Author

Jasmin Tanaja, recognizing the importance of advancements in stem cell research, began work on her own project in Professor LaFerla's lab. Her study is the first to show that stem cells can help improve cognitive performance after a focal brain lesion targeted specifically to a neuronal subpopulation. She describes her research experience to be one of "uncovering another mystery of science," and feels fortunate to have been a part of its discovery. Jasmin followed this research by beginning a new clinical research project on emergency medicine at the UCI Medical Center. She hopes to follow her years at UCI by moving on to graduate school.

Key Terms

- CaMKIIα
- Diphtheria Toxin A-chain
- GFAP
- Neural Stem Cells
- Tet-DTA/CaM Double Transgenic Mice
- Tetracycline Responsive Element
- tTA Tetracycline Transactivator

Investigation of Transplanted Neural Stem Cells: The Extent of their Ability to Improve Cognitive Deficit in Response to an Induced Lesion

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Abstract

Neuronal and synaptic losses are prominent in most clinical conditions. Many experiments have used site-specific lesioning methods to mimic such conditions. These approaches may cause non-specific damage, affect multiple cell types, or cause loss of cells. To study the effect of a targeted neuronal lesion, we generated a novel transgenic mouse model in which it is possible to ablate specific types of neurons by a system of induction. This approach takes advantage of the tet-off system to drive the expression of diphtheria toxin A-chain (DTA), which relies mainly on the presence or absence of a tetracycline analog—in our case doxycycline—to deactivate or activate the expression of DTA respectively. Post induction, neural stem cells (NSC) were transplanted in the hippocampal region of the mice. After histological and behavioral assessment, we found that the neural stem cells were multipotent, and differentiated into astrocytes and oligodendrocyte precursors, although a smaller number differentiated into neurons. Analysis indicated percentages of differentiation as follows: 1.78% ± 0.515 neurons, 15.39% ± 6.76 astrocytes, and 17.07% ± 3.49 oligodendrocyte precursors. Behaviorally, the NSC-transplanted-mice performed significantly better than the controls in hippocampal-dependent tests. These transplanted neural stem cells show therapeutic potential to improve cognitive deficit.

Faculty Mentor

Neurological diseases have devastating consequences for the quality of life, and among these diseases, perhaps none is as dire as Alzheimer's disease (AD), which robs individuals of their memory and cognitive abilities, such that they are no longer able to function in society or even interact with their family members. AD is the most common cause of dementia among the elderly and the most significant and costly neurological disorder. Surprisingly, it has not yet been established whether neural stem cells can play a role in ameliorating cognitive decline. This study provides the first experimental evidence that neural stem cells can indeed restore cognitive deficits, and therefore opens up novel therapeutic approaches for treating cognitive decline associated with neurological disorders.

Frank M. LaFerla
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Introduction

According to the American Heart Association, clinical conditions such as ischemia are among the leading causes of death in the United States. In such conditions, substantial losses of cells (such as neurons) are common. Previous experiments have shown that neurons within the CA1 subfield of the hippocampus are extremely vulnerable to degeneration from these clinical conditions (Zou et al, 2005). They have also shown that it has been a challenge to mimic such site-specific lesions, especially without interrupting the blood brain barrier. Many methods to induce lesions are invasive and lack the ability to precisely target specific cell types. Based on these criteria, we developed a novel transgenic mouse in which it is possible to target the degeneration of cells in a specific site, and also in a temporally regulated fashion. Our model is unique in that we are able to regulate the expression of the diphtheria toxin (DT_A). Region specificity is controlled via the calcium-calmodulin kinase II alpha (CaMKIIα)-regulatory system, which enables selective collection by targeting the forebrain neurons. Since CaMKIIα-expressing neurons are affected in a gradual fashion (*i.e.* CA1 region first, followed by the cortex, dentate gyrus, and so on), controlling the time allows us to control the extent of lesioning and amount of neuronal loss. This is the first time an inducible system has been used to ablate CaMKIIα neurons, and in that respect it is unique as a tool for precise ablation of neurons in the brain. Most other systems for ablation use techinques that are not genetically targeted, such as ischemic lesioning, which are less precise.

