Opinion in Pharmacology* 2012.

7. Shirai H, Mandai M, Matsushita K, et al. Transplantation of human embryonic stem cell-derived retinal tissue in two primate models of retinal degeneration. *Proceedings of the National Academy of Sciences* 2016;113:E81-E90.

8. Klassen H. Stem cells in clinical trials for treatment of retinal degeneration. *Expert opinion on biological therapy* 2016;16:7-14.

9. Schwartz SD, Hubschman J-P, Heilwell G, et al. Embryonic stem cell trials for macular degeneration: a preliminary report. *The Lancet* 2012;379:713-720.

10. Jagatha B, Divya MS, Sanalkumar R, et al. In vitro differentiation of retinal ganglion-like cells from embryonic stem cell derived neural progenitors. *Biochemical and Biophysical Research Communications* 2009;380:230-235.

11. Wang SW, Gan L, Martin SE, Klein WH. Abnormal polarization and axon outgrowth in retinal ganglion cells lacking the POU-domain transcription factor Brn-3b. *Molecular and Cellular Neuroscience* 2000;16:141.

12. de Melo J, Qiu X, Du G, Cristante L, Eisenstat DD. Dlx1, Dlx2, Pax6, Brn3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. *Journal of Comparative Neurology* 2003;461:187-204.

13. Prusky GT, Alam NM, Beekman S, Douglas RM. Rapid quantification of adult and developing mouse spatial vision using a virtual optomotor system. *Investigative ophthalmology & visual science* 2004;45:4611-4616.

14. Güler AD, Ecker JL, Lall GS, et al. Melanopsin cells are the principal conduits for rod–cone input to non-image-forming vision. *Nature* 2008;453:102-105.

15. Karali M, Peluso I, Marigo V, Banfi S. Identification and characterization of microRNAs expressed in the mouse eye. *Investigative ophthalmology & visual science* 2007;48:509.

16. Lee E-S, Lee J-Y, Jeon C-J. Types and density of calretinin-containing retinal ganglion cells in mouse. *Neuroscience research* 2010;66:141-150.

17. García-Crespo D, Vecino E. Differential expression of calretinin in the developing and regenerating zebrafish visual system. 2004.

18. DeParis S, Caprara C, Grimm C. Intrinsically photosensitive retinal ganglion cells are resistant to N-methyl-D-aspartic acid excitotoxicity. *Molecular vision* 2012;18:2814.

19. Moreno MC, Campanelli J, Sande P, Sáenz DA, Keller Sarmiento MI, Rosenstein RE. Retinal oxidative stress induced by high intraocular pressure. *Free Radical Biology and Medicine* 2004;37:803-812.

20. Yang Y, Mohand-Said S, Léveillard T, Fontaine V, Simonutti M, Sahel J-A. Transplantation of photoreceptor and total neural retina preserves cone function in P23H rhodopsin transgenic rat. *PLoS One* 2010;5:e13469.

21. Tucker BA, Park I-H, Qi SD, et al. Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. *PLoS One* 2011;6:e18992.

22. Pearson R, Barber A, Rizzi M, et al. Restoration of vision after transplantation of photoreceptors. *Nature* 2012;485:99-103.

23. Santos-Ferreira T, Postel K, Stutzki H, Kurth T, Zeck G, Ader M. Daylight vision repair by cell transplantation. *Stem cells* 2015;33:79-90.

24. Anderson MG, Smith RS, Hawes NL, et al. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. *Nature genetics* 2001;30:81-85.

25. Singhal S, Bhatia B, Jayaram H, et al. Human Müller glia with stem cell characteristics differentiate into retinal ganglion cell (RGC) precursors in vitro and partially restore RGC function in vivo following transplantation. *Stem cells translational medicine* 2012;1:188-199.

26. Li X, Kang Q, Gao S, et al. Transplantation with retinal progenitor cells repairs visual function in rats with retinal ischemia-reperfusion injury. *Neuroscience letters* 2014;558:8-13.

