Evaluation of the kidney-brain axis in mice: Effects of nephrotoxic serum nephritis on murine behaviour and structural brain changes

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Abstract

Acute kidney injury (AKI) and chronic kidney disease (CKD) are common disorders that are associated with a substantial morbidity and mortality burden. In spite of this, there is limited data available on the effects of impairments in renal function on behavioral performance, and the underlying mechanisms by which acute or chronic azotemia giving rise to abnormal behavior still remains poorly understood.

The purpose of this study was to elucidate the changes in brain structure and behavioral performance which occurs concomitant to a sudden decrease in renal excretory function.

Anti-GBM Glomeruli Nephritis (GN) was induced by the administration of a heat inactivated rabbit anti-mouse GBM antiserum called nephrotoxic serum nephritis (NTS) intravenously via the tail vein. A Urine Albumin ELISA & Creatinine Assay was conducted to confirm proteinuria.

To determine whether AKI results in adverse effects on behavior, C57BL/6N GN and control female mice were subjected to a battery of behavioral tests. Behavioral studies like the Open Field test (OF) that analyzes hyperactivity and locomotive

activity, Elevated Plus Maze (EPM) including the Light/Dark box experiment that tests for anxiety were performed. Our findings show that although GN mice are hypo active in the Open Field; they exhibit a hyperactive phenotype in the Light and Dark box test. GN mice also exhibited a depressive phenotype in the Tail Suspension Tail suggesting that C57BL/6N mice administered with NTS could potential cause brain injury thus causing neurobehavioral changes.

Introduction

In kidney disease it has been shown that the kidney-brain axis exists in a model of renal ischemia reperfusion injury (IRI) (4), but so far there is no data on inflammatory kidney disease models such as the experimental glomerulonephritis model.

The clinical symptoms seen in the changes that take place in acute kidney injury's infliction on the Central nervous system range from decreased mental status to obtundation including seizures in patients. In patients with acute kidney injury the symptoms of encephalopathy are generally more pronounced and progress more rapidly than with chronic kidney disease or end stage renal disease (5, 6). Although neurologic sequelae of acute kidney injury are well established, the pathogenesis of acute uremic encephalopathy is poorly understood. A great number of uremic toxins have been implicated in playing a role in the pathogenesis of uremic encephalopathy (7) and more recently Liu et al. 2008; provided compelling evidence that inflammation might be involved in mediating brain injury due to renal IRI (4). So far, there exists no evidence on the brain-kidney axis in an inflammatory kidney disease model such as NTS, which leads only to marginal elevations of serum-creatinine without progressing to terminal kidney failure during a follow up for more than 6 months (Eller K, unpublished observations). NTS is an inflammatory kidney disease that is induced by the injection of a rabbit anti-mouse glomerular basement

membrane (GBM) antibody and accelerated by a preceding immunization against rabbit IgG. Animals with NTS present with proteinuria, proliferative and inflammatory glomerular changes including crescent formation and leukocyte infiltrates, which are mainly located in the periglomerular and interstitial region (8, 9). It has been proven to be dependent on Th17- as well as Th1-cells (10, 11). Early treatment with immunsuppressive substances such as the mTOR inhibitor Rapamycin has been proven to protect from disease by suppressing both the production of antibodies as well as the T cell response (12). Our studies look at the effect of NTS and its relation to the brain-kidney axis. Although studies conducted by Liu et al. (4) provided evidence in regards to Acute Kidney Injury leading to inflammation and functional changes in the brain; our model provides an alternative approach where mice are not subjected to acute surgical procedures. Because this study focuses on the innate behaviour of mice to explore, surgery can introduce additional variables within the behavioural findings. Although Liu et al. (4) provide behavioural data in the Open Field in regards to locomotive activity, there is a need to conduct additional behavioural studies in order to appreciate the effects of acute kidney injury on the brain. Here, we provide preliminary data on the effects of NTS on the brain kidney axis by conducting a series of behavioural tests likes the Open Field test (OF) that analyzes hyperactivity and locomotive activity, Elevated Plus Maze (EPM) including the Light/Dark box experiment that tests for anxiety respectively.

