Altered Neurotransmission and Neuroimaging Biomarkers of Chronic Arsenic Poisoning in Wild
Muskrats (Ondatra zibethicus) and Red Squirrels (Tamiasciurus hudsonicus) Breeding near the
City of Yellowknife, Northwest Territories (Canada)

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Abstract

Chronic arsenic poisoning has been shown to be a risk factor for the development of intellectual disability. Numerous human and animal studies have also confirmed that low-level arsenic exposure has deleterious effects on neurotransmission and brain structures which have been further linked to neurobehavioral disorders. The aim of this present work was to comparatively assess structural brain volume changes and alteration of two (2) neurotransmitters, specifically dopamine (DA) and serotonin (5-HT) in the brains of wild muskrats and squirrels breeding in arsenic endemic areas, near the vicinity of the abandoned Giant mine site in Yellowknife and in reference locations between 52-105 km from the city of Yellowknife. The levels of DA and 5-HT were measured in the brain tissues, and Magnetic Resonance Imaging (MRI) was used to attempt brain volume measurements. The results revealed that the concentrations of DA and 5-HT was slightly increased in the brains of squirrels from the arsenic endemic areas compared to the reference site. Further, DA and 5-HT were slightly reduced in the brains of muskrats from the arsenic endemic areas compared to the reference location. In general, no statistically significant neurotransmission changes and differences were observed in the brain tissues of muskrats and squirrels from both arsenic endemic areas and non-endemic sites. Although MRI results showed that the brain volumes of squirrels and muskrats were not statistically different between sites after multiple comparison correction; it was noted that core brain regions were substantially affected in muskrats, in particular the hippocampal memory circuit, striatum and thalamus. Squirrel brains showed more extensive neuroanatomical changes, likely due to their relatively smaller body mass, with extensive shrinkage of the core brain structures, and the cortex, even after accounting for differences in overall brain size. The results of this present study constitute the first observation of neuroanatomical changes in wild small mammal species breeding in arsenic endemic areas of Canada.

Keywords: Chronic arsenic poisoning; brain volume; magnetic resonance imaging; and wildlife.
Arsenic (As) is a naturally occurring non-essential metalloid capable of inter-organ toxicity (Shameem et al., 2015). Primarily found in the Earth’s crust and bedrock, it can gradually leach into underground water, subsequently being distributed through the wider environment and taken up by biological organisms (Vahter, 2008). Exacerbated by anthropogenic activities such as gold mining, As has been shown to accumulate in groundwater, soil and vegetation across different mineralized environment in China, Korea and Canada (Huang et al., 2019; Kim et al., 2005; Ko et al., 2003). Humans and animals living in As-endemic areas are exposed to chronic As exposure through ingestion, inhalation and skin absorption and several adverse pathologies have been reported in animals and humans inhabiting these areas (Amuno et al., 2018; Clark and Raven, 2004; Shameem et al., 2015). While acute arsenic toxicity can result in death, chronic exposures have been linked to inter-organ carcinogenesis, mutagenesis, and apoptosis (Fry et al., 2007). Different arsenic species have been shown to cross the blood-brain barrier, likely through the activity of glucose transporter GLUT1 in mammals, allowing them to accumulate in brain tissues over time with the highest concentrations appearing at the pituitary gland and hypothalamus (Agrawal et al., 2015; Liu et al., 2006; Mejia et al., 1997). Analyses of brain images obtained with magnetic resonance imaging (MRI) indicate that past metal exposure to lead, another neurotoxic metal, can result in changes in brain volume (Cecil et al., 2008). While the relationship between brain volume and As exposure remains largely unexplored, the element has been linked to toxic encephalopathy and cognitive impairment in human subjects and animal models (Bolla-Wilson and Blecker, 1987; Fincher and Koerker, 1987; Hamadani et al., 2011). As exposure has additionally been linked to central nervous system (CNS) damage, resulting in gradual loss of brain function, Alzheimer’s disease (AD), Down’s syndrome (DS) and Parkinson’s disease (PD) (Bartolomé et al., 1999; O’Bryant et al., 2011; Shameem et al., 2015). As-induced neuropathology is likely driven by the metalloid’s ability to induce generation of reactive oxygen species (ROS) (Lebel et al., 1992; Samikkannu et al., 2003) whilst disrupting the endogenous antioxidant defence systems in affected cells (Argos et al., 2006; Breton et al., 2007).

Ultimately, As exposure results in elevated lipid peroxidation (LPO), a common biomarker of oxidative stress and neurotoxicity (Flora et al., 2005; Pi et al., 2002; Ramanathan et al., 2003). It is commonly believed that brain tissues are so susceptible to oxidative stress due to their relatively low level of antioxidant defence, high oxygen utilization and high iron content (Agrawal et al., 2015; Uttara et al., 2009). An additional mechanism of As-induced neurotoxicity is the ability of As to alter the organization and phosphorylation of cytoskeletal proteins, ultimately resulting in neuropathy (Vahidnia et al., 2008; Vahidnia et al., 2007). The accumulation of As in brain tissues has also been shown to impact concentrations of several monoamine neurotransmitters including serotonin (5-HT) and dopamine (DA) (Mejia et al., 1996; Uttara et al., 2009). Disruption in 5-HT and DA concentration has been linked to AD, PD, DS and other neurological diseases (Freed et al., 2001; Reinikainen et al., 1990; Shimohata et al., 2017). In addition to tissue concentration, monoamine neurotransmitter function is modulated by uptake rates, transporter ex-
pression and transporter affinity, albeit these remain poorly understood in both muskrats and red squirrels (Daws, 2009).

