

Research article

Abcam Monoclonal Egr-1 ab133695 is an effective primary antibody replacement for Santa Cruz sc-189 polyclonal Egr-1 in songbirds[☆]



Erin N. Scully^a, Juliana Montoya Sanchez^a, Christopher B. Sturdy^{a,b,*}

^a Department of Psychology, University of Alberta, Canada

^b Neuroscience and Mental Health Institute, University of Alberta, Canada

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ABSTRACT

Background: The immediate early gene ZENK (acronym zif268, Egr-1, NGFI-A, krox24) has been used extensively in songbird research (Mello et al., 1992; Jarvis and Nottebohm, 1997), as well as other research areas. ZENK has been used in assessing learning and memory, measuring neural activation, and identifying the cellular and molecular substrates involved in the first stages of memory formation (Watson and Clements, 1980). Previous songbird research has found that neurons located within the areas involved in auditory perception, namely the caudomedial nidopallium and caudomedial mesopallium, exhibit high levels of ZENK protein expression in response to conspecific songs and calls (Mello and Ribeiro, 1998; Avey et al., 2011).

New method: In large part due to its neuronal-specific labeling of ZENK protein, Santa Cruz Egr-1 sc-189 has been widely accepted as the standard primary antibody in songbird research. However, Santa Cruz Biotechnology Egr-1 no longer specifically labels and has also discontinued production of Egr-1 sc-189. Thus, the current study is focused on analyzing the effectiveness of alternative primary antibodies: Abcam polyclonal c-Fos, Abcam monoclonal ab133695 Egr-1, and Proteintech polyclonal Egr-1.

Results: Abcam monoclonal Egr-1 was successful in specifically labeling ZENK positive cells in the songbird auditory nuclei. Abcam polyclonal c-Fos and Proteintech polyclonal Egr-1 were found to have non-specific labeling.

Comparison with existing methods: Abcam monoclonal Egr-1 ab133695 was found to produce differential and specific labeling in the targeted auditory nuclei similar to previous studies successfully using Santa Cruz polyclonal Egr-1 (i.e. Mello and Ribeiro, 1998).

Conclusions: Abcam monoclonal Egr-1 effectively labels ZENK in the songbird auditory nuclei, making it a suitable primary antibody replacement for Santa Cruz polyclonal Egr-1.

1. Introduction

Immediate early genes (IEG) encode for transcription regulatory proteins, which have low expression when a neuron is not active. These proteins are thought to mediate long-term cellular changes involved in memory and learning (Watson and Clements, 1980; Jarvis and Nottebohm, 1997). In addition, IEGs have often been used as a tool to visualize neural activity using animal models. In songbird research specifically, the IEG protein product ZENK (zif268, Egr-1, NGFI-A, krox24) has been used to visualize how the brain responds to auditory information in the

auditory nuclei caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM; Knapska and Kaczmarek, 2004). Using immunohistochemistry, and the primary antibody Egr-1, the songbird research community has made great strides in understanding where auditory stimuli are processed and how different types of auditory stimuli are responded to (see Mello et al., 1992; Jarvis and Nottebohm, 1997; Avey et al., 2011).

Unfortunately, the most widely used primary antibody (182 citations according to product website; “Egr-1 Antibody (C-19): sc-189”, n. d.) used in the field, Santa Cruz Biotechnologies Egr-1 sc-189 (Santa Cruz,

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* Corresponding author.

E-mail address: esturdy@ualberta.ca (C.B. Sturdy).

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CA, USA), has recently been discontinued. Also, multiple reports of un-reliable labelling of ZENK positive cells have surfaced by researchers using sc-189 produced after 2015 (unpublished observations, birdsong-l@usc.edu [Electronic mailing list]). In order to evaluate the properties of possible replacement antibodies, we tested two new primary Egr-1 antibodies, as well as one c-Fos (another type of IEG) antibody to determine whether they were effective in marking neural activity in the songbird auditory nuclei NCM, dorsal (NCMd) and ventral (NCMv), and CMM. Previous studies have shown that there is robust ZENK expression in the auditory nuclei to conspecific songs and calls (Mello and Ribeiro, 1998; Avey et al., 2014). Following this previous research, we used conspecific songs and calls along with a silence control group, which has been shown to elicit minimal ZENK expression. In Part 1 of the experiment, we used zebra finches (*Taeniopygia guttata*) to test 11 possible protocols with novel primary antibodies, along with the Santa Cruz Egr-1 antibody as a control (12 groups total). In Part 2, we tested the generalizability of our findings by following the most successful protocol from Part 1, but using another songbird species (black-capped chickadees; *Poecile atricapillus*) as our subjects.