Methods

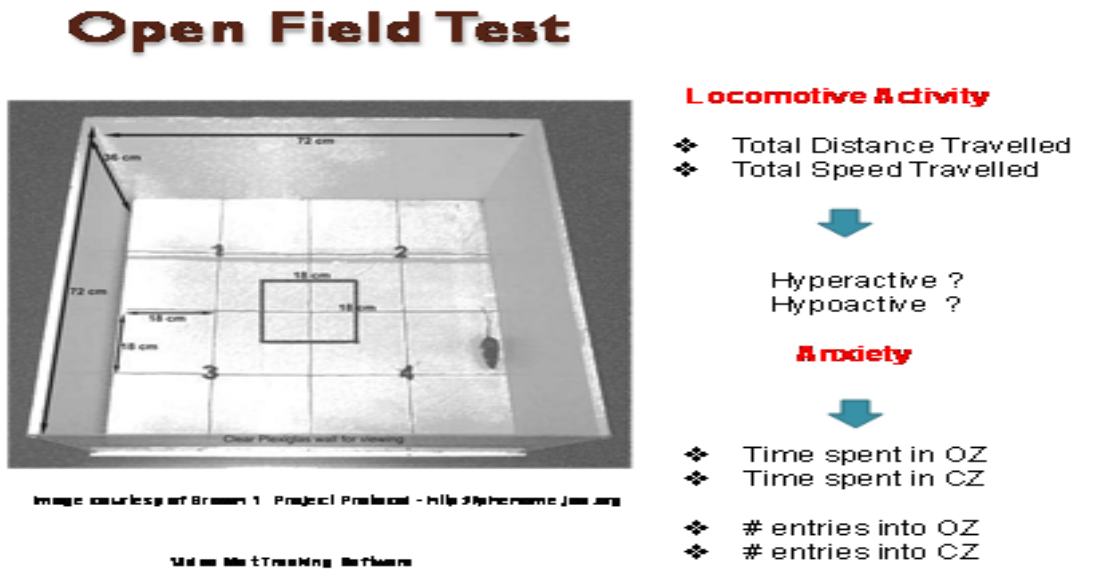
Subjects and handling procedures

Experimentally naïve C57BL/6N mice (*N* = 15 Females: Control *N* = 5; Immunized *N* = 9; and GN *N* = 9: were housed in groups of 4 or 5 per cage under controlled temperature (22°C) and humidity (50%), lights on at 6:00 h, lights off at 18:00 h, maximal light intensity 100 lux. Mice were given food and water *ad libitium* and were regularly handled at least an hour per day for one week prior to testing. Handling and testing of mice were conducted at the same time each day to avoid introducing external variables. Mice were subjected to behavioral testing at 3 months of age. All behavioral experiments were conducting during the light phase. An acclimation period of one hour in the same room where the behavior test was conducted was performed prior to the beginning of all behavioral tests. Mice remained in their home cages during these acclimation periods. Prior to the beginning of the test, the mice were transferred into a test cage and the testing room was illuminated with white light during the open field test (70 Lux) and during the Light/Dark box test (390 Lux). All mice were injected in the tail intravenously inorder to deliver the saline or drug on Day 1 and Day 7. The control C57BL/6N mice were injected with saline on Day 1 and Day 7. The immunized mice were injected serum on Day 1 and saline on Day 7. The GN mice were injected with serum on day 1 and day 7 and all three groups were monitored for a total or 21 days. Proteinuria was confirmed by ELISA conducted every week for a total of 3 weeks. All animal care and experimental procedures were approved by an ethical committee at the Federal Ministry of Science and Research of the Republic of Austria and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were

designed in such a way that the number of animals used and their suffering was minimized.