Despite the well-established link between As exposure and cognitive deficits, few studies have directly examined the association between environmental arsenic exposure and subsequent neurostructural features and neurotransmission in wildlife breeding in As contaminated areas. To this end, the present study aimed to assess how brain levels of 5-HT and DA and brain volume differ among two wild rodent populations, Muskrats (Ondatra zibethicus) and Red squirrels (Tamiasciurus hudsonicus), breeding at different proximities to As endemic areas near the abandoned Giant mine site and in reference locations from the city of Yellowknife, Northwest Territories, Canada. While mining operations ceased at the Giant Mine site in 2004, elevated concentrations of As continue to be observed in local water, vegetation and soil around the mine area and surrounding locations giving rise to public concerns about potential arsenic exposure and associated health effects (Amuno et. al, 2018; Jamieson, 2014).

Wild muskrats and red squirrels are widely distributed across Northern Canada, making them an appropriate choice for examining the As-induced toxicity associated with proximity to the Giant Mine site. Additionally, muskrats and squirrels exploit different ecological niches, with the former species being semi-aquatic and the latter fully terrestrial. As such, studying both species provides potentially important perspectives about different routes of environmental As exposure to mammalian communities in the Northwest Territories. The muskrat and red squirrel both exhibit reportedly narrow home-ranges, with the latter being highly territorial, implying any As exposure would have occurred within the site they were collected at (Ahlers et al., 2010; Gurnell, 1984).

Thus, the primary purpose of this study was to compare structural brain volume changes and alterations of neurotransmitters in wild population of muskrats and squirrels breeding in arsenic endemic areas and non-endemic areas near the city of Yellowknife, Northwest Territories, and to further assess the correlation between different brain parameters (volume variations and neurotransmission) and nail biomarker of arsenicosis. In addition to examining molecular markers, the application of MRI in this study allows us to evaluate the local changes in brain volume. MRI-based brain volume changes in murine are used extensively in studies of neuropsychiatric disorders and the effects of pharmaceuticals and as such offer a sensitive and whole brain assay for neurotoxin exposure (Guma et. Al, 2018).

2 Materials and methods

2.1 Sampling

Ethical clearance for wildlife handling was granted from the Department of Environment and Natural Resources, Government of the Northwest Territories as well from the University of Saskatchewan. Proper permits were obtained before commencing the study: a wildlife research permit (WLWL500561) and a general research licence (No. 16190) from the Aurora Research Insti-
Brain samples were isolated from a total of 15 muskrats and 15 squirrels sampled from different locations surrounding the mining area. Nails and gut contents were isolated from 15 additional animals into a total of 30 muskrats and 30 squirrels. These animals were trapped from three different locations surrounding the mining area: the vicinity of the abandoned mine (within ~2 km radius; referred to as site 1); an intermediate location 20 km (referred to as site 2) from the Giant mine site; and from a background/reference area (referred to as site 3). Reference area (site 3) for capturing muskrats ranged between ~53.4 km to 62 km from Yellowknife, and for squirrels it was between 95 km to 105 km from the city of Yellowknife.

Animals were not separated into male and female groups due to the unequal numbers of male and female animals captured during the field work. The ages of the squirrels and muskrats were generally estimated based on their overall body weight and length. For example, a squirrel was considered an adult if the body length was between 10-15 inches (25.4-38.1 cm) and weighed between 260-350 g (Pennsylvania State University 2002). A muskrat was considered adult if it weighed between 2.5 and 4 pounds with total body length ranging from 23-26 inches (58.42-66.04 cm) and with a tail length of 8-11 inches (20.32-27.94 cm) (New York Department of Environmental Conservation). All animal samples utilized for this study were generally within the adult range.

Animals were sampled between March and April 2018. All trapped animals were euthanized and dissected for the removal of target organs such as brain, liver, kidney, nails, gut content and bones were separated in individual falcon tubes and frozen. The scope of the present study included the examination of the neuroanatomical and biochemical status of the brain systems of the animals and did not specifically include observations of clinical or behavioural differences between individuals or sites. For MRI analyses, whole heads of muskrats and squirrels were collected immediately from the animals in each site (~5 per site) and fixed in formalin solution and shipped to McGill University for MRI scanning and assessment. For measurements of metal(loid), serotonin and dopamine, brain tissues were isolated from each frozen head sample and divided into two parts: metal(loid) analysis and biochemical measurement. Samples were then shipped back to the laboratory of University of Saskatchewan (Canada) for biochemical and metal analysis. All animal wastes and carcasses were stored in MS biobags (100% biodegradable) and transported to the landfill for final disposal.

### 2.2 Analysis of Arsenic and Cadmium in biological tissues and environmental samples

The protocol for tissue digestion for metal(loid) analysis is described Amuno et al, 2018). Briefly, brain and gut contents of muskrats and squirrels were digested in 5 volumes of 1N trace metal grade Nitric acid (EMD Millipore, Billerica, MA, USA) at 60 °C for 48 h. The method for digesting nail samples was similar except the concentration of nitric acid (16 N). After digestion, supernatants were collected from tissue digests by centrifuging at 2500 g for 10 minutes. The concentrations of As and Cd in supernatants were measured using a graphite furnace atomic absorption spectrometer (AAnalyst 800, Perkin Elmer, USA) after appropriate dilution of samples.
in 0.2% nitric acid. A reference material (DOLT-4; National Research Council of Canada) along with appropriate method blanks, certified Cd and As standards (Fisher Scientific, Canada) were included in the measurements for the purposes of quality control and assurance. The recovery percentage of Cd and As in the reference material was 96% and 92%, respectively. The trace metal concentrations in animal tissues were normalized on the basis of wet tissue weight. Environmental contamination of the study area was determined through measurement of total As and Cd in vegetation, surface water and soils samples, and contaminant exposure was determined through analyses of As and Cd in nails and brains of the animals. Detailed description of the study area has been reported previously (Amuno et al. 2018).