2. Methods

2.1. Subjects part 1

Three male zebra finches of at least one year of age acquired from Eastern Bird Supplies Inc(Thetford Mines Sud, Quebec, Canada) were used. Prior to the experimental procedure, birds were group housed in colony rooms that were kept on a 14:10 h light:dark cycle, and maintained at 20 °C. Birds were provided *ad libitum* access to food (Hagen Finch Staple VME Seed), water, and various environmental enrichment materials: perches, separators, and houses. Twice a week, birds were given a mixture of hard-boiled eggs with either spinach or parsley.

2.2. Subjects part 2

Two adult black-capped chickadees (one male and one female; DNA analysis of blood samples confirmed sex; Griffiths et al., 1998) were used. Chickadees were caught in Edmonton, Alberta, Canada (North Saskatchewan River Valley, 53.53N, 113.53W, Mill Creek Ravine, 53.52N, 113.47W) and were at least one year of age at time of capture (determined by examining the color and shape of outer tail rectrices; Meigs et al., 1983; Pyle, 1997). Prior to the experimental procedure, birds were housed in colony rooms were kept on the natural light:dark schedule for Edmonton, Alberta, Canada for the spring season (March 21, 2019–June 20, 2019), and maintained at 20 °C. Birds were given *ad libitum* access to food, water, and environmental enrichment materials: perches, separators, and houses. Twice a week birds were given a mixture of hard-boiled eggs with either spinach or parsley, and three times a week birds were given one superworm (*Zophobas morio*), as nutritional supplementation. This research was conducted with the approval of the University of Alberta Animal Care and Use Committee for Biosciences, meeting the standards of the Canadian Council on Animal Care.

2.3. Playback stimuli

In Part 1, subjects were randomly assigned to hear either male zebra finch songs ($n = 2$) or silence ($n = 1$). In Part 2, one black-capped chickadee heard silence while the other heard male black-capped chickadee calls. For both Part 1 and Part 2, stimuli were composed of two songs or two calls, with each call or song coming from different individual birds, played within the first 10 seconds of the stimulus, followed by 50 seconds of silence. Stimuli were created using SIGNAL software (version 5.05.02, Engineering Design, 2013) to edit the length of each stimulus and GoldWave (version 5.70; GoldWave, Inc., St. John's, NL,

Canada) to bandpass filter the stimuli (350–1,300 Hz). All stimuli were presented at approximately 75 dB with a Brüel & Kjær Type 2239 sound level meter (Brüel & Kjær Sound & Vibration Measurement A/S, Nærum, Denmark; A-weighting, slow response) as measured from the middle of the playback cage.

2.4. Playback procedure and equipment

Approximately 24 hours before experimental playback began, each bird was singly housed in a modified cage (80 × 30 × 40 cm, Jupiter Parakeet, Rolf C. Hagen Inc., Montreal, Canada) in a sound attenuating chamber (1.7 m × 0.84 m × 0.58m; Industrial Acoustics Corporation, Bronx, New York, USA), with free access to food and water. All birds were exposed to auditory playback on a loop for 30 min. To ensure maximum quantity and quality of ZENK preservation (Avey et al., 2011), birds were exposed to 1 h of silence in the dark following playback, then immediately transcardially perfused. Because previous research has shown that the ZENK protein accumulates over time, we isolated the birds in the dark and silence to ensure that the ZENK protein expressed was in response to the playback (Mello and Clayton, 1994). A lethal dose of 0.04 ml of 100 mg/ml ketamine and 20 mg/ml xylazine (1:1) was administered intramuscularly. The bird was perfused via the left ventricle using heparinized 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brain of each bird was then extracted and placed in a PFA solution for 24 hours, followed by a 30% sucrose PBS solution for 48 hours. Brains were fast frozen using isopentane and dry ice and stored at -80 °C until sectioned. This procedure followed the standard procedure for what other ZENK songbird research has used (Avey et al., 2011; Mello et al., 1992; Park and Clayton, 2002).

