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Novel objects alter immediate early gene expression globally for ZENK and regionally for c-Fos in neophobic and non-neophobic house sparrows

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ABSTRACT

Neophobia – an animal's reluctance to approach novel objects, try new foods, or explore unfamiliar environments – affects whether animals can adapt to new environments and exploit novel resources. However, despite its importance, the neurobiological mechanisms underlying this personality trait are poorly understood. In this study, we examined regional brain activity using the expression of two immediate early genes (IEGs), ZENK and c-Fos, in response to novel objects or control conditions in captive house sparrows (*Passer domesticus*, n = 22). When exposed to novel objects, we predicted that we would see differential IEG activity in brain regions involved in regulating stress and emotion (hippocampus, medial ventral arcopallium, lateral septum), reward and learning (striatum), and executive function (NCL) between neophobic and non-neophobic individuals. To classify birds by phenotype, we used behavior trials that tested willingness to approach a food dish in the presence of several different novel objects, habituation to one novel object, and willingness to try several different novel foods. We then exposed birds to a new novel object or a control condition and assessed protein expression of two IEGs in neophobic vs non-neophobic individuals after this final exposure. An analysis of average sparrow feeding times in the presence of novel objects showed a bimodal distribution of neophobia behavior. There was also high repeatability of individual novel object responses, and average responses to all three trial types (novel object, novel food, and habituation to a novel object) were significantly correlated. Although we saw no differences between neophobic and non-neophobic birds in IEG expression in response to novel objects in any of the 6 brain regions examined, there was a significant global decrease in ZENK expression and a significant increase in c-Fos expression in the medial ventral arcopallium and the caudal hippocampus in response to novel objects compared to controls, suggesting that these two regions may be important in novelty detection and threat perception. Additionally, there was no object effect in the rostral hippocampus, which supports the hypothesis that the avian hippocampus may have a rostrocaudal functional gradient similar to the septotemporal gradient in mammals.

1. Introduction

Novel urban and suburban environments are replacing natural environments worldwide, which creates strong selection pressure for many wild animals [1]. Neophobia, an animal's reluctance to approach a novel object, try a new food, or explore an unfamiliar environment, is a specific type of exploration-avoidance behavior with critical ecological and evolutionary relevance, because it affects whether or not animals will be able to adapt to new environments and exploit novel resources [2-4]. Being willing to explore novelty may positively affect an individual's fitness by increasing its chance of finding food and nest sites, but it may also increase exposure to predation and disease [5,6]. Thus, neophobia may be particularly important in determining which individuals, populations, and species are most capable of exploiting human-altered landscapes. However, despite the broad ecological importance of neophobia, the neurobiological basis for this personality trait is inadequately understood.

The mechanisms underlying neophobia remain a mystery partly because one behavioral output – often, reluctance to approach a novel object – could arise from different neurobiological processes [7]. For example, neophobia could arise due to differences in perception (i.e., the ability to recognize objects as novel and therefore potentially

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hazardous) or differences in judgement (i.e., similar recognition of novelty, but differences in whether objects are perceived as threatening). Examining neuronal activity in response to novel objects in neophobic and non-neophobic individuals could help distinguish between these two possibilities and provide insights into neurobiological differences between different behavioral phenotypes. In fact, a wide and diverse body of literature supports the idea that individual differences in behavior or personality can partly be attributed to individual differences in patterns of brain activity [8-10].

One common way to compare neuronal responses to different stimuli is by examining the expression of immediate early genes (IEGs) like ZENK (avian homologue of *zif-268*, *egr-1*, *ngfi-a*, and *krox-34*) and c-Fos [11-13]. The low expression levels of ZENK and c-Fos during rest and their high responsiveness to membrane depolarization make them highly sensitive markers for neuronal activity [14,15]. Further, ZENK and c-Fos induction are associated with neuronal plasticity (e.g., long-term potentiation induction in hippocampus) and they appear to play an important role in memory formation and learning [16]. However, it is uncommon for researchers to use more than one IEG to assess neuronal activity, even though there is evidence that different IEGs can be sensitive to different types of stimuli [17,18]. In songbirds, c-Fos and ZENK can be induced in the same neurons by the same stimulus [19], or the same stimulus can cause differential expression of the two IEGs [20]. Therefore, understanding how different IEGs in different brain regions respond to novel objects, and to what extent those responses are correlated or independent, was another goal of this research.

In this study, we assessed neophobia phenotypes using novel object and novel food trials, then used ZENK and c-Fos expression to examine neuronal activity in response to novel objects in house sparrows (*Passer domesticus*), a species exhibiting wide and repeatable individual variation in neophobia [21,22]. We focused on IEG expression in six brain regions shown to be involved in learning, executive function, problem solving, and threat perception in birds (Table 1): striatum, two regions of the dorsomedial hippocampus (one more rostral and one more caudal), medial ventral arcopallium (AMV, previously referred to as the nucleus taenia of the amygdala), caudolateral nidopallium (NCL, considered the avian "prefrontal cortex"), and the lateral septum. If part of what determines individual variation in neophobia is differential activity in reward circuits in the brain, we would expect to see differential IEG activity in the striatum in neophobic and non-neophobic individuals during novel object trials. If neophobic and non-neophobic individuals differ in executive function and higher order decision making, we expect to see differential IEG activity in the NCL when exposed to novel stimuli. Finally, if neophobic and non-neophobic individuals differ in neural circuits involved in regulating emotion and stress, we expect to see differential IEG activity in the dorsomedial hippocampus, AMV, and lateral septum in response to novel objects. Additionally, we examined whether object presence alone, or being fasted or fed, affected IEG expression in the brain.

Table 1
Brain regions hypothesized to differ in neuronal activity in neophobic and non-neophobic house sparrows exposed to novel stimuli.

Brain region	Function	Citations
Striatum	Learning, reward, cognitive flexibility	[23][24, 25]
Hippocampus	Memory, navigation, emotion, exploratory behavior, neophobia	[26-28]
Medial ventral arcopallium (AMV)	Social behavior, threat perception, novelty detection	[29,30]
Caudolateral nidopallium (NCL)	Executive functions, decision making, cognitive flexibility	[31,32]
Lateral septum	Social behavior, emotion, learning, memory, regulation of hypothalamic pituitary adrenal axis	[33-35]

2. Materials and methods

2.1. Study subjects

Adult house sparrows (n = 22, 15 males and 7 females) were captured using mist nets at bird feeders in East Baton Rouge Parish between 28 June and 16 July 2019. Sparrows were housed individually in cages in a vivarium at Louisiana State University with unlimited access to mixed seeds, grit, a vitamin-rich food supplement (Purina Lab Diet), and water. Because cage sides are solid and cages were placed side by side, birds were visually but not acoustically separated from neighbors. Sparrows had access to a variety of perches and a dish of sand for dust bathing. Animals were solo housed to avoid potential effects of social interactions on neophobia [36]. Sparrows were maintained at natural day length (12 L:12D) for a minimum of four weeks to acclimate to the captive environment before trials began. Animals were collected under Louisiana state permit LNHP-18–098, and all experimental procedures approved by the Louisiana State University Institutional Animal Care and Use Committee. We used approved methods for bird capture, transport, and husbandry as specified in the Ornithological Council's Guidelines to the Use of Wild Birds in Research [37], and approved methods of euthanasia for laboratory animals as specified in the 2020 American Veterinary Medical Association Guidelines for the Euthanasia of Animals.