2.3 Biochemical measurement of DA and 5-HT in the brain tissues of muskrats and squirrels

5-HT and DA concentration in the brains of muskrat and squirrel were measured using commercial ELISA kits, according to the manufacturer’s instructions (BioVision). Briefly, brain tissue was isolated from the animals, weighed and homogenized in PBS buffer using a polypropylene pestle attached to a portable handheld homogenizer. The homogenates were then centrifuged for 5 minutes at 5000 g and the supernatants were collected. Before starting the assay, all the reagents, standards and samples were brought to room temperature. Samples and standards were run in duplicate. A standard curve was run, and the values of unknown samples were determined from the standard curve. All readings were performed at 450 nm using Verioskan Flash™ (Thermo Fisher Scientific) plate reader.

2.4 Magnetic resonance imaging (MRI)

Squirrel brains in skull were removed from preservative and patted dry. Eye sockets were filled with a water-sorbitol gel to prevent magnetic susceptibility artifacts during imaging. MR images were acquired using a Bruker 7T 70/30 USR magnet with 30 cm bore and AVANCE electronics, using Paravision 5.1. The specimens were placed in a 40 mm, 1H transceive, volumetric radiofrequency (RF) coil (Bruker). A structural, 3D FLASH (Fast, Low Angle SHot) sequence was used with TE/TR of 4.894 ms / 20.750 ms, flip angle of 20° and zero-fill acceleration factor of 1.34. Final matrix size was 240 by 174 by 148, yielding 150 μm isotropic voxels. Total scan time was 24 minutes using 2 averages. Muskrat brains in the skull were imaged using a Bruker Pharmascan 7 T MRI system. The Pharmascan has a 16 cm clear bore and AVANCE II radiofrequency (RF) amplifier electronics. The Bruker software version employed for imaging the muskrat brains was Paravision 5.1. During imaging, specimens were placed in a 40 mm, 1H transceive, volumetric RF coil (Bruker). A three-dimensional, sagittal orientation, steady-state free precession (SSFP) sequence was used for imaging. The SSFP imaging sequence provides high contrast between gray and white matter and delineation of anatomical boundaries in the brain. Relevant SSFP MRI sequence parameters were as follows: TE/TR of 5 ms/10 ms, flip angle of 30°, 31
signal averages and an isotropic imaging spatial resolution of 200 μm. Total scan time for each brain was six hours and 54 minutes.

Squirrel and muskrat brains were processed iteratively in order to produce final brain difference maps in a standardized orientation. Briefly, raw scans were manually reoriented into standardized MINC animal orientation (RAS), bias field corrected with N4 to a minimum knot distance of 1.875 mm across the entire field of view, Otsu thresholded to determine a foreground-background mask, which was then used to re-perform N4 weighted on tissue (Tustison, et al., 2010; Otsu, 1979). This first round of MRI scans was then processed using the Mice-Build-Model (MBM.py) pipeline from pydpiper/2.0.12 with a bootstrap initialization, to create an unbiased average of the input population through a iterative linear and nonlinear image registration steps (Friedel et. al, 2014). The resulting bootstrap average was then rotated to align the brain axes to the primary directions of the MRI volume and the brain roughly manually segmented. The brain average was then registered to the original preprocessed scans via linear registration with “antsRegistration” and the average brain mask transformed onto the individual subjects to provide a new weighting for N4 correction (Avants et. al, 2011). The MBM.py model build was then repeated using the first-round average as a target for initialization and the preprocessing repeated. Finally, the second-round average was used again for a final run of MBM.py where the voxel-wise brain difference maps were used for further statistical analysis. Voxel wise brain differences are computed as “minimum deformations” from an unbiased average of the input subject scans. These minimum deformations represent the change in volume from the subject to the average, as computed by the Jacobian determinant of the deformation field. Two determinant maps are produced, one including any bulk volume differences estimated from the linear registration stage, and another where residual linear changes are estimated and removed, to produce a pure local volume difference map. The Jacobian maps were then log transformed and smoothed at 200 um full-width-half-maximum 3D gaussian for statistical regularity.

2.5 Statistical analysis

The brain volumes and brain levels of 5-HT and DA were compared between the reference site (site 3) and the As endemic sites (site 1 and site 2; 2 km and 20 km away from Giant mine, respectively) using t-tests. A non-parametric Mann-Whitney U test was used in case the assumptions of equal variance and normal distribution were not met for a particular analysis. All analyses were carried out in SigmaPlot11 (SystatSoftware Inc., San Jose, California, USA). Metal(loid) accumulation (from the environment samples and animal tissues was used for determining their correlation with the brain neurotransmitters by applying two tailed Pearson correlation analysis in R software (version 3.5.1) (R Core Team, 2018). Correlation tables were created by using ‘corrplot’ package in R. In all statistical procedures, a p-value of ≤0.05 was considered to be statistically significant. Voxel-wise Jacobian determinant maps were analyzed using R/3.5.1 and RMINC/1.5.2.1 using general linear modelling with site as a factor predictor and site 3 as the
reference, voxel-wise multiple comparisons were corrected for with false discovery rate (FDR) (R Core Team, 2018; Lerch et. al, 2017).

3 Results

3.1 Environmental Exposure Data

Measurements of As and Cd was conducted in the nail samples and other environmental samples (i.e. soil, surface water and vegetation samples) from the study area which generally showed elevated concentration of As in site 1 and 2 (Table 1). We have previously reported elevated concentrations of As in the soils and vegetation samples ~20 km radius of the vicinity of the Giant mine site (Amuno et al. 2018). Recent investigations have shown that As concentration in lakes surrounding the Yellowknife area exceeded the federal drinking water guideline of 10 µg/L (Palmer et al. 2015). In this study, total arsenic (As) concentrations in the nails of muskrats and squirrels from arsenic affected areas, near the Giant mine site, were significantly higher than those from the control areas. Muskrats sampled from near the mine area showed nail arsenic levels that ranged from 0.66 µg/g to 2.1 µg/g, while those from the reference site was generally below detection limit of 0.063 µg/g approximately ~53.4-62 km away from the city of Yellowknife. Cd was not detectable in the nails of muskrats and squirrels from the As endemic areas and the reference area. For the squirrel samples, total As concentration in the nails ranged from below detection limit to 1.4 µg/g for squirrels sampled nearest to the mine site ~2 km, but ranged from non-detectable to 0.2 µg/g for the squirrels from 20 km radius from the mine site, and was generally non-detectable in all the squirrel samples from the reference location. As was not detected in the brain samples of muskrats from the study area but Cd was detected in two brain samples of muskrats closest to the mine area, which ranged from 0.0018 µg/g to 0.0024 µg/g. As was detected in the brains of squirrels from the vicinity of the mine area (0.06-4.18 µg/g) and intermediate location 20 km away (0.072-0.95 µg/g) and was undetected in the squirrel brain samples from the reference site.

