COVER SHEET

TITLE: Nurr1 knockout in substantia nigra proposed to result in cognitive deficits in Rattus norvegicus

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DEPARTMENT(2):

YEAR: 2017

ABSTRACT

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Many Parkinson's disease (PD) patients suffer from cognitive impairment. Reduced secretion of dopamine (DA) from substantia nigra (SN) to striatum results in motor deficits in PD patients, but little is known about its effects on cognition. Nurr1 is a nuclear receptor expressed in SN neurons and regulates DA expression. The role of Nurr1 in PD-related cognitive deficits has not yet been studied. We hypothesize that reduced levels of Nurr1 in the SN, leading to decreased DA in striatum, will result in cognitive deficits in *Rattus norvegicus*. Knockdown of Nurr1 in SN with an anti-Nurr1 ribozyme was unsuccessful. CRISPR/Cas9 will be used to knockout Nurr1 before performing cognitive and behavioral testing to help reveal the role of Nurr1 in the cognitive behavior of PD patients and potentially implicate overexpression of Nurr1 as a treatment for cognitive impairments.

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Nurr1 knockout in substantia nigra proposed to result in cognitive deficits in *Rattus norvegicus*

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Abstract

Approximately seventy percent of Parkinson's disease (PD) patients suffer from cognitive impairment. It is known that the reduced secretion of dopamine (DA) from substantia nigra (SN) to the striatum results in motor deficits in PD patients, but little is known about its effects on cognition. The prefrontal cortex and hippocampal regions are involved in cognitive behaviors that are commonly affected in PD. These brain regions are connected to the striatum and indirectly to the SN. Nurr1 is a nuclear receptor expressed in SN neurons and regulates the expression of DA. The role of Nurr1 in PD-related cognitive deficits has not yet been studied. We hypothesized that reduced levels of Nurr1 in the SN, which results in decreased levels of DA in striatum, will affect prefrontal cortical and hippocampal function and result in cognitive deficits in the animal model. Nurr1 was knocked down in the SN of rats using an anti-Nurr1 ribozyme, but no significant knockdown was detected. Therefore, we have designed the guiding RNA to develop a Crispr/Cas9 Nurr1 knockout to inject for cognition testing using several behavioral tasks. The results from this study will help reveal the role of Nurr1 in the cognitive behavior of PD patients and implicate overexpression of Nurr1 as a potential treatment for cognitive impairments.

Introduction

Cognitive disabilities, such as memory deterioration and difficulty focusing, appear early in Parkinson's Disease (PD), prior to severe motor impairments [1]. Correlation between a deficiency of the neurotransmitter dopamine (DA) and these cognitive deficits, has been found [2,3]. The prefrontal cortex helps regulate cognitive functions, such as planning, decision-making, and memory formation [4]. The striatum has been found to be involved with solidifying habits and skills, while the hippocampus deals with encoding memories [5,6]. The substantia nigra (SN) contains projections to the striatum, which in turn projects to the hippocampus and prefrontal cortex [7]. Therefore, we propose that alterations in the SN will affect hippocampal and prefrontal cortical function. In fact, in animal models of PD, 6-hydroxy- dopamine (6-OHDA) lesions in the SN result in sensorimotor deficits and decreased length, branching, and density of dendritic spines in the prefrontal cortex and nucleus accumbens. Furthermore, this model is known to have reduced levels of DA in the striatum, resulting in spatial memory loss, depression, and impaired reaction time [8,9].