2.2. Neophobia trials

We conducted three different sets of behavior trials to determine whether individuals were neophobic or non-neophobic. For all trials, we removed food dishes from cages 30 min before lights off the previous evening to standardize the motivation to feed. The morning of trials, researchers entered the room 30 min after lights on, began video recordings, replaced the food dish with its treatment (or the normal food dish for control trials), and left the room. We recorded 1 h of behavior using pole-mounted cameras (ZOSI Z18.5.T.2) connected to a DVR (ANNKE Model DM310) to determine how long it took each sparrow to approach and feed. At the end of the hour, we stopped the video recording and replaced normal food dishes. Because sparrows do not eat in the lab when lights are out [38], this only represents an additional 2 h of fasting at maximum for birds that do not feed during neophobia trials.

For the first set of tests (novel object trials), we used five different novel objects that sparrows likely would not have encountered previously. The objects were: a purple plastic egg placed in the food dish, a white cover over part of the dish, a red-painted dish, yellow coiled pipe cleaners around the edge of the dish, and a blinking light hanging above the dish. Food was presented with all objects. Objects were selected to share few common features (e.g., red color) that might target ecologically-relevant cognitive biases [7] and have all been shown to significantly increase average latency to feed in house sparrows [36]. We conducted five trials over five days, and each sparrow randomly received three of the five objects and two days of control (no object) trials, in random order. The two objects not used during this week were used for habituation and IEG trials, see below.

For the second set of tests (novel food trials), we used four novel foods that sparrows would likely not have encountered previously. The foods were: grated cheddar cheese, diced kiwi, smooth peanut butter, and colorful fruit-flavored breakfast cereal. Foods were selected to maximize the diversity of food colors and textures, and were previously used in a study of house sparrow feeding neophobia [39]. Novel food trials were conducted over five days in which each sparrow received three of the four foods (randomly selected) and two days of control trials, in random order.

In the third set of tests (habituation trials), we evaluated whether house sparrows would habituate to repeated exposures of the same object. Testing took place over five days in which the first day was a control trial (no object presented). During the second day, each sparrow

was exposed to a new novel object they had not been exposed to previously, with the same object presented on three subsequent days (1 h exposure each day). Videos from the fourth day were lost due to accidental interruption by animal husbandry staff, so habituation data are from trial days 2, 3, and 5.

For the final IEG trial, sparrows were exposed to a new novel object they had not seen previously (n = 7 neophobic and n = 9 non-neophobic birds). Because neophobic birds do not approach the object and do not feed, and non-neophobic birds do approach and feed, we also randomly selected several sparrows who were either kept fasted with no food dish as a "fasted" control (n = 3 neophobic birds) or presented with the normal food dish as a "fed" control (n = 2 non-neophobic birds, 1 neophobic bird). We had not fully analyzed behavior data and had not yet determined which birds were neophobic and which were non-neophobic at the time of the final trials, hence the random selection of fasted and fed birds. All sparrows were euthanized after this final trial to assess IEG activity. Previous work in songbirds has shown that IEG proteins peak ~90 min after stimulus exposure [40]. Therefore, after a 1 h novel object (or fasted or fed) exposure, sparrows were captured and held in dark quiet cages for 30 min before being deeply anesthetized with ketamine and xylazine at doses appropriate for house sparrows [41]. Once animals were in a surgical plane of anesthesia, they were transcardially perfused with ice-cold heparinized saline, then fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde.

2.3. Behavior classifications

Videos from the three sets of initial trials (novel object, novel food, and novel object habituation) were scored for time to first feed from the food dish. Responses to novel object tests were significantly repeatable, and average novel object response was significantly correlated both with average novel food response and average response to a novel object during habituation trials (see Results). Therefore, we concluded that average novel object response was a consistent measure of behavior that reflected "neophobia" generally (rather than "object neophobia" as distinct from "food neophobia") and used data from the first week of 3 novel object tests to evaluate which house sparrows were neophobic or non-neophobic. We ranked sparrows in order of least neophobic (shortest average time to feed in the presence of novel objects) to most neophobic (longest average time to feed in the presence of novel objects). When birds did not feed during an object trial, we assigned them a maximum value of 1 h. A finite mixture model analysis using a parametric stochastic EM algorithm [42] estimated the Weibull distributions of our data and determined that an appropriate threshold to classify neophobia groups was 0.5, and a histogram of average feeding times shows a bimodal distribution (Fig. S1). Therefore, the 11 sparrows with the longest average time to feed in the presence of novelty were classified as "neophobic" (n = 8 males, 3 females) and 11 sparrows with the shortest time to feed were classified as "non-neophobic" (n = 7 males, 4 females). We also tried using average time to feed in the presence of novel objects as a continuous variable (see Results).

For the final IEG trial, we used video recordings of all sparrows to observe activity (i.e., number of hops, jumps, and flights) and to verify whether they fed during the final trial; video observers were blind to individual personality. There was no difference in activity between neophobic, non-neophobic, and fasted/fed sparrows in this final trial (ANOVA: $F_{3,6}$ =0.004, p = 1). All non-neophobic sparrows fed from the food dish during this final trial, whereas only 1 of 7 neophobic sparrows fed in the presence of novel objects during the final trial (10 min into the trial). Control sparrows given food dishes without novel objects also all fed.

2.4. Immunohistochemistry for immediate early gene proteins

Brains were post-fixed in 4% paraformaldehyde phosphate buffer for 24 h at 4 °C, then soaked in 0.1 M phosphate buffer containing 30% sucrose for cryoprotection. After sinking (~2 days), brains were flash-frozen in powdered dry ice and stored at − 80 °C until sectioning. Brains were cut at − 20 °C in the coronal plane in 40 μm sections using a ThermoFisher NX50 cryostat. To standardize the angle of the brain during sectioning, brains were frozen with the flat dorsal side down in OCT compound (Fisher Scientific). Starting at striatum, triplicate sections were collected in wells containing cryoprotectant (0.2 M phosphate buffer, 15 M PVP, 1.5 M sucrose, and 0.5 M ethylene glycol in distilled water) and stored at − 20 °C until the day of immunohistochemistry.