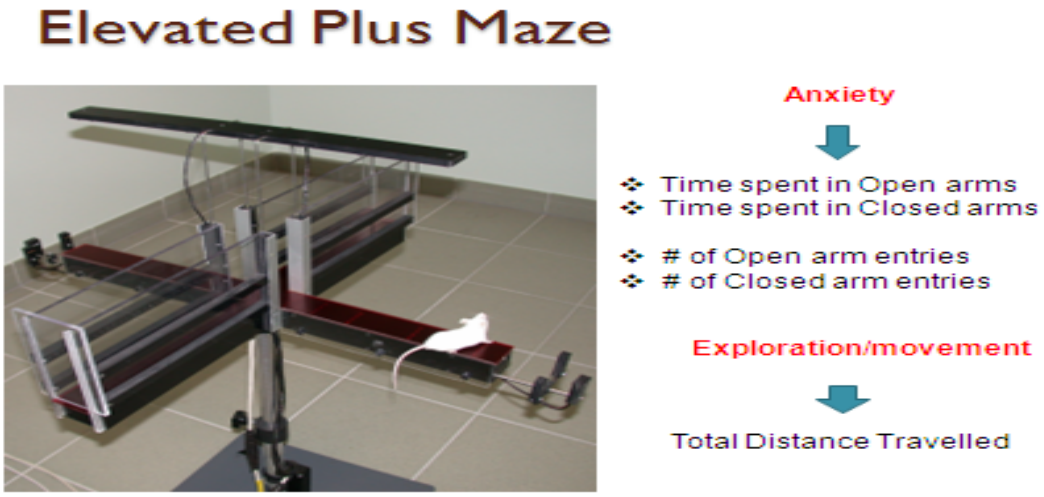
Open Field

The OF consisted of a box (50 x 50 x 30 cm) that was made of opaque grey plastic and illuminated by 70 Lux at floor level. The ground area of the box was divided into a 36 x 36 cm central area and the surrounding border zone. Mice were placed individually in the center of the OF and their behaviors were recorded for 10 minute period. In the OF test, anxiety is assessed by evaluating the level of activity in which mice present in response to a novel area under bright anxiety producing light. In addition, patterns of wall tracing around the perimeter are a typical pattern of behavior, that overtime, subsides as the mouse begins to habituate and explore the center of the arena. Lack of or delays in habituation, in addition to, absence of movement may be indicative of initial assessments for anxiety behavior. The data were subsequently analyzed using VideoMot 2® (TSE Systems) video-tracking software. Post testing, mice were removed and transferred back into their respective home cages and trace odors were removed using 70% ETOH.



Elevated Plus Maze (EPM)

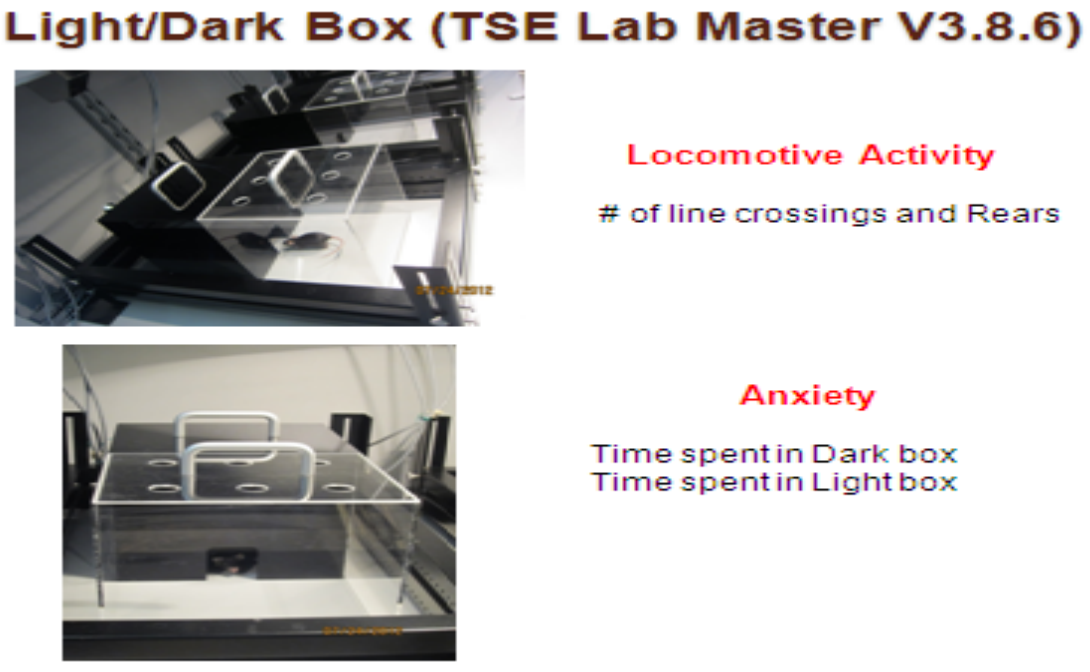
The mice were placed in the center of a maze (5 x 5 cm) with four arms (30 cm long, 5 cm wide) arranged in the shape of a plus- shaped maze equipped with 15 cm high walls at their sides and the far end. The EPM elevated 70 cm above the floor was made of opaque grey plastic. The light intensity at the central quadrangle was 70 lux. Mice were placed on the central platform of the maze facing an open arm and exploratory behavior on the plus-maze was recorded for 10 min by a vertically mounted video camera linked to a monitor and video recorder in an adjacent computer and subsequently tracked by a video camera above the centre of the maze and recorded with the software VideoMot2 (TSE Systems, Bad Homburg, Germany). The elevated plus maze rests on the naturalistic conflict between the tendency of mice to explore a novel environment and the aversive properties of a lit, open arena. Anxiety was measured by analyzing the amount of time spent and entries to the open arms verses the closed arms - decreases in these proportions corresponded to heightened levels of anxiety. Traces of odor were removed by the use of ETOH 70% after each run. All animals were tested during the light phase.



