CNS regeneration after chronic injury using a self-assembled nanomaterial and MEMRI for real-time in vivo monitoring

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Received 19 June 2010; accepted 7 December 2010

Abstract

To speed up the process of central nervous system (CNS) recovery after injury, the need for real-time measurement of axon regeneration in vivo is essential to assess the extent of injury, as well as the optimal timing and delivery of therapeutics and rehabilitation. It was necessary to develop a chronic animal model with an in vivo measurement technique to provide a real-time monitoring and feedback system. Using the framework of the 4 P's of CNS regeneration (Preserve, Permit, Promote and Plasticity) as a guide, combined with noninvasive manganese-enhanced magnetic resonance imaging (MEMRI), we show a successful chronic injury model to measure CNS regeneration, combined with an in vivo measurement system to provide real-time feedback during every stage of the regeneration process. We also show that a chronic optic tract (OT) lesion is able to heal, and axons are able to regenerate, when treated with a self-assembling nanofiber peptide scaffold (SAPNS).

From the Clinical Editor: The authors of this study demonstrate the development of a chronic injury model to measure CNS regeneration, combined with an in vivo measurement system to provide real-time feedback during every stage of the regeneration process. In addition, they determined that chronic optic tract lesions are able to heal with axonal regeneration when treated with a self-assembling nanofiber peptide scaffold (SAPNS).

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Key words: Chronic CNS regeneration; Nano contrast agent; Self-assembled materials; MEMRI; in vivo

The need for real-time measurement of axon regeneration in the central nervous system (CNS) in vivo is essential for CNS injury assessment to speed up the process of recovery, as well as optimal timing and delivery of therapeutics and rehabilitation. Previously, axonal regeneration accompanied by behavioral recovery in hamsters was demonstrated after acute injury and treatment with a self-assembling peptide nanofiber scaffold (SAPNS)\textsuperscript{1} (see Figure 1). SAPNS is a liquid that self-assembles into a 3D structure; it completely fills a cavity and creates a permissive environment that allows cells to migrate in and axons to grow through. It dynamically reconfigures based on the local charge environment and does not form covalent bonds. The material breaks down over time due to peptidase action or is used as building blocks for the body.

Using the framework of the 4 P’s of CNS regeneration (Preserve, Promote, Permit and Plasticity) as a guide, the next steps are: (1) assess the degree of injury to determine the optimal extent and duration of a permissive environment for axonal regeneration to occur; and (2) measure real-time axonal growth — in acute and chronic CNS injury — to determine the optimal time for therapeutics delivery and rehabilitation.

For the work to be clinically relevant, it was necessary to develop a chronic animal model with an in vivo measurement...
technique to provide real-time monitoring and feedback. It was also important to precisely pinpoint the time when regenerating axons attained a suitable stage of reinnervation at the target tissue to determine the correct timing for rehabilitation. In this study we hypothesized that magnetic resonance imaging (MRI) and a manganese (Mn2+) tracer could be used to show axon regeneration in hamsters in a chronic CNS injury model.

MEMRI has never been tested in hamsters, but Mn2+ can be toxic at high dosages in rats and mice, prompting a search to establish a correct dose range. In previous tracing studies, 1-2 μL of manganese chloride (MnCl2) at 1.0 M were injected into either the larger anterior chamber of a mouse eye or the posterior chamber of a rat eye. Doses of 1500–3000 nmol of MnCl2 caused the eye to turn gray, indicating toxicity on the eye structure. Recently it was shown that the optimal dose for MEMRI of the rat visual pathway was 300–600 nmol of MnCl2, providing sufficient signal-to-noise ratio (SNR) for a detailed assessment of the visual pathway; higher doses were toxic, causing retinal ganglion cell (RGC) death and loss of Mn2+ enhancement throughout the visual pathway. Because each species reacts differently to NCAs, a pilot study in hamsters was performed to determine optimal scan timing, postdose, to establish the optimal window for contrast agent loading and diffusion to maximize SNR in living animals.

Here we show a successful chronic injury model to measure CNS regeneration, combined with an in vivo measurement system to provide real-time feedback during every stage of the regeneration process. We also show that a chronic OT lesion is able to heal, and axons are able to regenerate, when treated with SAPNS.