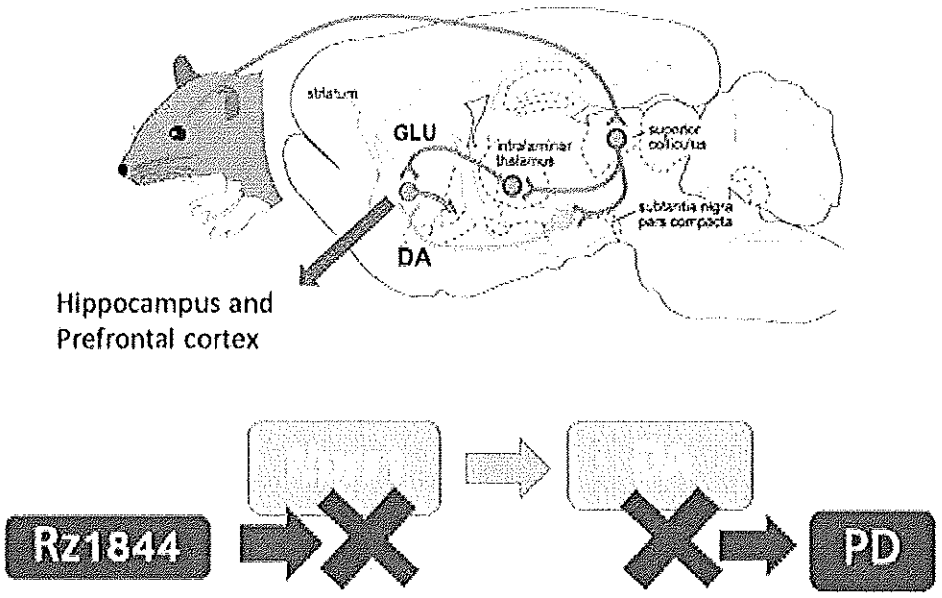

Figure 1. Diagram depicting the connections of the *Rattus norvegicus* brain. The substantia nigra (SN) has projections to the striatum which then projects to the hippocampus and prefrontal cortex. Alterations in the SN are thus thought to affect hippocampal and prefrontal cortical function. The anti-Nurr1 ribozyme Rz1844 knocks down Nurr1 mRNA production by about 60% and we hypothesized that reducing Nurr1 in the SN with this ribozyme would reduce striatal DA levels and thus prefrontal cortex and hippocampal function as seen in Parkinson's Disease (PD).

Nurr1 (*NR4A2*) is a transcription factor that regulates the production of DA in the SN. In addition, Nurr1 is involved in learning and memory formation in the hippocampus [10,11]. Decreases in the Nurr1 protein resulting from mutations in the *NR4A2* gene have been found to be associated with PD and Alzheimer's disease [12].

An anti-Nurr1 ribozyme (Rz1844) was developed by our lab that knocks down Nurr1 mRNA production in the targeted SN by approximately 60% (Figure 2 and [13]). Ribozymes are RNA enzymes that cleave mRNA in a sequence-specific manner, resulting in nonfunctional mRNA. Our lab has shown that expression of anti-Nurr1 ribozyme results in reduced extracellular DA in the striatum ([13] and Figure 2). Using this Nurr1 ribozyme we have generated an early-stage model of PD with cognitive impairments that have been found to precede motor deficits in PD patients [1].

We hypothesized that reduced levels of Nurr1 in the SN, which results in decreased levels of DA in striatum, will reduce prefrontal cortical and hippocampal function. In order to test this, we knocked down Nurr1 using Adeno-Associated viral vector delivery of anti-Nurr1 ribozyme (AAV5-Rz-1844) to the SN of rats. A control vector (AAV5-GFP) was injected into the SN of control groups [13]. Multiple behavioral tasks were performed and rats expressing the Nurr1 ribozyme were expected to display cognitive deficits relative to controls. The open field task was used to test exploratory, locomotor, and anxiety-like behavior. The Morris water maze (MWM), contextual fear conditioning task, and T-maze were used to study multiple forms of memory via hippocampal function. A working memory version of the MWM was used to evaluate prefrontal cortex function.

