Experimentally Altered Navigational Demands Induce Changes in the Cortical Forebrain of Free-Ranging Northern Pacific Rattlesnakes *(Crotalus o. oreganus)*

Matthew L. Holding Julius A. Frazier Emily N. Taylor Christine R. Strand

Abstract

The hippocampus of birds and mammals plays a crucial role in spatial memory and navigation. The hippocampus exhibits plasticity in adulthood in response to diverse environmental factors associated with spatial demands placed on an animal. The medial and dorsal cortices of the telencephalon of squamate reptiles have been implicated as functional homologues to the hippocampus. This study sought to experimentally manipulate the navigational demands placed on free-ranging northern Pacific rattlesnakes *(Crotalus o. oreganus)* to provide direct evidence of the relationship between spatial demands and neuroplasticity in the cortical telencephalon of the squamate brain. Adult male rattlesnakes were radio-tracked for 2 months, during which time 1 of 3 treatments was imposed weekly, namely 225-meter translocation in a random direction, 225-meter walk and release at that day's capture site (handling control) or undisturbed (control). Snakes were then sacrificed and the brains were removed and processed for histological analysis of cortical features. The activity range was larger in the translocated (Tr) group compared to the handled (Hd) and undisturbed control (Cn) groups when measured via 95% minimum convex polygon (MCP). At the 100% MCP level, Tr snakes had larger activity ranges than the Cn snakes only. The volume of the medial cortex (MC) was larger in the Tr group compared to the Cn group. The MC of Hd snakes was not significantly different from that of either of the other groups. No differences in dorsal cortex (DC) or lateral cortex volumes were detected among the groups. Numbers of 5-bromo-2′-deoxyuridine (BrdU)-labeled cells in the MC and DC 3 weeks after BrdU injection were not affected by treatment. This study establishes a causal relationship between navigational demands and greater MC volume in a free-ranging reptile.

Introduction

The mammalian and avian brains have been the targets of numerous studies exploring the relationship between learning and neuroplasticity, implicating the hippocampus as a major component in spatial memory and navigation-related tasks [Clayton et al., 1997; Patel et al., 1997; Gould et al., 1999; Maguire et al., 2000; Amrein et al., 2004a, b; Pravosudov and Omanska, 2005; recently reviewed in Barker et al., 2011]. Diverse experiments and comparative studies have demonstrated that aspects of

neuroplasticity, such as neurogenesis, synaptogenesis and altered rates of cell death, can be stimulated differentially depending on the environmental demands placed upon these animals [Kempermann et al., 1997; Gould et al., 1999].

Adult neurogenesis in rats is enhanced by spatial learning and navigation in Morris water mazes and enriched environments [Gould et al., 1999]. In wild voles *(Microtus spp.),* hippocampal volume and neurogenesis can be related to sex, seasonal and species differences in spatial demands [Jacobs et al., 1990; Galea and McEwen, 1999]. Maguire et al. [2000] showed that taxi drivers exhibit a larger hippocampal volume than control subjects and further that hippocampal size correlates positively with time spent driving a taxi. These authors concluded that environmental demands in the form of increased navigational requirements placed on taxi drivers were the likely cause of the observed hippocampal growth.

In congruence with the mammalian literature, heightened spatial requirements in birds are often matched to corresponding changes in hippocampal volume and rates of neurogenesis. Female brown-headed cowbirds presumably have higher demands for spatial memory than males of the species, given that they must locate and revisit the nests of the other bird species in which they lay their eggs. The females have a larger hippocampus than the males in this species [Sherry et al., 1993], and captivity results in smaller hippocampal volume in female cowbirds only [Day et al., 2008]. Food-caching behavior in birds represents a spatial memory requirement that can be readily manipulated in the laboratory, and, as predicted, the amount of food storing allowed is positively associated with both hippocampal size and rates of hippocampal neurogenesis [Clayton and Krebs, 1994; Patel et al., 1997; Biegler et al., 2001].

As one might anticipate based on their phylogenetic position in relation to mammals and birds, nonavian reptiles display neuroplasticity during adulthood. In fact, adult neurogenesis occurs in more telencephalic brain regions in nonavian reptiles compared to their mammalian and avian counterparts [Pérez-Cañellas and García-Verdugo, 1996; Font et al., 2001]. However, the functional significance of this widespread neuronal recruitment is poorly understood. The reptilian medial cortex (MC) and, to a lesser extent, dorsal cortex (DC) have been implicated as both structural and functional homologues of the hippocampus of mammals and birds [Bruce and Butler, 1984; Butler and Hodos, 1996; Rodriguez et al., 2002]. Again, associations with sex, seasonal and species-specific differences in spatial demands and measures of neuroplasticity in these brain regions have been demonstrated [Roth et al., 2006; Delgado-González et al., 2008; Sampedro et al., 2008]. Lesioning of the MC and DC of turtles produces deficits in spatial memory and the ability to navigate a maze [Rodriguez et al., 2002; López et al., 2003]. In squamate reptiles, lesioning of the MC causes similar disruption of the ability to navigate [Lopez-Garcia et al., 2002; Day et al., 2001]. Day et al. [1999a, b; 2001] suggested that relative size differences in both the MC and DC of two lizards of the genus *Acanthodactylus* with contrasting spatial demands may not be related to differences in the lizards' ability to carry out bird- or mammal-like spatial navigation, because no such differences were detected in laboratory tests aimed at quantifying such abilities. Holtzman et al. [1999] and Zuri and Bull [2000] showed that corn snakes *(Elaphe guttata)* and sleepy lizards *(Tiliqua rugosa),* respectively, could rapidly acquire the ability to perform a spatial task, but their spatial tasks did not require the use of distal cues as did those in Day et al. [1999a, b]. Roth et al. [2006] conducted the only study to date that related spatial requirements and cortex size in a snake. These authors showed that male cottonmouths *(Agkistrodon piscivorus),* which have a larger average home range size than females, have a larger relative MC, but not DC, than females.