27. Qu Z, Guan Y, Cui L, et al. Transplantation of rat embryonic stem cell-derived retinal progenitor cells preserves the retinal structure and function in rat retinal degeneration. *Stem cell research & therapy* 2015;6:219.

28. Hertz J, Qu B, Hu Y, Patel R, Valenzuela D, Goldberg J. Survival and Integration of Developing and Progenitor-Derived Retinal Ganglion Cells Following Transplantation. *Cell transplantation* 2014;23:855-872.

29. Becker S, Eastlake K, Jayaram H, et al. Allogeneic Transplantation of Muller-Derived Retinal Ganglion Cells Improves Retinal Function in a Feline Model of Ganglion Cell Depletion. *Stem Cells Transl Med* 2016;5:192-205.

30. Venugopalan P, Wang Y, Nguyen T, Huang A, Muller KJ, Goldberg JL. Transplanted neurons integrate into adult retinas and respond to light. *Nature communications* 2016;7.

31. MacLaren RE, Pearson R, MacNeil A, et al. Retinal repair by transplantation of photoreceptor precursors. *Nature* 2006;444:203-207.

Figure Legends

Figure 1: NMDA-injected animals show reduction in number of RGCs and consequent visual function deficits. A-F) Brn3a whole mount retina immunostaining showing reduced number of Brn3a positive cells in NMDA-injected animals compared to control animals. G) Graph represents quantitative analysis showing the percentage of Brn3a positive cells. H-I) Schematic of Optomotry and light avoidance test set up. J) Visual acuity of NMDA-injected animals is significantly low compared to that of control animals. K) NMDA-injected animals also showed less light avoidance behavior compared to that of control animals. Data are expressed as Mean ± SD from triplicates of three different experiments. Number of animals used, Control = 10 and NMDA injected = 10. Scale = 100µm.

Figure 2: NMDA-injected animals showed a significant reduction in RGC projections into the image forming centres in the brain, LGN and SC but not in non-image forming centre such as SCN. A-D&E) CTB-488 shows that axonal projections into the dorsal LGN are significantly reduced in the NMDA-injected animals compared to that of the control animals. F-I&J) Anterograde tracing experiment showed significant reduction in RGC projections to Superior Colliculus in NMDA-injected animals. CTB-488 staining, tracking RGC projections into SC was significantly low in NMDA-injected animals compared to that of control animals. RGC projections into non-image forming brain centres are not affected in NMDA-injected animals. K-N&O) RGC projections into SCN of NMDA-injected animals are almost intact similar to that of control. Data are expressed as Mean ± SD from triplicates of three different experiments. Scale = 100µm.

Figure 3: GFP-expressing ES-NPs showed extensive integration and functional connectivity in transplanted NMDA-injected mouse models. A-C) Whole mount immunostaining of retina showed extensive integration of GFP-expressing cells with typical neuronal dendritic processes indicating RGC morphology. D-G) Transplanted GFP-expressing cells co-expressed RGC marker Calretinin. H-J) GFP-expressing ES-NPs integrated into the GCL of NMDA-injected animals as shown by the immunostaining of retinal sections. K) Transplanted, NMDA-injected animals showed improvement in visual functions. Visual acuity of transplanted, NMDA-injected animals is significantly increased compared to that of NMDA-injected animals prior to transplantation. L) Transplanted, NMDA-injected animals also showed improved light avoidance behavior compared to that of

NMDA-injected animals prior to transplantation. M-P) GFP-expressing cells integrated into the retina after transplantation expressed c-Fos, an immediate early gene upon light induction confirming functional integration with photoreceptor expressing cells. Data are expressed as Mean ± SD from triplicates of three different experiments. Number of animals used, Control = 10, NMDA-injected = 10, Transplanted = 10. Scale = 100µm.