Methods

Young adult Syrian golden hamsters (Mesocricetus auratus), aged 6 to 8 weeks and weighing 90–100g were used. The eyes of anesthetized hamsters in Group 1 were injected with NCA; animals were scanned with a 7Tesla (7T) MRI (see Figure 2, B). MEMRI was performed on normal young adult hamsters to determine the optimal window for maximum contrast in the intact hamster visual system to establish a comparative baseline for the experimental results.

After establishing the normal visual pathway using 7T MRI scanning, a group of experimental animals was prepared. The investigation schedule followed the flowchart outline (see Figure 3, B). On day 1 the brachium of the superior

Figure 1. Self-assembling peptide nanofiber scaffold (A) Molecular model of the SAPNS or (RADA)4 (arginine, alanine, aspartate, alanine) molecular building block. (B) Molecular model of numerous (RADA)4 molecules undergoing self-assembly to form one half of the well-ordered nanofiber structure. (C) SAPNS 0.05% is examined using atomic force microscopy (scale bar = 5000 nm).
colliculus (BSC) was exposed and transected. On day 45 the visually guided behavioral assessment began. The 45-day delay was necessary to mimic the recovery period used in the acute transection and treatment model. On day 90 the contralateral eye was injected and then scanned to verify the extent and completeness of the surgery, as well as to assess the regeneration before treatment. On day 105 the transected brachium was re-exposed and treated with SAPNS. On day 152 the visually guided behavioral assessment was conducted, before the contralateral eye was injected and scanned, to assess the regeneration and reinnervation of the superior colliculus (SC) after SAPNS treatment in the experimental group of animals. Concurrently, the untreated animal group was injected and scanned to use as a control group to compare the effectiveness of the SAPNS treatment over the same period. Finally, following the last scan, the animals were injected with cholera toxin B subunit-fluorescein isothiocyanate (CTB-FITC), to trace RGC axons and then sacrificed four days later. The brains were dissected, sectioned and processed for histology and examined under a fluorescent microscope.

**Transection of BSC and SAPNS treatment**

The left OT in a group of experimental young adult hamsters was completely transected with a scalpel incision at the BSC, as previously described. Briefly, an incision extending 1-2 mm below the surface of the brain stem anterior to the SC was made from the midline of the exposed BSC to a point beyond the lateral margin of the SC. As previously described, for 1% SAPNS, 10 mg of RADA16-I dry powder was mixed in an Eppendorf tube containing 1 ml of Milli-Q water (Millipore, Billerica, Massachusetts), sonicated for 30 seconds and filtered.

**Behavioral assessment**

There were two rounds of visually guided behavioral assessment: (1) 45 days post transection of the BSC; and (2) 45 days post treatment. All adult animals were tested for visually elicited orienting movements, as previously described. Briefly, to elicit orienting movements, sunflower seeds were presented first by hand and later with the aid of a white metal wire on the end of which there was a small, black rubber ball, slotted for holding a seed. Animals were tested 2 to 3 times weekly for

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**Figure 2.** (A) Injection site diagram Location of the eyes and location of the right eye injection of NCA MnCl₂. Depiction of how the eyes are connected to the SC and location of the lesion in experimental animal. (BSC) Brachium of superior colliculus, (SC) Superior colliculus, (NCA) nano-contrast agent. (B-D) Visualization of intact visual system 48 hrs post MnCl₂ intravitreal injection in normal controls (B) Dorsal scan of the hamster brain showing the optic tract (OT) and the SC with the MnCl₂ clearly visible in white. Rostral is to the left and caudal is to the right. (C) Line drawing of the hamster brain with the cortex removed. Box corresponds to the scan to the left. Red line depicts the location of the future transection site in the OT (BSC). (D) The OT of hamster is visualized using a WG-HRP (WHEATGERM-HRP) reaction after injecting it into the eye (this produced a darker shade of blue, more like black, due to the intensity and concentration of the reaction products). This shows the spread and direction of the optic radiation and is similar to the signal from the MRI. (R) Rostral, (C) Caudal, Superior colliculus (SC), pretectal area (PT), lateral posterior nucleus (LP), medial geniculate body (MGB), lateral geniculate body (LGB) and inferior colliculus (IC).
15 days. Independently, two investigators tested each animal for 10–20 minutes every other day, or less frequently, before any responses were seen in the affected field. On each test day a minimum of 10 presentations were made on each side, with random choice of side. Visually elicited orienting movements were tested repeatedly by presenting a sunflower seed to part of the hamster’s visual field.14,15 Left and right visual fields were tested, and all trials were videotaped with an overhead camera. A trial was counted if a response occurred within 2 seconds of stimulus presentation. The completion of a turn was signaled by the animal’s head coming to a stationary position within 5° of the stimulus for at least one-third of a second.