Brain regions were identified based on visible landmarks. We used sections of striatum ~120 μm after the first appearance of the striatum. We used rostral dorsomedial hippocampal and lateral septum sections from slices where both the lateral ventricle and septomesencephalic tract (TrSM) were visible. We used caudal dorsomedial hippocampal sections where the cerebellum first became visible and the mesopallium began to disappear. The dorsomedial hippocampus region has also been suggested based on anatomical and functional studies to be homologous to the Ammon's horn and subiculum of the mammalian hippocampus [43], making it a particularly strong candidate region to be involved in integrating sensory and emotional information to produce appropriate behavioral outputs. We targeted both caudal and rostral portions of the dorsomedial hippocampus because there is evidence from chickens that the caudal and rostral poles show functional differences, analogous to septotemporal differences in the mammalian hippocampus [44,45]. We targeted AMV based on the visibility of the cerebellum and arcopallium. Sections used for NCL were 40 μm after AMV sections, in a pallial area where we have confirmed the presence of dense basket fiber staining for tyrosine hydroxylase in house sparrows, consistent with NCL in other songbird species [46]. For each region and IEG, we ran immunohistochemistry for all 22 animals in the same assay.

Two series of targeted sections were stained separately, one for ZENK and one for c-Fos immunoreactivity. Sections were washed in Tris-buffered saline (TBS, pH 7.6) 3 times and incubated in 0.5% hydrogen peroxide for 30 min followed by 3 more TBS washes. All washes were a minimum of 10 min and all wash steps used 72 μm mesh well inserts. To block background immunoreactivity, sections were incubated in 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) for ZENK or 10% normal goat serum (Aurion, Wageningen, Netherlands) for c-Fos in 0.3% Triton in TBS (TBS-T) for 1 h. After washing 3 times in TBS, sections were moved out of mesh well inserts and incubated with primary antibodies. For ZENK, we used monoclonal mouse anti-ZENK (1:500 in TBS-T and 1% normal horse serum; antibody 7B7-A3) donated by Dr. David Keays, Research Institute of Molecular Pathology in Vienna, Austria and raised against an N-terminal fragment 260 amino acids in length (1−260) of rock pigeon ZENK [47]. For c-Fos, we used polyclonal rabbit anti-c-Fos (1:5000 in TBST-T and 1% normal goat serum; Abcam, Cambridge, MA, USA, ab190289), raised against a human c-Fos N-terminal fragment 380 amino acids in length (1−380). For both IEGs, we used a 20 h incubation at 4 °C. After washing 3 times in TBS, sections were then incubated at room temperature for 1 h in biotinylated goat anti-rabbit IgG (for c-Fos, Vector Laboratories) or biotinylated horse anti-mouse IgG (for ZENK, Vector Laboratories) diluted 1:500 in TBS-T, followed by three more washes in TBS-T. Sections were incubated in avidin–biotin horseradish-peroxidase complex (Vectastain ABC, Elite kit, Vector) 1:100 for 1 h and washed two times in TBS. Sections were visualized with DAB (Sigma Fast-DAB), mounted onto slides, dehydrated in ethanol, cleared in HemoDe (Scientific Safety Solutions, Keller, TX, USA), and cover-slipped using Permount (Electron Microscopy Sciences, Hatfield, PA, USA).

Both antibodies were validated for specificity in house sparrows using Western immunoblots and preadsorption controls. For ZENK, dark bands appeared at 75 kDa and 150 kDa, indicating that a ZENK subtype is present (Fig. S2). For c-Fos, dark bands appeared at 80 kDa and 150 kDa, with lighter bands at 60, 30, 20 and 10 kDa (Fig. S2). Extra bands were likely ZENK- and c-Fos-related isoforms [47,48]. For preadsorption

controls, primary antibodies were incubated with antigen (c-Fos protein: Abcam, ab56280; ZENK protein: donated by Keays lab) in 5X molar excess for 1 h at room temperature prior to an overnight incubation with sample tissue at 4 °C. In the presence of excess antigen, no staining was detectable in tissue sections for either c-Fos or ZENK (Fig. S3).

2.5. Immediate early gene quantification

Four sections per region of interest per individual were used to quantify ZENK and c-Fos. Each section included both right and left hemispheres unless the hemisphere was damaged during processing, although we were unable to distinguish between right and left hemispheres because we used free floating immunohistochemistry. Images of each region were captured using a digital camera (Olympus DP74) mounted on an Olympus TH4-100 microscope using a 20x objective lens. Three images were used for larger regions (hippocampal regions and striatum) and 1–2 images for smaller regions (AMV, lateral septum, and NCL) (Fig. S4). One fed control sparrow did not have enough dark c-Fos staining to identify the lateral septum, so there is one missing data point in c-Fos density for the lateral septum. We used ImageJ [49] to measure immunopositive cell density in each image using a procedure adapted from Mischler et al. [50]. Images were cropped to include only the region of interest (except for striatum, which took up the entire field of view), and the area of each region was measured. We converted images to 8-bit grayscale, increased the contrast, and used manual thresholding to make immunopositive nuclei white against a black background, ensuring that only nuclei were included and not artifacts. We next defined particle circularity, and size range which differed by region: caudal and rostral hippocampus= 9.07–65 μm^2; striatum= 9.07–40 μm^2; NCL, AMV, and lateral septum= 9.07–50 μm^2. We then used the count function to quantify the number of immunopositive nuclei and calculated cell density. Image analysis was done by individuals blind to phenotype (neophobic vs. non-neophobic) and treatment (novel object vs. fasted vs. fed).

2.6. Data analysis

We used R Studio v 4.0.8 for behavior analyses [51]. Cox proportional hazard models were used to investigate patterns in feeding behavior among the two groups using the "coxme" function in the coxme package [52]. A preliminary analysis of novel object, food, and habituation trials did not detect an effect of sex on neophobia (all $p \geq 0.84$), therefore sex was excluded from subsequent analyses. We also ran preliminary analyses for object and food neophobia trials and did not detect an effect of presentation order on neophobia (all $p > 0.16$), therefore trial number was excluded from subsequent analyses. To evaluate whether our three behavioral tests elicited neophobia (novel stimuli compared to controls), we created models using object type (novel object trials), food type (novel food trials), or trial type (habituation trials: control, object presentation 1, 2, or 4) as independent variables, individual ID as a random effect, and time to feed as the dependent variable (3 models; one for each set of neophobia trials).

