CNS regeneration after chronic injury using a self-assembled nano material

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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 non-invasive magnetic resonance imaging (MRI) and a manganese ($MnCl_2$) contrast agent we show a successful chronic injury model to measure CNS regeneration. 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). With the combination of the injury model and the time series of scans we were able to show how they can be combined to produce a system that can be used to follow regenerationin vivo to provide real-time feedback during every stage of the regeneration process. We also were able to show that rengeration can be effected 100 days post injury when applied to the chronic wound.

Keywords: chronic CNS regeneration, $MnCl_2$, self-assembled materials, MEMRI, in vivo

1 INTRODUCTION

Real-time measurement of axon regeneration in the CNS in vivo is essential to assess the state of the CNS and to speed up the process of recovery and rehabilitation, We previously demonstrated axonal regeneration, accompanied by behavioral recovery in hamsters, after acute injury and treatment with SAPNS [1]. 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.

It was necessary to develop a chronic animal model with an in vivo measurement technique to provide real-time monitoring and feedback. In order to determine the correct timing for rehabilitation it was also important to precisely pinpoint the time when regenerating axons attained a suitable stage of reinnervation at the target tissue. We hypothesized that MRI and a $MnCl_2$ tracer could be used to show axon regeneration in hamsters in a chronic CNS injury model.

MRI is a non-invasive approach to study details of the body's inner depth in vivo and nano contrast agent (NCA) $MnCl_2$ is able to label the nervous system intra-axonally[2]. $MnCl_2$-enhanced MRI (MEMRI) has been widely used in rats and mice for visualizing activated regions of the brain [3]; detecting optic nerve lesions in vivo [4]; and tracing axons [5] and neurons [4, 5]. It had also been suggested that $MnCl_2$ could be a good contrast agent in a chronic CNS injury model.

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.

2 MATERIALS AND METHODS

Young adult Syrian golden hamsters (Mesocricetus auratus), aged 6 to 8 weeks, 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. MEMRI was performed on normal young adult hamsters to establish a baseline upon which the experimental results can could be compared. Following establishment of the normal visual pathway using 7T MRI scanning, a group of experimental animals was prepared. Day 1 – the brachium

of the superior colliculus (BSC) was exposed and transected; day 45 - visually guided behavioral assessment began [6, 7]. Day 90 - the contralateral eye was injected and scanned to verify the extent and completeness of surgery as well as assess regeneration prior to treatment. Day 105 - the transected brachium was re-exposed and treated with SAPNS. Day 152 – 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. Finally, following the last scan, the animals were injected with cholera toxin B subunit-fluorescein isothiocyanate (CTB-FITC), to trace retinal ganglion cell axons, then sacrificed four days later. The brains were dissected, sectioned and processed for histology and examined under a fluorescent microscope.

2.1 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 [8].

2.2 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 [7].

2.3 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; while 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.

2.4 Intravitreal $MnCl_2$ injections

Approximately 24 hours prior to MRI, the hamsters were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital. 50mg/kg. 2 µl of $MnCl_2$ (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. The experimental animals received one $MnCl_2$ injection 24 hours before scanning for each of three time points days 0, 90 and 152.

2.5 MRI and HRP OT compariosn

For comparison of the optic tract with the MRI, 5 µl of 25% wheat germ agglutinin conjugated to the enzyme horseradish peroxidase (WGA-HRP) (Type VI, Sigma) 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 post-fixed in the same fixative for another four hours at 4^oC. In anther group of animals $MnCl_2$ was injected into one eye and the animals were scanned at different times to determine the optiumum time and cocntration post injection. The eyes were injected with NCA and imaged in a 7T Bruker scanner to determine the optimal post injection scan time and the intactness of the visual system at several time points up to 100 hours post injection. The experimental animals were scanned three times: (1) before transection; (2) just before SAPNS treatment; and (3) before the animals were injected with CT-b and euthanized.

2.6 Data acquisition and analysis

Maximum intensity projection was performed on a segmented volume covering the SC and their brachii in order to investigate the $MnCl_2$ enhancement at the dome-shaped SC globally.

2.7 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. This was accomplished with a glass micropipette (tip diameter, ~10 µm) [9].

2.8 Immunolabeling of OT axons

The mounted sections were air-dried, then washed three times with PBS (pH 7.4) at 10 min intervals and pre-blocked in PBS (pH 7.4) containing 2% Triton 100, 2% normal rabbit serum, and 2.5% BSA for 30 min at room temperature. Slides were then incubated with goat anti-choleragenoid (List Biological Laboratories) (1:3,000 dilution)/2% Triton 100/2% normal rabbit serum/2.5% BSA for 48 hrs at room temperature. Slides were then washed four to five times in PBS (pH 7.4) at 5 min intervals and coverslipped with DAKO mounting medium (DAKO). Sections were examined under a fluorescence microscope; pictures were taken with a Kodak DCS 520 digital camera.