**Treatment surgery**

Two-thirds of the animals were randomly selected as the experimental group (n = 10) and treated with SAPNS, which was applied in the new cuts; the other one-third were cut (n = 5), but not treated and were used as sham controls. The BSC was re-exposed 105 days after the first surgery and three perpendicular cuts were made across the old transected region; 30 μL of 1% SAPNS solution was injected into the channels.

**Intravitreal MnCl2 injections**

Approximately 24 hours prior to MRI, the hamsters were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital: 50mg/kg. 2 μL of MnCl2 (0.2 M), dissolved in Milli-Q water mixture, was injected into the vitreous chamber of the eye through a glass micropipette inserted into the right contralateral eye to the inflicted OT lesion at the BSC, at the temporal corneal-scleral junction (see Figure 2, A). The micropipette was held inside the eyeball for ~20 seconds before removing it to prevent leakage of the injected solution. The experimental animals received one MnCl2 injection 24 hours before scanning for each of three time points on days 0, 90 and 152.

**HRP OT tracing**

In addition, 5 μL of 25% wheat germ agglutinin conjugated to the enzyme horseradish peroxidase (WGA-HRP) (Type VI,
Sigma, St. Louis, Missouri) solution was injected into the posterior chamber of a normal animal’s left eye to trace the retinofugal projections. Two days later, the animal was perfused with Karnosky’s fixative; the brain was dissected out from the skull and postfix fixed in the same fixative for another 4 hours at 4°C. The isolated brain was rinsed; the meninges covering the brain were removed with a soft brush to avoid damaging the surface until it was clean to improve the penetration of the chromogen into the brain tissue. The whole brain was reacted for HRP histochemistry using tetramethyl-benzidine (TMB) as the chromogen (Mesulam, 1978). The reacted brain was rinsed several times before photographs were taken to reveal the HRP-filled retinofugal tract coursing towards, and covering, the entire SC (see Figure 2, D).

3D MRI

In a group of normal control animals, the eyes were injected with NCA and imaged in a 7T MRI to determine the optimal postinjection scan time and the intactness of the visual system. All MRI measurements were acquired using a 7T Bruker scanner. Under inhaled isoflurane anesthesia (3% induction and 1.5% maintenance), animals were kept warm on a ∼35°C heating pad and were placed in the prone position during scanning. Images were acquired using an isotropic T1-weighted 3D FLASH sequence with the following settings: TR/TE = 21.7/3.1ms, 30-degree flip angle, acquisition matrix = 168 × 168 × 128 and voxel resolution 226 × 226 × 226 μm.3 Four averages were used; the total acquisition time was 23.5 minutes for each animal. Three months after the SAPNS, or 6 months after the initial BSC transection, MEMRI was performed again to assess the effect of the SAPNS treatment on the regeneration and reinnervation of the axotomized retinal fibers.

Data acquisition and analysis

Maximum intensity projection (MIP) was performed on a segmented volume covering the SC and their brachii to investigate the MnCl2 enhancement at the dome-shaped SC globally. Regions of interest (ROI) were drawn manually in the SC and the area proximal to the BSC on both sides; the mean and standard deviation of the ROIs were found using Image-J software for microscopy (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland). The ROIs were copied for each sample and each value was compared using both ratio-between-mean of opposite and standard deviation of the ROIs was used to ensure uniform pixel density depending on the capture objective.