To determine whether our two phenotypes significantly differed in their response to novelty, we created two additional Cox proportional hazard models with individual ID as a random effect, object/food and phenotype as independent variables, and time to feed as the dependent variable with a dataset including only novel object or food trials (no control or habituation trials). Presentation order was included as an independent variable for habituation trial analyses, but not for object or food trial analyses because there was no significant effect in initial models. A final set of Cox proportional hazard models examined whether phenotypes differed in their ability to habituate to novel objects for each exposure trial that included individual ID as a random effect and phenotype as the main effect. We created Kaplan-Meier survival curves of house sparrow approach times with the "survfit" command in the survival package [53] and visualized them using the "ggsurvplot" command in the survminer package [54].

We also used regression analysis to examine possible correlations between average responses to novel object trials, average responses to novel food trials, and average responses to multiple presentations of the same object during habituation trials for all birds. We calculated the repeatability of individual responses of all birds using among and within sum of square values from an ANOVA in which individual ID was the independent variable and time to feed was the dependent variable [55] for: (1) the four novel object trials (three from novel object trials and the first object exposure of the habituation trial), and (2) the three novel food trials.

We used JMP Pro 16.0 (SAS Institute) for all IEG analyses. We performed three different linear mixed model analyses, or six models total (i.e., three models for two different IEGs). A first round of analyses assessed whether fed or fasted status affected the density of c-Fos or ZENK staining. For this analysis, we grouped birds that ate from their normal food dish ($n = 3$), neophobic birds that ate in the presence of novel objects during this final trial ($n = 1$), and non-neophobic birds that ate in the presence of novel objects ($n = 9$) into a "fed" group ($n = 13$) and birds that were deliberately fasted ($n = 3$) and neophobic birds that did not eat in the presence of a novel object ($n = 6$) into a "fasted" group ($n = 8$). These models used c-Fos or ZENK density as the dependent variable and fed status (fed vs. fasted), brain region (rostral and caudal dorsomedial hippocampus, striatum, AMV, lateral septum, and NCL), and a fed status*brain region interaction as a fixed effect. Individual ID was included as a random effect in these models.

We next ran models comparing the effect of a novel object vs. no novel object. For this analysis, we grouped fed and fasted birds as a "no novel object" group ($n = 6$) and neophobic and non-neophobic birds as a "novel object" group ($n = 16$). These models used c-Fos or ZENK density as the dependent variable and novel object presence, brain region, and a novel object presence*brain region interaction as fixed effects. Individual ID was included as a random effect in these models. In cases where there was a significant interaction between novel object presence and brain region, we compared novel object and non-novel object groups for each of the 6 brain regions separately using an ANOVA (or Welch's ANOVA when Bartlett's test indicated significant differences in between-group variance).

We then assessed whether there were differences between the neophobic and non-neophobic birds in IEG response to novel objects. Because fed status did not have a significant overall effect on c-Fos or ZENK density for any brain region (see Results), we ran simplified models that excluded fasted and fed birds and simply compared c-Fos and ZENK density in neophobic ($n = 7$) and non-neophobic birds ($n = 9$) exposed to novel objects with the food dish. These models used c-Fos or ZENK density as the dependent variable and personality type (neophobic or non-neophobic), brain region, and a personality*region interaction as fixed effects and individual ID as a random effect. For all IEG models, we assessed normality of the residuals using normal quantile plots and checked for homoscedasticity by inspecting plots of studentized residuals against predicted values of dependent variables. Sex was initially included as a fixed effect in IEG models, but because the effect of sex was not significant (all $p \geq 0.23$), we excluded it from the final analyses. Finally, we also used Pearson correlations to examine correlations between ZENK and c-Fos density within each region. We corrected for multiple testing by controlling the false discovery rate (FDR) using the Benjamini-Hochberg procedure with an FDR of 0.25. Exact p- and r-values are reported, with text and footnotes in each table indicating the results of FDR corrections.

3. Results

3.1. Neophobia behavior

During novel object trials, time to feed was not associated with presentation order ($\beta = 0.07$, HR(95% CI)= 1.07(1.23–0.94), $z = 0.95$,

$p = 0.34$) and all novel objects significantly increased the time to feed in comparison to control trials in which no novel object was presented (Fig. S5; Table 2a). Further, there was no difference in latency to feed among the different objects (Fig. S5). Neophobic sparrows significantly differed from non-neophobic sparrows in the time to feed in the presence of a novel object (Fig. 1; $\beta = 1.01$, HR(95% CI)= 24.24(77.20–7.64), $z = 5.36$, $p < 0.0001$), as would be expected from the fact that neophobia phenotype was assigned based on novel object trials.

During novel food trials, time to feed was not significantly associated with presentation order ($\beta = 0.14$, HR(95% CI)= 1.15(7.55–0.18), $z = 1.42$, $p = 0.16$) and all novel foods, except for the cereal ($p = 0.06$), significantly increased the time to feed in comparison to control trials in which the regular seed mixture was presented (Fig. S6; Table 2b). Neophobic sparrows differed from non-neophobic sparrows in time to taste a novel food ($\beta = 1.01$, HR(95% CI)= 2.27(6.65–1.13), $z = 2.24$, $p = 0.025$; Fig. S7).

During habituation trials, time to feed was not significantly associated with object type ($\beta = 0.05$, HR(95% CI)= 0.93(1.45–0.60), $z = -0.35$, $p = 0.75$). Time to feed was significantly affected by the presence of a novel object during the first and second exposures but not during the final, fourth exposure (Fig. S8, Table 2c). Phenotype significantly affected exposure number, revealing that neophobic sparrows took significantly longer than non-neophobic sparrows to feed during habituation trials (Table 3; Fig. S9).

Individual responses to novel objects were highly repeatable during novel object trials ($r = 0.72$) but not during novel food trials ($r = 0.17$). However, when we averaged responses to each trial type within an individual (e.g., average response to the three novel objects, average response to the three novel foods, etc.), regression analyses revealed significant correlations in average responses to novel objects and novel foods (adjusted $r^2 = 0.19$, $F_{1,20} = 5.80$, $p = 0.026$), novel objects and the same object presented multiple times (adjusted $r^2 = 0.19$, $F_{1,20} = 5.83$, $p = 0.026$), and novel foods and the same object presented multiple times (adjusted $r^2 = 0.22$, $F_{1,20} = 6.78$, $p = 0.017$).

Table 2
Results of three Cox proportional hazard models of house sparrow feeding probability during (a) novel object, (b) novel food, and (c) habituation trials. Individual (n = 22) was included as a random effect, trial as a main effect, and either object type (a), food type (b), or exposure number (c) as a main effect. Sample sizes for each group were: non-neophobic: n = 11, neophobic: n = 11, except for habituation trials which were non-neophobic= 11, neophobic= 9 where two sparrows were excluded for not approaching during control trials. Contrasts are with respect to control trials.

Object/food/ trial type	β coefficient	Hazard ratio (95% confidence interval)	z-score	p
(a) *Novel object effects*				
Cover	-2.34	0.10 (0.25 – 0.04)	-4.79	< 0.0001
Egg	-2.69	0.07 (0.16 – 0.03)	-5.97	< 0.0001
Light	-2.22	0.11 (0.25 – 0.05)	-5.33	< 0.0001
Pipe cleaners	-2.63	0.07 (0.19 – 0.03)	-5.26	< 0.0001
Red dish	-1.99	0.14 (0.29 – 0.06)	-5.12	< 0.0001
(a) *Novel food effects*				
Cereal	-0.76	0.47 (1.04–0.21)	-1.87	0.062
Cheese	-1.95	0.14 (0.41–0.05)	-3.61	0.0003
Kiwi	-1.61	0.20 (0.58–0.07)	-2.99	0.0028
Peanut butter	-1.13	0.32 (0.39–0.27)	-2.84	0.0045
(a) *Habituation trials*				
First exposure	-2.47	0.08 (0.2 – 0.04)	-5.60	< 0.0001
Second exposure	-1.05	0.35 (0.74 – 0.17)	-2.79	0.006
Fourth exposure	-0.57	0.56 (1.17 – 0.27)	-1.54	0.12

3.2. Immediate early gene expression

In linear mixed models examining the effects of being fasted or fed on IEG protein expression, we found a significant overall effect of brain region on c-Fos cell density ($F_{5,99} = 11.57$, $p < 0.0001$) but no effect of feeding status ($F_{1,20} = 0.14$, $p = 0.71$), or interaction between feeding status and brain region ($F_{5,99} = 1.85$, $p = 0.11$). Similarly, we also found a significant overall effect of brain region on ZENK cell density $F_{5100} = 89.95$, $p < 0.0001$) but no effect of feeding status ($F_{1,20} = 0.18$, $p = 0.68$), or interaction between feeding status and brain region ($F_{5100} = 0.58$, $p = 0.72$).

When we examined the effects of novel object presence on c-Fos expression, we found a significant effect of brain region on cell density (Fig. 2; F5100 =14.58, $p < 0.0001$), and a significant interaction between novel object presence and brain region ($F_{5100} = 3.7$, $p = 0.0043$), although no global effect of novel object presence on c-Fos cell density (Fig. 2h, $F_{1,21} = 1.48$, $p = 0.24$). Region by region post-hoc analyses revealed that there was increased c-Fos density in the AMV (Fig. 2g, $F_{1,20} = 12.38$, $p = 0.0022$) and caudal hippocampus (Fig. 2b, $F_{1,20} = 12.61$, $p = 0.0020$) of sparrows exposed to a novel object on, in, or near the food dish compared to sparrows exposed to the normal food dish or no dish. There was also a trend towards decreased c-Fos cell density in the lateral septum of birds exposed to novel objects (Fig. 2e, $F_{1,19} = 3.76$, $p = 0.067$), but this was primarily driven by one individual with very high c-Fos density in the lateral septum. When this individual was excluded, this trend disappeared ($F_{1,18} = 0.66$, $p = 0.43$). We found a significant effect of brain region on ZENK cell density (Fig. 3; $F_{5100} = 76.10$, $p < 0.0001$), as well as a significant effect where novel object presence caused decreased ZENK cell density across all 6 brain regions (Fig. 3h, $F_{1,20} = 4.63$, $p = 0.044$), but no significant interaction between novel object presence and brain region ($F_{5100} = 1.04$, $p = 0.40$).

In models comparing only neophobic and non-neophobic sparrows exposed to novel objects, we found a significant overall effect of brain region on c-Fos expression ($F_{5,70} = 6.65$, $p < 0.0001$) but no effect of personality ($F_{1,14} = 0.066$, $p = 0.80$), or interaction between personality and brain region ($F_{5,70} = 1.56$, $p = 0.18$). Similarly, we found a significant effect of brain region on ZENK expression ($F_{5,70} = 61.2$, $p < 0.0001$) but no effect of personality ($F_{1,14} = 0.47$, $p = 0.50$), or interaction between personality and brain region ($F_{5,70} = 0.21$, $p = 0.96$). When average time to feed in the presence of novel objects was used as a continuous measure of neophobia and included as a fixed effect, we still found no effect of neophobia on c-Fos ($F_{1,14} = 0.51$, $p = 0.49$) or ZENK expression ($F_{1,14} = 0.09$, $p = 0.77$), and no interaction between neophobia and brain region for c-Fos ($F_{5,70} = 0.98$, $p = 0.43$) or ZENK expression ($F_{5,70} = 0.25$, $p = 0.94$).

Finally, we found that c-Fos and ZENK expression was significantly correlated in lateral septum, which was true whether or not we excluded the individual with very high c-Fos staining (Table 4). After FDR correction, this correlation was still significant. In AMV, there was a trend towards significant co-expression of c-Fos and ZENK.

4. Discussion

The overall goal of this research was to investigate if the perception of novel objects for neophobic and non-neophobic individuals is reflected in different patterns of neuronal activity, assessed via protein expression of two different IEGs. We predicted that we would see differential IEG activity in brain regions involved in regulating stress and emotion (hippocampus, medial ventral arcopallium, lateral septum), reward and learning (striatum), and executive function (NCL) between neophobic and non-neophobic individuals. Although we saw clear and repeatable differences in neophobia behavior, we found no differences in IEG expression between neophobic and non-neophobic sparrows in response to novel objects in any of the six brain regions examined. This result suggests that neophobia is not caused by different patterns of overall activity in these brain regions involved in decision making,

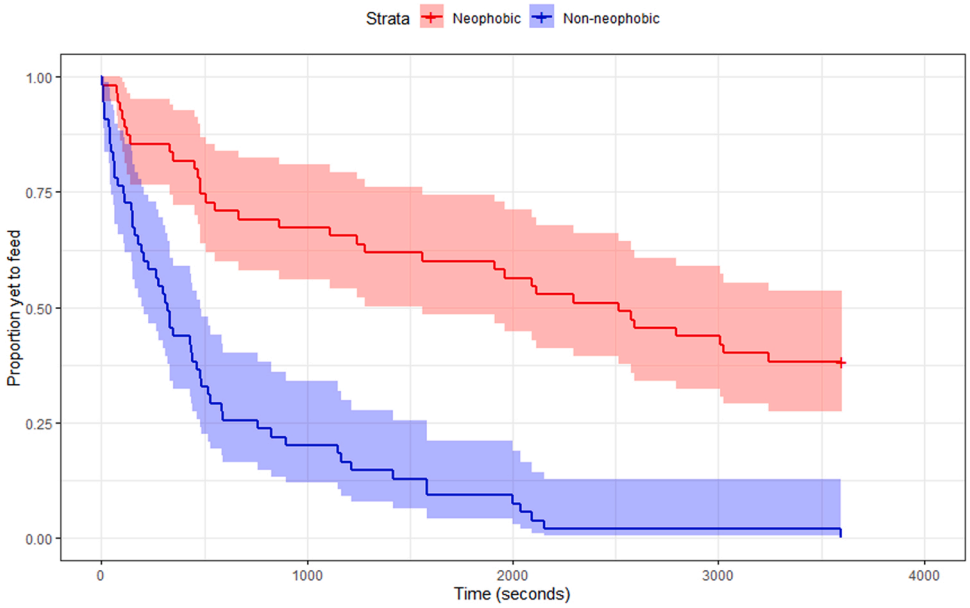

Fig. 1. Non-neophobic (n = 11) and neophobic house sparrows (n = 11) differed in their latency to approach the food dish with a novel object present ($p < 0.0001$). Kaplan-Meier survival curves of average house sparrow feeding likelihood in the presence of three different novel objects on, near, or in the food dish. See main text for novel objects used.

Table 3
Results of four Cox proportional hazards models examining the effect of phenotype (non-neophobic or neophobic) on feeding response time for each habituation exposure trial. Individual was included as a random effect. Sample sizes for each group were: non-neophobic: n = 11, neophobic: n = 9. Contrasts are with respect to neophobic birds. Fig. S8 presents differences among exposure number and Fig. S9 presents the phenotype-specific responses for each trial.

Trial	β coefficient	Hazard ratio (95% confidence interval)	*z*-score	*p*
(a) Control (no exposure)				
Non-neophobic	0.30	1.35(3.42–0.54)	0.64	0.52
(b) First exposure				
Non-neophobic	0.84	2.30(9.66–0.55)	1.14	0.25
(c) Second exposure				
Non-neophobic	1.15	3.15(9.22–1.08)	2.09	0.036
(d) Fourth exposure				
Non-neophobic	1.12	3.05(1205.87–0.01)	2.04	0.042

learning, memory, and responding to threats, although it is possible there are other brain regions where IEG activity would differ in neophobic and non-neophobic birds exposed to novel objects. Sample sizes were also relatively low, and an ideal study design would have included larger control groups of fasted non-neophobic and fed neophobic birds. However, one of the main reasons we included these fasted and fed controls was to ensure that any differences we saw between neophobic and non-neophobic birds was not merely due to them being fasted or fed, respectively. Because we did not see any differences in immediate early gene density between neophobic and non-neophobic birds for any brain region, this is less of a concern.

Additionally, it is possible that baseline (unstimulated) IEG expression could have differed among neophobic and non-neophobic sparrows, and thus there could have been a difference between the groups in their *change* in IEG expression in response to an object. However, avian studies have shown that there is generally less IEG induction in baseline or familiar conditions [56,14,30] and in conditions where the individual has habituated to the stimulus [57,58]. The sparrows in this study were maintained in their normal housing during object tests (where they had been housed for >6 weeks) and had been previously exposed to the same test procedure for ~3 weeks (food removal the previous evening, followed by return of the food the following morning either with a control or novel condition). Therefore, we think baseline IEG induction in both neophobic and non-neophobic birds should have been relatively low, though future studies could test this possibility.

Further, IEGs are often used as a proxy for general neuronal activity, but IEG induction does not give information about the identity of active neurons; e.g., IEGs can be induced by both excitatory and inhibitory neurons [59]. Although overall neuronal activity might not have differed by phenotype, the types of neurons that were active may be phenotype specific. Because emerging evidence suggests that differences in the expression and density of receptors for different neurohormones and neurotransmitters can play a role in avian behavioral phenotypes [60,38,61], future studies should examine co-expression of IEGs and relevant receptors to determine whether there could be differences in the types of cells active in response to novel objects in neophobic and non-neophobic birds.

Novel object and novel food trials have been widely used to understand how neophobia varies with respect to age [62], sex [22], social environment [36], diet [63], invasion success [64], urbanization [21], domestication [65], and circulating hormone concentrations [66]. However, what is often missing from these studies is an evaluation of whether an animal's response to one type of neophobia trial is reflective of its response to other neophobia trials – i.e., does a novel object test measure "neophobia" generally or simply a novel object response specifically? Indeed, novel object, novel food, and habituation trials all revealed significant and consistent differences between the house sparrows that were fastest (non-neophobic) and slowest (neophobic) to feed in the presence of novelty, where neophobic birds were slower to feed in the presence of a novel object, slower to taste a novel food, and took longer to habituate to a novel object. Individual responses to novel objects were repeatable, although novel food responses were not, which may be partly due to one of the novel foods (the cereal) eliciting a different response. However, average responses to all three of these trial types were significantly correlated, similar to an earlier study where novel object and novel food responses were also correlated in house sparrows [21]. Thus, novel object tests using several unique novel

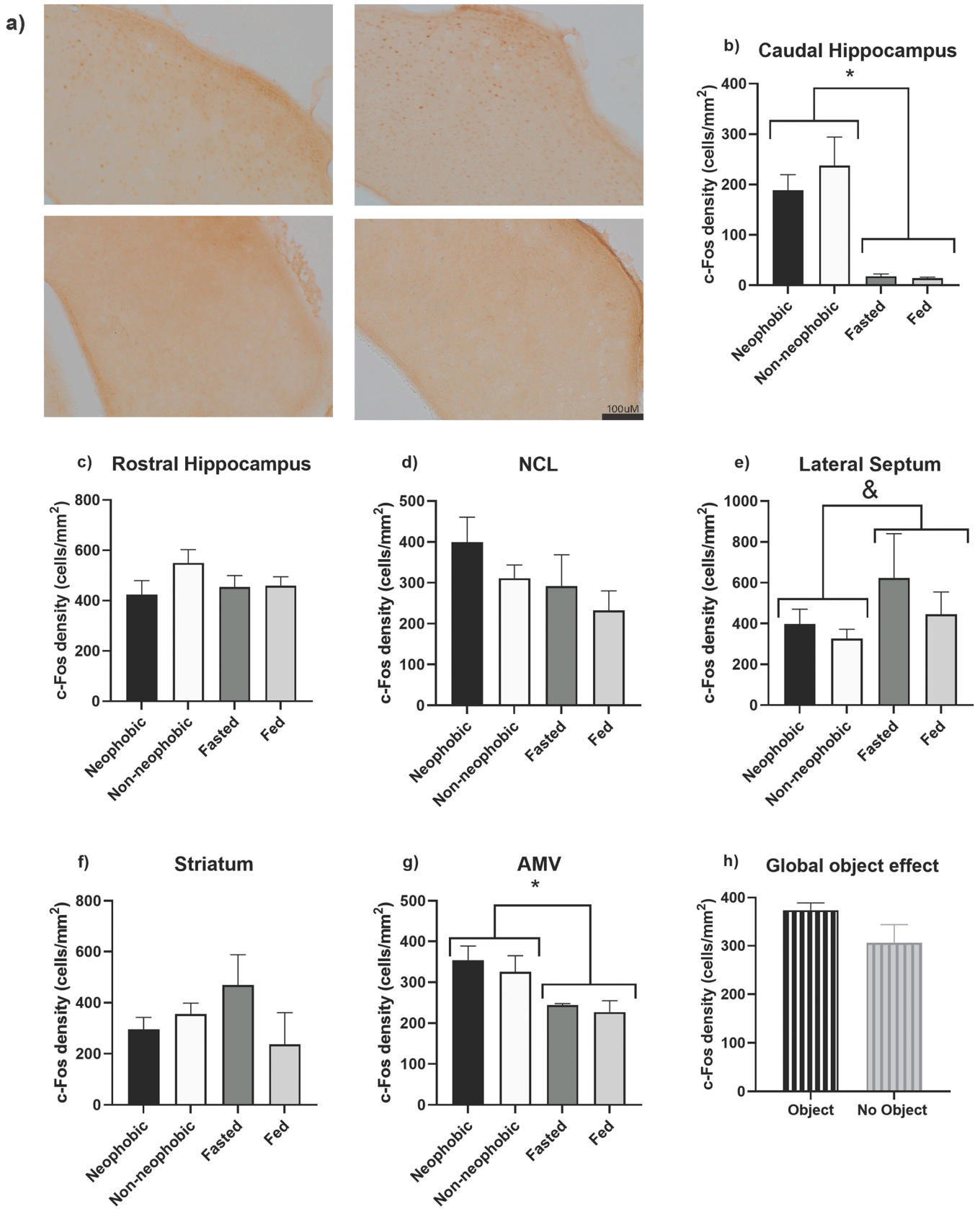

Fig. 2. Effect of behavioral phenotype and object presence on c-Fos expression. (a) Representative staining of the caudal dorsomedial hippocampus for each phenotype: top left=neophobic, top right=non-neophobic, bottom left=fasted, and bottom right=fed. Scale bar= 100 μm. (b-g) Expression of the immediate early gene c-Fos (mean ± SE cell density) in six brain regions of neophobic (n = 7), non-neophobic (n = 9), fasted (n = 3) and fed (n = 3) house sparrows. (g) Effect of novel object presence (n = 16) or absence (n = 6) on global c-Fos density. NCL=caudolateral nidopallium, AMV=medial ventral arcopallium. *$P < 0.05$ &$P = 0.07$.

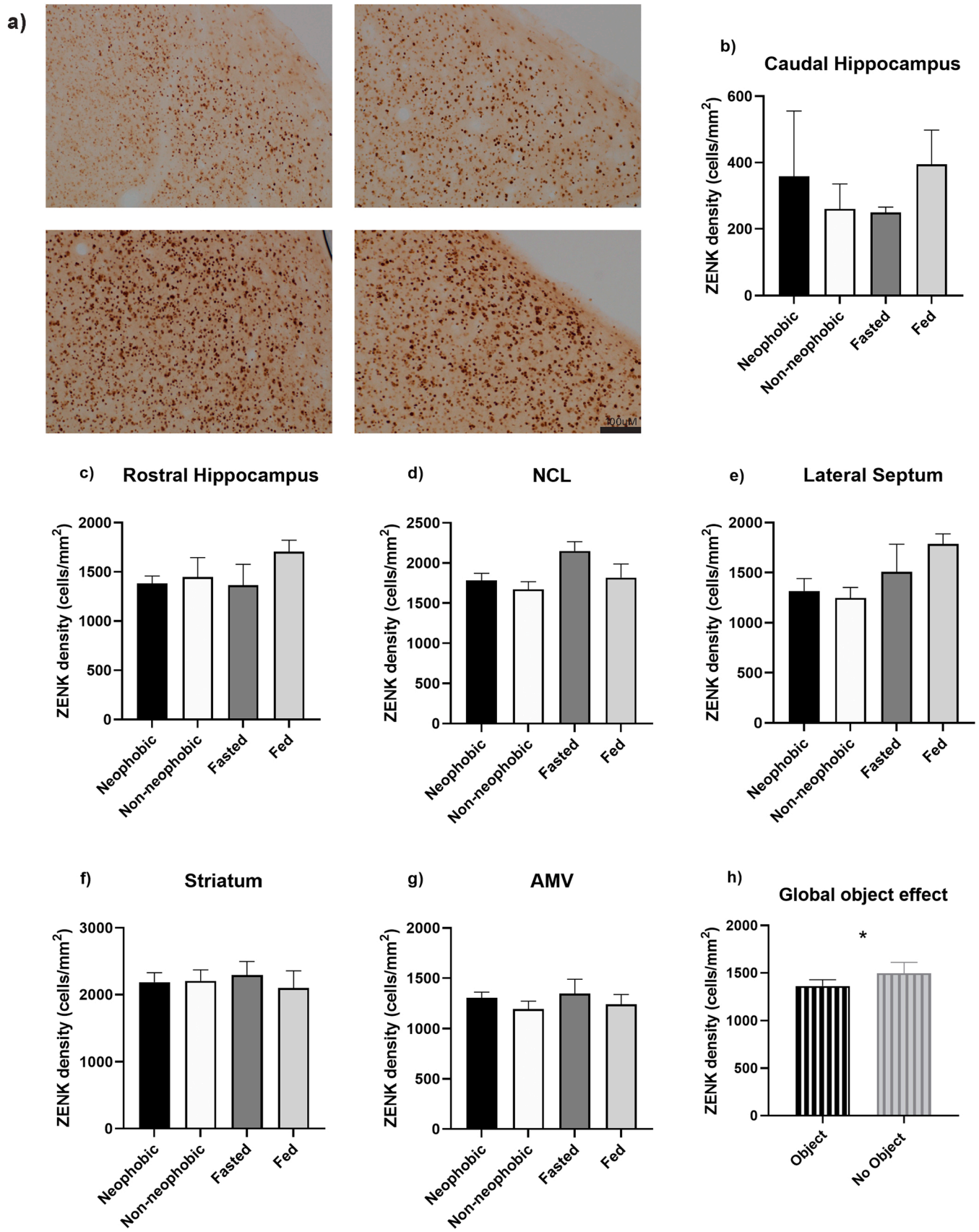

Fig. 3. Effect of behavioral phenotype and object presence on ZENK expression. (a) Representative staining of the caudolateral nidopallium (NCL) for each phenotype: top left=neophobic, top right=non-neophobic, bottom left=fasted, and bottom right=fed. Scale bar= 100 μm. (b-g) Expression of the immediate early gene ZENK (mean ± SE cell density) in the nuclei of neophobic (n = 7), non-neophobic (n = 9), fasted (n = 3) and fed (n = 3) house sparrows. (g) Effect of novel object presence (n = 16) or absence (n = 6) on ZENK density. NCL=caudolateral nidopallium, AMV=medial ventral arcopallium. *$P < 0.05$.