We elected to use a tetracycline inducible system to regulate and control the damage produced. This system is ideal, not only because it meets the requirements of a conditional system (*i.e.* reversible, inducible), but it is also widely used. It provides tight regulation, ensuring that all transcription of interest only occurs as intended, and controlling the length of induction (Gossen and Bujard, 1992).

In our experiment, we used a subtype of the tetracycline inducible system called the tet-off system, which means that as long as the Tet-DTA/CaM mice are provided with a tetracycline analog (doxycycline) in their water, no transcription occurs. Other studies have used the opposite mechanism, the tet-on system, under which transcription occurs when doxycycline is added to their diet. We did not use the tet-on system due to its relatively high error rates (e.g. unreliable time sensitivity, etc.).

The primary components of this system are the CaMKIIα promoter, tTA, tet operon that has been fused to a minimal promoter, and DT_A. Figure 1 shows an overview of the system.

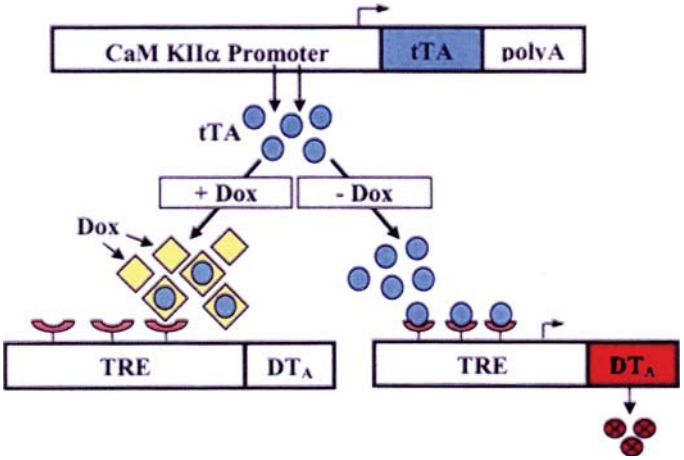

Figure 1

Schematic diagram of the tet-off inducible system. The Tetracycline Responsive Element (TRE) drives the expression of Diphtheria Toxin A-chain and the CaMKIIα promoter targets the Tetracycline Transactivator (tTA) to basal forebrain neurons. The presence of doxycycline (available ad libitum in the drinking water and food) precludes transgene expression, whereas withdrawal of doxycycline leads to transgene induction and Diphtheria Toxin A-chain expression (Yamasaki, 2007).

The Tetracycline inducible system relies on the expression of a transactivator molecule (tTA), which activates transcription from a specific transgene in the absence of doxycycline (a Tetracycline analog). The CaMKIIα promoter drives the expression of tTA. These tTA molecules bind to a specific sequence of the tet operon, which, along with a minimal promoter, makes up the Tetracycline Responsive Element (TRE); when tTA binds to this TRE, it activates transcription of the gene of interest. Functionally, when doxycycline is present, it binds to tTA, and prevents it from binding to TRE. However, when doxycycline is not present, tTA molecules are free to bind to TRE. This binding results in the transcription of diphtheria toxin A-chain, which is what causes the lesions in our Tet-DTA/CaM mice.

Our results will allow us to assess the potential of transplanted neural stem cells in Tet-DTA/CaM mice. The assessment includes the extent of their ability to survive and migrate, and how cognition is affected in response to an induced lesion. We assessed measures such as behavior, histology and immunohistochemistry.

Neural stem cells hold great potential to improve deficits and stunt the progression of many diseases. Within the last

few decades, research in stem cells has grown exponentially, and has shown promise as a therapeutic approach. Although extensive studies have been done in using stem cells as a therapy to improve cognition, the results remain inconclusive. Our study attempts to further clarify the properties of neural stem cells in response to an induced lesion, and the potential of neural stem cells as a therapeutic solution. Until recently, scientists strongly believed that the neurons that exist in adults eventually die and cannot be regenerated (Ramon y Cajal, 1928). However, recent findings have reinforced the newly accepted concept that neurogenesis is an ongoing and natural occurrence. Further, neural stem cells are promising as a possible future therapeutic solution, mainly because of their basic properties, such as their capability to renew themselves and differentiate into one or more specialized subtypes (Rao and Mattson, 2001; Nowakowski and Hayes, 2001). Also, stem cells have been shown to help in treating ischemia and other neurodegenerative diseases (Lindvall et al. 2004).