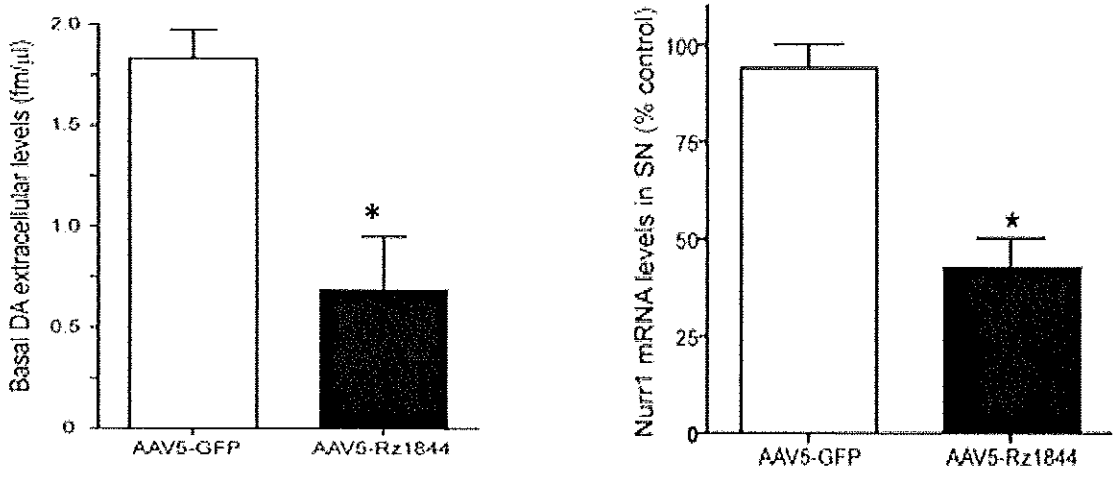

Figure 2. Nurr1 knockdown in the substantia nigra (SN) leads to decreased dopamine (DA) in striatum. Left. Basal extracellular DA levels in striatum found to be significantly lower in ribozyme experimental groups injected with AAV5-Rz-1844 as compared to AAV5-GFP controls. Relative amounts based on injected vs uninjected hemisphere ($p = 0.0005$). Right. Relative amount of Nurr1 mRNA in SN of controls injected with AAV5-GFP and ribozyme experimental groups injected with AAV5-Rz-1844 ($p=0.0079$).

Methods

Experimental Groups: Sprague-Dawley rats were housed in groups of 2 under 12/12-light/dark cycles and given access to food and water as necessary in accordance with animal care protocols. There were four experimental groups: (10 rats with unilateral ribozyme injections, 10 control rats with unilateral injections, 10 rats with bilateral ribozyme injections, and 10 control rats with bilateral injections). Unilateral injections of AAV-Rz-1844 into the SN allowed for identification of possible motor asymmetries from the knockdown manipulation. This is important to ensure that animals do not have motor deficits that might affect their performance. If the Nurr1 ribozyme results in motor deficits, the animals would show motor impairments on the contralateral side of the injection, since motor cortex in one hemisphere controls movements on the opposite side of the body. Preliminary data from our lab indicated that these Nurr1 Rz-1844 animals or injected controls did not suffer any obvert motor deficiencies. Next, we tested the groups of animals that received bilateral injections of AAV-Rz-1844 or AAV-GFP into both SN hemispheres. Knocking down both hemispheres was important to completely block behavior. Three weeks after the injections, the animals underwent several learning tasks to test cognitive functions as related to PD including anxiety, prefrontal cortex (working) memory, striatal (procedural) memory, and hippocampal memory.

Behavioral Tasks: The *limb-use asymmetry test* was used as a control for motor deficits. This test involved videotaping the animals as they reared on a Plexiglas wall. Touches of the left and right paws were recorded for 20 total contacts. If motor function was not affected, rats used both paws equally.

The *open field* task tested exploratory, locomotor, and anxiety-like behavior. Animals were placed in a Plexiglas box (40.65cm x 40.65 cm x 30.5 cm) and videotaped while they freely

explored for 15 minutes. Distance ambulated in 15 minutes and the time spent in the center of the box/ total area was measured. Animals with motor impairments did not ambulate as much as normal animals.

The *Morris water maze* was used to study both spatial memory, a hippocampal function, and working memory, a prefrontal cortex function. Distance swam while seeking the platform in the allotted amount of time was measured. In order to study spatial memory, the rat was placed in a cylindrical pool of water where a hidden platform (5cm underneath the surface) was placed in one section as the only escape from the water [14]. The animal was placed in the water in a different quadrant of the pool each training trial and allowed to find the platform using spatial cues in the room. If the animal did not find the platform in 90 seconds, it was placed on the platform and allowed to rest for 10 seconds. Then, the rat was picked up and dropped from the next point. This training was performed over five days with four trials per day. After the fourth trial on the fifth day of hidden platform training, the platform was removed and the animal could swim for 60 seconds. If approximately 25% of the swim distance was spent in the target quadrant, the animal was not considered to have learned the task, but was instead behaving due to chance. This tests spatial memory. The testing of working memory was performed for the subsequent 7 days with two trials each day. Platform location was changed each day, but remained the same within one day for both trials. Release point of the animal however was different for each trial.

Contextual fear conditioning. The first day was dedicated to training in which the rat was placed in a closed box and a series of events occurred. Training began with 2 minutes of exploring followed by 30 seconds of a tone and a shock for the last two seconds. The process was repeated and then one minute was allowed for exploration. Hippocampal function was then measured 24 hours after training using context testing. The animal was placed into the same box and allowed to

explore for 6 minutes. The amount of time the animal freezes without movement was measured to test for learned fear from the environment only.