3.2 Brain DA levels

For squirrels, the general trend in the data indicated a slight reduction in DA levels with increasing distance from the mining area. For squirrels, the highest DA level was found at site 1, with a mean of 27.37 ng/mg protein, followed by site 2 with mean value of 26.05 ng/mg protein and finally at site 3, with mean value of 20.05 ng/mg protein (Figure 1b). Although there was minor reduction in the DA levels away from mine site, none of these reductions were statistically significant, as revealed by t-tests among different sites (Table 2). On the other hand, for muskrats, there was a slight increment in brain DA levels with increasing distance from the mining area. The lowest DA level was found at site 1, with a mean of 169.25 ng/mg protein, followed by a slightly higher DA levels at site 2 with mean value of 175.96 ng/mg protein and finally the highest DA levels at site 3, with mean value of 232.86 ng/mg protein (Figure 1a).
there were minor changes in DA levels of the muskrats with site, but none of these changes were statistically significant, as revealed by t-tests among different sites (Table 2).

### 3.3 Brain 5-HT

For muskrats, the general trend in the data indicated no relationship between 5-HT levels with the distance from the mining area. For muskrats, the mean 5-HT levels were 942.84, 839.14, and 1072.13 ng/mg protein, for site 1, 2, and 3, respectively (Figure 2a). As expected, there were no statistically significant difference in the 5-HT levels between different sites, as revealed by t-tests among different sites (Table 2). For squirrels, the mean 5-HT levels were 8501.69, 1770.02, and 2143.61 ng/mg protein, for site 1, 2, and 3, respectively (Figure 2b). The mean values in squirrels showed an extremely high 5-HT levels at site 1; and this is due to the presence of an outlier as indicated in Figure 2b with the value of approximately 10 times higher than the median. Removing the outlier brings the mean value of 5-HT at site 1 to 2840.74, which is still slightly higher than those of sites 2 and 3. Nonetheless, there were no statistically significant difference in the 5-HT levels between different sites, as revealed by t-tests among different sites (Table 2).

### 3.4 Brain volume changes

For muskrats, the mean total brain volumes were 4734.62 mm$^3$, 4790.14 mm$^3$, and 4816.66 mm$^3$, for site 1, 2, and 3, respectively (Figure 3a). As expected, there were no statistically significant difference in the brain volumes between different sites, as revealed by t-tests among different sites (Table 3). For squirrels, the mean brain volumes were 4941.96 mm$^3$, 5046.995 mm$^3$, and 4879.013 mm$^3$. Similarly, there were no statistically significant difference in the total brain volumes of squirrels between different sites (Figure 3b and Table 3). For both muskrats and squirrels, the general trend in the data indicated no relationship between total brain volume and the distance from the mining area.

Local brain differences represented by log-transformed voxel-wise Jacobian local deformation maps did not show any differences between sites after correcting for multiple comparisons in FDR. Considering this dataset is an environmental sample with substantial unaccounted for variation between subjects, we present below the site-wise local brain differences statistical maps thresholded at <0.05 uncorrected.

Figure 4 shows a series of coronal sections comparing muskrat relative brain differences for the 2 km (4a) and 20 km (4b) sites relative to site 80 km. While no anatomical atlas exists for this population, their similarity to other murine makes general interpretation possible. All regions inferred from anatomy are based on 7th Edition Paxinos rat brain atlas. Table 4 lists brain regions and direction of effect for the 2 km and 20 km vs 80 km group difference. The unthresholded maps for muskrats show a few regions of increased volume (cerebellar lobules, superior colliculus and fornix) but substantial regions of decrease include the hippocampus, subcortical structures, amygdala and somatosensory and motor cortices. Effects reduce at the 20 km site, but
many remain. In addition, volumetric differences are noted just outside the inferior midbrain, indicating a possible bulk reduction in the volume across all structures.

Figure 5 shows a series of coronal sections comparing squirrel relative brain differences for the 2 km (5a) and 20 km (5b) sites relative to site 80 km. Again, while no anatomical atlas exists for this population, their similarity to other Murine makes general interpretation possible. All regions inferred from anatomy are based on 7th Edition Paxinos rat brain atlas. Table 5 lists the brain regions and direction of effect for the 2 km vs the 80 km group difference in squirrels.

The unthresholded maps for squirrels show similar regions of increased volume to muskrats, with cerebellar and some white matter regions. We also see broadly distributed increase in the volume of the dentate gyrus. There are again peak reductions in hippocampal, subcortical and amygdaloid regions, but in addition, a broad reduction in the volume of cortex across most regions.

3.5 Correlation between distance, exposure status, and brain neurotransmitters

In a two-tailed Pearson correlation for the muskrat and squirrel, DA and 5-HT did not show any significant correlation with metal(loid) concentrations in vegetation, distance and soil. However, serotonin (5-HT) and DA showed a significant negative correlation with the As concentration in nails. In squirrels, brain DA showed significant negative correlation with nail As concentration (-0.43, p = 0.04). DA in squirrel did not reveal significant correlations with any of the parameters tested (Figure 6). In muskrats, brain 5-HT showed significant negative correlation with nail As concentration (-0.75, p = 0.02) (Figure 7). DA in squirrel did not reveal significant correlation with any of the parameters tested.