Based on this potential and other supportive evidence, we expect that the transplanted neural stem cells will migrate and differentiate in response to the lesion induced by the diphtheria toxin A-chain. We hope that our novel transgenic model in conjunction with the Tetracycline inducible system will facilitate studies involving injuries characterized by specific cell loss, and provide insight to recovery in cell death and possible therapies for stimulating the regenerative processes.

Materials and Methods

Animal Background

Double transgenic mice were generated by crossing CaMKIIα-tTA mice to TRE-DT_A mice [generous donation from Dr. Glen Fishman (NYU, NY) for the TRE-DT_A mice]. Mice were maintained on 2mg/ml doxycycline (Sigma, St. Louis, MO) supplemented with 5% sucrose in deionized, filtered water to prevent the induction of DT_A. During the induction of DT_A expression, water was administered without doxycycline. All procedures were performed in accordance with the regulations of the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine under protocol 1999-1706.

Genotyping Methods : PCR

Mice were genotyped by PCR. For the CaMKIIα-tTA transgene, the following primer was used: 5'-TCGCGATGACTTAGTAAAGC-3' and 5'-CGCATTAGAGCTTAATG-3'. The primer 5'-CCGCAGCGTCGTATTTATTG-3' and 5'-TCTTCGTACCACGGGACTAA-3' was used for the TRE-DT_A transgene. These primers are essential for initiating DNA replication from the single template strand.

Reverse Transcriptase PCR

To amplify the expression of diphtheria toxin, RNA samples isolated from hemibrains underwent reverse transciption using the above primers and PCR. RNA samples were first homogenized in GuCNS solution (4M guanidine thiocyanate, 0.5% sarkosyl, 0.025M NaCitrate, 100mM β-mercaptoethanol) and phenol/chloroform was extracted. The samples were further treated with RQ1 RNase-free DNase (Promega, Madison, WI) for 15 minutes in the presence of RNasin (Promega, Madison, WI). During the reverse transcription with Superscript II RT, Oligo dT primers (Invitrogen, Carlsbad, CA) were added.

Perfusion Methods

Mice were anesthetized with Nembutal Sodium Solution (Ovation Pharmaceuticals, Deerfield, IL). They were then perfused transcardially with PBS at a rate of 12mL/min, followed by 4% paraformaldehyde in 0.01M phosphate buffer (pH 7.4).

Tissue Sections Maintenance and Processing

After perfusion, the brains were fixed in 4% paraformaldehyde for approximately 48 hours. For further immunohistochemistry processing, brains were sectioned at 40 μm using a vibratome (Pelco, Redding, CA).

Immunohistochemistry

Four primary antibodies were used: anti-GFAP (1:500 DAKO, Denmark), anti-NeuN (1:10,000, Chemicon), anti-NG2 (1:200, Chemicon), and anti-GFP (1:2000, Chemicon). For anti-NeuN (1:10,000, Chemicon, Temecula, CA) staining, tissue sections were washed with 3% MeOH/Hydrogen peroxide for 30 minutes, followed by 0.1% Triton X and 3% normal serum. On the second day, the sections were developed with SG-1 (Vector Labs). For anti_CNPase (1:20,000, Sigma), signals were amplified using the Tyramide Signal Amplification System (Perkin Elmer, Waltham, MA).

