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Short communication

Hypoxia ischemia affects ultrasonic vocalization in the neonatal rat

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ABSTRACT

Perinatal hypoxic-ischemic encephalopathy results in a spectrum of pathologies related to the degree of initial infarct and environmental factors, including maternal interactions. Infants actively influence their environment by crying; rat pups produce ultrasonic vocalizations (USVs). Our study observed that ischemic pups engage in less time producing USVs and make fewer USVs overall, with male ischemic pups experiencing reductions in more categories than females. Future studies should consider whether alterations in mother–pup interactions result from these reductions.

Although stroke is popularly conceptualized as a disease affecting the elderly, cerebrovascular accidents occur in individuals of all age groups [4]. In fact, in preterm and/or low birth weight infants the rate of cerebrovascular accidents ranges from 20 to 30% [11]. Perinatal hypoxic-ischemic encephalopathy is associated with a spectrum of neuroanatomical abnormalities involving both subcortical white matter, as well as cortical and subcortical grey matter structures [26,27]. Phenotypically, these present themselves with behavioural outcomes ranging from cognitive delays of mild developmental delay to mental retardation, seizures, attention deficit disorder and school readiness problems, and motor delays manifesting as spastic or dyskinetic cerebral palsy [10,17,20]. The range in outcomes can be related to the degree of initial infarct, the developmental maturity of the infant and the environment that the infant is located within [22].

Neither human infants nor rat pups are passive recipients of maternal care, as they actively pursue interactions with their mothers/dams [12,31]. One way infants solicit interactions is through vocalization, including crying [for review see 23]. Crying can increase maternal solicitude, and rapid maternal solicitude is effective in reducing crying [2,14]. Although brain injury was not associated with major effects on the duration of crying in human infants, infants with moderate brain damage exhibited longer durations of aversive vocalizations (non-crying) than neurologically normal infants [21]. Infants with prenatal and perinatal complications also exhibited cries that were rated as more aversive and discomforting when compared with normal healthy infants [34].

Rat pups also solicit vocalizations via the production of ultrasonic vocalizations (USVs). USVs occur in response to temperature change [30] and in certain situations, such as isolation from the litter [30,29]. USVs solicit maternal interactions [1] and can be differentiated into 10 categories associated with distress and/or maturity [5,6].

Of the few studies that have investigated the relation between USV production and perinatal experiences, the results are far from conclusive. For instance, immersion in a hypoxic environment significantly reduced the number of USVs made by 7–9-day-old rat pups [3]. However, Blumberg and Alberts only investigated the pup's behaviour when in the hypoxic environment, and did not determine whether these differences extended into the recovery period. Further, not all neurologically invasive treatments result in reductions in USVs. As an example, febrile convulsions induced on postnatal day 7 (P7) increased the number of USVs made by pups on postnatal day 10 (P10) and on postnatal day 12 (P12) [16]. Thus, the goal of this study was to investigate how a hypoxic-ischemic insult, similar to a human neonatal cerebrovascular insult [32], induced on P10 affected USV production in Wistar rat pups

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on P12. Unlike Blumberg and Alberts [3] who only counted the total number of occurrences of USVs, we further categorized the types of USVs that the pups made to determine if the hypoxic-ischemic pups were less able to make more mature calls than the controls.

1. Subjects

Wistar rats were bred in the colony at the University of Saskatchewan, and received food and water *ad libitum* (12:12 day–night cycle). Rats delivered vaginally and litters were culled to 10 rats/litter. Pups (28 males, 20 females) remained with their dams until they were weaned and then they were maintained in standard lab housing in same sex groups with their littermates.

2. Induction of ischemic brain injury

On P10, 31 rats (11 females, randomly chosen from 6 litters) were given an ischemic injury by vasoconstriction of the middle cerebral artery using the technique described in [33]. The remaining 13 rats (7 females from the same 6 litters) were assigned to the control group. Briefly, rats were anesthetized with an induction concentration of 4% halothane (30% oxygen–nitrogen balanced atmosphere) and anesthesia was maintained at 1.25% halothane. Core body temperature was monitored rectally, and maintained at $36.5 \pm 0.5\,^{\circ}C$ for the duration of the surgery. Rats were placed in a neonatal stereotaxic instrument (Kopf Instruments) and a midline incision was made. A small hole was drilled into the skull (A/P: 0.0; M/L: ± -3.2, measurements taken from bregma) and a guide cannula was inserted to -5.5 mm depth. One microlitre of Endothelin-1 (120 pmol concentration) was injected into the brain at a rate of 15s/μL. 10 min after the injection was completed, the guide cannula was removed and the incision was sutured closed. Control rats were anesthetized for the same duration as the ischemic rats, during which time their scalp was incised and then closed with sutures.