4 Discussion of Results

4.1 Influence of arsenic and Cd exposure on DA and 5-HT levels in muskrats and squirrels

Both inorganic as well as organic form of As are known to accumulate in brain of many animal models (Sánchez-Peña et al., 2010; Tyler and Allan, 2014). Several studies have suggested that As could cause cognitive and neurological deficits in humans (Tolins et al., 2014; Tyler and Allan, 2014). Laboratory studies have shown that exposure to moderate levels of As over long term exposures could lead to negative impact on the DA and 5-HT regulation in mice (Kim et al., 2014; Liu et al., 2013). However, available evidences on As induced toxicity seems to suggest species and sex dependent differences in the effect level (Bardullas et al., 2009; Liu et al., 2013; Rodríguez et al., 2010). Cd has also been shown to affect the levels and release rates of 5-HT and DA in brain of mammals (Gutiérrez-Reyes et al., 1998; Lafuente et al., 2003, 2001). Moreover, As and Cd exposure has been linked with various types of brain damages. For example, exposure to inorganic As caused encephalopathy and peripheral neuropathy in many patients (Jenkins, 1966). Cd, on the other hand, has been hypothesised to be a causal factor in Myalgic Encepha-
lomyelitis/Chronic Fatigue Syndrome and associated decrease in the volume of the gray matter (Pacini et al., 2012). We have shown that a relatively higher concentration of As and Cd are present in the soil, vegetation and water near the vicinity of Giant mine in Yellowknife, which may increase the risk of neurotoxicity in exposed animals due to chronic arsenic poisoning.

The results presented in this work is the first documentation of 5-HT and DA content of the brains in wild rodent species in As endemic areas of Canada. The metal(loid) contents in the brain of squirrels and muskrats demonstrated a species-specific difference (part 1 results). Squirrels collected from site 1 and 2 showed relatively higher levels of As and Cd in their brains compared to the reference site. On the other hand, muskrats collected from As contaminated locations did not show any significant increase in brain levels of either metal (loid)). It is possible that the integrity of the blood-brain barrier in muskrats remained intact and prevented the metal(loid)s from penetrating into the brain despite chronic arsenic exposure. More efficient detoxification of metal (loids) in muskrats could also explain the lower levels of Cd and As in the brain. However, evaluation of the species-specific differences in the blood-brain barrier integrity and As detoxification was not in the scope of this present study; hence, not performed. Exposure of brain to As and Cd in the squirrels may have contributed to the significant increase in the activity of glutathione peroxidase, along with minor although non-significant increase in catalase in the squirrels captured near the mining site.

Despite significant accumulation of As and Cd, and minor disturbance in the oxidative environment in the brains of squirrels, this study did not observe any significant changes in the DA and 5-HT content in the squirrels captured near the Giant mine area compared to background locations. Similarly, muskrat brains from the As endemic area did not show any evidence of significant disturbance in the content of DA and 5-HT compared to control location.

### 4.2 Proximity to Giant mine and alteration of neurotransmission in muskrats and squirrels

A negative correlation between distance from the mine area and concentrations of DA and 5-HT levels in the brains tissues of squirrels was observed (Figure 6); however, because these correlations were not statistically significant; the relationship or impact between distance from the mine area and the brain DA and 5-HT levels cannot be established conclusively. Research has shown that people with neurobehavioral disorders and mental disturbances have altered neurotransmitters function (Zangen et. al, 2001). Several studies have shown that abnormal neurotransmitters activities, particularly DA and 5-HT have roles to play in schizophrenia and other mental disorders (Remington, 2008. Laruelle et. al 2000). Elevated and 5-HT concentrations in the brain have been linked to anxiogenic behaviours in both lab mice and the Meriones shawi desert rodent (Yamada et al. 2000; Bouytas et al. 2019). Upregulation of 5-HT receptors in the zebrafish brain have also been linked to loss of social and antipredator behavior (Attaran et al. 2019). Studies have also shown that abnormalities in 5-HT activity also play an important role in a variety of...
mental illnesses (Arango et al., 2002). Excessive activation of DA signaling pathways may be linked to psychotic disorders (Tost et al. 2010).

Recognizing that the concentrations of dopamine and serotonin in the animals from the As endemic areas did not differ significantly from the control location, it was difficult to determine the extent of neurotransmitter dysfunction due to the influence of chronic arsenicosis. Given that 5-HT showed a significant negative correlation with the As concentration in nails for muskrats (-0.75, p = 0.02) and DA showed negative correlation with As concentrations in nails for squirrels (-0.43, p = 0.04), it is likely that chronic As exposure may have a direct impact on the dopaminergic and serotonergic system in a species-specific manner which may induce depression-like or anxiety-like disorders in the exposed animals (Ressler and Nemeroff, 2000). While not part of the present study, it is likely that behavioural analysis carried out in squirrels from site 1 may exemplify some of the same anxiogenic behaviours associated with 5-HT disruption reported in other rodent species.