Table 4
Pearson correlation coefficients and non-adjusted p values between c-Fos and ZENK density for all regions of interest in house sparrow brains (n = 22). NCL=caudolateral nidopallium, AMV=medial ventral arcopallium.

Region	Correlation between c-Fos and ZENK
Rostral Hippocampus	r = 0.31, p = 0.14
Caudal Hippocampus	r = 0.20, p = 0.36
NCL	r = 0.020, p = 0.93
Lateral septum	**r = 0.51, p = 0.013** * *without outlier: **r = 0.47, p = 0.027** * *
Striatum	r = 0.24, p = 0.26
AMV	r = 0.39, p = 0.058

* *Significant after FDR correction

objects appear indicative of a general "neophobia" phenotype in house sparrows, and this behavioral paradigm alone may be sufficient to reliably distinguish between neophobic and non-neophobic individuals.

Although we did not find an effect of phenotype on IEG activity, we found a strong effect of novel object presence, an effect that was global for ZENK and region-specific for c-Fos. We found no effect of fasting vs feeding on IEG activity, and no difference in overall activity levels between neophobic, non-neophobic, and fasted/fed groups, indicating that these brain responses were specific to the presence of a novel object and not because of differences in feeding or activity. Neophobic sparrows that did not approach objects and non-neophobic sparrows that approached and fed in the presence of a novel object both showed increased c-Fos activity in AMV and caudal dorsomedial hippocampus, and decreased ZENK activity across all six brain regions compared to fasted and fed controls. These results are consistent with previous IEG work in male chicks that found higher density of c-Fos positive cells in the TnA (called AMV in our study) in individuals exposed to novel objects [30], though it should be noted that there is some debate about whether TnA/AMV in songbirds is truly homologous to the region defined as such in chickens and pigeons [67]. In contrast, Perez et al. [30] found a positive relationship between neophobia and c-Fos expression, with neophobic chicks showing increased c-Fos density in the nucleus accumbens and posterior amygdala, two brain regions we did not examine in this study. Overall, our results suggest that a non-neophobic behavioral response is not the result of sparrows ignoring objects or failing to recognize them as novel [68], in which case we would have expected non-neophobic birds to show similar patterns of IEG expression as fasted and fed controls.

Our study also provides evidence that the six brain regions we examined may be involved in responding to novel objects in house sparrows, and that the AMV and caudal dorsomedial hippocampus may play a particularly important role. However, it is important to note that IEG induction during exposure to a novel object trial does not conclusively demonstrate a causal role for these regions in neophobia, and further research is required, e.g., using lesions or inactivation of these regions. The mammalian amygdala is involved in perception of novelty [69], and processing fear and expression of fear behaviors [70,71], though there is still no clear consensus about which portions of the songbird brain constitute the "avian amygdala" [72,73]. Studies in both pigeons and chicks suggest that the TnA (here called AMV) is involved in neural processing of threatening and novel stimuli [29,30]. Our findings also suggest that the AMV is also involved in responding to novel objects in a songbird species.

The caudal hippocampus in birds has been proposed to be analogous to the ventral portion of the rodent hippocampus [74], and this analogous region in rodents is involved in anxiety and novelty recognition [75,76]. Studies in chickens have found that the caudal hippocampus can be more sensitive than the rostral hippocampus to stress-induced decreases in adult neurogenesis [45], and our c-Fos findings clearly suggest that the caudal hippocampus is more sensitive to novel stimuli than the rostral hippocampus. Future work should further explore the role of these regions and others in the social-decision making network (SDMN) involved in aversive non-social behaviors [77].

One goal of our study was to compare responses of the two different IEGs in different brain regions. Interestingly, we found unique effects depending on which IEG was used. As mentioned above, novel object presence induced a regional increase in c-Fos density, whereas it induced a global decrease in ZENK density. Correlation data suggest that for lateral septum, c-Fos and ZENK responses were more similar within an individual. However, the IEG responses in NCL, rostral and caudal dorsomedial hippocampus, AMV, and striatum were not significantly correlated. Previous research has also showed varying results with different IEGs depending on regions of interest and stimulus [17,18]. In avian research, ZENK and c-Fos have been shown to increase or decrease in song control nuclei in response to socially-relevant song treatments depending on the focal individual's sex, song novelty, and source of songs [20,10,78]. ZENK induction has also been associated with conditioned fear memory in the pigeon hippocampus [26]. Most studies examine either ZENK or c-Fos as an indicator of general neuronal activity; however, our data show that researchers should carefully consider relevant brain nuclei and stimulus type when choosing a marker of neuronal activity.

5. Conclusion

We did not find differences in neophobic and non-neophobic sparrows at the level of IEG expression, although we found a strong effect of novel object presence on IEG expression, which differed between ZENK and c-Fos. Because neophobia directly influences an animal's ability to explore novel environments and exploit novel resources, it is important to understand the neurobiological basis of this behavior. Future studies should assess whether the repeatable differences in behavior seen between neophobic and non-neophobic sparrows may be due to differences not in *what* brain regions are active, but in *how* active regions respond to novel stimuli, e.g. through different patterns of neurotransmitter or neurohormone release in neophobic and non-neophobic individuals.

CRediT authorship contribution statement

Melanie G. Kimball: Data curation, Formal analysis, Visualization, Funding acquisition, Writing – original draft. **Eve B. Gautreaux:** Data curation, Writing – review & editing. **Kaitlin E. Couvillion:** Data curation, Writing – review & editing. **Keegan R. Stansberry:** Data curation, Writing – review & editing. **Tosha R. Kelly:** Data curation, Formal analysis, Writing – review & editing. **Christine R. Lattin:** Conceptualization, Investigation, Methodology, Formal analyses, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

We declare we have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2022.113863.

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