For immunofluorescence, tissues were blocked with 3% normal serum with 2% BSA and 0.1% Triton-X in TBS, followed by incubation in primary antibody overnight at 4 °C. Post-incubation, they were rinsed and quenched with secondary Alexa Fluor-conjugated antibodies (1:200 Molecular Probes, Carlsbad, CA) in block, rinsed, and mounted in Fluoromount G (Southern Biotech, Birmingham, AL). Lastly, for the terminal transferase dUTP nick end labeling assay (TUNEL), sections were incubated with 0.02 mg/ml

proteinase K (Promega) at 37 °C for 15 minutes. Incubation was done with terminal transferase reaction mix (Roche, Indianapolis, IN) at 37 °C for 45 minutes, and developed with diaminobenzidine substrate using the avidin-biotin horseradish peroxidase system (Vector Labs, Burlingame, CA).

Differentiation Quantification Methods

Tissue sections were double stained for NeuN/GFP (neurons marker), GFAP/GFP (astrocytes marker), or NG2/GFP (oligodendrocytes precursors marker). Quantification of GFP positive cells at dentate gyrus were taken using a 10x10 counting grid at 10x, and colocalization at 20x. Immunofluoroscent markers were assessed using a confocal microscopy (Olympus) coupled with LaserSharp 2000 software.

Neural Stem Cells Surgeries

Tet-DTA/CaM mice (4–6 months of age) were induced for 25 days by withdrawing doxycycline from their diet. After 25 days, doxycycline was re-introduced into their diet to limit the extent of the lesion. Eleven days after doxycycline was re-supplemented, stem cell surgeries were performed. Mice were anesthetized with Avertin 0.6mL/25g body weight. Stem cell injections were performed in the following coordinates: A/P -2.06, M/L ±1.75, and D/V -1.75 (coordinates were determined by the mouse brain atlas by Franklin and Paxinos, 1997). NSCs and vehicles were stereotactically injected bilaterally with 2 μL at the rate of 1 μL/min. Separate syringes were used for stem cell and vehicle injections. Syringes were thoroughly rinsed with deionized water, then buffer after each injection. Because the brain size of the induced mice was relatively reduced due to neuronal loss, pilot attempts were performed to determine the appropriate coordinates of the injection sites. We decided that M/L ±2.00, and D/V -1.85 would be comparable to the non-induced coordinates.

Post surgeries, surgery sites were sealed with bonewax, closed with tissuemend, and antibiotic ointment was applied. Mice were then returned to their cages, which were placed on heating pads.

Behavioral Paradigm and Procedures

Habituation. Each behavioral group contained n=8–13 mice. Mice were handled on three consecutive days. On day 4, mice were habituated in groups of three or fewer for 15 minutes each. The following day, mice were caged individually for 10 minutes each, and 5 minutes on day 6. On day 7, half of the mice received the place test and the rest received the object test. On day 9, the groups received the opposite tests.

Objects used were similar in height (approximately 1.5 in), and were plastic or metal. Two rectangular cages 18" x 9.5" x 10" were used (Figure 2). All explatory activities were videotaped and followed well-known and documented behavioral procedures specifically for object and place memory tests (Mumby et al., 2002).

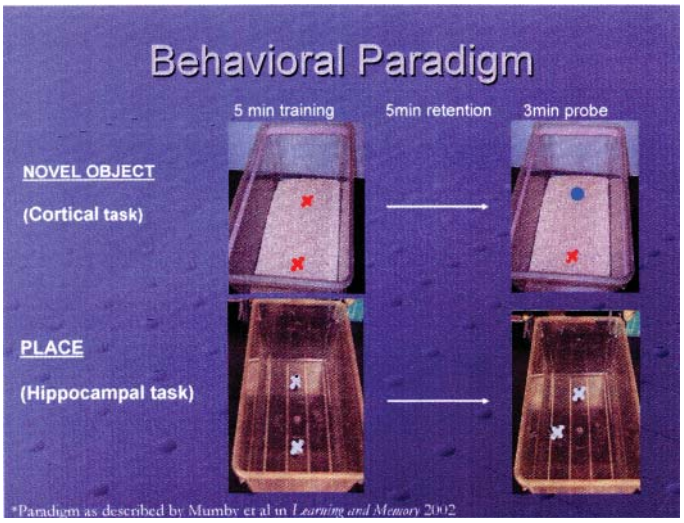

Figure 2

The cages used to test object and place recognition tasks (Yamasaki, 2007).