4.3 Nail biomarker of chronic arsenicosis and relationship with neurotransmission

External tissues such as nails and hair have been considered as important tools for monitoring chronic arsenic poisoning and can provide good estimation of exposure (Jenkins, 1979). Several studies have demonstrated significant correlation between trace element concentrations in nails and a variety of adverse outcomes such as changes in body mass index, changes in blood pressure, and peripheral vascular diseases (Grashow et al., 2014; Lin et al., 1998; Mordukhovich et al., 2012). In confirmation with these previous studies, our study, for the first time, showed a negative correlation between nail As concentration and brain 5-HT and DA concentrations in muskrats and squirrels, respectively. Hence, as described above, nail As concentrations could have a profound effect on neurotransmission function in the exposed muskrats and squirrels inhabiting the vicinity of the mining area. On the contrary, As contents in the brains of squirrels did not show significant correlation with the neurotransmitter levels in the brains (Fig. 4). Therefore, the results of this study pose a question as to why brain As levels did not show strong correlation with the neurotransmitters despite the chronic exposure to arsenic from the natural environment. It is likely that the excessive metal(loid)s in brain could have been sequestered by small proteins such as metallothionein (MT). MT proteins are present in abundant quantities in the brain of mammals (Ebadi et al., 1995). Sequestration of metals by MT is expected to provide protection to neurotransmitter releasing neurons. Finally, in this study, we only measured the neurotransmitter concentrations and not their release pattern from brain neurons. A few studies have shown that both As and Cd can affect the release or the ability of neurons to sustain the release of neurotransmitters (Gutiérrez-Reyes et al., 1998; Rodríguez et al., 1998). Changes in neurotransmitter release patterns could lead to a variety of behavioural, motor, and cognitive malfunctions in mammals (Beninger, 1983). Unfortunately, it is logistically not possible to conduct the neurotransmitter release studies in a field setting. Nonetheless, significant negative correlation between nail As concentration and brain 5-HT and DA concentrations in muskrats and squirrels clearly indicate
that dopaminergic and serotonergic systems in the muskrats and squirrels populations in this
mining area might be at the risk of potential adverse effects.

4.4 Environmental exposure and Brain volume changes in muskrats and squirrels

Changes in brain volume have been observed in many neurodegenerative diseases (Oxtoby et al.,
2017; Tascone et al., 2017), and metal(loid)s like Cd and As have been linked to neurodegenera-
tive disorders (Escudero-Lourdes, 2016; Lee et al., 2018). Based on high concentration of Cd and
As in the soil and vegetation near the study area, and a known association of these metal(loid)s
with neurodegenerative disorders, we hypothesised that the brain volume of muskrats and squir-
rels will be affected near the mining area. However, our study did not find any evidence of sig-
nificant decrements in brain volume associated with chronic As poisoning in the animals from
the As endemic areas and background area. It is likely that low sample size may have affected
the statistical robustness of our results as an environmental sample has a large number of uncon-
trolled variables. It also remains unknown whether the brains of animals from the study area
have been affected by demyelination, neuronal and axonal loss due to exposure to As and Cd
from the natural environment. Therefore, more studies are needed to conclusively establish
whether there is a causal relationship between As and Cd contamination in the Yellowknife envi-
ronment and brain volume changes in wildlife species inhabiting the vicinity of the mining site.

In both muskrats and squirrels, we observed volumetric increases in some common areas, and
decreases in core brain regions and the cortex. At the level of MRI volumetry such as this, we are
unable to definitively confirm if volume increases and decreases are due to cell growth, cell
death, or inflammatory processes without the aid of microscopic cellular analysis. Given the ex-
tensive changes across the brain, including the midbrain regions, however, we surmise that
chronic arsenic poisoning and metal exposure are likely having system-wide effects on the brain
of muskrats and squirrels. The measures of both increased and decreased volume indicate that a
disruption of neural circuitry is likely, with some regions having compensatory increases to bal-
ance changes elsewhere. This is most clearly seen with the somatomotor cortex and cerebellum,
which play complementary roles in motor control.

A finding that deserves further consideration is the lack of the observed statistical relationship
between the role of toxicants and brain volume. There may be several reasons why this has not
been observed, including: the duration of the exposure, route of exposure, sample size consider-
ations, and overall risk and resilience to exposure to toxicants. A further major contributor is the
unknown population variability of brain structure, a factor typically controlled via inbred labora-
tory strains in pre-clinical animal studies. Further, unlike the well-established methods in comput-
tational methods that we have leveraged here, we lack a validated MRI-based atlas for the squirrel
and muskrat which may have also added some error in our analyses. Unlike laboratory neu-
roscience experiments, it is also difficult to have a true control group in this type of a natural en-
vironmental setting that would allow us to fully elucidate this relationship. As this type of ex-
perimental work proceeds, increased group sizes and additional descriptive information for each
sample will enable develop statistical methodologies that can be robust to the type of variation described here.

5 Conclusion

This is the first study to directly examine the effects of chronic arsenic poisoning and Cd exposure on neurotransmission and brain volume changes in wildlife species from As endemic areas of Canada. The statistical analysis did not reveal any significant changes in the levels of brain DA and 5-HT in the muskrats and squirrels across sampling locations. However, nail As concentration showed significant correlation with brain 5-HT and DA concentrations in muskrats and squirrels, respectively. Overall, evidence of Cd and As accumulation in the brain samples of some animals, and observation of extremely high Cd/ and As accumulation in soil, vegetation, and water from the vicinity of the mine area suggests there could be a potential risk of neurological or neurobehavioural changes in the animals breeding in arsenic affected areas. This is supported by our observation of significant negative correlation between nail As concentrations and brain 5-HT and DA levels in these species. There is also need for collection more samples in order to generate increased understanding of the prevalence of brain volume anomalies and altered neurotransmission in wildlife species from the Yellowknife area. We suggest that future studies focus on monitoring neurotransmitter release patterns as a part of neurological effect analysis of metal(loid).

Disclaimer

Dr. Amuno is an adjunct professor at the School of Environment and Sustainability, and his participation in this study was undertaken independently and apart from his current work with the Nunavut Impact Review Board (NIRB). The analysis and views expressed in the study remain solely those of the authors and do not constitute the views of NIRB.

References


Jenkins, D.W., 1979. USEPA. Toxic trace metals in mammalian hair and nails. EPA-600/4-79-049.


Table 1: Measured concentrations (maximum-minimum) of As and Cd in nails of animals, surface soils, vegetation, and water from three different sites near mining area. Site 3 represents reference site, site 2 represents intermediate location (~20 km from mine), and site 1 represents locations in the vicinity of mine (within ~2 km radius).