2.5. Histology part 1

Starting from the midline, 40 µm sagittal sections were collected from each brain and stored in PBS. Sections were stored in 24 well trays, with two sections per well, two trays per brain. Each tray was divided into sets of four wells that would receive the same treatment, with six groups per tray, for a total of 12 treatment groups. A) 1:1000 Santa Cruz Erg-1 sc-189 1-day incubation (Santa Cruz Biotechnology, Santa Cruz, CA, USA), B) 1:1000 Abcam Egr-1 ab133695 1-day incubation (Abcam Inc, Toronto, ON, Canada, C) 1:1000 Abcam Egr-1 ab133695 2-day incubation, D) 1:2000 Abcam Egr-1 ab133695 1-day incubation, E) 1:2000 Abcam Egr-1 ab133695 2-day incubation, F) 1:5000 Abcam Egr-1 ab133695 1-day incubation, G) 1:5000 Abcam Egr-1 ab133695 2-day incubation, H) 1:1000 Proteintech Egr-1 55117-1-AP 1-day incubation (Proteintech, Rosemount, IL, USA), I) 1:1000 Proteintech Egr-1 55117-1-AP 2-day incubation, J) 1:500 Proteintech Egr-1 55117-1-AP 1-day incubation, K) 1:1000 Abcam c-Fos ab209794 2-day incubation, L) 1:500 Abcam c-Fos ab209794 2-day incubation. All primary antibody concentrations refer to the amount of stock primary diluted in the vehicle 0.3% 0.1M PBS/Triton X-100 (i.e. 1:1000 is 1 µl of primary in 1000 µl of vehicle). Groups with a 1:1000 concentration (A, B, C, H, I, and K) were run together first, and based on preliminary results, the remaining groups (D, E, F, G, J, and L) were run at a later date with concentrations modified from the stock 1:1000 concentration depending on the preliminary results. We used a starting concentration of 1:1000 in an attempt to maintain our laboratory's protocol as closely as possible.

All sections were run using the same immunohistochemistry protocol, as follows. Sections were first washed twice in 0.1 M PBS for a minimum of 5 min, transferred to a 0.5% H₂O₂ solution (135 µl of 30% H₂O₂ in 7.5 ml dH₂O per tray) and incubated for 15 minutes. Three 5 min washes in 0.1 M PBS followed with an incubation in 10% normal goat serum (0.835 ml of NGS in 7.5 ml 0.3% 0.1M PBS/Triton X-100 per tray). Depending on treatment group, sections were incubated at room temperature for either 1 h in 10% normal goat serum and 2-days in primary antibody, or