For scoring, the timing did not begin until the mice moved from their initial position. Exploration was scored if the mouse was within one inch of the object. Activities such as being close to the object (but farther than one inch), or chewing or standing on the object did not count.

Object and Place Recognition Tests. Mice were placed in separate cages with two identical objects. Both mice were allowed to explore the object for 5 minutes, then were returned to their home cages for a five-minute retention period. For the object recognition, after 5 minutes lapsed, the mice were returned to their testing cages. However, one of the objects was removed and replaced with a novel object. This part of the testing lasted for 3 minutes. Similarly for the place recognition, the mice were returned to their individual testing cages after the retention period. However, instead of introducing a novel object, one of the objects was relocated from its original position.

Results

We developed a novel transgenic mouse in which it is possible to ablate a specific subtype of neurons in a tightly regulated fashion. This approach uses the tet-off system to inducibly drive the expression of diphtheria toxin in the absence of doxycycline. The inducible property of this system allows us to control the extent of the lesions. Further, controlling the length of induction allows us to monitor the extent of recovery. In this study, we used the system as a model to determine the potential of stem cell transplantation as a therapy for cognitive deficits from an induced lesion.

Reverse Transcriptase Assay

Strict transcriptional regulation of the diphtheria toxin expression is extremely important. Our tet-off system meets this requirement to ensure there is no detectable leakiness of the toxin transcription when mice are on doxycycline water. The diphtheria toxin acts to inhibit RNA translation, which may have a detrimental effect on living cells. To analyze this, we used a sensitive reverse transcriptase assay to determine the precise time point of diphtheria toxin expression after the doxycycline was removed. We found that diphtheria toxin expression was first apparent at eight days post induction (Figure 3 left). Thus, we concluded that it takes eight days for a sufficient amount of the toxin to be detected in the brain after transgene induction. This is the same amount of time that is required to remove the doxycycline from the system.

In addition, our tet-off system provides the option to disrupt the toxin expression when doxycycline is re-introduced into the diet. Our reverse transcriptase PCR showed that diphtheria toxin was no longer detectable seven days following the addition of doxycycline back into the diet. Similarly, we assume that seven days is the time point at which sufficient doxycycline has accumulated in the system to effectively suppress expression (Figure 3 right). More importantly, the reverse transcriptase PCR data also showed that no diphtheria toxin was detected in non-induced mice that had been supplemented with doxycycline water (data not shown).

Histopathology

We evaluated the spatial control on a histopathological level. We analyzed the extent of diphtheria toxin expression and its effects at the cell level. Using the H&E stain, we found that at 20 days of induction, neuronal loss became apparent (Figure 4B,E,H,K). At 30 days of induction, substantial neuronal loss in the CA1 region was evident (Figure 4C,F,I,L).

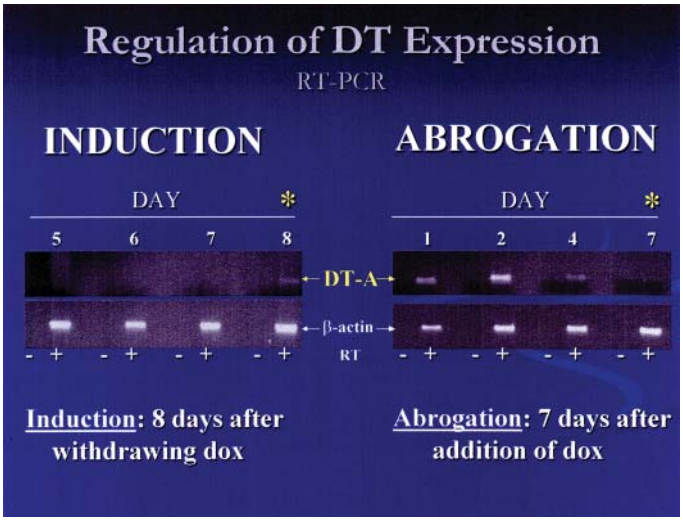

Figure 3

The reverse transcriptase image on the left shows that the expression of diphtheria toxin is first apparent by the eighth day after doxycycline withdrawal. The image on the right shows that diphtheria toxin is no longer detectable by the seventh day after re-introducing doxycycline to the water (source: Dr. Tritia Yamasaki).