<table>
<thead>
<tr>
<th>Sites</th>
<th>Surface soils (mg/kg) N=36</th>
<th>Vegetation (mg/kg) N=43</th>
<th>Water (μg/L) N=12</th>
<th>Muskrat nails (μg/g) N=15</th>
<th>Squirrel nails (μg/g) N=15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As</td>
<td>Cd</td>
<td>As</td>
<td>Cd</td>
<td>As</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>74.6 - 400.2</td>
<td>0.2 - 0.7</td>
<td>21.9 - 727.2</td>
<td>0.04 - 1.13</td>
<td>110.3 - 213.6</td>
</tr>
<tr>
<td>2</td>
<td>0.6 - 54.3</td>
<td>Bdl - 0.8</td>
<td>0.2 - 7.8</td>
<td>Bdl - 1.13</td>
<td>10.14 - 23.58</td>
</tr>
<tr>
<td>3</td>
<td>0.9 - 10.3</td>
<td>Bdl - 0.7</td>
<td>Bdl - 0.6</td>
<td>0.34 - 3.15</td>
<td>bdl</td>
</tr>
</tbody>
</table>

Bdl = below detection limit; N= number of samples

Table 2: Statistical analysis of dopamine and serotonin levels in the brains of muskrat and squirrel, compared with t-tests between sites 1, 2 and 3. Analysis is reported as t(df)=; p= for
parametric tests and U=; p= for non-parametric Mann-Whitney U tests (if the assumptions of normality or equal variance between sites were not met). * indicates statistically significant difference (if p < 0.05)

<table>
<thead>
<tr>
<th></th>
<th>Site 1 vs 2</th>
<th>Site 1 vs 3</th>
<th>Site 2 vs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dopamine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(muskrat, brain)</td>
<td>t(8) = -0.13; p = 0.9</td>
<td>t(8) = -1.001; p = 0.35</td>
<td>t(8) = -0.94; p = 0.38</td>
</tr>
<tr>
<td><strong>Serotonin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(muskrat, brain)</td>
<td>t(8) = 0.66; p = 0.53</td>
<td>t(8) = -0.71; p = 0.5</td>
<td>t(8) = -1.2; p = 0.26</td>
</tr>
<tr>
<td><strong>Dopamine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(squirrel, brain)</td>
<td>t(8) = 0.15; p = 0.89</td>
<td>t(8) = 0.78; p = 0.46</td>
<td>t(8) = 0.94; p = 0.37</td>
</tr>
<tr>
<td><strong>Serotonin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(squirrel, brain)</td>
<td>t(7) = 1.59; p = 0.16</td>
<td>t(7) = 0.84; p = 0.43</td>
<td>t(8) = -0.48; p = 0.65</td>
</tr>
</tbody>
</table>

**Table 3:** Statistical analysis of the brain volume from muskrat and squirrel, compared with t-tests between sites 1, 2 and 3. Analysis is reported as t(df)=; p= for parametric tests and U=; p=
for non-parametric Mann-Whitney U tests (if the assumptions of normality or equal variance between sites were not met). * indicates statistically significant difference (if p < 0.05)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Site 1 vs 2</th>
<th>Site 2 vs 3</th>
<th>Site 1 vs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muskrat</td>
<td>t(8) = -0.369; p = 0.722</td>
<td>t(8) = -0.267; p = 0.796</td>
<td>t(8) = -0.562; p = 0.589</td>
</tr>
<tr>
<td>Squirrel</td>
<td>t(8) = 0.327; p = 0.752</td>
<td>U=6 p=0.222</td>
<td>t(8) = -0.711; p = 0.497</td>
</tr>
</tbody>
</table>

