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Evaluation of Inosine Effects on Functional Recovery from Stroke in a Rat Model

DATE

This study was conducted as part of a Services Agreement between NeuroDetective Inc. and Company, entitled as above and dated XXX.

SUMMARY

- Chronic administration of inosine, beginning immediately after unilateral devascularizing lesion of the right somatosensory cortex in adult rats, accelerated recovery of tactile placing as well as recovery of forepaw inhibition in swimming, increased reaching attempts in a food retrieval task, and enabled increased extension of the tongue, compared to untreated lesioned animals.

- Inosine had no effect on the lesion-induced impairments in forepaw reaching success, forepaw asymmetry, body orientation, and toenail grooming.

- Across the four tests where there was an overall inosine effect, there were no consistent differences among the different doses of inosine.

- There were no significant effects of immediate inosine treatment on the amount of remaining brain areas in the lesioned animals, either in terms of absolute size, relative hemisphere area remaining, or relative cortical area remaining. Delayed treatment with inosine actually produced a slight but significant reduction in the size of the lesioned hemisphere, relative to immediate treatment, but was not significantly different than saline controls.

Design

This study examined the functional and morphological effects of three doses of inosine administered chronically for 56 days following a right-side lesion of the somatosensory cortex by pia stripping in adult rats. Three groups of rats received one of three doses of inosine beginning immediately following the lesion, and a fourth group received the highest of the three doses beginning 48 hours following the occlusion. Lesion control animals received chronic normal saline, and a group of untreated, unlesioned animals was also included. All groups had been trained prior to surgery to perform in a demanding forepaw-reaching task. Post-surgery, all animals were tested at multiple time points on seven tests of motor function, until 42 days post-surgery. At that point one-half the animals in each group received injections of biotinylated dextran amine beads into the left somatosensory cortex, to assess sprouting, while the remaining half of the animals continued to be tested functionally for two more weeks, for a total of 56 days dosing with inosine. These latter animals then were examined histologically to measure infarct size.

Surgeries were conducted by at the facilities of NeuroDetective Inc. in Lethbridge, Alberta, Canada. Functional testing was conducted in the NeuroDetective facilities in Lethbridge by and under the supervision of Dr. Bryan Kolb. Morphological analyses of infarct size were also conducted under the supervision of Dr. Bryan Kolb at NeuroDetective (Lethbridge).

Subjects and Surgery

Seventy male Sprague-Dawley rats (*Rattus norvegicus*, Charles River) were housed in groups of 3-6 animals in wire-bottom cages with *ad libitum* access to food (5001 Rodent Diet, LabDiet, Brentwood, MO.) and water, except as noted below. Housing arrangements, surgical procedures, and testing regimens were approved by the institutional Animal Welfare Committee and adhered to the guidelines of the Canadian Council on Animal Care.

Prior to pre-operative training, and again post-operatively on testing days, animals were placed on restricted food rations (15 g/animal) for ~18 hours prior to training or testing, so that the animals would be motivated in the Tray Reaching task. Water was available *ad libitum* during food restriction.

<u>Pre-training.</u> Pre-operatively, all animals were trained on the Tray Reaching task for a period of 14 days. Initially, animals were placed for 30 minutes in pairs into wire-bottom acrylic-walled cages (26.5 l x 26.5 w x 19.0 h cm) with the front (19.0 x 25.5 cm) panel consisting of 3.0 mm metal bars spaced at 1.3 cm distances (center-to-center). The 1.0 cm spacing between the bars allows an animal to easily project its forelimb(s) through the bars and grasp food (Pullet Developer, New Life Feeds, Lethbridge, AB.) from outside of the cage. The food is presented on a 5.0 cm wide by 0.5 cm deep tray that runs the width of the front panel and can be moved so that it is 0.3 cm to 3.0 cm from the outside of the cage. If animals were inclined to use their tongues to retrieve food from the tray, the tray was moved away from the cage front so that use of the forelimbs would be necessary. The initial use of animals in pairs increased the opportunity that animals would learn to reach through the bars from a cage mate. Once most animals had demonstrated that they had learned the task, training continued with animals placed individually in these cages. Those animals not demonstrating acquisition of the skill when most were already successful, were placed in the reaching cages on one or two separate occasions for 8 hours (with water) so that more severe food deprivation would motivate acquisition of the task. By 14 days, all animals had acquired the task and their reaching success rate (number of reaches that resulted in food being brought to the mouth/total number of reaches, expressed as a percentage) was assessed from a 5 min. videotape. Limb preference (left or right) was recorded for the side that was used most often in the task, although separate scores for the left and right paws were not kept. Where

animals showed no clear paw preference, a designation of ambidextrous was assigned.

Initial Surgery. Under sodium pentobarbital anesthesia (Somnitol, 60 mg/kg), the rats to be treated all received devascularizing lesions of the right sensorimotor cortex, as described in Kolb et al. (1997). There were 12 rats in each of five groups: vehicle[normal saline, "NS"); 2, 10, or 50 mM of inosine, beginning immediately post-lesion; and a so-called "delay" group [50 mM inosine beginning 48 hrs post-surgery]. There were six unoperated controls. All surgical animals were implanted with a cannula attached to a 28 day osmotic minipump (Alza model #2004; 250 ml volume, 0.25 ul/hr flow rate) that contained one of the three doses of inosine cited above, or the saline vehicle. At the manufacturer-specified flow rate (see previous), effective daily doses of inosine were 3.2 ug/day (2 mM group), 16.08 ug/day (10 mM group), or 80.4 ug/day (both 50 mM groups). All NDI personnel at the test site were blinded to the group affiliations of the animals until the experiment was complete.

Within the first post-op week, two animals died from post-surgical complications.

Second Surgery. On Day 28 post-surgery, all operated animals received a second implant of the same type of osmotic pump as in the first surgery, containing the same concentration of inosine or vehicle as in the first surgery. The animals were placed under light isoflurane anesthesia prior to receiving the second pump implant.

Third Surgery. Two weeks following the second surgery (referred to hereafter as Day 42 [the time following the first surgery]), following completion of behavioural testing on that day, half the lesioned animals were given injections of biotinylated dextran amine beads (BDA). The surgical procedure used was that described in Benowitz et al. (1999, PNAS).

Behaviour Testing

Testing Schedule. The behaviour of the animals was evaluated post-operatively at Days 4, 7, 14, 21, 28, 35 and 42 following the first surgery. In addition one-half the animals (those receiving BDA injections) were evaluated at Day 56 post-operative.

On each testing day, six assessments were made. A seventh assessment of fine motor control was made post-mortem (efficacy of toenail grooming).

1. *Tactile Placing*

Sensory-motor integrity was tested twice using slightly different procedures. In both, the animal is held gently by the torso, with its forelimbs hanging freely. Each forelimb is tested separately by orienting one side of the animal toward a tabletop, then moving the animal slowly and laterally toward the edge of the tabletop until either the dorsal surface of the forepaw (test 1) or the vibrissae on that side (test 2) make contact with the tabletop. An intact forelimb is typically placed quickly onto the tabletop, whereas an impaired forelimb is not. Ten trials are performed with each endpoint measure, using each forelimb, in a balanced order. If the speed and accuracy of the affected forelimb placement was similar to that of control animals, then a score of 3 was given. If there was a significant delay in placement of the limb, but the limb was placed successfully, then a score of 2 was given. A marked delay in successful placement resulted in a score of 1, and failure to place the limb on the surface of the bench was scored as 0.

By the end of the fifth week many animals were placing almost as well with the affected limb as with the unaffected limb. It was clear, however, that there was a difference in latency to initiate the movement. Thus, on week 6 the latency to make the placing response with each paw was calculated by videotaping the behavior and then conducting frame-by-frame analysis of the tape to calculate latency. With 30 frames per second it was not possible to score the normal (right) limb because it was too fast but it was possible to get an accurate measure of response latency on the affected (left) limb. Because the speed of the camera is a fixed rate of 30 frames per second, each frame represented $1/30^{th}$ of a second. The number of frames elapsed from touch to placement by the affected (left) limb was averaged over 6 trials, and then converted into a number which represented the mean latency in milliseconds for that animal.

2. *Forepaw Asymmetry*

Forepaw asymmetry of the animals was determined by filming them from below while in an acrylic cylinder 25 cm in diameter, on an acrylic platform. Preference was determined by separately counting the number of times in 5 minutes that an animal reared and placed the left or right forepaw on the surface of the cylinder in a gesture of postural stabilization.

3. *Tail Hanging*

Body orientation was scored as left, right or normal based on how the animal turned when hung several centimeters above a surface while being held by the tail. If an animal showed a preference for turning in one direction, then that direction was recorded. If no turning was observed, or if there was no preference in direction (equal turns to left and right), then a score of normal was recorded. While hanging, the position of the hindlimbs was also noted. If the hindlimbs were extended in a normal fashion then a score of 0 (zero) was recorded. If the hindfeet were closely apposed, touching, or crossed, or if either limb was flexed during hanging, then an abnormal score of 1 was recorded.

4. *Forepaw Inhibition*

In normal rats, swimming is accomplished by propulsion from the hindlimbs. The forelimbs are normally inhibited from any stroking and are held immobile and together under the animal's neck, a behavior under control of the motor cortex. This inhibition of the forelimbs was assessed by filming animals during swimming. Animals were introduced into one end of an aquarium (30 w x 90 l x 43 h cm) filled to a depth of 25 cm with room temperature water (~25^{o}C) and filmed as they swam to a 9.5 cm square visible platform projecting 2 cm above the surface of the water placed at the opposite end. Scoring of inhibition was done by counting the number of strokes, if any, made by each forelimb in three swims along the length of the aquarium. A swim was deemed scorable only if the animal did not touch the sides of the aquarium during the swimming trial. Thus, attempts by the animal to escape from the water using the aquarium walls were not included in the counts.

5. *Tray reaching*

Beginning at Day 10 post-op, animals were trained 30-40 min daily (on non-test days) to reach for food pellets, initially allowing unrestrained use of either the impaired or unimpaired paw. When the animal began repeated reaching with at least one paw, the right (unimpaired) paw was given a bracelet, and training continued with the left (impaired) paw only. Training continued until Day 27 post-operative. The bracelet used consisted of fabric adhesive strips of bandage, applied to the wrists of the forelimb, effectively blocking the animals from extending that limb through the bars.

On each designated post-op test day, the animal received single 7-min test trials. The behavior of the rats was videorecorded and performance scored from the tapes. Scoring was in terms of the absolute number of reaches (when the paw is inserted through the bars of the box) and percent of these reaches that were successful (when the animal retrieves a piece of food and consumes it) per 7 min test period. On Day 21 the animals were tested only with their impaired paw. On Days 28, 35, 42 and 56 the animals were tested with both their impaired (left) and unimpaired (right) paws, in separate 7 minute test sessions. For the test of the left paw, the right paw was fitted with a cuff to prevent that paw from being inserted between the metal bars of the reaching box. For the test of the right paw, the animals were given a free reaching test in which they could use either paw. All animals used their right paw in such a test but some also attempted to use their left paw. In this case the animals invariably had a preoperative left paw preference. This outcome provided another measure of recovery, at least in the animals with a left paw preference.

6. Tongue Protrusion

While in their home cages, animals were given a slurry of chocolate chip cookie (cookie bits mashed in warm water), placed on a spatula. One hour later each animal was placed in a test cage alone, and the slurry presented again, this time on a ruler placed perpendicular to the cage bars. How far the slurry was licked off the ruler is recorded as a measure of tongue protrusion. The degree of tongue protrusion is known to reflect the extent of damage to the area of the motor cortex where the tongue is functionally represented. This test was only given at Days 28, 35, and 42.

7. *Toenail length*

The length of all ten hindlimb toenails was measured when animals were anesthetized for perfusion. Normal animals continually groom their toenails, a behavior that requires fine motor control. Nail length was measured as the linear distance from the point of emergence of the nail from the cuticle to the most distal extent. If the nail was broken or otherwise irregular, then no measurement was recorded. This measure of toenail length is known to reflect the functional integrity of the motor cortex.

Histology

Following the last series of behavior testing (at 42 days post-lesion) half the animals received injections of biotinylated dextran amine beads into the intact sensorimotor cortex (see above). Fourteen days following these injections, the animals were perfused and their brains removed and sent the client's laboratory for analysis of sprouting.

The remaining half of the animals (N = 36) had CSF samples taken from their cisternae magna, followed by perfusion with 0.9% saline and 4% paraformaldehyde. The brain, lung, liver, kidney, and heart were dissected. All organs except the brain were shipped to the client for analyses, along with the CSF samples.

The brains were weighed and stored individually in 30% sucrose in 4% paraformaldehyde for at least 24 hrs. The brains were then cut frozen in 40µm coronal sections. Every tenth section was saved and stained in cresyl violet.

<u>Brain Measurements.</u> Eight sections of each brain were measured. The planes are illustrated in Figure 1. The sections were identified and defined using the following criteria:

Section 1. The last section before the olfactory bulbs join the cerebral hemispheres (Bregma +4.5 mm in the Paxinos & Watson atlas);

Section 2. Two sections before the corpus callosum crosses between the hemispheres (Br +3.0 mm);

Section 3. Corpus callosum (Br +1.5 mm);

Section 4. Anterior commisure (Bregma);

Section 5. First hippocampal section (Br –1.5 mm);

Section 6. First section in which the medial habenular nuclei are visible (Br –3.0 mm);

Section 7. Posterior commisure (Br –4.5 mm);

Section 8. Last hippocampal section (Br –6.0 mm).

Slides were placed on top of a light table and a digital video camera was used to capture an image of the sections on a computer using Scion Image software (ver. 1.62, NIH). From these images the cross sectional area of both the neocortex and the whole hemisphere (including cortex) were measured, separately for each side of the brain. The resulting 4 areas per section (cortex and whole hemisphere, left and right sides) were then analyzed using a statistical software package (Statview, version 5.0). The results were converted into square millimeter measurements based on the calibration of 466 pixels to 1 cm. Analysis of variance (ANOVA) was used to detect significant ($P < 0.05$) differences between and among treatment groups, and Fisher's exact test was used to determine where those differences occurred.

RESULTS

Statistical analyses of the behavior data were performed in two different ways: 1) using each of the inosine-treated groups as a separate treatment group, and 2) collapsing all groups that received inosine immediately after the surgery into one "inosine" group. This latter manipulation was done because there appeared to be few, if any, reliable effects that were related to dose, and collapsing across dose gave greater robustness to the statistical conclusions. All statistical tables are included.

1. Tactile Placing

The vibrissae and paw tests were identical so a single score was calculated for each animal, as the mean of the scores in the two tests. All animals in all lesioned groups were severely impaired at tactile placing by their affected (left) paw one week following the lesion. There was a clear improvement with increasing post-lesion time in all groups, but the Delayed (inosine) group clearly improved the least, being significantly worse than the vehicle group ("NS," normal saline) at Week 5 (see pp. TP-2, TP-13). The immediate-inosine groups all recovered slightly faster than the NS group, and by week 3 both the 2 mM and 50 mM inosine groups performed significantly better than the NS and Delay groups (pp. TP-2, TP-11).

The severity of the lesion was evident statistically in the performance of the "unaffected" (right) paw, which in fact was affected: placing by the right paw in the lesioned but untreated (NS) group was significantly worse than normal (p. TP-6), and both the low (2 mM) and high (50 mM) doses of inosine significantly improved performance by the right paw as well (p. TP-6).

Analysis of the latency to place revealed a particularly interesting pattern. At Week 6 (the first post-lesion time point at which latency was examined), the inosine group (all immediate-inosine doses combined) was significantly faster than the Delayed-inosine group, and almost significantly faster than the NS group (p. TP-25). By week 8 this pattern had disappeared as the other lesion groups had improved (p.TP-27), although the 2 mM and 10 mM groups were still trending to place faster (p. TP-26).

In sum, taken together the data suggest that inosine both speeded up recovery and lead to more complete recovery compared to both the no treatment (NS) and Delayed treatment groups.

2. Tongue Extension

All lesion groups had significantly reduced tongue extension relative to the normal control group. All inosine groups improved over the test period and by week 6 the immediate-inosine (all doses combined) and delayed inosine groups performed significantly better than the vehicle (NS) group (pp. TE-2, TE-5). Analysis of the different dosage effects showed that, by Week 6, only the 2 mM dose group was not significantly better than the NS group (p. TE-9).

3. Forepaw Inhibition

When the animals were placed in a water tank and their swimming pattern analyzed, a release of forepaw inhibition in the forelimb contralateral to the lesion was seen in all lesion groups. There was a gradual recovery in all groups over the study, with the inosine group (all immediate-inosine doses combined) showing slightly better performance than the other lesion groups (NS, Delay) beginning at Week 4 and continuing through Week 8 (p. FI-1). This slightly better performance did not achieve statistical significance over Weeks 1-6, when all animals were tested, but a separate analysis of Week 8 (half the animals tested) showed that the immediate-inosine animals did attempt significantly fewer swimming strokes with their impaired forepaw (i.e., exhibited greater inhibition), compared to the NS group (p. FI-6), and in fact were not significantly different from normal. Animals receiving either 2 mM or 50 mM inosine tended to show more inhibition than 10 mM and Delayed-inosine animals (p. FI-2), but sample sizes were too small for meaningful statistical analysis of the separate inosine-treatment groups.

4. Tray Reaching

Banding of the good (RIGHT) paw began on week 3 and continued through week 6, so that over this time period the animal was forced to use its impaired paw during testing. In addition, after testing the impaired paw on weeks 4, 5, and 6, and again on week 8 for those animals getting this testing, the animals received a free-choice test, in which they could use either paw.

The intended principal measure was Percent Successful Reaches, i.e. the percent of paw reaches that resulted in successful retrieval of the food piece. These data are presented on pages R-3 through R-50, except that Week 3 results are for the banded test (free-choice was not tested at that time point) and on Week 8 both free-choice and banded test results are given. Statistical analyses are also presented for these data, separately for each Week. There were no significant effects with either analysis, with all groups being equally impaired (around 40% successful reaches), and the degree of impairment did not improve significantly.

We also analyzed the data from both free-choice and banded tests in terms of whether the animals reached with their impaired paw at all. Specifically, a percentage of animals that reached at all with their LEFT limb was calculated. By this measure there was a large, significantly positive effect of inosine (all immediate-doses combined) in the banded tests, i.e. when the animals were forced to use their impaired (LEFT) paw (p. R-1). In other words, although the animals were still impaired, the immediate-inosine animals did *initiate* reaching with their impaired (LEFT) paw, which was rare in the other lesion groups (NS, Delay).

When animals were tested in free-choice reaching, all animals in the Delay (inosine) group reached with their unimpaired (RIGHT) paw on every test day. Of the immediate-inosine group (all doses combined), only 40% reached with either paw at all on week 1, and this improved to about 70% on week 2 and 90% on week 4 (the overwhelming choice was to reach with the unimpaired paw).

Another difference between the immediate-inosine and the Delay (inosine) groups in the free-choice test was that about 20% of the immediate-inosine animals eventually used their bad paw (which they had previously preferred), even though they were not good at it (p. R-2, bottom). No animal in the NS or Delay groups ever did this. (In our experience with this model, it is rare indeed for any animal with a large motor cortex lesion to use its bad paw voluntarily.)

In sum, in the Tray Reaching test the lesioned animals receiving inosine immediately after the lesion were much more likely to attempt to use their bad paw at all, even though they were not good at it, compared to vehicle treated animals and to animals receiving inosine after a 48-hour delay. Analysis of the reaching success data, with the bad paw, showed no significant effect of inosine at any dose.

The fact that even a small percentage of immediate-inosine animals did attempt to use their impaired forepaw does suggest that a worthwhile future study might take advantage of this and factor in extensive physiotherapy in an attempt to improve reaching performance.

5. Forepaw Asymmetry

All groups showed a strong, and significant, forepaw placement asymmetry favoring the intact paw throughout the study (pp. FA-2, FA-3, FA-5). There was significant improvement across all lesion groups with increasing post-lesion time, but no significant interaction of Week (post-lesion time) and Treatment, indicating there was no effect of inosine.

6. Tail Hanging

All lesion groups showed a strong bias to make body turns to the left, contralateral to the lesion, biases that produced significant Chi-square test results when compared to Normals at all post-lesion test times (pp. TH-1 through TH-16). Inspection of the data showed clearly there were no consistent dose differences vs. the NS group.

7. Toenail length

There was a consistent lesion effect on claw cutting, leading to significantly longer toenails in all lesion groups compared to control (normal) animals in both the left hindpaw (p. TL-1) and right hindpaw (p. TL-8). Separate analyses for each of the five nails on the left and right hindpaws produced a significant inosine effect with only one of the nails, the lateral-most (5^{th}) digit on the right side (p. TL-14), a result that we do not consider meaningful.

Gross anatomy. The extent of motor cortex damage was grossly similar in all groups, but there were large individual differences in the extent of temporal and posterior parietal cortex involvement. Figure 1 p. 7) presents a series of photomicrographs illustrating a representative lesioned animal. There were no obvious effects of treatment on the lesion size, nor any suggestion that the lesion size varied in any systematic way across different batches of surgeries.

Areal measurements. There was no effect of inosine dose on areal measurements for the three immediate-treatment groups, so the data were collapsed across these groups.

Two types of analyses were performed. First, ANOVAs were conducted on the ratios of left to right cortical areas, and left to right hemisphere areas, with treatment group and rostral-caudal level as factors. Secondly, separate ANOVAs were conducted on the absolute left and right hemisphere area measures, and the absolute left and right cortical area measures, again with treatment group and rostral-caudal level as factors.

(a) Ratios.

The critical analysis for an inosine effect is the relative effect of the ischemia on the lesion versus intact cortices in particular, and hemispheres overall. Results showed no effect of inosine treatment with either the specific cortex area measure, or the overall hemisphere area measure (see p. 8), although the expected effect of rostral-caudal level remained. That is, areal measures varied with rostral-caudal level. ANOVAs on the right cortex/left cortex area measure, and the right hemisphere/left hemisphere area measure, respectively, showed no main effects of treatment ($F(2,25) = 0.3$, $p = 0.76$; $F(2,25) = 0.96$, $p = 0.40$) but there were significant rostral-caudal level differences ($F(7,175) = 111.3$, $p < 0.0001$; $F(7,175) = 145.3$, $p < 0.0001$). The interactions were not significant (p's > 0.5). See pp. 9–12.

(b) Absolute areas.

ANOVA on the left (normal) cortex and hemisphere revealed significant main effects of Treatment ($F(2,25) = 9.9$, $p < 0.0007$; $F(2,25) = 10.7$, $p < 0.0004$, respectively), as well as rostral-caudal level ($F(7,175) = 104.2$, $p < 0.0001$; $F(7,175) = 375.5$, $p < 0.0001$, respectively). The interaction was significant only for the hemisphere measure ($F(14,175) = 2.3$, $p < 0.0072$). The Treatment effect reflects the fact that the delayed inosine group had slightly, but significantly, smaller brains than the other groups. The significant interaction reflects the fact that the delayed inosine effect varies with rostral-caudal level. See pp. 13-16.

ANOVA on the right (lesioned) cortex and hemisphere area measures showed a similar pattern of results. There were significant main effects of Treatment ($F(2,25) = 3.3$, $p < 0.0515$; $F(2,25) = 3.6$, $p < 0.04$, respectively), as well as rostral-caudal level ($F(7,175) =$

57.9, $p<.0001$; $F\,(7,175) = 214.4$, $p<.0001$, respectively). Again, the interaction was significant only for the hemisphere measure ($F\,(14,175) = 2.2$, $p < 0.008$). See pp. 17-20.

Raw data are appended (pp. 21-27).