Light & Dark Box Test

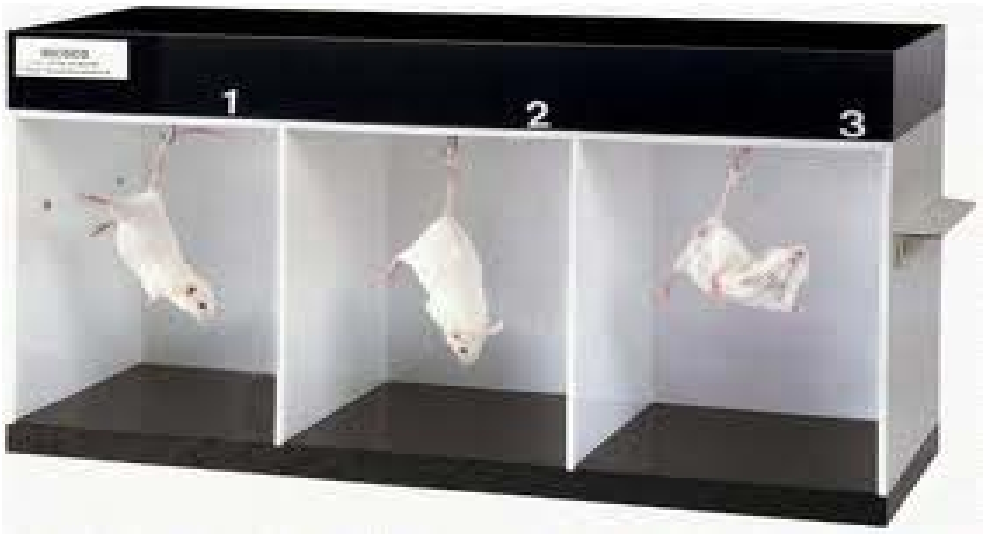
The Light & dark box test comprises of two chambers of equal length (50cm x 50cm) Mice were placed in the Light part of the apparatus in between the door way of the light chamber while facing the dark chamber. The light chamber was illuminated by 390 Lux, while the dark chamber read 0 Lux. The orientation of the mouse facilitated the breaking of the infrared beams which automatically started the recording of the animal's behavior for 10 minutes using the Labmaster setup/Special Analysis-Acti Mot 2 program (TSE systems).

The Light & dark box apparatus tests the innate aversion of mice to brightly illuminated areas and the natural conflict to explore a novel environment. Anxiety was measured by analyzing the amount of time spent in the dark chamber and entries to the light chamber verses the dark chamber – decreases into the light chamber indicates heightened levels of anxiety. Traces of odor were removed by the use of ETOH 70% after each run. All animals were tested during the light phase.



Tail Suspension Test

The tail-suspension test is a behavioral test that accesses and screens for depression related like behaviors. Mice are suspended by their tails with adhesive tape in a position far from nearby surfaces while preventing mice from observing other animals being tested. The mice's behavior is quantified for six minutes and behavior of time spent curling, time spent swinging and time spent immobile are scored.



Tail Suspension Test: Image - Compliments of Science Direct.com

Statstical analyses

Statistical data were computed in *Statistica* V. 6.1 (Statsoft, Inc. Tulsa, OK). All data were conducted using an ANOVA. Significant differences were determined by Tukey's HSD post hoc comparisons test. Significance levels were set at alpha 0.05 with a confidence level of 95%. Data are presented as mean ± SEM