Figure 4: DBA/2J animals did not show any improvement in visual functions after transplantation. A) Though there was a significant decrease in visual acuity of DBA/2J animals compared to that of control ($p<0.001$), no significant improvement in visual acuity was seen after transplantation in DBA/2J animals. B) Light avoidance behavior was also very poor in transplanted animals indicating that there was no significant vision recovery after transplantation in DBA/2J animals. Data are expressed as Mean ± SD from triplicates of three different experiments (Control = 3, DBA/2J = 6, Transplanted = 6). C) DAPI staining of control eye section showing intact GCL layer of the retina D) NMDA-injected animals showed disorganized GCL layer with less number of RGCs compared to that of control wild type animals. E) Pre-clinical glaucoma model, DBA/2J also showed a highly disorganized GCL layer with scattered RGCs. Scale = 100µm.

Figure-1

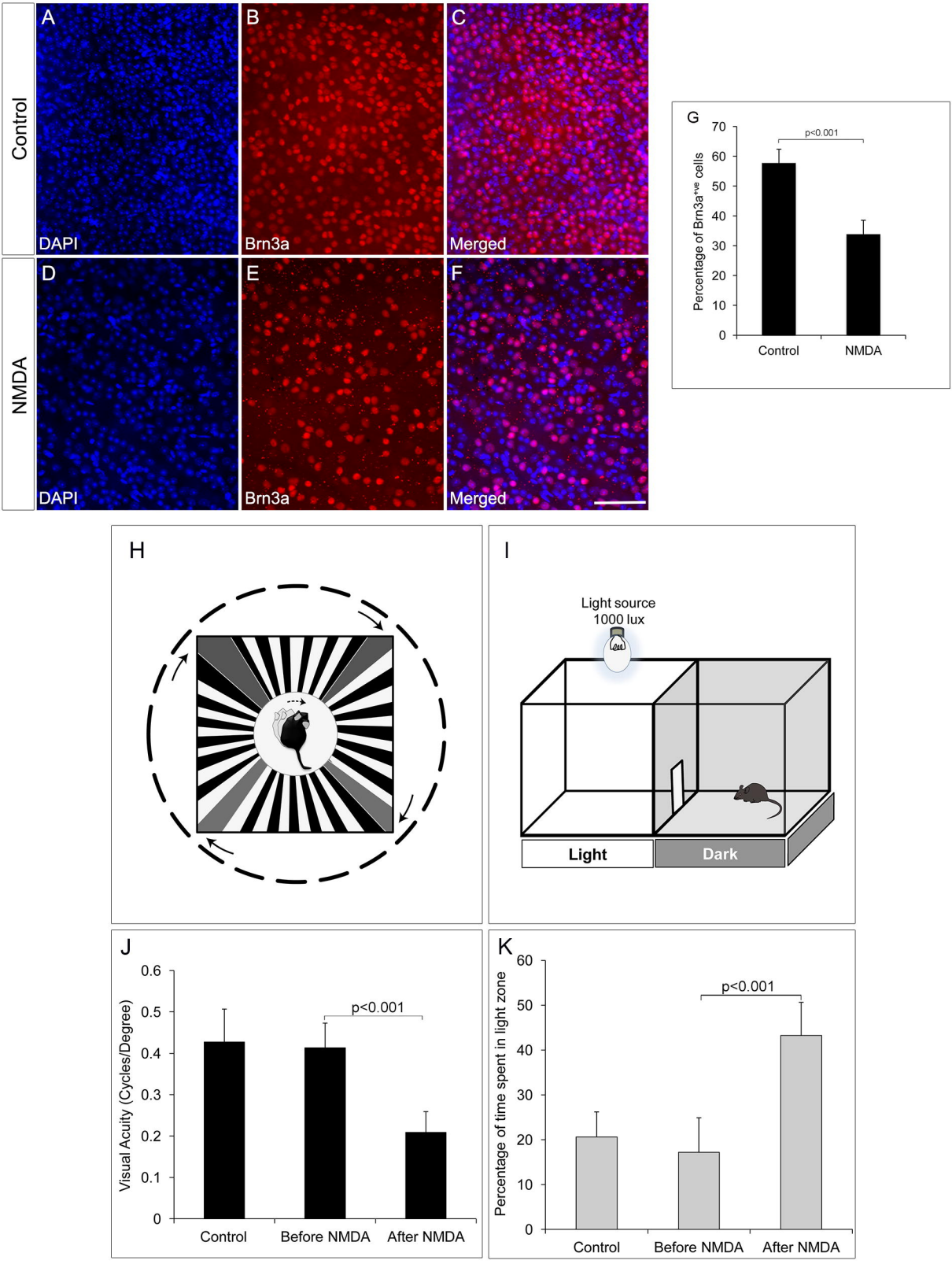
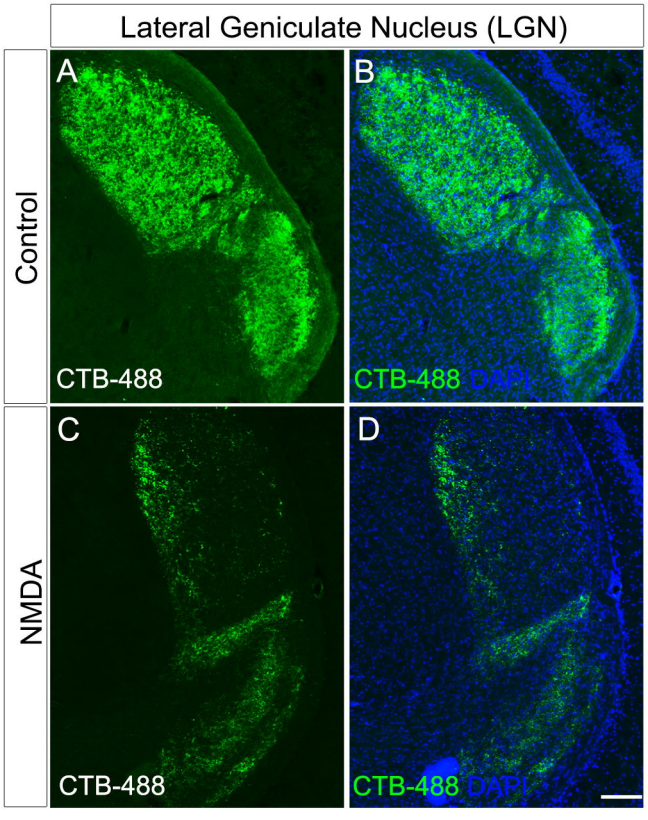
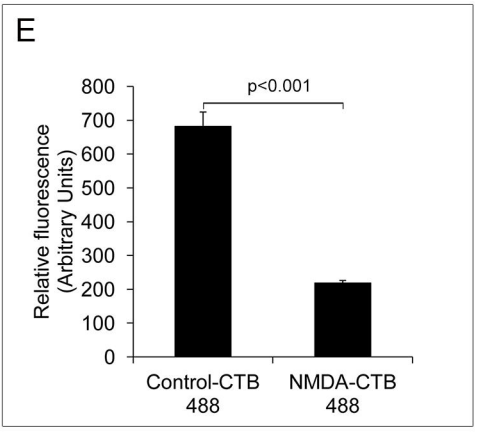
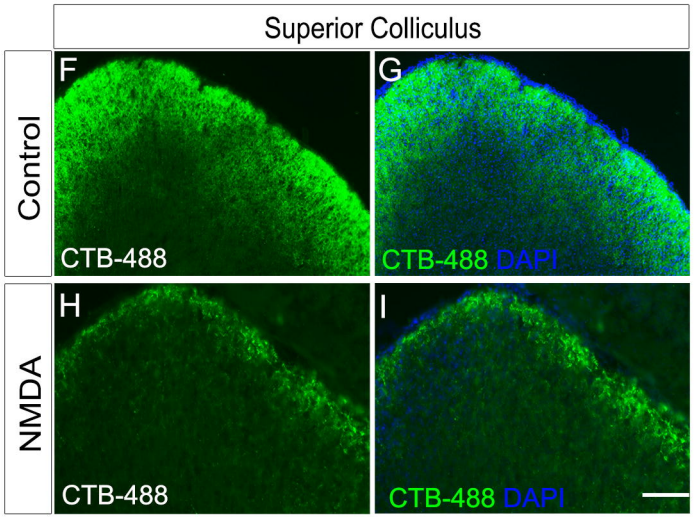
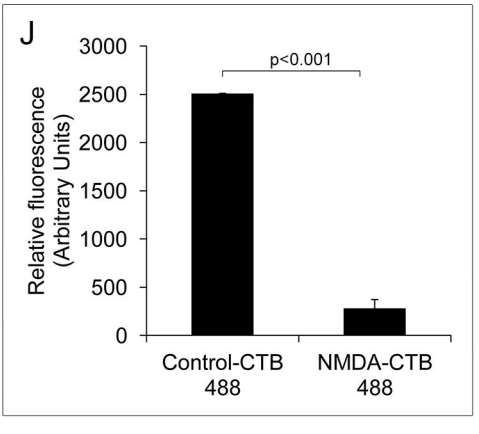