Preparation for tracing regenerated axons

Following the last scan, the animals received intraocular injections of 1 μL of 1% CTB-FITC into the vitreous humor of the right eye using a glass micropipette (tip diameter, ∼10 μm). The animals were placed in an incubator for warmth and to recover fully before returning to their cages for postoperation monitoring. Four days after intraocular injection, the animals were sacrificed with an overdose of anesthetics and perfused transcardially with chilled 0.9% NaCl and 0.25% NaNO3 (pH 7.4), followed by 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains and eyes were removed and postfix fixed in 2% paraformaldehyde at 4°C for 4 days. For cryoprotection, the dissected brains were kept in 30% sucrose at 4°C until they sank. Next, 30 μm parasagittal sections were cut on a cryostat and mounted directly on gelatin-coated slides.

Immunolabeling of OT axons

The mounted sections were air dried, then washed three times with PBS (pH 7.4) at 10-minute intervals and pre-blocked in PBS (pH 7.4) containing 2% Triton 100, 2% normal rabbit serum and 2.5% BSA for 30 minutes at room temperature. Slides were then incubated with goat anticholeragenoid (List Biological Laboratories, Campbell, California) (1:3000 dilution)/2% Triton 100/2% normal rabbit serum/2.5% BSA for 48 hours at room temperature (20°C to 25°C). Slides were again washed three times in PBS (pH 7.4) and incubated with fluorescent donkey anti-IgG antibodies Alexa-488 (secondary antibody from Invitrogen–Molecular Probes) (1:200 dilution) for 1.5 hours at room temperature (20°C to 25°C) in a light-protected chamber. Slides were then washed 4 to 5 times in PBS (pH 7.4) at 5-minute intervals and coverslipped with DAKO mounting medium (DAKO, Carpinteria, California). Sections were examined under a fluorescence microscope and pictures were taken with a Kodak DCS 520 digital camera. Montages of the images were constructed to study locations and to analyze the extent of regenerated axons in the SC.

Cross-section measurement of optic nerves

Each optic nerve was cross sectioned and mounted, then photographed in bright field using a Zeiss Axiophot microscope (Carl Zeiss MicroImaging GmbH, Munich, Germany) and spot camera. These photographs were used to measure the area of the cross section. Multiple sections were used, and the average was taken to determine the cross-section area. Photoshop (Adobe Corp., San Jose, California) measurement feature was used to measure the area of the cross section. To standardize the units, a scale was used for each of the objectives and the standard was used to ensure uniform pixel density depending on the capture objective.

Results

Pilot study for dose in hamster

Image intensity varied depending on the dose of the NCA injected into the eye, on the time between the administration of the NCA, and on the scanning. Time was needed for the uptake of NCA by the RGCs after injection and transportation from the optic axons along the optic nerve and OT to the eventual visual target in the SC. The 400-nmol dosage of the MnCl2 was sufficient to visualize any sparse connections that may have been spared during surgery. We later discovered that the 400-nmol dose showed some toxicity through the formation of a cataract and subsequent RGC depletion with multiple doses.
MEMRI visual system morphology

Morphologically, though the images were not as clear and sharp as those obtained through conventional stained histology sections, the visual pathway could be traced along the OT from the ventral aspect of the midbrain as it coursed first laterodorsally and then mediadorsally through the brachium of SC and terminated in the SC (see Figure 2, B). Other secondary visual targets, such as the lateral posterior nucleus, pretectal nucleus and lateral geniculate nucleus (LGN), were also visible in the scanned images. In the horizontally scanned image, the injected eye appeared lighter with the presence of NCA, in contrast with the left non-injected eye, indicating the successful application of the NCA to label the RGCs (see Figure 4).

Histological confirmation

Using HRP we could visualize the same basic morphology in the brainstem and the SC after reaction. The dark band traveling from the LGN through the BSC and finally into the SC confirms the basic density and location of the scan where the right eye was injected (see Figure 2, D). The whole mount of the HRP reacted brain also aided visualization of the MEMRI images.