The *T-Maze* was used to test habit learning [15]. Animals had a choice of two arms and the correct one contained a reward. Once they were trained to find the reward, the T-maze was rotated 180 degrees. Place learners chose to enter the location in space in which they previously received a reward (e.g. turn west despite T-maze position; Hippocampal function). Response learning was indicated by the rat choosing to go left or right within the T-maze, depending on where it last received a reward (Striatal function). Animals with hippocampal deficits use a response strategy.

Analysis: Behavioral tests were analyzed using a t-test with significance set at $p < 0.05$. Levels of Nurr1 knockdown in the SN of control and experimental animals was determined by reverse transcription Real-Time PCR, as described previously [13]. Following the protocol from the Applied Biosystems StepOnePlus Real-Time PCR manual (http://www.mbl.edu/jbpc/files/2014/05/ABI_StepOnePlus_qPCR_ReagentGuide.pdf), data was analyzed using a double delta Ct analysis to find an expression fold change for Nurr1.

Results

Behavioral Tasks: We did not find statistical differences in any of the behavioral tests (Limb-use asymmetry test, T-test, $p = 0.87$, 0.26, and 0.39 for left paw, right paw and both paws respectively; Open field task, T-test, $p = 0.78$ and 0.64 for time spent in the center and periphery respectively; Morris water maze, T-test, $p = 0.35$, 0.94, and 0.78 for time spent finding the visible platform, hidden platform, and the quadrant where the platform was previously placed respectively; Contextual fear conditioning, T-test, $p = 0.4286$; T-maze, T-test, $p = 0.4327$). Ten animals were used in each group (experimental and control).

Analysis: Following the behavior experiments, Real-Time PCR data of the substantia nigra showed no significant difference in levels of Nurr1 ($p > 0.40$) (Figure 3).

CRISPR/SaCas9: Four guide RNAs were developed for the CRISPR/Cas9 construct, and two were found to have annealed properly after sequencing (Figure 4,5). Currently, we are working on cutting the regions from a pBlue shuttle vector and cloning them into the AAV p601 vector which will be tested in cell lines for Nurr1 knockdown before injection into animal models [16].

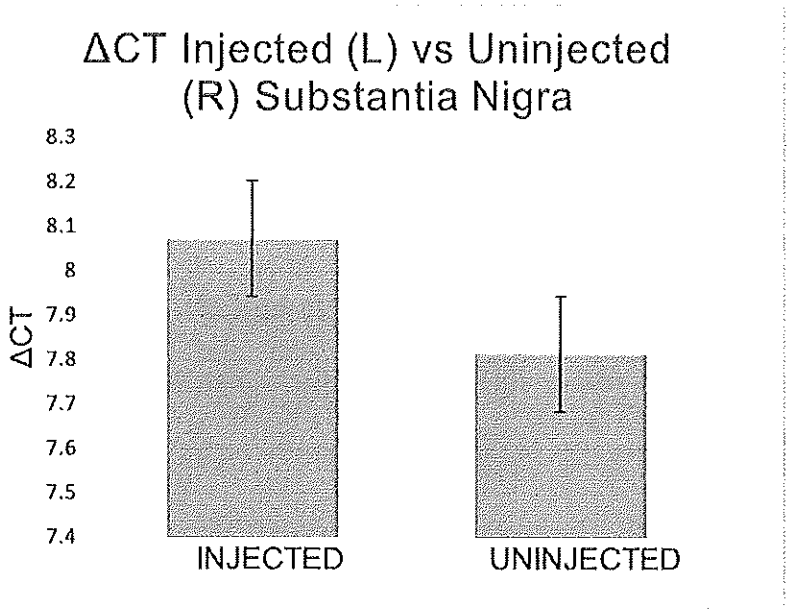

Figure 3. Nurr1 levels in the substantia nigra (SN) not significantly different in injected versus uninjected rats. Mean ΔCT value for ribozyme experimental groups injected with AAV5-Rz-1844 as compared to AAV5-GFP controls (uninjected) found to have no significant difference ($p > 0.40$).