Control
NMDA
A
B
C
D
E
F
DAPI
Brn3a
Merged
DAPI
Brn3a
Merged
G
Percentage of Brn3a+ve cells
p<0.001
Control
NMDA
H
I
Light source
1000 lux
Light
Dark
J
Visual Acuity (Cycles/Degree)
p<0.001
Control
Before NMDA
After NMDA
K
Percentage of time spent in light zone
p<0.001
Control
Before NMDA
After NMDA

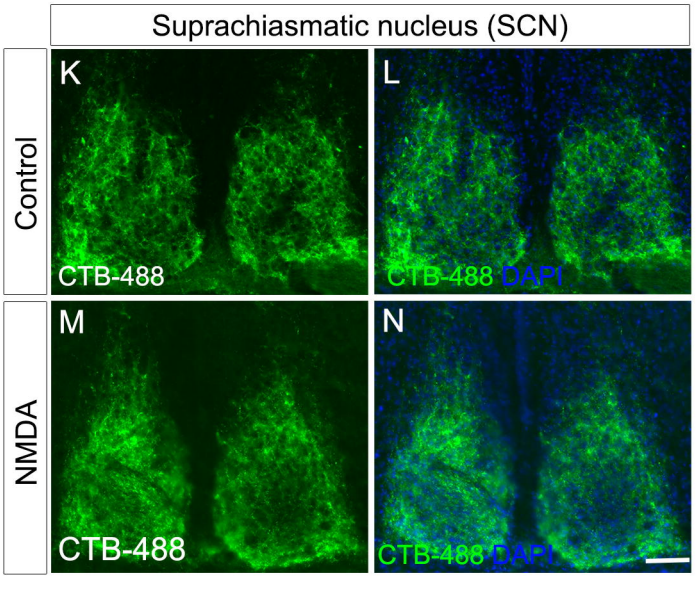
Figure-2


Lateral Geniculate Nucleus (LGN)
Control
NMDA
A
B
C
D
CTB-488
CTB-488 DAPI
CTB-488
CTB-488 DAPI


E
p<0.001
Relative fluorescence (Arbitrary Units)
800
700
600
500
400
300
200
100
0
Control-CTB 488
NMDA-CTB 488


Superior Colliculus
Control
NMDA
F
G
H
I
CTB-488
CTB-488 DAPI
CTB-488
CTB-488 DAPI


J
p<0.001
Relative fluorescence (Arbitrary Units)
3000
2500
2000
1500
1000
500
0
Control-CTB 488
NMDA-CTB 488