Pretreatment behavioral assessment

The complete transection of the OT was confirmed with behavioral assessment. None of the transected animals (n = 15) showed any response to a visual stimulus presented to the affected eye above normal spontaneous turning, indicating that the eye was completely disconnected.

First MEMRI scan 90 days after surgery

Ninety days after the successful transection of BSC, the contralateral right eye was injected with NCA 24 hours before scanning. Figure 4, B shows a large cavity in the cerebral cortex overlying the region of the BSC and SC. A white band indicating the presence of the NCA was clearly seen rostral to the SC. Medially, a gap was seen between the NCA label and the anterior border of the SC, indicating the position/location where the BSC was transected. It was apparent that the optic fibers were completely severed at the BSC before entering the SC, and no evidence of the NCA was present in the SC, suggesting that the optic fibers did not regenerate and reinnervate the visual target before treatment. This appears to be the case for all 15 animals (see Figure 4, B).

Post-treatment behavior assessment

Behavioral assessment was performed in the experimental animals with no response above the normal spontaneous turning seen in controls. The animals’ inability to respond to visual stimulus correlated with the absence of regenerated optic fibers in the nontreated sham groups of animals. It was also reflected in the treated group of animals because the affected SCs of these animals were only sparsely repopulated by the regenerating fibers.

Post-treatment MEMRI

Eight of the 10 SAPNS-treated animals showed that the SAPNS-treated channels were healed, confirming previously reported cases that SAPNS would allow for wound healing in the brain. The signal intensity was compared before and after treatment, showing a significant difference in the signal intensity and indicating some regeneration had occurred. The ratio of the left SC to right SC showed a significant signal increase after treatment: P < 0.01 of the 8 animals that showed reconnection. Moreover, some fibers containing NCA did regenerate and extend their axonal processes into the vacated SC through the newly created channels across the original BSC transected site (see Figure 5, B and C). The control animals showed enlarged gaps at the injury site (see Figure 5, A) with no NCA detected posterior to the original BSC transected site similar to Figure 4, B.

Maximal SI

Maximal SI, measured between the right control SC and the left labeled SC, was achieved in not less than 24 hours and more than 48 hours after injection of the NCA intravitreally into the right eye. It appeared that 4 days post-NCA injection the SI deteriorated. This could be due to the removal of the NCA from the system or some other diffusion factors. The SI ratio remained relatively constant between 1 and 2 days and did not show any significant difference. This correlates with Watanabe’s findings in rats.

Gross anatomical changes in the visual system

We later noticed that the MnCl2-injected eyes had a marked decrease in size in comparison with the un.injected control eyes, as well as in comparison with the contralateral eye in the same animal. The lenses of the injected eyes were clouded, appearing to have formed cataracts. After one injection, the eye appeared to be the same size; however, by the third injection the eye size had significantly decreased and the lens was completely opaque.

Optic nerve measurements

The optic nerve cross-section measurement of the treated animals was 52% smaller in cross-sectional area than that of the normal control animals that did not receive a MnCl2 injection.

Discussion

A. new chronic CNS injury model

This work created a chronic model of CNS injury that would have multiple readouts, one that is reproducible and employs three separate modes for measuring CNS regeneration. The first mode provides real-time feedback before, during and after OT transection through MRI; the second, behavioral testing, uses visually-guided orienting behavior; and the third mode is the histological assessment. The treatment method created a channel at the site of injury; the material was deposited into the channel to give the axons a growth path to follow during the regeneration process, as opposed to attempting to apply the treatment in the original wound site. Using MRI the continuity of both the
connection and the subsequent disconnection was tested. We were able to follow the development of the injury site and the subsequent reconnection in some animals.

We created this model using the hamster visual system for several reasons: Because the visual pathway is near the surface of the brain, it is easily observable by MRI and is accessible to make a controlled disconnection syndrome. In addition, it allowed for a behavioral readout showing real-time regeneration with enough fidelity to measure the changes caused by various treatments, such as growth factors, and to reveal how the system reacts to them at different stages of the regeneration process.