**Table 4**: Summary statistics of peak uncorrected relative brain differences in muskrats for 2 km and 20 km site relative to 80 km site.
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Site</th>
<th>t-statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left Lobule 2/3 Cerebellar Vermis</td>
<td>2 km</td>
<td>8.519</td>
</tr>
<tr>
<td>Left Middle Cerebellar Peduncle</td>
<td>2 km</td>
<td>8.293</td>
</tr>
<tr>
<td>Left Superficial Gray Layer Superior Colliculus</td>
<td>2 km</td>
<td>7.903</td>
</tr>
<tr>
<td>Right fornix</td>
<td>2 km</td>
<td>7.275</td>
</tr>
<tr>
<td>Left Fimbria</td>
<td>2 km</td>
<td>-8.804</td>
</tr>
<tr>
<td>Left anterior commissure, anterior part</td>
<td>2 km</td>
<td>-8.669</td>
</tr>
<tr>
<td>Left nucleus accumbens core</td>
<td>2 km</td>
<td>-7.117</td>
</tr>
<tr>
<td>Right subiculum, transition region</td>
<td>2 km</td>
<td>-6.928</td>
</tr>
<tr>
<td>Right caudate putamen (striatum)</td>
<td>2 km</td>
<td>-6.884</td>
</tr>
<tr>
<td>Right external capsule</td>
<td>2 km</td>
<td>-6.837</td>
</tr>
<tr>
<td>Right fimbra</td>
<td>2 km</td>
<td>-6.67</td>
</tr>
<tr>
<td>Left primary somatosensory cortex barrel field</td>
<td>2 km</td>
<td>-6.533</td>
</tr>
<tr>
<td>Left primary somatosensory cortex dysgranular cortex</td>
<td>2 km</td>
<td>-6.399</td>
</tr>
<tr>
<td>Right fornix</td>
<td>2 km</td>
<td>-6.092</td>
</tr>
<tr>
<td>Left caudate putamen/corpus callosum/external capsule</td>
<td>2 km</td>
<td>-6.057</td>
</tr>
<tr>
<td>Left primary somatosensory cortex, dysgranular cortex</td>
<td>2 km</td>
<td>-5.905</td>
</tr>
<tr>
<td>Right CA1</td>
<td>2 km</td>
<td>-5.878</td>
</tr>
<tr>
<td>Left primary somatosensory forelimb region</td>
<td>2 km</td>
<td>-5.871</td>
</tr>
<tr>
<td>Dorsal hippocampal</td>
<td>2 km</td>
<td>-5.763</td>
</tr>
<tr>
<td>Brain Region</td>
<td>Site</td>
<td>t-statistic</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td>commissure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left superficial granular layer</td>
<td>20 km</td>
<td>7.184</td>
</tr>
<tr>
<td>Left lobule 2/3 cerebellar white matter</td>
<td>20 km</td>
<td>6.964</td>
</tr>
<tr>
<td>Right postsubiculum</td>
<td>20 km</td>
<td>6.376</td>
</tr>
<tr>
<td>Right oriens layer/CA1 hippocampus</td>
<td>20 km</td>
<td>-7.863</td>
</tr>
<tr>
<td>Left posterior hypothalamic nucleus</td>
<td>20 km</td>
<td>-6.701</td>
</tr>
<tr>
<td>Right basomedial amygdal nucleus, posterior part</td>
<td>20 km</td>
<td>-6.131</td>
</tr>
<tr>
<td>Right ventral pallidum</td>
<td>20 km</td>
<td>-6.047</td>
</tr>
<tr>
<td>Left molecular layer of the dentate</td>
<td>20 km</td>
<td>-6.013</td>
</tr>
<tr>
<td>Right CA1</td>
<td>20 km</td>
<td>-5.882</td>
</tr>
</tbody>
</table>
Table 5: Summary statistics of peak uncorrected relative brain differences in squirrels for 2 km and 20 km site relative to 80 km site.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Site</th>
<th>T-statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right molecular layer of the dentate</td>
<td>2 km</td>
<td>8.809</td>
</tr>
<tr>
<td>Left cerebellar primary fissure</td>
<td>2 km</td>
<td>7.353</td>
</tr>
<tr>
<td>Right inferior cerebellar peduncle</td>
<td>2 km</td>
<td>6.484</td>
</tr>
<tr>
<td>Left ventrolateral hypothamic tract</td>
<td>2 km</td>
<td>6.265</td>
</tr>
<tr>
<td>4th ventricle</td>
<td>2 km</td>
<td>6.252</td>
</tr>
<tr>
<td>Right medial geniculate nucleus</td>
<td>2 km</td>
<td>6.061</td>
</tr>
<tr>
<td>Left spinal vestibular nucleus/nucleus X</td>
<td>2 km</td>
<td>5.892</td>
</tr>
<tr>
<td>Left dorsolateral intermediate entorhinal cortex</td>
<td>2 km</td>
<td>-9.947</td>
</tr>
<tr>
<td>Left ventral intermediate entorhinal cortex</td>
<td>2 km</td>
<td>-9.215</td>
</tr>
<tr>
<td>Left dorsal subiculum</td>
<td>2 km</td>
<td>-7.687</td>
</tr>
<tr>
<td>Left basolateral amygdaloid nucleus</td>
<td>2 km</td>
<td>-7.107</td>
</tr>
<tr>
<td>Left primary somatomotor, forelimb</td>
<td>2 km</td>
<td>-6.49</td>
</tr>
<tr>
<td>Left central amygdaloid nucleus</td>
<td>2 km</td>
<td>-6.427</td>
</tr>
<tr>
<td>Right primary motor cortex</td>
<td>2 km</td>
<td>-5.898</td>
</tr>
<tr>
<td>Right intermediate gray superior colliculus</td>
<td>2 km</td>
<td>-5.768</td>
</tr>
<tr>
<td>Left lateral posterior nucleus</td>
<td>20 km</td>
<td>5.88</td>
</tr>
</tbody>
</table>
Figure 1: Boxplots on the dopamine (DA) in the brains of (a) muskrats and (b) squirrels collected from three different sites, where site 3 represents reference site, site 2 represents intermediate location (~20 KM from mine), and site 1 represents locations in the vicinity of mine (within ~2 KM radius). The sample size (n) was 5 for each group. The closed circles represent outliers.
Figure 2: Boxplots on the complete data for serotonin (5-HT) in the brains of (a) muskrats and (b) squirrels collected from three different sites, where site 3 represents reference site, site 2 represents intermediate location (~20 KM from mine), and site 1 represents locations in the vicinity of mine (within ~2 KM radius). The sample size (n) was 5 for each group. The closed circles represent outliers.
Figure 3: Boxplots for the data on brain volume changes in a) muskrats and b) squirrels collected from three different sites, where site 3 represents a distance of approximately 80 KM from mine, site 2 represents ~20 KM from mine, and site 1 represents locations in the vicinity of mine (within ~2 KM radius). The sample size (n) was 15. The closed circles represent outliers.
Figure 4: Coronal views of a) 2 km vs control site and b) 20 km vs control site, uncorrected t-statistic relative brain volume differences of muskrats.
Figure 5: Coronal views of a) 2 km vs control site and b) 20 km vs control site, uncorrected t-statistic relative brain volume differences of squirrels.
Figure 6: Correlation between different parameters in squirrels. Positive correlation is depicted with blue colour and negative correlation is depicted with red colour. Correlations which are not significant at the 0.05 level are denoted by a cross. In the figure, accumulation of arsenic (As) and cadmium (Cd) are shown in different compartments analysed in this study (soil; vegetation (veg); brain; gut; nail). Parameters of neurotransmitters are also included as: DA – dopamine; 5-HT – serotonin
Figure 7: Correlation between different parameters in muskrats. Positive correlation is depicted with blue colour and negative correlation is depicted with red colour. Correlations which are not significant at the 0.05 level are denoted by a cross. Accumulation of arsenic (As) and cadmium (Cd) are shown in different compartments analysed in this study (soil; vegetation (veg); brain; gut; nail). Parameters of neurotransmitters are also included as: DA – dopamine; 5-HT – serotonin.
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: