

Brain activity underlying American crow processing of encounters with dead conspecifics

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ABSTRACT

Animals utilize a variety of auditory and visual cues to navigate the landscape of fear. For some species, including corvids, dead conspecifics appear to act as one such visual cue of danger, and prompt alarm calling by attending conspecifics. Which brain regions mediate responses to dead conspecifics, and how this compares to other threats, has so far only been speculative. Using 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) we contrast the metabolic response to visual and auditory cues associated with a dead conspecific among five *a priori* selected regions in the American crow (*Corvus brachyrhynchos*) brain: the hippocampus, nidopallium caudolaterale, striatum, amygdala, and the septum. Using a repeated-measures, fully balanced approach, we exposed crows to four stimuli: a dead conspecific, a dead song sparrow (*Melospiza melodia*), conspecific alarm calls given in response to a dead crow, and conspecific food begging calls. We find that in response to observations of a dead crow, crows show significant activity in areas associated with higher-order decision-making (NCL), but not in areas associated with social behaviors or fear learning. We do not find strong differences in activation between hearing alarm calls and food begging calls; both activate the NCL. Lastly, repeated exposures to negative stimuli had a marginal effect on later increasing the subjects' brain activity in response to control stimuli, suggesting that crows might quickly learn from negative experiences.

1. Introduction

In humans, the observation of a dead body can prompt a variety of responses including disgust, grief, stress, fear, sadness and empathy, depending on the familiarity of the individual and the context in which it is being observed [1,2,3,68]. To better understand how such a diversity of responses is mediated, functional neuroimaging studies have been used to examine what areas of the brain process images of the deceased. Through these studies, several areas have been implicated including the sublenticular/extended amygdala region, the anterior insula, the right superior parietal cortex, and the orbitofrontal cortex [4–6]. In contrast, which emotional states are prompted by the observation of a dead conspecific remain unknown in non-human animals, but there is mounting evidence that some animals express intense interest in their dead [7].

Animals including some social insects, birds, and mammals exhibit a diversity of responses following the discovery of a dead conspecific. In some animals such as eusocial insects and rats, the presence of a

conspecific corpse prompts stereotyped responses such as burial, removal or ingestion [8,75]. Among other animals, particularly social mammals with large relative brain sizes such as cetaceans, primates, and elephants, observations of captive or free ranging individuals responding to conspecific corpses are both numerous and variable. These animals may explore, guard, transport, attack, engage in caretaking behaviors such as grooming, or become sexually aroused ([9,10]; Stewart et al. 2011; [11]). Although observations of animals responding to their dead are key to elucidating the range of resulting behavioral expressions, such accounts cannot reveal more nuanced aspects of how animals perceive their dead or the neural mechanisms that underlie resulting behaviors.

How the brain responds to different types of sensory information related to dead conspecifics, for example audio and visual, can reveal much more about how humans and other animals process information. For example, dogs are well known for their attentiveness to humans, but functional magnetic resonance imaging (fMRI) studies were the first to reveal that dogs identify human faces with a specialized, face-

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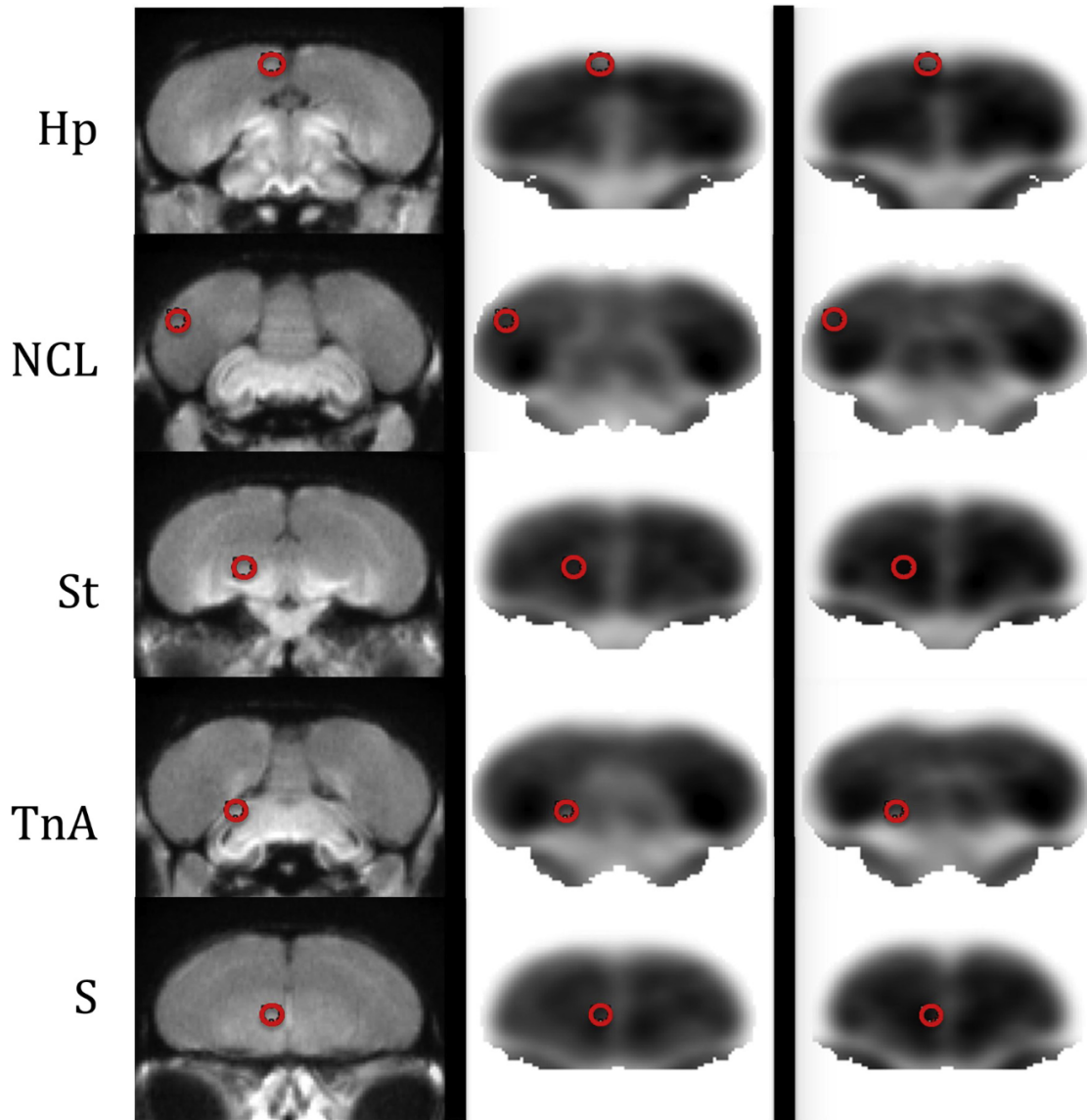


Fig. 1. Regions of interest. Left image is a structural Magnetic Resonance Image (MRI) of the crow brain. Center and right images are co-registered Positron Emission Tomography (PET) images from two subjects in the current study. Red circles indicate *a priori* regions of interest: Hp = Hippocampus, NCL = Nidopallium caudolaterale, St = Striatum, TnA = Nucleus taeniae of amygdala, S = Septum.

selective region in the temporal cortex similar to one in primates, sheep and crows ([12]; Perrett et al. [13]; [14,15]). In addition to revealing specialized areas or allowing for cross-species comparisons, functional imaging studies can reveal important within-species insights into the differences in the brain's response even when the behavioral response appears similar.

When presented with a red-tailed hawk (*Buteo jamaicensis*) or a human holding a dead crow, wild American crows (*Corvus brachyrhynchos*) alarm call, often resulting in the recruitment of other crows to the area to form a mob [16,17]. Existing whole brain neural imaging studies on crows demonstrate that these threats are not equal and in fact trigger distinct neural circuits. Whereas unfamiliar humans holding dead crows show high activation in a network connecting distinct telencephalic structures including the hippocampus, red-tailed hawks also activate some of these nuclei and others, but not the hippocampus or amygdala [18,19]. These studies demonstrate that behavioral observations provide an incomplete picture of the complexity in how crows perceive different dangers.

Like some mammals, some species in the Corvidae family (crows, jays, ravens, etc.) also respond strongly to dead conspecifics. Following the discovery of a conspecific carcass, American crows (*Corvus brachyrhynchos*), California scrub-jays (*Aphelocoma californica*), and common ravens (*Corvus corax*) will alarm call, often resulting in the recruitment of nearby individuals. Among wild crows, the presentation of a dead sympatric heterospecific (*Columba livia*) does not result in such strong consistent responses [17]. While the produced alarm calls clearly attract conspecifics, unlike some other animals, including Campbell's monkeys (*Cercopithecus cambelli*), vervet monkeys (*Cercopithecus aethiops*), and red squirrels (*Tamiasciurus hudsonicus*), crows do not appear to produce referential, threat-specific alarm calls, though they can vary the duration and rate of alarm calling as a means of communicating urgency (Seyfarth et al. [20]; Greene and Meagher [21]; [22,23]). Crows, jays and ravens subsequently avoid or show wariness in areas where a dead conspecific was discovered [17,24,25]. In some cases, crows may even make exploratory, aggressive or sexual contact with unfamiliar dead crows [26]. Crows demonstrate the ability

to learn features associated with the dead crow after only one exposure, including the location where a dead crow was discovered [17]. In addition, American crows and common ravens learn the faces of people they witness handling dead conspecifics [17,27]. How the auditory and visual information associated with dead conspecifics is actually processed in these, or any non-human animal, remains unknown, however. Given this complexity of responses, crows make a viable model for a functional neuroimaging study designed to explore what areas of the brain in a non-human animal facilitate diverse responses to conspecific bodies.

Utilizing a longitudinal 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) imaging study, we aim to reveal neurological aspects of how American crows process the presentation of a dead crow. Given that, in the wild, attendance to the body of a dead crow appears to be facilitated by individuals responding to alarm calling, we also explore patterns of activation associated with the auditory cues that follow the discovery of a dead crow. To meet these objectives, we present crows with cues associated with dead conspecifics and corresponding control stimuli including: 1) an unfamiliar dead crow; 2) a dead song sparrow; 3) alarm calls recorded from unfamiliar free ranging birds during encounters with dead crows; and 4) unfamiliar crow begging sounds. We compare brain activity levels (as measured by the relative FDG uptake) between the paired dead conspecifics and control stimuli in regions associated with associative learning (the hippocampal complex and the striatum), expressions of social behavior (the subpallial limbic network), and executive function (the nidopallium and mesopallium). Among these regions we selected five *a priori* determined brain structures including the striatum, the hippocampus, the nucleus taeniae of amygdala (TnA), the septum, and the nidopallium caudolaterale (NCL), to examine for increased brain activity in response to the stimuli (Fig. 1). Specifically, we explore the following hypotheses: 1) If cues associated with dead crows primarily stimulate fearful recall and learning, we expect to see activation in areas associated with these processes including the striatum and the hippocampus [28–30]. Given its role in associative learning we believe it is crucial to include the hippocampus in this hypothesis, however, we do so cautiously as the avian hippocampus is a smaller, more peripheral structure relative to the other four structures we investigate, and therefore more prone to type II errors resulting from misregistration errors. 2) If cues associated with conspecifics trigger social behaviors such as recognition circuits or affect we expect activation in the subpallial limbic network which includes the amygdala complex, the extended amygdala and the septum ([31]; [32]). Given the complexity of this system, we will examine two regions as representative indicators of activity in this area: the septum and the nucleus taeniae of amygdala. 3) If cues associated with dead crows stimulate integration from multiple sensory inputs resulting in higher order decision making, we expect activation in the nidopallium and mesopallium. Specifically, we will examine the nidopallium caudolaterale (NCL), which is known in birds as the primary mediator of executive function [18]. 4) Lastly, if carry-over effects occur between imaging sessions, we expect effects from the number of previous imaging sessions on activation levels. Specifically, we expect that it will primarily manifest as an increase in responsiveness to stimuli as indicated in this study by increased brain activity (i.e. increased FDG uptake,) rather than a decrease in response as a result of repeated stimulation, as indicated in this study by decreased brain activity (i.e. decreased FDG uptake). Given that observations of dead crows and the vocalizations associated with them indicate a mortal threat, we expect that the number of previous experiences with dangerous stimuli will have a greater effect than the number of previous neutral stimuli.

2. Material and methods

2.1. Animal collection and housing

In the summer of 2014 we captured $N = 7$ American crows ($N = 2$

sub-adult females, $N = 1$ sub-adult male, and $N = 4$ sexually mature males) using a net launcher from a bait site located near a large roosting center in Bothell, Washington, USA. We based age and sex determinations on body weight, tarsus width, and mouth lining color [33]. None of the crows had dependent young and all had completed their annual molt. Following capture, we housed the crows individually for two weeks in adjacent outdoor aviary cages measuring $1 \times 2 \times 2$ m. We provided a diet of meat, fruit, eggs, seeds and dried dog food *ad libitum*. We collected and cared for the crows in accordance with University of Washington Institutional Animal Care and Use Committee Protocol 3077-01, 1, Washington Scientific Collection Permit 11–359, and US Scientific Collection Permit MB761.

2.2. Stimulus presentation protocol

We imaged three subjects per day. The night before imaging, we moved each test subject to a smaller, covered $0.5 \times 0.5 \times 1$ m cage housed within the PET laboratory room to allow for acclimation. We did not provide food in the 12 h prior to imaging, but did provide water *ad libitum*. The day of imaging, one researcher reached under the covered cage and removed the subject while covering its face with a hood after which, a second person administered one mCi of 18F-fluorodeoxyglucose (FDG) via i.p. injection (see Fig. S1 in [14] for a time-activity curve of FDG uptake in the American crow). The subjects appeared to remain calm during this procedure and did not exhibit signs of struggle. We then returned the subject to the covered cage for a two-minute rest. Following the rest period, a single researcher removed the cover and exposed the bird to one of four stimulus options presented on top of a white sheet of cardboard placed on a stool one meter in front of the cage. The stimuli included two “dangerous” stimuli and two corresponding control stimuli (Fig. 2). The visual dangerous/neutral stimuli included a prepared “dead” crow skin and a prepared “dead” song sparrow skin. We used multiple exemplars of each skin in a random order ($N = 3$ dead crows, $N = 7$ dead song sparrows). The skins were prepared at different times. The audio dangerous/neutral stimuli included an audio recording of crows giving alarm calls in response to a dead crow and an audio recording of a juvenile crow begging sound. We recorded stimulus calls from free ranging birds at $N = 14$ different sites at a minimum of 32 km from the site where we collected the subjects and other recordings, within the greater Seattle area. We assumed that all calls and skins were from birds unfamiliar to the test subjects. We used Syrinx (John Burt, www.syrinxpc.com, Seattle, WA, U.S.A) to removed background noise from recordings and Audacity 2.1.3 to normalize peak amplitude to 0 dB. For each audio stimulus type, we created $N = 7$ unique tracks, one for each subject. All tracks lasted one minute and were spaced to mimic natural call patterns. We played all tracks at approximately 75 dB using a Bem wireless HL2022A speaker. For both call types, each recording was sourced from a unique site and featured multiple vocalizing individuals, as is typical in natural settings. The total number of individual callers for each recording is unknown. The average call rate for each recording (as assessed to the best of our ability given multiple callers) is 1.28 ± 0.08 begging calls/sec and 1.72 ± 0.04 scolding calls/sec. The experimental stimulation period lasted 10.5 min. During this time, the stimulus was presented for one min, followed by 30 s of rest in the cloaked cage, for a total of 7 test/rest pairs. During auditory experiments the speaker was turned off during the rest period. Following each rest period, the cloak was removed, rendering the test space visible to the subject and allowing them to see or hear the stimulus. Over the course of four separate testing days, each crow was exposed to all four stimuli in a balanced design ($N = 4$ exposure trials per subject; $N = 28$ total trials). Three of the subjects received one of the two dangerous stimuli first and the other four the control stimulus. Across the seven subjects we presented stimuli in every possible sequence.

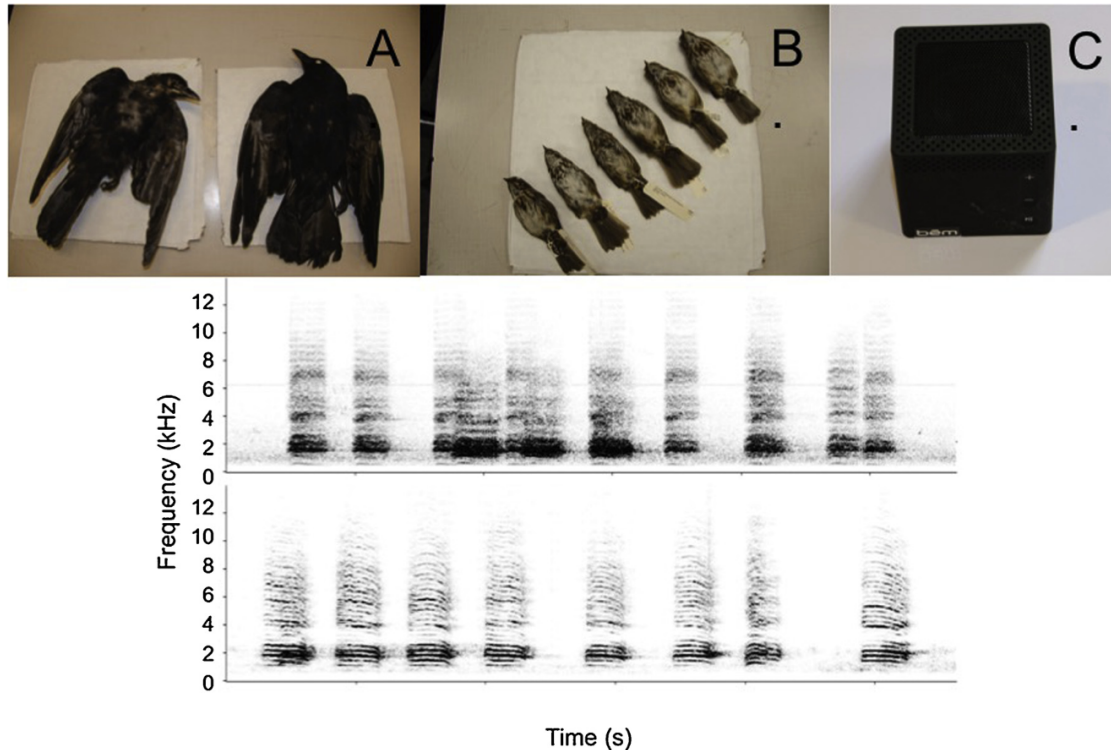


Fig. 2. Experimental stimuli. All subjects were exposed to four stimuli: A) an unfamiliar dead crow, B) an unfamiliar dead song sparrow, and C) a speaker playing one of two conspecific vocalizations including alarm calls given in response to a dead crow and food begging calls. The spectrograph shows differences in call structure between alarm calls (top) and food begging calls (bottom).

2.3. Imaging and image processing

Immediately following the terminal rest period of the experimental stimulation protocol, we hooded and removed the subject from its cage, anesthetized it with five percent isoflurane in oxygen with a flow rate of 300–800 mL/min via a special nose

cone manufactured from a 50-mL syringe tube, and then positioned it in the scanner. We obtained high resolution FDG-PET images using a Siemens Inveon PET system for 10 min, starting 25 min after FDG injection (except for one subject, which was imaged after 27 min). Following imaging, we executed a 13 min attenuation scan and then reconstructed using vendor supplied 3D OSEM/MP algorithm with attenuation and scatter corrections applied to the data. The image matrix was $128 \times 28 \times 159$. We stereotaxically aligned the PET images to the jungle crow (*Corvus macrorhynchos*) brain atlas ([14]; Izawa and Watanabe 2007). For consistent stereotactic transformations of scans from the same subject, we estimated and applied nine affine parameters to the images using algorithms for automated human brain image analysis adapted for crow brains (NEUROSTAT, University of Utah; [34]). We estimated that aligned precision was one to two millimeters.

2.4. Statistical procedure

After normalizing to global brain FDG uptake [35], we conducted volume of interest (VOI) analysis to extract FDG uptake values from our five *a priori* selected regions across each subject's four stimulus tests. As the amount of metabolic FDG uptake is used to infer the relative level of activation, moving forward we will refer to these data as "brain activity", "activation level" etc., in the main text, rather than as "FDG uptake". To account for the experimental design of exposing subjects to multiple stimuli, we used repeated-measures ANOVAs to compare activations across individuals between the sight of a dead crow and a dead sparrow, or in a separate model, as activations between hearing alarm

and begging calls across each region.

To account for possible carry-over effects between trials, we included a covariate in the repeated-measures ANOVAs to test for the interaction between the main (stimulus) effect and the effect of the previous number of exposures. For example, a subject that received stimuli in the order dead crow, begging, alarm calling, dead sparrow, would have seen one dangerous stimulus prior to the control trial when evaluating responses to auditory stimuli, but two dangerous stimuli prior to the control test when evaluating responses to visual stimuli. Thus we included either the number (0–2 possible) of previous dangerous exposures or the number of (0–2 possible) previous control exposures, to determine which experience had a greater effect on regional brain activity.

For each stimulus type (visual or auditory), considering the stimulus and possible carry-over effects resulted in a repeated measures ANOVA with a single fixed, 2-level factor, which compared the stimulus (the independent variable) against the resulting regional brain activity (the dependent variable). The two levels owe to the two stimuli being compared; the dangerous and control stimuli. In addition, the model contained a covariate which accounted for the number of dangerous or neutral exposures prior to seeing the control or dangerous stimulus. The difference in regional brain activity between the control and neutral pair, is reported as the within-subjects main effects. A statistically significant within-subjects main effect therefore indicated a difference in activation between the two stimuli. The carry-over effects resulting from repeated exposures are reported as the between-subjects effect, which is functionally a linear regression of the covariate on the activation level. We used the between-subjects parameter estimates (reported in SPSS v.19 as beta values) associated with each stimulus to infer whether brain activity was increased or decreased, as a result of the repeated exposures. We discuss the outcome of both these covariate approaches in detail in the "Carry-over from prior experience during experimentation" section in the results. Because we found that the

number of dangerous exposures was more often significant or marginally significant when looking at the between-subjects effect, we only report main effects with tests using this as a covariate, as this seemed more conservative (see “Differential response to dangerous and control stimuli” in the Results).

In addition to these tests, we used multivariate ANOVAs to compare brain activity in response to each dangerous and neutral stimulus against the activity from three crows looking at an empty room from an initial baseline study [14]. To facilitate this comparison, the stimulus presentation and imaging protocol were designed to match the protocol from this initial study.

We adopted an approach to statistical inference as proposed by Fisher [36]. We considered p values < 0.05 as evidence that an effect should be confirmed by other studies, and p values between 0.05 and 0.20 as evidence of effects that should be tested in future studies with increased replications or other design improvements. We consider p values > 0.20 to indicate that the effect is too small to be detected by the experimental design.

We used SPSS v.19 (IBM, Armonk, NY, U.S.A.) to conduct all statistical tests.

3. Results

3.1. Carry-over from prior experience during experimentation

Crows showed changes in brain activity in response to repeated exposure to stimuli in the amygdala (TnA) and the striatum, but in no other areas. These responses did not appear to be more common during auditory vs. visual tests. Only in the left amygdala, but not in the right, did we find a significant overall effect of presentation order across both stimulus groups (Dead crow/dead sparrow between-subjects $F_{1,5} = 22.30$, $p = 0.005$, Fig. 3; alarm calling/begging, $F_{1,5} = 16.08$, $p = 0.010$, Fig. 4). In both of these cases, we found evidence of increased brain activity indicated by the positive relationship between the number of dangerous exposures (a dead crow or playback of alarm calls) and the activation level during exposure to a control stimulus (dead sparrow beta = 17.81, dead crow beta = 46.97; begging beta = 86.21, alarm calling beta = 34.96). Increased brain activity was also suggested by some evidence of effects in the right striatum, but not in the left (Between-subjects $F_{1,5} = 5.16$, $p = 0.072$; dead crow beta = 12.05, dead sparrow = 55.31) during visual experiments. In the hippocampus, however, we found some evidence for decreased brain activity during auditory presentations bilaterally (Left hemisphere:

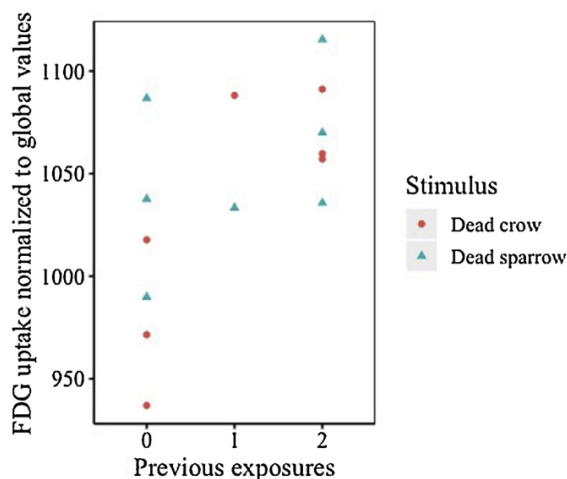


Fig. 3. Effect of number of previous exposures to dangerous stimuli (either dead crow, alarm calls, or both) on brain activity (FDG uptake) in the left amygdala during experiences with visual stimuli.

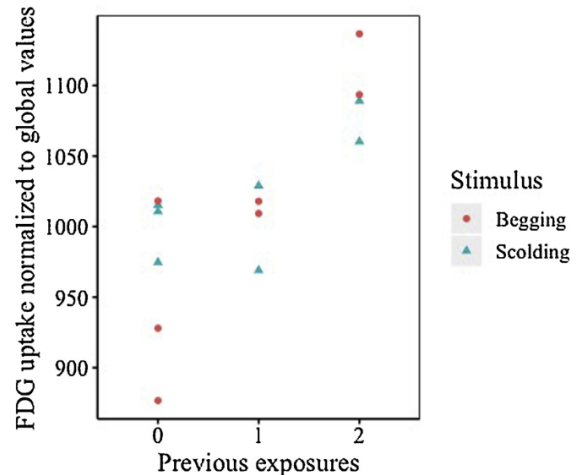


Fig. 4. Effect of number of previous exposures to dangerous stimuli (either dead crow, alarm calls, or both) on regional brain activity (FDG uptake) in the left amygdala during experiences with auditory stimuli.

Right hemisphere $F_{1,5} = 4.10$, $p = 1.0$, alarm call beta = -39.78, beg beta = -51.87; Right hemisphere $F_{1,5} = 3.40$, $p = 0.12$, alarm call beta = -50.10, beg beta = -58.88).

Exposure to control stimuli prior to dangerous stimuli was associated with more muted changes in regional brain activity than the reverse. We found some evidence of depressed brain activity in the left amygdala during auditory tests (Between-subjects $F_{1,5} = 4.57$, $p = 0.087$, alarm call beta = -16.56, begging = 69.48). We also found some evidence of increased brain activity in the right striatum (Between-subjects $F_{1,5} = 5.16$, $p = 0.072$; dead sparrow beta = 55.31, Dead crow = 12.10).

3.2. Differential responses to dangerous and control stimuli

When confronted with a dead conspecific, crows showed higher relative activity in the NCL than when presented with a dead song sparrow (Repeated measures within-subjects main effects Right-hemisphere: $F_{1,5} = 22.6$, $p = 0.005$; Left-hemisphere: $F_{1,5} = 19.22$, $p = 0.007$; Fig. 5). In neither case did carry-over effects from prior trials strongly influence the differential response of the NCL (Right-hemisphere: interaction of covariate with main effect $F_{1,5} = 0.84$, $p = 0.40$, Dead sparrow beta = 7.78; Left-hemisphere: $F_{1,5} = 4.36$, $p = 0.09$, Dead sparrow beta = -11.89). Likewise, we found significant right NCL activity when comparing presentations of the dead crow to crows that had seen only an empty room (multivariate ANOVA $F_{1,8} = 22.47$, $p = 0.001$). We found some evidence of increased activity in the right septum when crows viewed a dead conspecific relative to when they viewed a dead sparrow (Repeated measures within-subjects main effects $F_{1,5} = 3.77$, $p = 0.11$; no significant interaction with prior experience; Fig. 5). However, the activity in the septum elicited by the dead crow did not differ from the activity from an empty room (Multivariate ANOVA Right-hemisphere $F_{1,8} = 0.35$, $p = 0.57$; Left hemisphere: $F_{1,8} = 1.19$, $p = 0.31$; Fig. 5). We found some evidence of activity in the right striatum when comparing dead crows and dead sparrows (Repeated measures within-subjects main effects $F_{1,5} = 3.87$, $p = 0.11$; no significant interaction with prior experience) but not when comparing either to the empty room. We did not find that viewing a dead crow stimulated significantly higher activity in either the hippocampus or amygdala relative to either a dead sparrow or the empty room.

Comparing presentations of crow alarm and begging vocalizations, we found some activity in the septum bilaterally (Repeated measures within-subjects main effects Right hemisphere: $F_{1,5} = 14.98$,

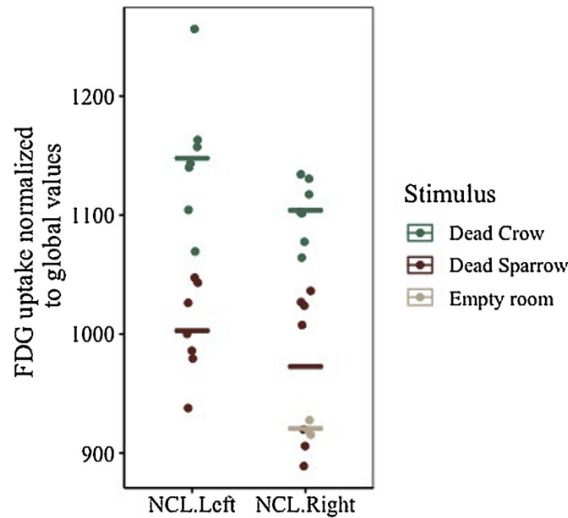


Fig. 5. Individual values for FDG uptake normalized to global values in each structure and corresponding hemisphere where activation in response to the stimulus (i.e., dead crow in the case of dead crow vs. dead sparrow or dead sparrow in the case of dead sparrow vs. empty room) met the threshold for statistical significance ($P < 0.05$). Horizontal lines indicate group mean. Individual data points represent uptake values for each subject ($N = 7$ Dead crow, $N = 7$ Dead sparrow, and $N = 3$ Empty Room subjects). In all cases, Dead Crow was significantly higher than the control stimuli shown (Dead Sparrow and/or Empty Room). In one case (right NCL) Dead Sparrow was not significantly different from Empty Room subjects.

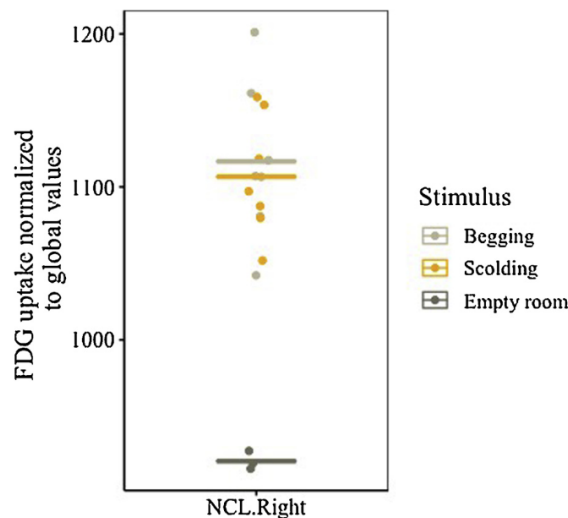


Fig. 6. Individual values for FDG uptake normalized to global values in the right NCL where activation in response to the stimulus met the threshold for statistical significance ($P < 0.05$). Horizontal lines indicate group mean. Individual data points represent uptake values for each subject ($N = 7$ Begging, $N = 7$ Alarm calling, and $N = 3$ Empty room subjects). Begging and Alarm calling showed higher activation relative to the empty room, but not each other.

$P = 0.012$; Left hemisphere: $F_{1,5} = 4.5$, $p = 0.087$). This activity was influenced by prior experience (Right hemisphere: interaction of covariate with main effect, $F_{1,5} = 6.94$, $P = 0.046$; Left hemisphere: interaction of covariate with main effect: $F_{1,5} = 7.97$, $p = 0.037$). Crows had increased septal activity when exposed to the control begging call (Right: begging beta = 33.82, alarm call beta = -1.09; Left: begging beta = 48.36, alarm call = -19.56) following previous exposures to dangerous stimuli (dead crow and alarm vocalization). We found some differences between the auditory stimuli and the empty room control;

crows showed significant activation in the right, but not the left, NCL during both types of playback (Multivariate ANOVA: Alarm call: $F_{1,8} = 62.49$, $P < 0.001$; Beg: $F_{1,8} = 39.8$, $P < 0.001$; Fig. 6). See supplementary figures S1-S5 for mean activation across all tested regions.

4. Discussion

Some nonhuman animals including cetaceans, elephants and primates have repeatedly been observed expressing a diversity of responses towards conspecific carcasses, but the drivers of these behaviors are unknown though a variety of explanations have been offered including grief, learning opportunities, confusion, or agitation [7,37,64]. An understanding of brain activity during these encounters may help provide clarity, but no such studies have been undertaken in nonhuman animals. Likewise, wild American crows also express a diversity of responses to dead conspecifics including alarm, rapid, long-term memory formation, and a variety of physical interactions [17,26]. They do not show such strong responses towards dead heterospecifics including rock pigeons (*Columba livia*) and Eastern grey squirrels (*Sciurus carolinensis*), however [17,26]. In the present study we sought to explore how such a diversity of responses may be explained by what brain areas process visual and auditory information associated with dead crows, and corresponding control stimuli including dead song sparrows and crow begging calls. In response to visual stimuli, we found that crows showed no significant activity in the amygdala and hippocampus. Instead, observations of dead crows, but not dead sparrows, provoked activation in the NCL, an area that has been compared to the mammalian prefrontal cortex and purportedly associated with higher-order cognitive functions. In addition, we found some support for activation in the striatum and septum. In response to auditory stimuli, we found that hearing alarm calls corresponded with higher levels of activation in the septum, relative to hearing begging calls, but this finding was influenced by the longitudinal design, where birds exposed to control stimuli showed increased brain activity due to previous exposure to dangerous stimuli. We found no differences in the other four regions. When comparing the two calls types against the empty room, we found activation of the NCL. During both visual and auditory tests, we found evidence of a lateralized response across most regions, where either the left or right hemisphere was more strongly activated. Finally, we found that crows showed some evidence of carry-over effects with each trial, most commonly manifesting as increased regional brain activity. Together, these results suggest that cues associated with dead crows activate the NCL allowing for context dependent decisions about how to respond, along with other areas important for regulating predictions and conspecific recognition. This process may help explain why, in the wild, crows display such a diversity of responses towards visual and auditory cues associated with dead conspecifics [26].

Wild American crows are wary in areas where a conspecific body was previously discovered and remember people they saw handling them [17]. That they learn threats associated with dead crows was further demonstrated in a previous FDG-PET study wherein crows showed significant activity in their hippocampus, an area involved in associative and spatial learning [28], when viewing an unfamiliar person holding a dead crow [19]. Given these findings, we predicted that the observation of a dead crow, even in the absence of a predator, would activate the hippocampus. Contra to this prediction, we did not find higher levels of hippocampal activation relative to either seeing a dead song sparrow or to birds that saw only the empty room. Perhaps to detect a stronger learning response, presentations of the dead crow needed to include an element that was unique to only those trials, such as an unfamiliar person, or possibly even an object, since birds may exhibit attendance to the imaging room across all trials. It is also possible that low detection of hippocampal activation is due to the fact that the structure itself is quite small and located near the periphery of the telencephalon, which makes it more vulnerable to type II errors

resulting from slight misregistration between scans.

In humans, the amygdaloid complex plays a central role in reward and affiliation, including mediating responses to familiar people or pets, and the ability to interpret human facial expressions (Stoeckle et al. [38]; [39], 79). The avian amygdala, the nucleus taeniae in particular, has been suggested to play a role in social memory and behavior including discrimination of familiar and unfamiliar individuals [31, 71]. A previous imaging study on crows indicated that the observations of a dead crow did not result in activation of the amygdala, however in this study dead crows were presented as being held by an unfamiliar person [19]. Here we found that even in the absence of a discrete predator the presentation of a dead crow did not result in detectable activation of the nucleus taeniae of the amygdala in comparison to either the observation of a dead song sparrow or when compared to birds that saw an empty room. Our findings may show that the observation of an unfamiliar dead crow does not trigger social memory centers or affiliative behaviors.

Crows observing a dead conspecific showed significant activation in the NCL when contrasted against the empty room and the dead sparrow. This result suggests that NCL activity is not stimulated simply by the observation of carcasses. The NCL has been proposed as an analogous region to the mammalian prefrontal cortex (PFC) and is suggested to mediate executive functions in birds ([18]; [40]). For example, the ability of corvids to demonstrate insight, episodic-like memory, and other features of cognitive flexibility has been largely attributed to their NCL [41]. In humans, the prefrontal cortex plays an important role in the circuit responsible for the regulation of fear and negative affect, including the withdrawal system, which organizes appropriate responses to threat cues [42]. When crows are exposed to another innate threat, a red-tailed hawk, they also show activation of NCL [19]. These findings suggest that, rather than simple stimulus-response behaviors, unconditioned threats prompt complex and integrative decision making allowing crows to respond in a variety of ways. This hypothesis may explain why a broad diversity of responses including no response, alarm calling, and different kinds of touching have all been observed among wild crows in response to a dead, unfamiliar conspecific [26].

When exposed to a crow alarm calls given in response to a dead crow, subjects showed some activity in the right-hemisphere of the septum, relative to when they heard conspecific begging calls. That we saw activity in the septum is consistent with previous studies demonstrating the role of the avian septum in modulating social behavior, particularly aggression [31,32]. Because birds were collected during the breeding season when territorial aggression among crows is highest [43], they may show a stronger aggressive reaction to conspecific calls, especially when the calls are associated with a threat, than if we had tested birds outside of the breeding season. It is known, for example, that the presence of testosterone increases alarm calling in response to aerial predators by male domestic chickens (*Gallus gallus*; [44]). Relative to their effects on call production, the hormonal influences on acoustic perception are more poorly understood [45]. However, there is some evidence among vertebrates, including birds, that circulating hormones influences acoustic perception, though these studies are generally limited to responses toward sexual signals rather than other kinds of communication such as alarm calls [46–48]. Although we controlled for breeding status during the initial capture event, sex-based differences in acoustic perception warrant further study.

We found no other differences among our four other brain regions between trials where birds heard alarm calls produced in response to a dead crow and food begging calls. Previous field studies have shown that, among free ranging crows, playback of conspecific alarm calls and food-begging calls produce distinct behavioral responses [49]. Unlike some other social animals, however, including black-capped chickadees (*Parus atricapilla*), Japanese great tits (*Parus major minor*) and black-tailed prairie dogs (*Cynomys ludovicianus*) American crows do not seem to encode specific predator-based information in their alarm calls such

as predator size or identity [23,50–52]. Consequently, crows may not show the same kinds of neural diversity to alarm calls that they do in response to different kinds of visual threats [19]. Our results suggest that whereas crows have a variety of calls used for communicating different aspects of their state or environment, and can respond in variety of ways, similar pathways ultimately mediate these auditory cues. Further studies focusing on specific auditory and vocal centers may be useful.

Although we did not find significant NCL activation when comparing alarm calls and begging calls, we did find that both these stimuli resulted in increased NCL activity relative to the empty room. This suggests that crows may not respond to particular calls in fixed ways but rather integrate information from a variety of sensory modalities and respond in a context-dependent way. Instead of dead-crow stimulated alarm calls or juvenile begging calls triggering specific respective responses, each can provoke a variety of behaviors depending on the additional contextual information provided, such as visual information or physical constraints. For example, during the stimulus exposure protocol when crows are in small enclosures where movement is limited, crows do not alarm call in response to even the most salient threats [14,19], suggesting that in addition to the auditory information that might normally provoke alarm calling, they incorporate other context-dependent information to arrive at a more appropriate response. In the wild, the ability to use executive functions when processing alarm calls may allow crows to ignore calls given inappropriately to benign threats, or when responding may be more costly, such as during the breeding season when attending to alarms given in neighboring territories might result in physical altercations [16,43,53].

Across both visual and auditory trials we found evidence of hemispheric lateralization among all tested brain regions. Lateralization is widespread among vertebrates and aids in a variety of tasks including object discrimination, predator aversion, and the ability to attend to multiple stimuli ([54]; Roger et al. [55]). Although visual lateralization has been the most predominately studied form, there is also evidence of other sensory lateralization including audio and olfactory ([56]; Mooreman et al. 2012; [57]). Here we found that when comparing dead crows and sparrows, NCL showed unilateral activation, but when comparing dead crows to the empty room, we found a clear right-hemisphere bias. Likewise, we found marginal right-hemisphere activity in the septum when comparing dead crows and sparrows. We also found consistent activity in the right NCL when comparing alarm calling and begging calls against the empty room, as well as in the septum when comparing alarm calls and begs against one another. These findings are consistent with previous studies showing that the right-hemisphere controls fear and escape responses [56]. For example, Dharmaretnam and Rogers [58] found that chicken chicks (*Gallus gallus domesticus*) will reorient in order to be able to view predators with their left eye (right-hemisphere). Marzluff et al. [14] also found general right-hemisphere biased activation in the crow brain among subjects viewing familiar faces of threatening people; however, as in our study, lateralization was inconsistent in the nidopallium. Why we found right-hemisphere-biased activity during conspecific playbacks is somewhat puzzling though, considering the robust literature on left-hemisphere processing of conspecific calls. For example, previous studies show that harpy eagles (*Harpia harpyja*) and California sea lions (*Zalophus californianus*) orient to familiar conspecific sounds with their right ear (left-hemisphere; [59,60]). Furthermore, auditory processing as well as song and speech learning generally appear to be left-side biased in both birds and humans [61].

We found some evidence of carry-over effects occurring between stimulus exposures over the course of our longitudinal study. The effect of previous experiences appeared to be more influential if they were negative rather than a control. This pattern was most evident in the left amygdala and to some extent the right striatum. The amygdala and striatum showed increased sensitivity in response to the number of previous dangerous exposures. We also found marginal evidence for

decreased brain activity occurring during auditory tests in the hippocampus, but this result needs to be tested further. Given that repeated exposures to cues of danger may indicate that a particular space (the imaging room) or experience (stimulus presentation protocol) is of mortal threat, it is unsurprising that crows more often showed increases in regional brain activity following exposures to dangerous stimuli. While these findings do not assure complimentary changes in behavior, they are consistent with what we would expect to occur if the subjects were learning from previous experiences. That crows can rapidly learn from dangerous experiences has been previously demonstrated. For example, crows will learn the faces of people who capture them or who are seen holding dead crows after only a single exposure [17,62]. Such rapid learning is also of social value to crows and other corvids. After only a single experience, common ravens (*Corvus corax*) will learn and remember people who were unfair during an exchange task, and will choose not to work with them in the future [63]. Our finding that crows show changes in regional brain activity and may therefore be learning from previous experiences, underscores the care with which longitudinal studies using crows must be conducted. Therefore, as in our study, future experiments with crows must fully balance the order in which stimuli are presented and statistically account for previous experiences.

To our knowledge this is the first functional imaging study to examine the response of a non-human animal to the presentation of a dead conspecific, or to conspecific alarm calls. In response to viewing a dead crow, we found activation patterns consistent with higher-order decision-making but not learning or a social response. Further studies presenting sedated, familiar individuals may help reveal if in this study the unfamiliarity of the presented individual influenced the resulting brain activity. In response to dead crow induced conspecific alarm calls, we found no activation when compared to a neutral conspecific call, suggesting that auditory information may be processed by similar pathways regardless of the context. Future studies examining the activation response towards alarms calls collected during encounters with different kinds of threats would help clarify how crows process conspecific alarm calls. Together these results demonstrate that, among crows, the range of behavioral response to cues associated with different threats may be somewhat limited but their response is far from fixed.

CRedit authorship contribution statement

Kaeli N. Swift: Data curation, Formal analysis, Visualization, Funding acquisition, Writing - original draft. **John M. Marzluff:** Conceptualization, Investigation, Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing. **Christopher N. Templeton:** Conceptualization, Investigation, Methodology, Resources, Visualization, Writing - review & editing. **Toru Shimizu:** Methodology, Supervision, Validation. **Donna J. Cross:** Conceptualization, Formal analysis, Resources, Investigation, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:<https://doi.org/10.1016/j.bbr.2020.112546>.

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