This chronic model appears to be robust and can be used to carefully monitor the progress of regenerating axons as well as establish a timetable to implement each of the four P’s:

- **Preserve** - using a contrast agent will help determine the density of cells that are still alive and able to regenerate. This can be used to monitor success in keeping the cells alive.

- **Permit** - the correct time can be determined to create a permissive environment in the target tissue, serving to signal when the various forms of rehabilitation are needed to maximize return.

- **Promote** - as the axons grow through the injury site the need to create a permissive environment will be reduced and the new focus will be on promoting the axons toward the target tissue for reinnervation.

- **Plasticity** - when the axons have entered the target tissue this will signal the correct time to stop the promotion of growth and help the axons increase plasticity triggered by either behavioral rehabilitation or, in some cases, a combination of chemical factors that have been shown to increase chemical plasticity.

**Extent of CNS injury can be quantified and correlated to behavior**

Previously, after surgery disconnecting the OT from the SC through the sectioning of the BSC, it was very difficult to determine the extent of injury until histology was performed on the brain. MEMRI enabled us to both identify and quantify the extent of the injury site. Performing behavioral assessments also confirmed that there was a complete disconnection without sparing. Both the behavior and the MRI data confirmed that the disconnection was complete. Though this appears to be an obvious conclusion, behavioral testing had never been performed to establish a baseline before treatment.

**Tissue healing and regeneration can be visualized in vivo**

We have shown that the visual system is traceable in vivo using a NCA, MnCl₂ injected in the eye, and that the visual
Pathways can be mapped from the retina through the optic nerve, optic chiasm, lateral geniculate and finally to the SC. The label, although toxic, was easily visible in the 7T MRI. It clearly showed the intact visual system before the operation; after the operation, it showed a clear disconnection. Even after the SAPNS injection, we were still able to visualize the disconnection because the nanomaterials did not allow the fast diffusion of the label across the lesion. After 6 weeks we were able to see some reconnection across the BSC. We also saw a decrease in volume of the lesion over time, and the saline controls had an increase of the same area.

**Chronic OT lesions are able to regenerate**

The results show that the newly inflicted cuts heal when treated with SAPNS. Axonal regrowth, though sparsely distributed and not visible in every case, can be seen crossing the cut regions into the SC in each of the two of three testing modes: MRI, in vivo and postmortem histology. Though treatment appeared to ‘knit’ the two cut edges of the brain tissue together, closing the gap to enable the regenerating axons to get through the injury site, we did not observe this in every case. This is probably due to the NCA toxicity.

We had previously reported that when SAPNS was applied soon after the transection, regeneration was achieved and function was recovered.1 We anticipated that in a chronic case, when the treatment was delayed, regeneration might be slow or inhibited due to lost regeneration potential; this did not appear to be the case. In the axons of the surviving RGCs, the NCA was observed in the regenerating axons posterior to the lesion site after treatment with SAPNS. However, these were sparse, due to the toxicity of the NCA. In spite of the toxicity, however, there was some reconnection, and this study proved that MRI allows regeneration to be visualized even before the return of functional behavior.

This new paradigm used MRI to detect axons in the OT in hamsters before, during and after regeneration in a chronic injury treatment model. Though the contrast agent was toxic, we...
showed regeneration after a chronic injury even with the loss of many of the RGCs. The next step is to determine the optimal contrast agent, possibly reducing the concentration of the MnCl₂ to reduce the toxic side effects.

Combining chronic injury and treatment with SAPNS, along with the use of an NCA to monitor the progress of regenerating axons in a mammalian model, has laid the groundwork for future study to explore the timing and the treatment duration for each of the 4 P’s of regeneration. The ability to follow the progress of healing and the reinnervation of the vacated target area within the CNS opens another venue for studying the time period within which promotion of wound healing and axonal regeneration can be enhanced under this noninvasive approach.

It may be possible to use this approach in humans to provide real-time feedback of regenerating tracts, as opposed to current methods, which can take months or years to show behavioral return. Measurement is the key to enhancing feedback so that patients do not lose hope and MRI appears to be more sensitive than other traditional behavioral methods of measurement to determine future return of function. This is a way to change and tailor treatments, receive real-time feedback and not have to wait for function as a sign of recovery.

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