Results

Open Field

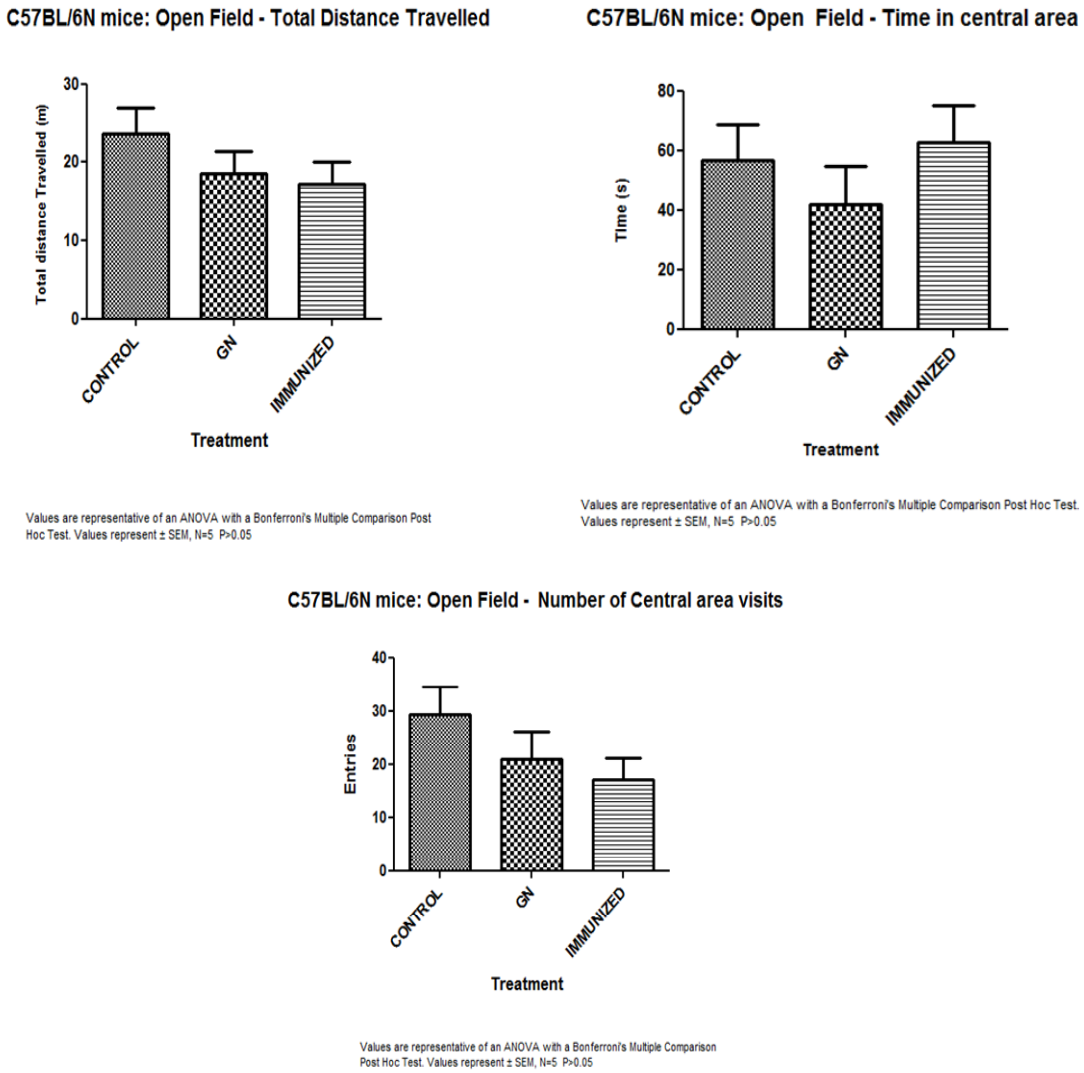
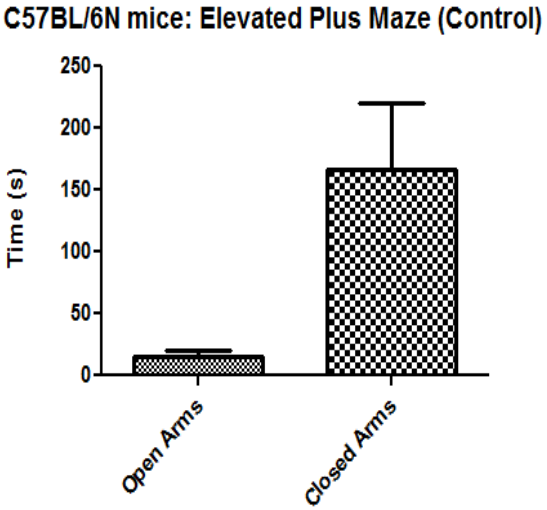


Figure 1: GN C57BL/6N mice were hypoactive in Open Field Test a) GN mice were hypoactive compared to control mice b) GN mice spent less time in the centre zone compared to control and immunized mice c) GN mice entered less into the centre zone compared to control mice.