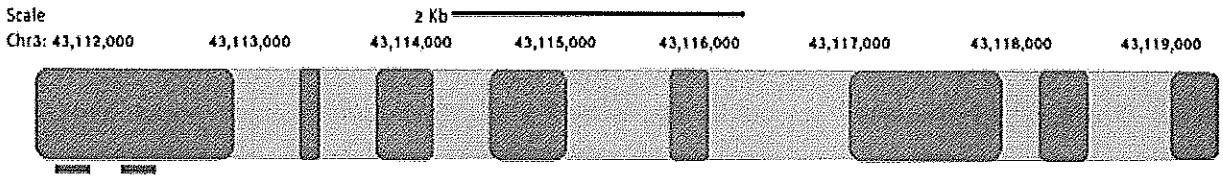

Figure 4. Nurr 1 gene with exons displayed in blue and introns in gray. Red dashes indicate region of primer design for CRISPR/Cas9 construct. Both are at the beginning of exon 1 and consist of 21 nucleotides plus the 6 base pair PAM sequence.

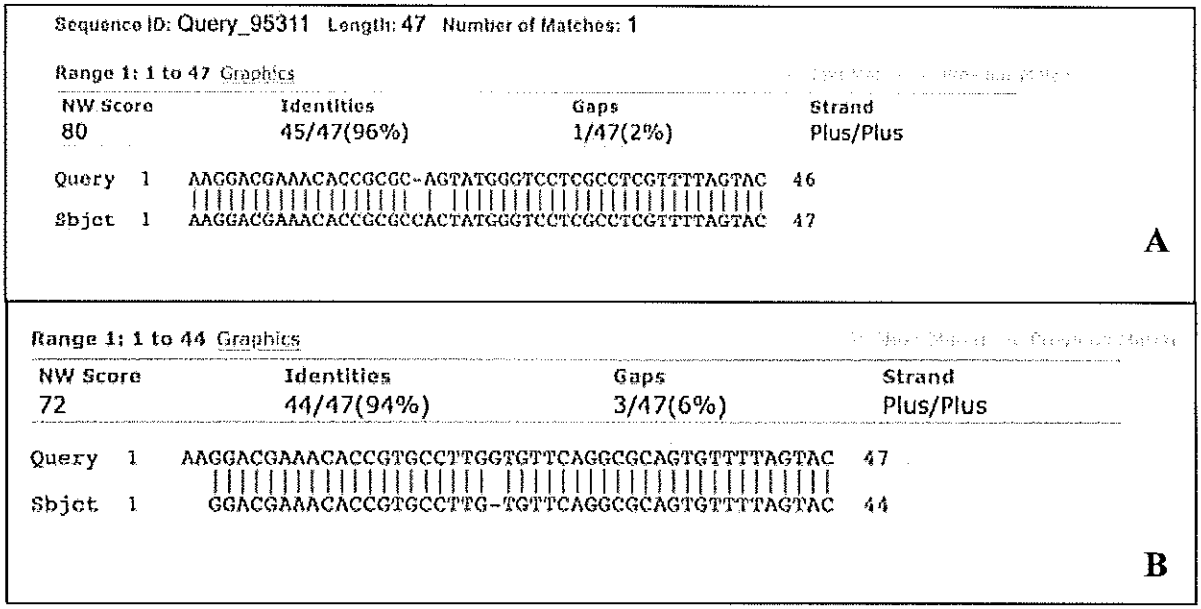

Figure 5. Sequence alignments for guide RNA primers 1 (A) and 2 (B) of CRISPR/Cas9 system and Nurr1 RNA. Primers consist of 21 nucleotides plus a 6 base pair PAM sequence in exon 1 of Nurr 1.

Conclusion/ Future Directions

Even though we have shown the AAV5-Rz-1844 ribozyme to knock down Nurr1 previously, we did not obtain similar results this study. We were surprised to not see any phenotypic behavioral changes in the animals and proceeded to recheck expression by RT-PCR which showed that there was no significant Nurr1 knockdown in experimental versus control groups. The lack of downregulation could be due to problems with the viral vector degrading or technical problems with stereotaxic injections into the SN. Due to the 60% knockdown usually accomplished by AAV5-Rz-1844 and the lack of any knockdown in our previous experiment, we decided to take a different approach and create a CRISPR/Cas9 construct to obtain complete knockout of Nurr1 in order to determine the impact of this protein on cognition. We have cloned these sequences to test in cell lines and then generate a viral preparation for injection into the SN of rats. In addition, we may overexpress alpha synuclein, a protein believed to help regulate the release of DA from presynaptic terminals, in another genetic model [17]. Comparing the severity

of cognitive deficits in various models may reveal why PD patients often show different types and degrees of cognitive alterations. However, the findings in this study will further elucidate the role of Nurr1 in the SN and cognitive deficits observed in PD neuropathy. Implications for a neuroprotective treatment of PD by overexpression of Nurr1 in the SN may result.

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