On P60, the rats were euthanized (overdose of halothane) and the brains prepared for histological examination. The brains were imbedded in paraffin and 6 μm sections were collected every 0.5 mm. Sections were stained with hematoxylin and eosin. Three sections anterior to bregma and every one until 6 sections after bregma were digitized (Sony Hyper HAD CCD camera, Scion CG-7 capture board) and volume of damage induced by treatment was calculated. As reported in Yager et al. [33], endothelin injection results in predominantly cortical damage to areas that are served by the right middle cerebral artery. In short, the damage is unilateral and more severe anteriorly, involving the entirety of the cortex and subcortical grey matter (caudate nucleus) [33]. Posteriorly, involvement is largely restricted to the cortical mantle, and to a lesser extent, the thalamus. As the injection of Endothelin-1 was unilateral and on the right side, percent damage of the right hemisphere was calculated as: ((left hemisphere volume − right hemisphere volume)/left hemisphere volume) × 100.

Four ischemic pups (1 females) and 1 control pup (female) experienced complications (seizures, rejection by the dam) that required their euthanasia prior to completion of the study on P60. As well, histology revealed that 2 (1 female) Endothelin-1-treated pups failed to exhibit any evidence of ischemia. As such, these pups were excluded from the subsequent analyses rendering the final composition of the groups as 12 control pups (6 females) and 25 ischemic pups (10 females).

As predicted, the ischemic rats exhibited significant reductions ($M = 23.460\%$, S.D. = 16.038, range 3.000–59.000%) to the right hemisphere, when compared to controls ($M = -0.810\%$, S.D. = 1.351, range −3.00–2.00%), $F(1, 33) = 26.663$, $p < .001$. There was no significant difference in percent damage between the sexes, $F(1, 33) = 0.245$, $p = .624$, nor was there a significant interaction between group and sex, $F(1, 33) = 0.227$, $p = .637$.

3. Ultrasonic vocalizations (USVs)

On P12, USVs were monitored (Petterson model D100 Ultrasound Detector) and recorded as a digital *.wav* file (PIII 450 computer). The pups were placed individually into a small isolation chamber (20 cm × 20 cm × 25 cm) for 3 min. During the first minute, the entire ultrasonic range was scanned to determine the individual frequency of USVs made by each pup, and the ultrasound detector was calibrated to detect USVs in the range of approximately 20–30 kHz. Once the calibration procedure was completed, a 2-min recording was initiated, after which the pups were returned to their litter.

A separate coder, who was blind to the treatment groups and hypotheses, reviewed the digital recordings (Avisoft sonograph and spectrogram software). The total number and duration of isolated calls were calculated for each pup. As well, each USV was characterized for structure and duration according to the system defined by Brudzynski [6], as either category: 0 (a '-' shaped call with constant frequency for the duration of the call); 1 (a '.' shaped call of brief duration); 2 (a '\' shaped call of rising frequency); 3 (a '/' shaped call of decreasing frequency); 4 (a 'U' shaped call of falling then rising frequency); 5 (a '∩' shaped call of rising and then falling frequency); 6 (a '⋀⋀' or '⋁⋁' shaped call made of three sweeps of frequency in the pattern of fall–rise–fall or rise–fall–rise); 7 (a 'W' or 'M' shaped call made of four sweeps of frequency); 8 (a wavy line shaped call made of multiple sweeps of sound frequency without a clear tendency to rise or fall); or 9 (a complex shaped call that does not resemble any of the other patterns). The duration and number of each of these categories of calls were summed.

4. Total number and duration of USVs

A 2 × 2 ANOVA was performed on the total number of USVs produced, using sex (male, female) of the pup and treatment (ischemic, sham) as the independent variables. ANOVA revealed that there was a main effect of treatment, $F(1, 33) = 4.851$, $p = .035$, with ischemic rats ($M = 120.40$, S.D. = 87.43) making significantly fewer calls overall than their sham littermates ($M = 193.69$, S.D. = 138.48). The main effect of sex failed to reach significance, $F(1, 33) = 2.402$, $p = .131$. Similarly, the sex by treatment interaction also failed to reach significance, $F(1, 33) = 0.526$, $p = .473$.

A 2 × 2 ANOVA was performed on the total duration of USVs produced, using sex (male, female) of the pup and treatment (ischemic, sham) as the independent variables. ANOVA revealed that there was a main effect of treatment, $F(1, 33) = 5.999$, $p = .020$, with ischemic rats ($M = 7247.70$ ms, S.D. = 4307.99) calling for less time overall than their sham littermates ($M = 11464.00$ ms, S.D. = 6801.48). The main effect of sex failed to reach significance, $F(1, 33) = 3.718$, $p = .062$. Similarly, the sex by treatment interaction also failed to reach significance, $F(1, 33) = 1.779$, $p = .191$.

The correlation between the percent damage to the right hemisphere and total number of vocalizations failed to reach significance, $r(35) = -.254$, $p = .129$. Considering the ischemic pups alone, this correlation was further reduced to $r(23) = -.035$, $p = .968$. Interestingly, when considering the control pups alone, the correlation was $r(10) = -.559$, $p = .059$, which, although not significant, suggests that larger volumes of the right hemisphere were associated with greater number of vocalizations in controls.

The correlation between the percent damage to the right hemisphere and total duration of vocalizations failed to reach