Elevated Plus Maze



Control Trial: (N=5)

% Time in Open Arms =

$$\frac{\text{(Time in Open Arms)}}{\text{(Time in O/Arms + Time in C/arms)}}$$

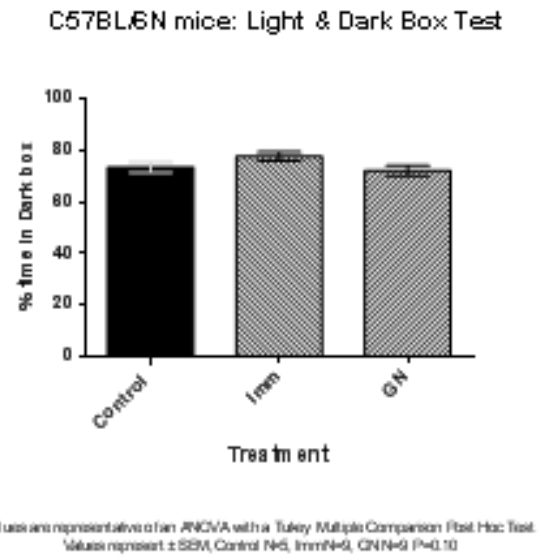
% Time in Open Arms =

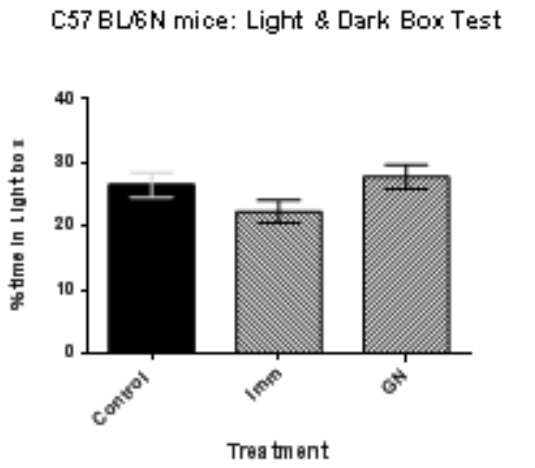
15.3803/(15.383 + 166.065)

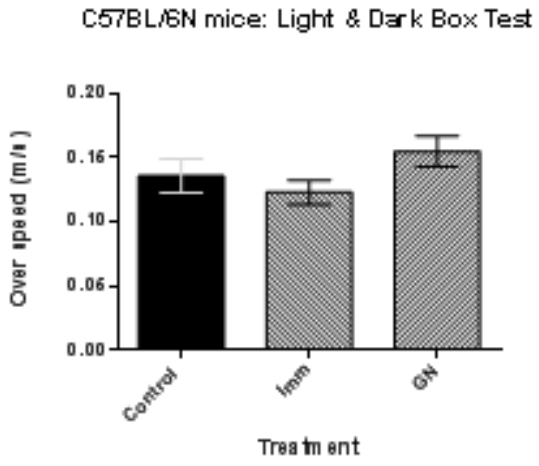
% Time in Open Arms = 8.48

Figure 2: C57BL/6N control mice exhibited a higher anxiety phenotype when placed into the Elevated Plus Maze for 10 minutes b) C57BL/6N mice exhibited higher anxiety by failing to enter into the open arm at least 20% of the entire trial interval

Light & Dark Box Test







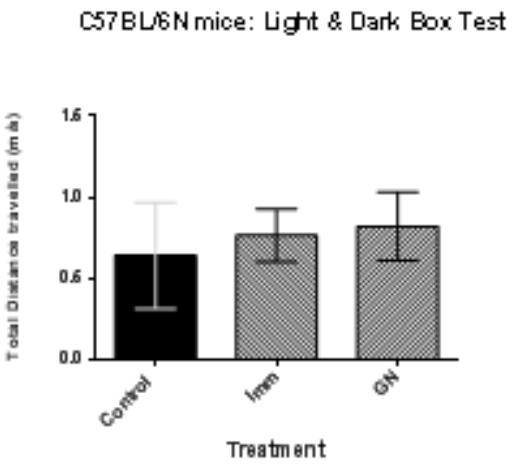
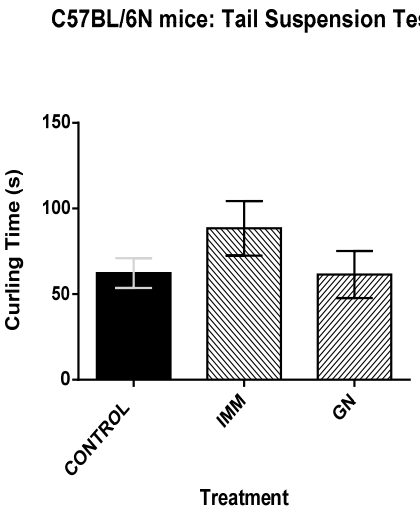
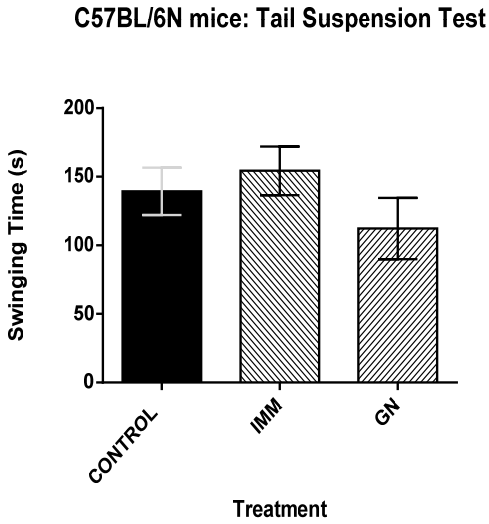


Figure 3: a) There were no significant differences in the percent time spent in the dark chamber between all treatment groups b) No significant differences in the percent time spent in the light box although a slight trend is seen in the GN compared to the both control and Immunized group c) GN mice were hyperactive in the Light & dark box test compared to the immunized and control mice, however the data was not significant d) GN mice travelled more in the total distance exhibiting hyperactivity activity that is context specific – data not significant.

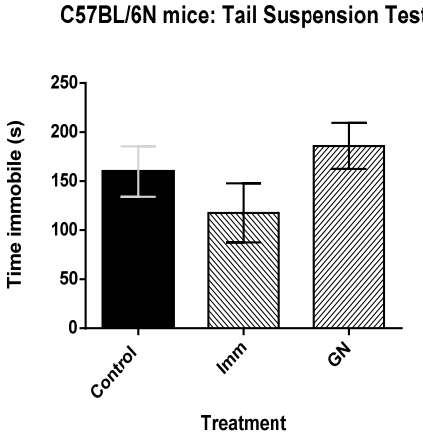
Tail Suspension Test



Values are representative of an ANOVA with a Tukey Multiple Comparison Post Hoc Test.
Values represent ± SEM, Control N=5, Imm N=9, GN N=9 P=0.33



Values are representative of an ANOVA with a Tukey Multiple Comparison Post Hoc Test.
Values represent ± SEM, Control N=5, Imm N=9, GN N=9 P=0.31



Values are representative of an ANOVA with a Tukey Multiple Comparison Post Hoc Test.
Values represent ± SEM, Control N=5, Imm N=9, GN N=9 P=0.19

Figure 4a & b: GN mice spent less time curling and less time swinging exhibiting a depressive phenotype **c)** GN mice spent more time immobile compared to the immunized and control mice

Discussion

Acute and chronic kidney injury affects the brain leading to encephalopathy with symptoms reaching from decreased mental status to seizures (5, 6). A kidney-brain axis exists in case of acute kidney injury seems to be mediated by inflammatory cells (4). So far, there is no evidence whether this is also the case in an inflammatory kidney disease model such as NTS. The administration of NTS to C57BL/6N mice in our findings resulted in behaviour changes observed in a battery of behavioural tests that were conducted. C57BL/6N GN mice exhibited hypo activity in the Open Field (Figure 1a) compared to the control mice but not the immunized mice. The GN mice also exhibited anxiety like phenotype by spending less time in the centre zone (Figure 1b) and fewer entries into the centre zone compared to the control mice. Concurrent with these findings, Liu et al (4) reported moderate to severe declines in locomotor activity compared with sham-operated mice in the Open Field of C57BL/6J mice subjected to renal ischemia or bilateral nephrectomy. Although the data published by Liu and colleagues showed a significant difference between locomotive activity conducted 45 minutes post ischemia compared to 60 minutes post ischemia, the limitations found with the study eluded to the fact that behavioral testing performed soon after surgery was not the ideal model. Our model provides an alternative approach to acute surgical procedures by intravenous drug delivery of NTS; this alternative allows the mice the ability to explore the open arena of the Open Field without impedance, thus giving well defined supportive data. In regards to the mechanism involved by which motor activity is modulated in uremia, Adachi et al studies (21) investigated changes in monoamine metabolism in the brain and motor activity in uremic rats and reported findings of impaired motor activity caused by uremia that suppressed rat central dopamine metabolism. These findings eluded to

the fact that alterations in monoamine metabolism, with particular emphasis on dopamine may potentially be the mechanism by which uremia alters motor activity.

The Elevated Plus Maze data was replaced by the Light and Dark box test in the analysis of anxiety in the C57BL/6N mice. Mice are required to enter the Open Arm of the EPM at least 20% of the time as part of their innate behavior in exploring a novel environment. Our findings showed that C57BL/6N mice were extremely anxious and thus displayed an anxiogenic phenotype. Concurrent with these findings, Post et al (3) investigated Gene-environment interaction influences anxiety-like behavior in ethologically based mouse models. Their studies reported that the two strains BALB/c and C57BL/6N mice displayed anxiety related behaviors with a significant increase in anxiety seen in the C57BL/6N mice in the Elevated Plus Maze. Although Post and colleagues conducted the Elevated Plus Maze for 5 minutes, our testing parameters of 10 minutes showed no changes to their anxious phenotype although given slightly more time to explore the Maze. Thus, the experiment was replaced with an alternative behavioral anxiety testing apparatus called the Light and Dark Box test.

The Light and Dark box test showed no significant findings as the population size N=9 would need to be increased in order to achieve significance.

The Light and Dark box test data showed no significant difference or trends across all treatment groups with respect to time spent in the dark chamber (Figure 3 a). A slight increase in trend was observed in regards to time spend in the light box where the GN exhibit a slight increase in percent time spent in the light arena (Figure 3b). The GN mice have a slight increase in speed compared to all the other treatment groups. Within this context, it may appear that the GN mice are slightly hyperactive. Interestingly, the GN mice travelled more in regards to the total distance traveled in

the Light and Dark box test compared to all treatment groups when compared to the Open Field that displayed a hypoactive phenotype confirming that movement in these test paradigms are context specific (Figure 3d). The Light and Dark Box test exhibits a phenotype of hyperactivity and decrease in anxiety seen by the C57BL/6N mice as they spend more time in the light arena. These results prove very interesting they exhibit the contrary of the initial findings in the Elevated Plus Maze. These findings could elude to the fact although both apparatus (Light and dark Box test & Elevated Plus Maze) both test the anxiety profile for mice, C57BL/6N may be anxious due to the Elevation of the maze 70cm above floor level and perhaps not necessarily to the novelty of the EPM arena. Interestingly a similar phenotype was seen in the study conducted by Post et al (3) investigating Gene-environment interaction influences anxiety-like behavior in ethologically based mouse models. In the study that looked at the difference between BALB/c and C57BL/6N in the EPM and Open Field conducted during the morning with lighting (120 Lux) verses at night (120 Lux), Post et al. reported findings that lightening condition has major influences on both behavioral tests as the C57BL/6N mice spend more time in the center area of the Open Field, but not at night. Thus, in the context paradigm of the Light and Dark Box test, lightening conditions (390 Lux) seemed to have created a less anxious phenotype by spending slightly more time in this arena.

Finally the GN mice exhibited depressive tendencies by swinging and curling less than the control and immunized groups (Figure 4 a & b). These phenotypes can also be seen in the increase in immobility episodes seen in the GN mice compared to the all other treatment groups (Figure 4c). The Tail suspension test is a predictive test of depressive activity observed when mice are suspended by the tail and subjected to a short term inescapable stress where they adopt an immobile posture. Increase in immobility compared to time spent swinging and curling is an indication of a

depressive state (24). In regards to the possible mechanism, Adachi et al studies (21) investigating the changes in monoamine metabolism in the brain reported findings that uremia alters monamine by suppressing central dopamine metabolism in rats. In a human study review by Paul Willner (23), reports predominantly clinical, studies, eluded to the fact that depression involves reduced levels of dopamine (DA) function and that the nigro-striatal DA system is underactive in retarded depressions. Thus, DA is a critical neurotransmitter that is involved in depression and potential changes to the monamine metabolism in the brain as the result of AKI, can create a depressive phenotype in the C57BL/6N mice tail suspension test.

Taken together, this study demonstrates major behavioral changes in the brain of mice administered NTS.

These findings could potentially open up a new line of investigation for more detailed studies to explore mechanisms including specific pathophysiologic pathways that may be the link between the brain – kidney axis. Additional studies could further elucidate the effect of nephrotoxic serum nephritis on murine behaviour and structural brain changes in C57BL/6N mice.

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