In spite of the overall paucity of literature on the subject, discrepancies such as those discussed by Day et al. [1999a, b; 2001] have already arisen with regard to the roles of the MC and DC in spatial memory and navigation in reptiles. The combination of controlled laboratory experiments and comparative neuroecological studies that relate brain morphology and neuroplasticity to selection pressures and species' life histories have begun to elucidate the trends previously discussed [Barker et al., 2011], but more work is clearly required. Experimental manipulation of the navigational demands placed upon a reptile would be of use in determining which brain regions play a role in navigation in reptiles, especially if this were accomplished in a natural setting with a free-ranging animal. Additionally, such an experiment would help determine whether previously observed differences in the volume and cell proliferation rates of reptiles with higher spatial demands are genetically determined or the result of cortical growth stimulated by the increased navigation itself. Lindsey and Tropepe [2006] highlighted the need for such field-based studies in truly understanding the function of neuroplasticity and 'bridging the gap' between ecology and associated changes in the brain. In this study, we used short-distance translocations of a free-ranging reptile in an attempt to unite the findings from

the laboratory and comparative studies. To our knowledge, this type of field-based experimental manipulation has not been carried out before, even in the more extensive mammalian and avian literature.

Translocation (the movement of an animal from one area to another by human action) is a commonly used management tool for mitigating interactions between rattlesnakes and humans [Reinert, 1991; Hardy et al., 2001; Nowak et al., 2002; Brown et al., 2009]. Short-distance translocations (translocations near or within an animal's home range) increase the area over which snakes are found and the amount of movement they undertake, while resulting in few to no ill effects for the animal [Brown et al., 2009]. We utilized short-distance translocation to experimentally increase the navigational demands of free-ranging northern Pacific rattlesnakes *(Crotalus o. oreganus).* Rattlesnakes are suitable for repeated capturing and translocation because their large bodies permit them to carry radio transmitters with little to no impact on normal behavior, and also because they spend much of their time above ground. We hypothesized that these animals would respond to increased navigational requirements with neuroplasticity in the MC only, potentially via neurogenesis. We predicted that translocated (Tr) snakes would have a larger MC than handled (Hd) and undisturbed control (Cn) snakes. Further, we predicted that Tr snakes would have higher numbers of new cells born in the MC. No such differences should be observed in the DC or lateral cortex (LC).

Materials and Methods

Field Experiment Procedures

Twenty-two adult male *C. o. oreganus* were captured during visual searches spanning a 1-month period from mid-March to mid-April 2010 on the Chimineas Ranch unit of the Carrizo Plain Ecological Reserve, San Luis Obispo County, Calif., USA (35°N, 119°W, altitude 750 m). The site consists primarily of rolling, grassy hills with scattered rocky outcrops and blue oaks *(Quercus douglasii).* All snakes lived in a part of the ranch under moderate grazing pressure from cattle and horses. The collection of snakes for research was carried out under the California Department of Fish and Game California Scientific Collection Permit No. 801072-05. The use and treatment of snakes was conducted under the guidelines of the California Polytechnic State University IACUC protocol No. 910.

Snakes were transported to the laboratory at California Polytechnic State University following initial capture. They were anesthetized via inhalation of isoflurane (Halocarbon Production Corp., USA) and implanted intracoelomically with 11- or 13.5-gram SI-2 radio transmitters (Holohil Systems Ltd., Carp, Ont., Canada). While anesthetized, we recorded the snakes' snout-to-vent length (SVL; ±0.2 cm) using a cloth measuring tape and mass (±5 g) using a Pesola spring scale. We defined adult males as those with an SVL greater than 80 cm, and only those snakes that met this requirement were placed in the study. Snakes were released at their original capture sites following 1–3 days of recovery from surgery.

Following the release of the final snake, each snake was randomly assigned to one of three experimental groups, namely Tr (n = 8), Hd (n = 8) or Cn (n = 6). Tr snakes were subjected to weekly (every 7–8 days) 225-meter translocations in the form of a randomly chosen straight-line displacement in an opaque plastic bucket. The distance was intended to represent a short-distance translocation and was chosen using data on the home ranges of adult male *C. o. oreganus* from the same population taken 3 years prior to this study [Putman B., pers. commun.]. Hd snakes were captured weekly, carried in an opaque plastic bucket over a straight-line distance of 112.5 m, returned to the site of capture and released. These snakes were therefore captured and carried a distance equal to that experienced by the Tr snakes in order to control for the potential effects of being handled. The bucket used for carrying Tr and Hd snakes was closed with a screw-top lid during transport, which would have eliminated the snakes' abilities to track celestial cues and limited the influx of olfactory cues during experimental movement of the snakes. The Cn snakes were radio-tracked only.

The direction of displacement for both the Tr and Hd animals was chosen using a random numbers table containing only integers between 1 and 360. These were translated to compass bearings used to move the snakes, with 360 representing due north. We wished to move the snakes to as many different areas as possible while avoiding the potential of moving a snake up to 500 m or more in a single direction if it happened not to have moved far from the previous week's release point. Therefore, a single restriction was placed on the random selection of direction for moving the snakes. Random numbers were eliminated until a bearing was selected that did not fall within 45° of the previous week's bearing. The appropriate distance was then measured using a handheld GPS unit (Garmin Legend, Garmin), and Tr snakes were placed in the nearest suitable cover within 10 m of their new location.

Treatment began on 23/4/10. All snakes were located 4 times weekly, on average, from 23/4/10 to 19/6/10. This is a relatively high frequency for tracking ectothermic, sit-and-wait predators such as rattlesnakes, which often go many days between movements. Each location was recorded to an accuracy of 10 m with a Garmin Legend GPS unit. Between 29/5/10 and 2/6/10, all snakes were captured and received subdermal injections of 5-bromo-2′-deoxyuridine (BrdU; MO744, Sigma-Aldrich Co., St. Louis, Mo., USA) in the field at a dose of 100 mg/kg. A concentration of 20 mg/ml BrdU was delivered dissolved in 0.9% NaCl solution. The Cn snakes were immediately released. The Tr and Hd snakes received their respective movement treatments following injection. Application of treatments was completed on 5/6/10, resulting in 6 translocations or handlings per snake. Snakes were captured and brought to the laboratory between 19/6/10 and 24/6/10.

Tissue Preparation and Measurement

Snakes were sacrificed 24–36 h after capture. Following the onset of deep anesthesia via inhalation of isoflurane, each snake was transcardially perfused with a 0.9% NaCl, 0.1% $NaNO_2$, 0.1 M phosphate buffer (PB) wash at 10 ml/min for 10 min followed by

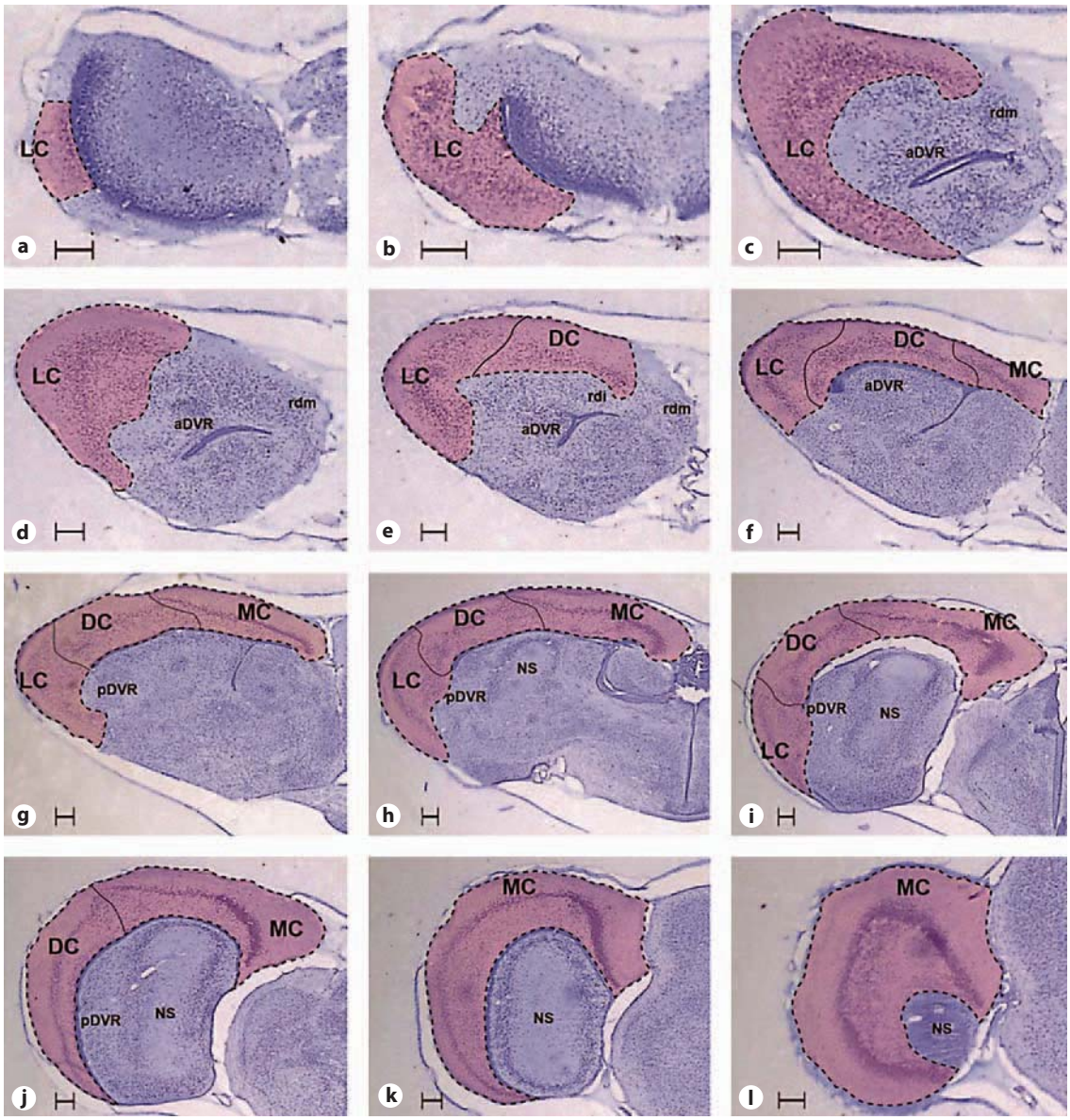

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Fig. 1. Images of cresyl violet-stained sections moving caudally from **a** to **l** through one hemisphere of the telencephalon of *C. o. oreganus*. The images represent areas in which major changes occur in the morphological features of the cortical regions under consideration. The area shaded and outlined by dashed lines represents the area measured during volumetric analysis, and the solid lines show the divisions between the MC, DC and LC. All scale bars are 0.2 mm. **a** Measurement began here, as the first cells of the cell layer of the LC become visible. **b** The LC expands dorsally and ventrally. **c** The LC expands medially. **d** The DC is present but difficult to discern from the anterior dorsoventricular ridge and retrobulbular region pars dorsomedialis. **e** Measurement of the DC began at the point where the lateral extension of the lateral ventricle first appears as the DC runs dorsal to the retrobulbular region pars dorsalis internus. **f** The MC cell layer first appears. **g–i** The MC expands laterally, while the DC expands ventrally in conjunction with the lateral ventricle. **j** The lateral ventricle completely encircles the posterior dorsoventricular ridge and nucleus sphericus. **k** The disappearance of the medial superposition marks the disappearance of the DC. All cortical area is now attributed to the MC. **l** After the disappearance of the nucleus sphericus, all remaining telencephalon is attributed to MC. aDVR = Anterior dorsoventricular ridge; rdm = retrobulbular region pars dorsomedialis; rdi = retrobulbular region pars dorsalis internus; pDVR = posterior dorsoventricular ridge; NS = nucleus sphericus.

4% paraformaldehyde in 0.1 M PB, pH 7.2, with 0.1% $NaNO_2$ for 10 min. The skulls were then removed and placed in 4% paraformaldehyde for at least 2 h, then the brains were carefully removed from the skulls and postfixed for 24 h in 4% paraformaldehyde. Next, brains were transferred to a 0.1 M PB solution for 24 h prior to embedding in 8% gelatin. The gelatin was allowed to solidify overnight, then the block was placed into 4% paraformaldehyde for 24 h and then into a 30% sucrose solution until it sank, at which time it was frozen in dry ice and stored at –80°C.

Four series of parallel transverse sections were obtained at a thickness of 35 μm using a cryostat. The first series was mounted onto slides directly from the cryostat, hydrated with mounting solution, allowed to dry and stained with cresyl violet. The remaining series were stored in cryoprotectant at –20°C until use.

Cortical brain regions in the cresyl violet-stained sections were photographed, and brain regions were measured using NIH ImageJ software. The measurements were made by a single researcher blinded to the treatment group of the snake. The MC, DC, LC and total telencephalon (TT) were identified in accordance with Halpern [1980]. Measurement began at the first appearance of the LC cell layer and continued until the disappearance of the telencephalon. Telencephalon rostral to the first appearance of the LC cell layer was not included in the measurement of TT volume. As in Roth et al. [2006], subdivisions of the MC and DC were not considered separately. However, we did not follow Roth et al. [2006] in considering the rostral and caudal MC separately. Representative photographs show the boundaries for the MC, DC and LC as measured, along with important morphological benchmarks for identification of each cortex (fig. 1). Volumes were calculated by summing the areas from both hemispheres of each region, then multiplying by section thickness (0.035 mm) and by the number of series (n = 4). Missing or badly damaged sections were accounted for by using the average area of the two adjacent sections.

A subset of a second transverse series was obtained for detection of BrdU-labeled cells, such that every eighth brain section was represented. Sections were drawn from the same region in which volume was measured, beginning at the appearance of the LC cell layer. Free-floating sections were washed in phosphate-buffered saline (PBS) 3 times for 5 min prior to denaturing DNA in 4 N HCl for 15 min. After 5 min in PBS, acid was neutralized in 3.8% sodium borate wash adjusted to pH 8.5 for 10 min. Sections were then washed 3 times for 10 min in PBS followed by blocking of nonspecific binding and endogenous peroxidases for 1 h in a solution of 5% normal horse serum (S-2000, Vector Laboratories Inc., Burlingame, Calif., USA), 1% bovine serum albumin and 0.5% H_2O_2 in PBS + 0.3% Triton X-100 (PBST). Sections were then left shaking for 24 h in a 1:1,000 dilution of anti-BrdU (clone Bu20a, DakoCytomation, Glostrup, Denmark) in PBST. Next, sections were washed 3 times for 5 min in PBST and placed in a 1:200 dilution of biotinylated antimouse IgG (BA-2000, Vector Laboratories) for 1 h. Another three 5-min washes in PBST were followed by 1 h of incubation in avidin-biotin-peroxidase complex solution (PK-6100, Vector Laboratories). A final series of three 5-min washes in PBST was followed by detection of bound peroxidase complexes by 4 min of incubation in a solution of Vector SG chromagen (SK-4700, Vector Laboratories), after which the reaction was stopped by two 5-min washes in PBS.

Labeled nuclei were counted in the ependymal layer, inner plexiform layer, cell layer and outer plexiform layer in the MC and DC (fig. 2). Cells were said to be associated with the ependymal layer if they were within two nucleus widths from the ventricle, and only cells on the cortical side of the ventricles were counted. Cell shape was not recorded.

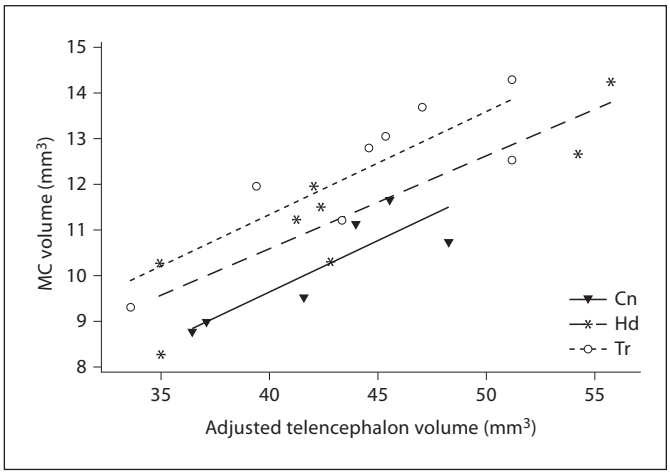
Data Analysis

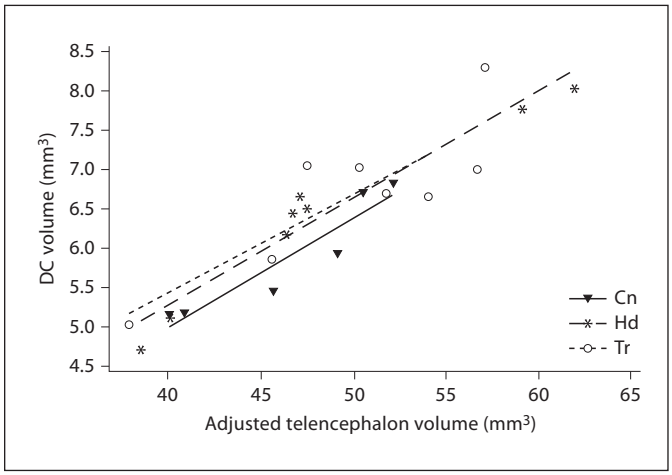
Arcview version 3.3 (ESRI, Redlands, Calif., USA) was used to analyze the spatial data obtained from each snake. The term activity range will replace home range for our purposes, since Tr animals were moved to areas not of their own choosing and potentially slightly outside of their home ranges. The activity range of each snake was measured by minimum convex polygon (MCP) at the 100 and 95% levels, which estimates activity range by encircling points to which a snake was tracked by a polygon with convex sides and the smallest possible area. Total distance moved (TDM) was calculated as the summed distance between all successive locations, minus translocation distances for the Tr group. It is a proxy for the total distance travelled by each animal on its own throughout the study period.

Either analysis of variance (ANOVA) or analysis of covariance (ANCOVA) were used to analyze all of the results, with treatment as a factor and multiple potential covariates considered. The MCP activity ranges data required log transformation to satisfy the normality assumption and were modeled using ANCOVA with SVL as a covariate. These data are presented in terms of the original variables. One-way ANOVA was used to test for an effect of treatment on the TDM of the snakes once ANCOVA using SVL and body condition (defined as residuals of a regression of log-transformed SVL vs. log-transformed mass) showed that these additional predictors were nonsignificant and did not improve the variation accounted for by the model.

ANCOVA was used to test for differences in volumes of the MC, DC and LC among the three experimental groups. Following Roth et al. [2006], the volumetric analyses were carried out by creating a unique covariate to analyze the volumes of each cortical region. Covariates were calculated by subtracting the region of interest (MC, DC or LC) from the TT volume (henceforth referred to as adjusted TT volume). This is the most appropriate way to build this model, as not doing so would result in a covariate partly made up by the response of interest itself, resulting in a falsely inflated R^2 value. Using these unique covariates instead of overall TT volume did not change the results of any tests. The SVL of snakes was also considered as a covariate, but this measure of animal size did not add information to the model in addition to what was provided by the adjusted TT volume ($p > 0.1$ when both covariates were included). One-way ANOVA was used to model the BrdU-labeled cell counts from the MC and DC, once SVL, TT volume and respective cortex volume were found to be nonsignificant as covariates.

The ANCOVA assumption of homogeneity of covariate slopes was violated when analyzing the activity range data, so the interaction between treatment and covariate was included in the final model. Alpha was set at 0.05 for all tests. The default procedure used for making pair-wise comparisons among treatments when the F test yielded a significant p value was Tukey's procedure. All analyses were conducted with Minitab Statistical Software (version 16, Minitab Inc., State College, Pa., USA). One snake in the Hd group received a second surgery halfway through the study to replace a failing radio transmitter and was never observed to move again following this surgery. As a result, all data collected from this animal were excluded from all analyses, leaving the Hd group with 7 snakes.





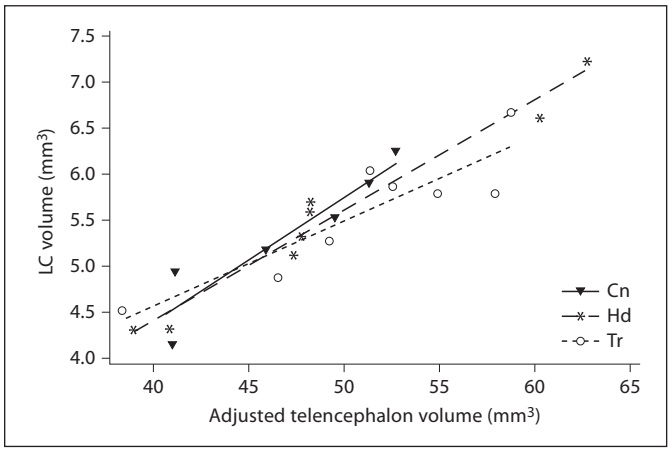


Fig. 2. The volume of the MC, DC and LC for each snake is plotted in relation to adjusted telencephalon volume (TT volume minus respective cortex volume) and separated by treatment group. Tr snakes had significantly larger MCs compared to Cn snakes. Treatment had no other significant impact on cortical volumes.

Results

Snake Ranges and Movement

Our choice of translocation distance appeared to satisfy the description of a short-distance translocation. Snakes never displayed the concentric circling behaviors observed during long-distance translocations that are believed to be attempts to locate familiar areas [Reinert and Rupert, 1999; Nowak et al., 2002]. Snakes navigated back to the exact site of the capture by the day after translocation on a number of occasions. On multiple other occasions, a previously translocated snake was found moving directly toward its previous site of capture.

The activity ranges of Cn snakes in this study were much larger than those from previous years that had been used in choosing the distance for translocation (current study mean 12.16 ha, previous study mean 5.03 ha [Putman B., pers. commun.]). Despite this discrepancy and in concurrence with the results of another study that used short-distance translocation in rattlesnakes [Brown et al., 2008], the MCP activity ranges were significantly impacted by treatment at the 100% ($F_{2,15} = 9.10$, $p = 0.003$) and 95% levels ($F_{2,15} = 10.04$, $p = 0.002$; table 1). While SVL did not have a significant main effect on activity range size ($p > 0.2$), it interacted significantly with treatment to affect activity range size ($p < 0.005$). Post hoc analysis of

Table 1. Mean 100 and 95% MCP areas and TDM of adult male *C. o. oreganus* in each treatment group

Treatment	100% MCP, ha	95% MCP, ha	TDM, m
Cn	12.16 ± 4.65^{a}	10.55 ± 3.93^{a}	5.326 ± 0.303^{a}
Hd	$14.51 \pm 4.61^{a, b}$	13.41 ± 4.28^{a}	5.520 ± 0.360^{a}
Tr	27.33 ± 5.74^{b}	25.56 ± 5.40^{b}	5.601 ± 0.243^{a}

Values represent means ± 1 SEM. Significantly different means are indicated by different superscript letters.

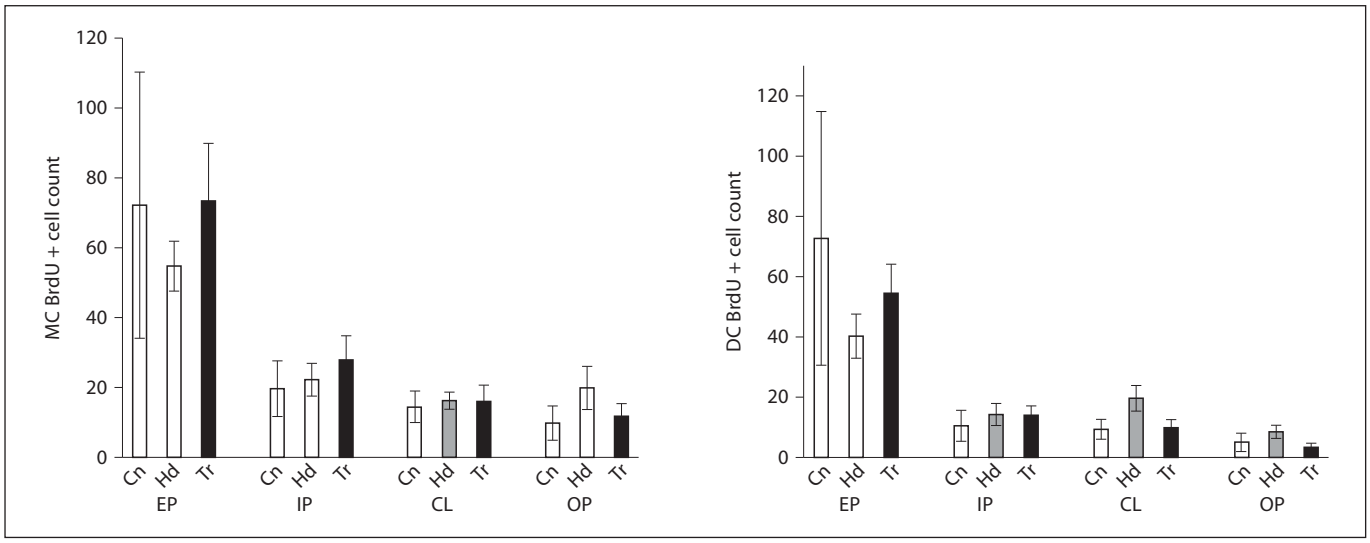


Fig. 3. Numbers of BrdU-labeled cells (means ± 1 SEM) counted in the ependymal (EP), inner plexiform (IP), cell (CL) and outer plexiform (OP) layers of the MC and DC of Cn, Hd and Tr adult male *C. o. oreganus* sacrificed 3 weeks after BrdU injection. There was no effect of treatment on the number of BrdU-labeled cells in any region of the MC or DC.

Table 2. Mean volumes of the cortical regions and TT for adult male *C. o. oreganus* in each treatment group

Treatment	MC, mm^3		DC, mm^3		LC, mm^3		TT, mm^3
	raw	size-corrected	raw	size-corrected	raw	size-corrected	
Cn	10.13 ± 0.49	10.43 ± 0.32	5.884 ± 0.304	6.17 ± 0.16	5.326 ± 0.303	5.60 ± 0.12	52.27 ± 2.36
Hd	11.30 ± 0.63	11.17 ± 0.30	6.419 ± 0.406	6.41 ± 0.15	5.520 ± 0.360	5.49 ± 0.11	54.81 ± 2.30
Tr	12.34 ± 0.55	12.15 ± 0.28	6.701 ± 0.337	6.49 ± 0.14	5.601 ± 0.243	5.40 ± 0.10	56.79 ± 2.56

Raw mean volumes and size-corrected mean volumes (least-squares means accounting for telencephalon volume) are presented for each cortical region. Values represent means ± 1 SEM.

the 100% MCP activity ranges revealed that Tr snakes had larger 100% MCP activity ranges than Cn snakes ($p = 0.0297$), while Hd snakes did not differ significantly from Tr ($p = 0.0619$) or Cn snakes ($p = 0.8826$). Post hoc analysis of the 95% activity ranges showed that Tr snakes had larger activity ranges than both Hd ($p = 0.05$) and Cn snakes ($p = 0.0166$). The TDM was not affected by treatment ($F_{2,19} = 0.76$, $p = 0.482$; table 1).

Cortical Volumes

The TT volume with the respective region of interest subtracted was a highly significant predictor of the size of each region ($p < 0.0001$ for all cortical regions analyzed). The MCP size and TDM were not significant predictors of MC, DC or LC volume ($p > 0.3$). An ANCOVA using the adjusted TT volume as a covariate to control for overall brain size revealed that treatment caused significant changes in MC volume ($F_{2,17} = 8.23$, $p = 0.003$; fig. 2). Treatment did not affect DC volume ($F_{2,17} = 1.17$, $p = 0.334$; fig. 2) or LC volume ($F_{2,17} = 0.84$, $p = 0.449$; fig. 2). Tukey's procedure revealed that Tr snakes possessed significantly larger MCs than Cn snakes ($T = 4.002$, $p = 0.0025$), while Hd snakes did not differ significantly from Tr snakes ($T = 2.397$, $p = 0.0692$) or Cn snakes ($T = 1.689$, $p = 0.2378$). Table 2 shows the average size of each brain region by treatment group.

BrdU-Labeled Cells

One small snake in the Cn group displayed counts of BrdU-labeled cells in its ependymal layer that were large

outliers for both the MC and DC measures, increasing the variability of this group greatly. We analyzed these data both with and without this individual, and since no discrepancies arose we present only the full data set here. Neither the SVL nor the volume of the respective cortical regions were significant predictors of BrdU-labeled cell numbers in the MC and DC. One-way ANOVA revealed that treatment did not affect the numbers of BrdU-labeled cells in the MC (ependymal layer: $F_{2,18} = 0.11$, $p = 0.899$; inner plexiform layer: $F_{2,18} = 0.37$, $p = 0.695$; cell layer: $F_{2,18} = 0.06$, $p = 0.942$; outer plexiform layer: $F_{2,18} = 1.46$, $p = 0.259$; fig. 3) or DC (ependymal layer: $F_{2,18} = 0.38$, $p = 0.688$; inner plexiform layer: $F_{2,18} = 0.28$, $p = 0.758$; cell layer: $F_{2,18} = 2.70$, $p = 0.094$; outer plexiform layer: $F_{2,18} = 1.85$, $p = 0.186$; fig. 3). The TDM and MCP size did not impact any facet of BrdU-labeled cell counts ($p > 0.25$ in all cases).

Discussion

In partial support of our hypothesis that reptiles respond to increased navigational demands with neuroplasticity in the MC, Tr snakes had significantly larger MCs than Cn snakes, with no differences in the DC or LC. Furthermore, the spatial demands of the animals followed the same pattern as the observed MC volume differences, as Tr snakes had larger 100% MCP activity ranges than Cn snakes. These results are in agreement with the finding that male *A. piscivorus* have larger MCs than females, in addition to larger home ranges [Roth et al., 2006]. It may be that the increased navigation of male snakes in that study due to searching for females was the cause of larger relative MC volumes in males compared to females. In light of our results, it is possible that such sex differences are not a genetically determined dimorphism but rather result from MC growth in response to differing navigational demands between the sexes. An interesting next step might be to test whether Tr snakes or male snakes can better solve a field maze or navigate back from a longer-distance translocation compared to non-Tr or female snakes, respectively. By testing such a hypothesis it might be possible to determine whether the ability to navigate, and not just MC volume, is itself plastic in squamates.

Clayton [1995] suggested that hippocampal size in food-storing birds is determined in large part by the act of storing food. However, the actual amount of food-storing allowed did not correlate positively with hippocampal size, and it is suggested that a threshold for the effect of storing on hippocampal size may exist [Clayton and Krebs, 1994]. In the present study, Tr snakes had larger MCP activity ranges than Cn snakes, but TDM was not affected by translocation, nor did MCP size or TDM show a significant positive correlation with MC volume. Thus, Tr snakes carried out the same amount of movement as Cn snakes but did so over a larger area. It may be that traveling over a larger area caused a threshold change that did not differ depending on how much area a snake actually traversed. Alternatively, the act of orienteering and navigating, especially if the area is novel or somewhat unfamiliar, could be the stimulus for MC growth, regardless of activity range size or TDM. As all Tr snakes were translocated the same number of times, they were equal in terms of the demand to emerge from hiding, determine exactly where they were and begin moving to where they wished to be next.

It is important to note that while the differences in both the MCP activity range results and the MC volumes did not turn out as predicted, the relationships among the groups were the same for both variables. The Hd snakes had activity ranges and MC volumes that were not significantly different from those of either the Tr or Cn snakes. The position of Hd snakes in terms of MC volume means that the support for our hypothesis is partial, but the similar relationship between activity ranges and MC volumes is conducive to concluding that MC growth was due to increased navigational demands as the snakes moved over larger areas. If navigational demands were somehow being impacted by our handling treatment, and translocation was a further extension of that increase, this could help explain the position of the Hd snakes in terms of MC volumes. This possibility is supported by the fact that there was a significant interaction between SVL and treatment in determining MCP activity range area. Larger Cn snakes appeared to cover less ground, while the Tr and Hd snakes both showed the opposite pattern. If larger snakes were being stimulated to move by handling alone, then a slight increase in the MC volume may have occurred that obscured potential differences induced by our translocation treatments.

However, additional explanations for the MC volumes of Hd animals must be considered. Given that Tr and Cn snakes were shown to have significantly different relative MC volumes, a type II statistical error may simply have been made in regard to the true effect of handling alone on MC volume. The unresolved position of Hd snakes could be a product of small sample size and the presence of uncontrollable variability, which is a downside of a field experiment like this one and certainly a possibility considering that the p value for comparing Tr and Hd MC

volume approached significance. Another potential explanation is that the stress associated with capture, handling and translocation had a positive effect on MC volume in both Tr and Hd animals and that the additional stressor of translocation further increased MC size in the Tr group. Snyder et al. [2009] showed that restraint stress causes a transient increase in the survival of newly born hippocampal neurons in mice. However, in contrast to these results, Gould et al. [1991] showed that treatment with the stress hormone corticosterone decreased rates of both cell birth and cell survival in rat pups. Likewise, Heine et al. [2004] found that both acute and chronic stressors suppress proliferation and stimulate apoptosis in the hippocampus of adult rats. Furthermore, natural stressors, such as the stress of losing a territorial conflict, have also been shown to result in marked decreases in neurogenesis [Gould et al., 1997].

Since different stressors appear to affect the brain differently, it is impossible to tell whether or not our captures and translocations impacted MC volumes via a stress-related pathway. Studies examining the effect of stress on the cortical forebrain of squamate reptiles would be most informative but have not been conducted as yet. Evidence to suggest that stress did not play a role in MC size in *C. o. oreganus* in this study was obtained in the form of baseline concentrations of corticosterone upon final capture of the animals in the field before sacrifice. Treatment did not significantly affect baseline concentrations of corticosterone [unpubl. observations]. Thus, the differences in MC volume observed are unlikely to be due to differences in circulating corticosterone concentrations indicative of a chronically stressed state. Even if capture stress had some impact on MC volume, the Tr snakes differed further in that following a dive into a hole or shrub near their point of release, they presumably had to emerge and orient themselves. As Tr snakes were often found at their original capture locations, they were receiving repeated stimuli in the form of navigational demands. Hd snakes did not have to orient themselves in the environment before embarking on their next movement, as their original location had not changed. Since corticosterone concentrations did not differ among treatments, we find the most appropriate conclusion to be that the increase in MC size in the Tr snakes compared to Cn snakes was due to the navigational demands incurred through translocation.

The BrdU injection was carried out in the field, followed immediately by application of the appropriate treatment. The labeling period following BrdU injection lasts about 2 h in mammals [Phuphanich and Levin, 1985]. It may be longer in a rattlesnake, a large-bodied, drought-tolerant ectotherm with a generally low standard metabolic rate [Beaupre, 1993]. Still, we do not suspect that labeling lasted for more than 1–2 days. Given a sacrifice time of around 3 weeks following injection, the numbers of BrdU-labeled cells observed in this study were likely a product of both rates of cell proliferation and cell survival.

Our hypothesis that altered navigational demands would result in differential rates of neurogenesis was not supported, as no effect of treatment on numbers of BrdU-labeled cells was observed in any layer of the MC. This is particularly interesting in light of the fact that volumetric differences were generated in the MC as a result of translocating snakes. It is notable that our BrdU injections coincided with the first bout of extremely hot weather of the year. The onset of these inclement weather conditions during the last few weeks of the study caused the snakes to stay below ground, move little (as evidenced by tracking the snakes to the same place over many successive days) and become much harder to catch. As a result, the snakes were only translocated/handled once after BrdU injection prior to sacrifice. Thus, their navigational demands during this period were not as intense as in the previous weeks of the experiment, lacking both experimental treatment and much natural movement of the snakes. Clayton and Krebs [1994] showed that in marsh tits, hippocampal growth stimulated by food storing ceased when food storing was brought to a halt, and a reduction in hippocampal size followed such that halted food storers had hippocampi whose volumes were not significantly different from those that were never allowed to store food. If this type of attrition can occur in snakes in a 2-week period with no treatment and little movement, then the potential effects of our treatments on neuronal recruitment may have been masked. Further, if overall MC volume regressed during this period, then the effect of translocation on MC volume may actually be greater than reported, and the unresolved position of the Hd snakes with respect to MC size may be partly due to the extended period without application of treatment. If the current amount of navigation and space mapping a snake undergoes is directly responsible for concurrent rates of neurogenesis, then we may have missed the period during which rates of neurogenesis were different.

Other potential means by which the MC could have grown include synaptogenesis, decreased neuronal attrition and size and spacing changes in existing neurons. Learning has been shown to increase synaptogenesis in rats, and the link between spatial memory and synaptogenesis is well-established [Black et al., 1990; Moser et al., 1998; Ramírez-Amaya et al., 2001]. Quantification of syn-

aptic density in mature neurons in the MC of these snakes represents a potential future direction to help explain the means by which the MC increased in size. Such an analysis would also provide valuable information regarding neuroplasticity in reptiles, as no studies of reptilian synaptogenic capacity are currently available. Marchioro et al. [2005] showed that most of the BrdU-labeled cells in the tropical iguanid lizard *Tropidurus hispidus* were fated to become neurons, especially in lizards with survival times longer than a few days following injection. If this trend holds across all squamate reptiles, then we can safely say that the rates of neuronal recruitment did not differ significantly among the treatment groups. If neuronal proliferation rates in *C. o. oreganus* differ from those of *T. hispidus,* the possibility that rates of neurogenesis and/or neuronal survival actually did differ between the treatments is not fully excluded either. A double-labeling study using anti-BrdU and anti-β-tubulin III or doublecortin, both markers of immature neurons, would be useful in determining exactly how many neurons were born during the BrdU-labeling pulse. Future studies that focus on periods of high activity in free-ranging animals and cover longer time periods would likely provide much useful insight.

Manipulating the navigational demands of a free-ranging vertebrate represents a unique approach to relating an animal's ability to orient itself and journey within its environment to brain morphology and the ecological significance of neuroplasticity. We have demonstrated that it is possible to experimentally manipulate space use and navigational demands in a manageable fashion in rattlesnakes. Although experiments such as ours do carry the limitation of reduced control, these costs are offset by gaining the valuable ability to elucidate cause and effect in a natural setting. Foremost among our goals in exploring brain function should be to understand the functional significance and adaptive value of a particular trait for the organisms in which it occurs. It is in this light that we suggest that these results have greatly aided in solidifying the role of MC neuroplasticity in responding to the varying demands for space use and navigation reptiles encounter naturally, such as those related to seasonal differences in spatial ecology. This study was limited to one season, one sex and one species of rattlesnake, leaving open the possibility for further informative studies in the same system. Additional studies that attempt to manipulate navigational demands in free-ranging vertebrates would be most useful to fine-tune our understanding of neuroplasticity and its role in navigation and spatial memory, especially if those studies can determine the specific types of cues being used to navigate (e.g. spatial, place, olfactory). Although our transport buckets were closed to the surroundings and sky and likely permitted little exposure of olfactory cues, we may have induced changes in other senses (e.g. magnetoreception) in both our Tr and Hd treatments, should such senses exist in these snakes. A further exploration of this issue would be exceptionally useful. Lastly, the discovery of adequate mammalian and avian models, along with additional reptilian models, would greatly expand the knowledge we can gain from field-based neuroecological experiments.

Acknowledgements

We wish to thank Scott Dorr for assistance in the field, Sloane Henningsen for assistance with field work and graphics, Kelsee Buskirk and Amanda Wagner for assistance with tissue preparation and Jason Blank for comments on the manuscript. This study was funded by a National Science Foundation Graduate Research Fellowship awarded to M.L.H., the Herpetologists' League's E.E. Williams Research Grant to M.L.H., an Aryan I. Roest Memorial Scholarship from California Polytechnic State University to M.L.H. and the California Polytechnic State University Biological Sciences Department.

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