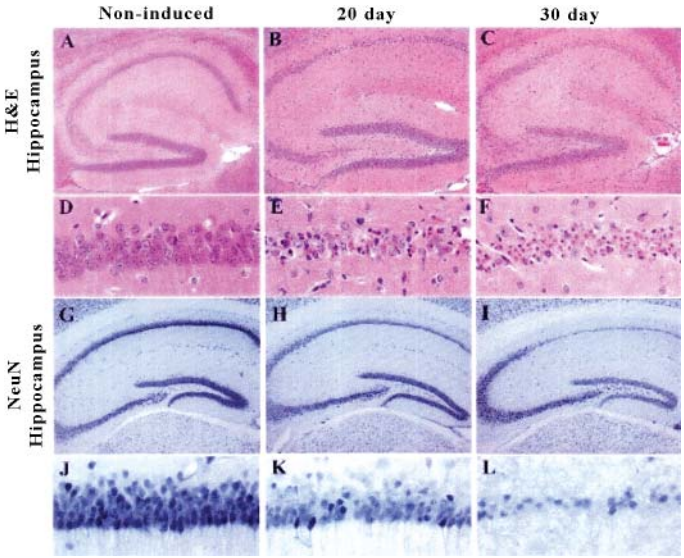

Figure 4

(A, D, G, J) Hippocampal region tissues were stained using H&E and NeuN. These non-induced mice show no apparent neuronal loss. (B, E, H, K) At day twenty, induced mice hippocampal regions show selective neuronal loss especially in the CA1.(C, F, I, L) After 30 days of induction, the hippocampal region depicts substantial neuronal loss (Source: Dr. Tritia Yamasaki, Jasmin Tanaja, Colin Tran, and Tom Tu)

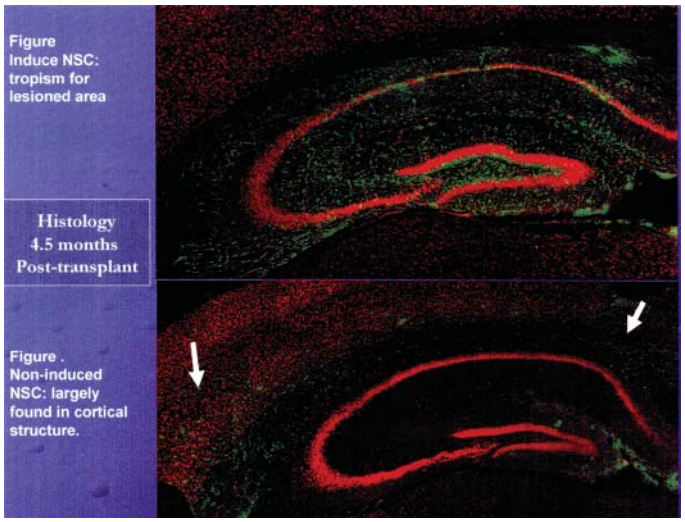

Figure 5

(Top) The non-induced image shows neural stem cell migration, mostly toward the cortical and white matter regions. (Bottom) In induced mice, the neural stem cells' migration route shows tropism for areas with neuronal loss. (source: Dr. Yamasaki and Jasmin Tanaja)

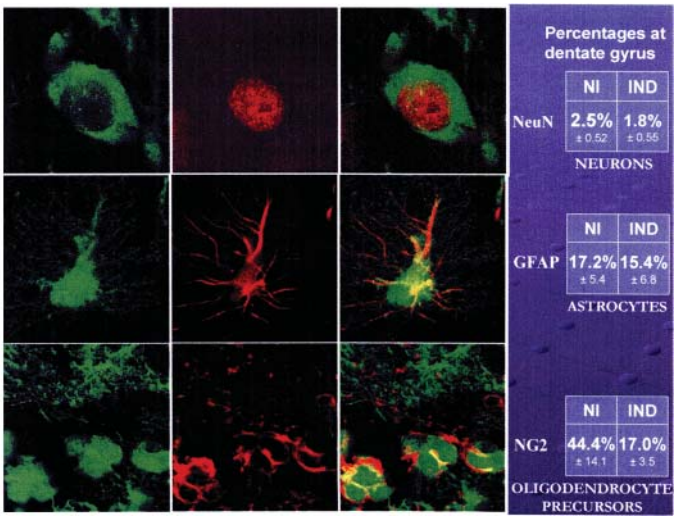

Figure 6

The transplanted neural stem cells differentiated into neurons, astrocytes, and oligodendrocytes precursors (source: Dr. Yamasaki, Jasmin Tanaja, and Colin Tran).

Migration Trend

We also assessed the migration trend of transplanted neural stem cells. A number of studies have shown that transplanted stem cells show tropism for degenerating sites, and our results support such a proposal. At 4.5 months post-transplantation, the transplanted neural stem cells survived robustly. The difference in migration is clearly evident between the induced and non-induced mice. In induced mice with neuronal loss, neural stem cells populate the hippocampus (Figure 5, bottom); in non-induced mice, neural stem cells migrated to the cortical and white matter region (Figure 5, top).

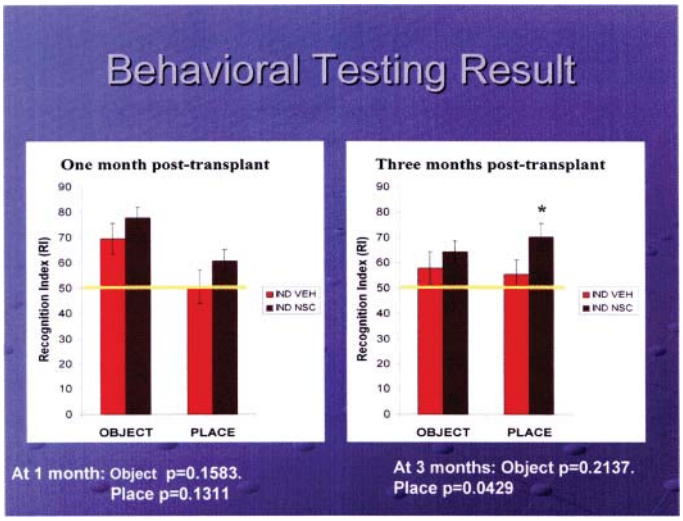

Figure 7

Behavioral results for object and place recognition tasks. Tet-DTA/CaM mice were induced for 25 days. (Left) At one month, although the NSC-transplanted mice outperformed the vehicle injected mice, p values do not show that they are statistically significant. (Right) At three months, the NSC-transplanted mice did significantly better than the vehicle injected mice in the place recognition task (p=0.0429) (Source: Dr. Tritia Yamasaki, Jasmin Tanaja, Colin Tran).

Statistical Analysis

Our data has shown that the transplanted stem cells have a tropism for regions with extensive neuronal loss. In addition, they are also able to differentiate to specialized subtypes. Semi-quantitative analyses indicate the result as follows: 1.78% ± 0.515 neurons, 15.39% ± 6.76 astrocytes, and 17.07% ± 3.49 oligodendrocyte precursors (Figure 6).

We hoped to show that the transplanted stem neural cells offer a therapy means to improve cognitive deficit (in brain lesions characterized by neuronal loss). We assessed whether there is a significant difference between the induced and non-induced mice in the performance of hippocampal and cortical dependent tasks. We tested our Tet-DTA/CaM mice at one and three months post transplantation. At one month, although the results do not show a significance difference, the NSC-transplanted mice performed better than the vehicle-injected-mice in both object and memory tasks (pobject=0.1583, pplace=0.1311, Figure 7 left). In contrast, at three months post-transplantation, NSC-mice performed significantly better for place recognition than the vehicle mice. It is interesting to note that place recognition is heavily dependent on the hippocampus (pplace=0.0429, Figure

7 right). Further, at three months post-transplantation, the NSC mice did not perform significantly better than the vehicle mice in object recognition task (pobject=0.2137). This is not surprising because object recognition is a cortical-dependent task.

Discussion

The purpose of this study is to determine the ability of the transplanted neural stem cells to improve cognitive deficit in response to an induced lesion. In many neurological conditions, where neuronal loss is prominent, cognition impairments are often profound. Neural stem cells offer promise as a way to replace these neuronal losses, mainly due to their basic properties of self-renewal and ability to differentiate into one or more subtype cells. Further, previous studies have shown that these stem cells display tropism for damaged regions. Our novel animal model allows us to explore whether a cognitive deficit can in fact be improved following a lesion (characterized by substantial neuronal loss). In collaboration with our Tet-off system, we are able to perform a precise spatial and temporal regulation. Our results show that there is no diphtheria toxin leakiness during the non-induced period. Further, the ability to regulate the length of induction enables us to control the extent of lesion. These are important factors in establishing how successful the transplanted neural stem cells are in improving cognition following extensive and selective neuronal loss.

There were several significant findings in this study in terms of the ability of neural stem cells to survive, migrate, and differentiate. We found that the transplanted neural stem cells survived for at least 4.5 months. At this time point, we also found that the transplanted stem cells had migrated and differentiated into neurons, astrocytes, and oligodendrocyte precursors. It is interesting to note that previous studies show a down regulation of GFP expression with differentiation (Vroemen et al., 2005; Zhang et al., 2004). Given this speculation, our semi-quantitative analysis may greatly underestimate the numbers present.

Our data also shows remarkable differences in the migration pathway the transplanted neural stem cells followed. In the non-induced mice, we found that the stem cells favored the cortical and white matter regions. However, in the induced group, we found that the stem cells had an unusually strong preference for the area of lesion. This is consistent with previous studies in which transplanted stem cells have a tropism for neurodegenerative regions (Aboodi et al., 2000; Imitola et al., 2004; Kelly et al., 2004). Areas with extensive neuronal loss or damage of normal tissue are believed to release trophic cytokines, which may act as a signaling cue to attract the stem cells.

We would eventually like to show that the transplanted neural stem cells make a significant difference in improving cognition impairments. We therefore assessed the induced and non-induced mice behaviorally. Previous studies report conflicting results; however, our experimental design is unique for three important reasons. First, we took advantage of the innate tendency of mice to explore novel objects without introducing a stressful environment. Second, the test we used did not introduce the level of stress that is found in more commonly used paradigms of contextual fear conditioning or the Morris Water Maze. Finally, our precise method of induction targets focal and specific subtypes of cells and gives us tight control over the induction length.

We assessed both groups (induced and non-induced) at two different time points. At one month, although the mice transplanted with neural stem cells outperformed the vehicle injected mice, the difference was not significant (in both object and place recognition). Studies suggested that transplanted stem cells take approximately one month *in vivo* to develop electrophysiological responsiveness of mature neurons (Auerbach et al., 2005; Englund et al., 2002). This implies that one month is the smallest amount of time for the neural stem cells to establish themselves. At three months post-transplantation, our data showed a significant difference between the NSC transplanted mice and vehicle-injected mice during the place recognition task (which is hippocampal dependent). We found no significant difference in the object recognition task, which depends more strongly on cortical regions. This is consistent with the fact that we noticed a significant improvement in the place recognition task—a hippocampal-dependent task—at three months. Finally, although we noticed an improvement trend, the object-recognition task at three months did not show a significant difference. This is not surprising because object-recognition is a largely cortical-dependent task (Clark, 2000; Tang 1997).

Our findings provide strong evidence that transplantation of neural stem cells may be an effective therapeutic approach in improving conditions characterized by impaired cognition or memory loss following extensive damage to normal tissue. Further experiments may involve the use of trophic factors to study their effects in influencing the trend of neural stem cell differentiation.

Acknowledgements

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