2.9 Cross section measurement of optic nerves

Each optic nerve was cross-sectioned and mounted. The cross-sections were photographed in both bright field and fluorescent illumination 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.

3 RESULTS

3.1 Pilot study for dose in hamster

Image intensity varied depending on the dose of the NCA injected into the eye, the time between the administration of the NCA, and the scanning. Time was needed for the uptake of NCA by the retinal ganglion cells (RGCs) after injection and transportation from the retinal ganglion cell axons, along the optic nerve and OT, to the eventual visual target in the SC. The 400 nmol dosage of the $MnCl_2$ 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.

3.2 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 laterodorsally first, and then mediodorsally, through the brachium of SC and terminating in the SC. Other secondary visual targets, such as the lateral posterior nucleus, pretectal nucleus and lateral geniculate nucleus were also visible in the scanned images. In the horizontally scanned image, the injected eye appeared lighter with the presence of NCA, in contrast to the left non-injected eye, indicating the successful application of the NCA to label the RGCs.

3.3 Histological confirmation

Using HRP we visualized the same basic morphology in the brainstem and the SC after reaction in a group of control animals. The dark band traveling from the LGN through the BSC and finally into the SC confirmed the basic density and location of the scan when the right eye was injected.

3.4 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, indicateding that the eye was completely disconnected.

3.5 MEMRI scans 0, 90, days after surgery

On day zero the animals were scanned showing they were intact. They were scanned again at ninety days after transection of BSC showing a complete transection of the optic tract. 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 prior to treatment.

3.6 Post-treatment behavior assessment

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

3.7 Post-treatment MEMRI

Eight of the ten 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 [1]. The signal intensity was compared before and after treatment showing a significant difference in the signal intensity, indicating some regeneration had occurred. The ratio of the left to right SC showed a significant signal increase after treatment: $p<0.01$ of the 8 animals that showed reconnection. The MRI scanned images of the sham animals showed enlarged gaps at the injury site with no NCA detected posterior to the original BSC transected site.

3.8 Maximal SI

Maximal SI, measured between the right control SC and the left labeled SC, was achieved in not less than 24 hours, and not 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 one and two days and did not show any significant difference. This correlates with Watanabe's findings in rats [10].

3.9 Anatomical changes in the visual system

It was later noticed that the $MnCl_2$-injected eyes had a marked decrease in size when compared to the uninjected control eyes, as well as 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.

3.10 Optic nerve measurements

The optic nerve cross-section measurement of the treated animals was 52% smaller than the normal control animals that did not receive a $MnCl_2$ injection.

4 DISCUSSION

This study demonstrates the development of a new chronic CNS injury model; 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.

Our method of treatment created a channel at the site of injury; the material was deposited into the channel to give the axons a 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. MEMRI enabled us to both identify and quantify the extent of the injury site. Performing behavioral assessments also confirmed a complete disconnection without sparing.

We demonstrated that the visual system is traceable in vivo using $MnCl_2$ 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. The newly inflicted cuts healed when treated with SAPNS. Axonal regrowth, though sparsely distributed and not visible in every case, could be seen crossing the cut regions into the SC in each of the three testing modes: MRI, in vivo and post-mortem histology.

We anticipated that regeneration might be slow or inhibited in a chronic injury case, due to lost regeneration potential, but this was not the case. In spite of the toxicity there was some reconnection and this study proved that MRI allows regeneration to be visualized even before the return of functional behavior.

This is a new paradigm: using a 7T 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 were able to show regeneration after a chronic injury even with the loss of many of the RGCs. The next step is to work out the optimal contrast agent, possibly reducing the concentration of the $MnCl_2$ to reduce the toxic side effects and using MRspec to measure the lack of, and then the restoration of, the glutamate signal to the SC [11].This method, 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 ground work for future study to explore the timing and the treatment duration for regeneration. The ability to follow the progress of healing, and the reinnervation of the vacated target area within CNS, opens up another avenue for studying the time-window within which promotion of wound healing and axonal regeneration can be enhanced under this non-invasive approach.

CNS regeneration with objective measures would allow treatments to be assessed before behavioral outcome. A treatment that is not effective could be changed much faster, helping patients see an outcome more quickly. Shorter measurement and feedback times would fundamentally change how CNS regeneration is approached and could change the pace of clinical trial outcomes.

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