Suprachiasmatic nucleus (SCN)
Control
NMDA
K
L
M
N
CTB-488
CTB-488 DAPI
CTB-488
CTB-488 DAPI

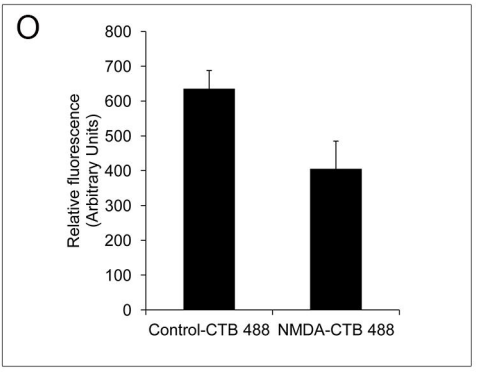
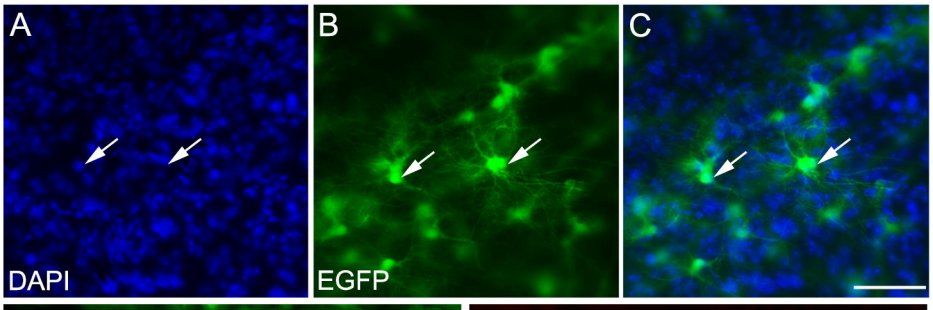
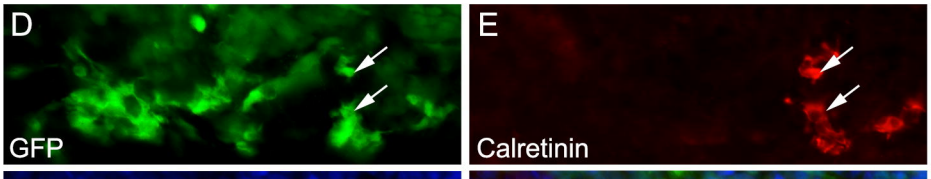
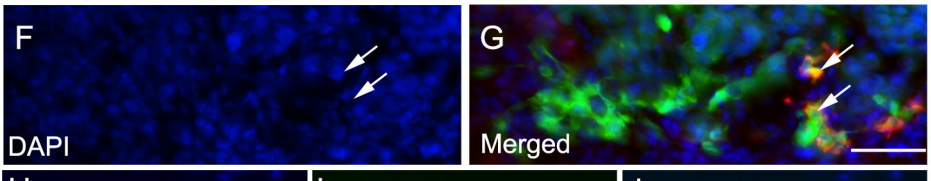
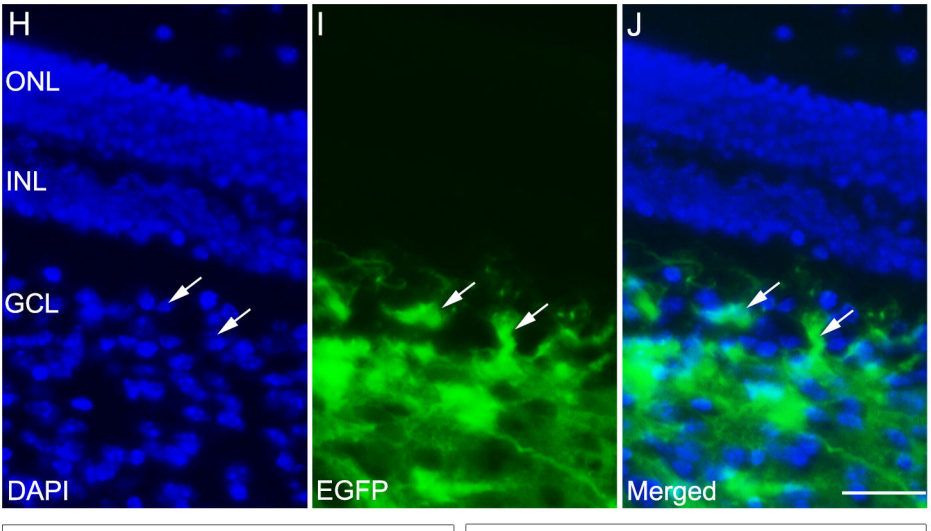

O
Relative fluorescence (Arbitrary Units)
800
700
600
500
400
300
200
100
0
Control-CTB 488
NMDA-CTB 488

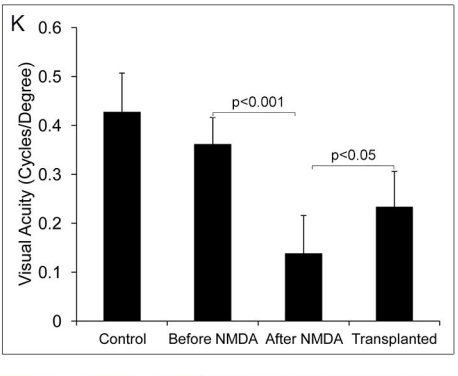
Figure-3

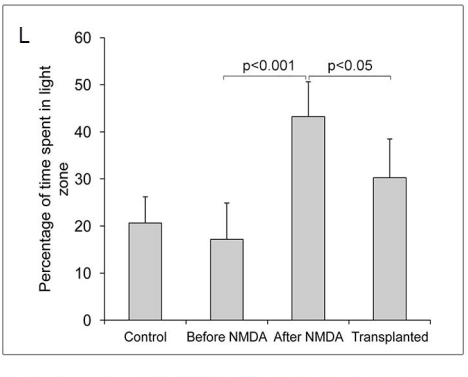

A
DAPI
B
EGFP
C


D
GFP
E
Calretinin


F
DAPI
G
Merged


H
ONL
INL
GCL
DAPI
I
EGFP
J
Merged


K
Visual Acuity (Cycles/Degree)
0.6
0.5
0.4
0.3
0.2
0.1
0
p<0.001
p<0.05
Control
Before NMDA
After NMDA
Transplanted


L
Percentage of time spent in light zone
60
50
40
30
20
10
0
p<0.001
p<0.05
Control
Before NMDA
After NMDA
Transplanted

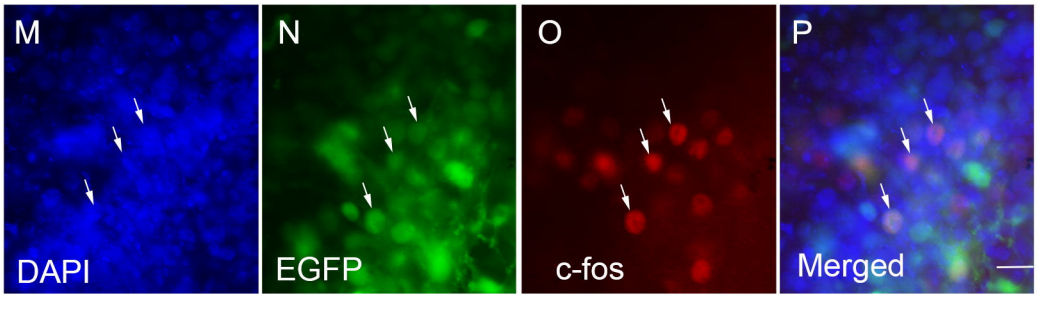
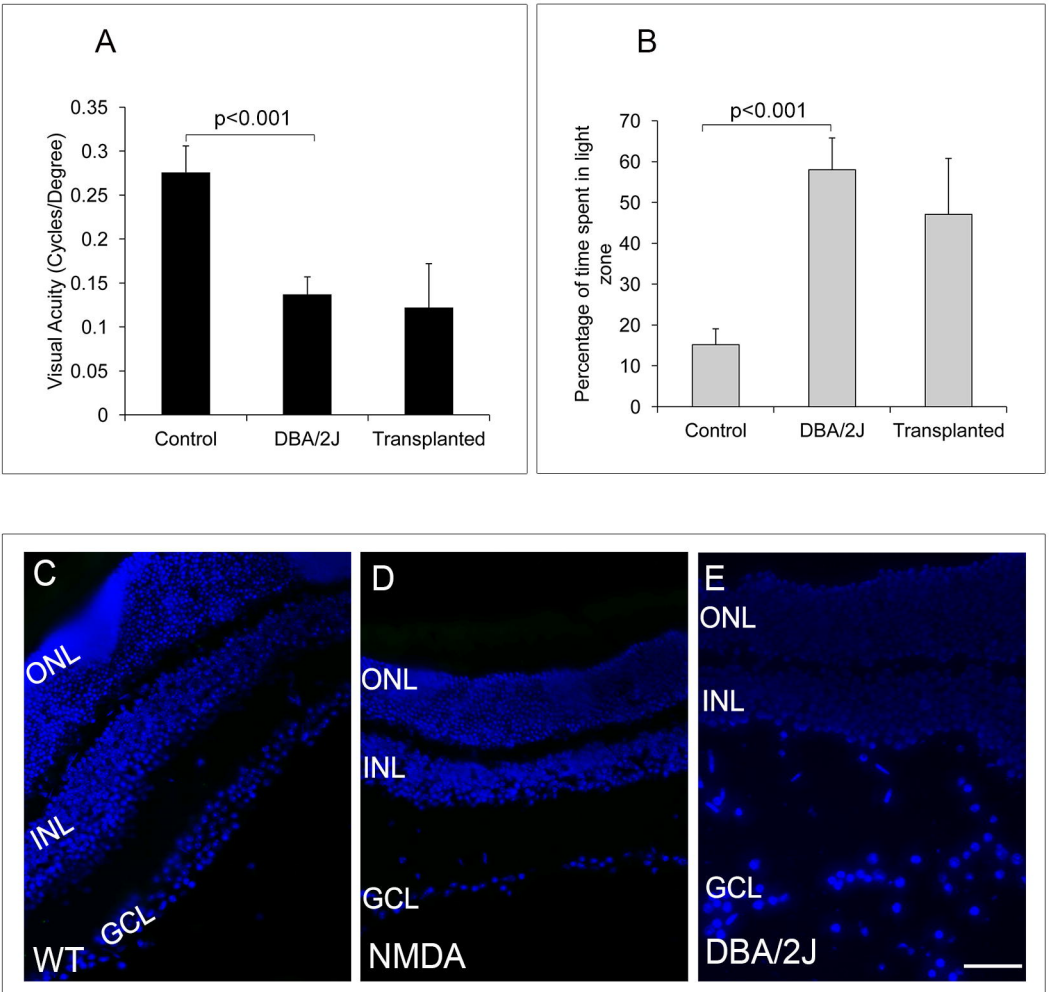

M
DAPI
N
EGFP
O
c-fos
P
Merged

Figure-4


A
p<0.001
Visual Acuity (Cycles/Degree)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
DBA/2J
Transplanted
B
p<0.001
Percentage of time spent in light zone
70
60
50
40
30
20
10
0
Control
DBA/2J
Transplanted
C
ONL
INL
GCL
WT
D
ONL
INL
GCL
NMDA
E
ONL
INL
GCL
DBA/2J