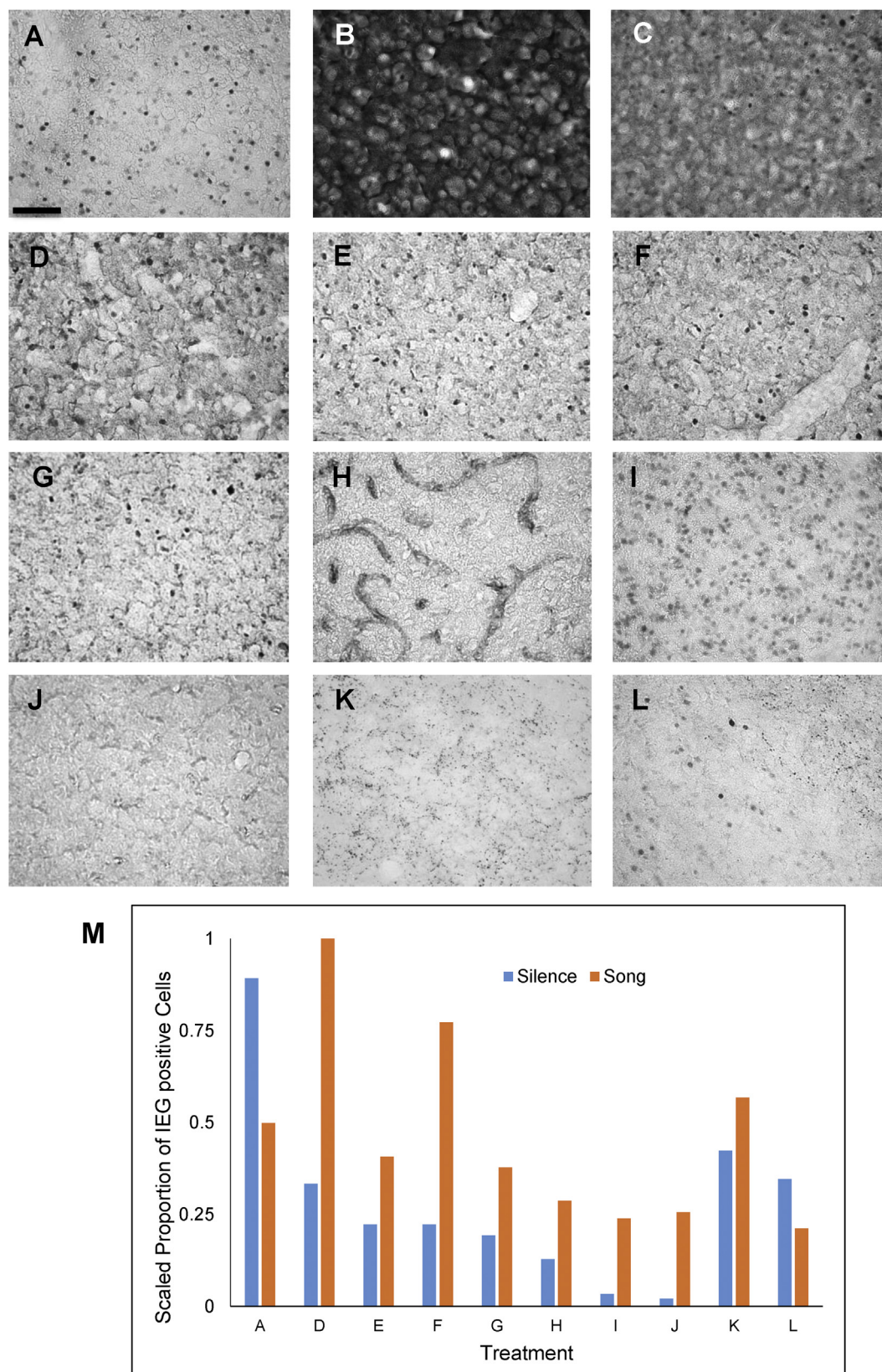


Figure 1. Effectiveness of primary antibody protocol. IEG labeling in the CMM of a song-exposed male zebra finch for each treatment; (A) 1:1000 Santa Cruz erg-1 1-day incubation, (B) 1:1000 Abcam Egr-1 ab133695 1-day incubation, (C) 1:1000 Abcam Egr-1 2-day incubation, (D) 1:2000 Abcam Egr-1 1-day incubation, (E) 1:2000 Abcam Egr-1 2-day incubation, (F) 1:5000 Abcam Egr-1 1-day incubation, (G) 1:5000 Abcam Egr-1 2-day incubation, (H) 1:1000 Proteintech Egr-1 55117-1-AP 1-day incubation, (I) 1:1000 Proteintech Egr-1 2-day incubation, (J) 1:500 Proteintech Egr-1 1-day incubation, (K) 1:1000 Abcam c-Fos ab209794 2-day incubation, (L) 1:500 Abcam c-Fos 2-day incubation (M) Scaled proportion (scaled to the highest overall count) of IEG marked cells per treatment in silence and song-exposed zebra finch males. Counts from (B) and (C) were not included in the graph (M) as the tissue was burned during the immunohistochemistry procedure rendering them unscorable. Scale bar = 50 μ m, same for all images.

Table 1. Cell counts for each treatment group before and after scaling (A) 1:1000 Santa Cruz erg-1 1-day incubation, (B) 1:1000 Abcam Egr-1 ab133695 1-day incubation, (C) 1:1000 Abcam Egr-1 2-day incubation, (D) 1:2000 Abcam Egr-1 1-day incubation, (E) 1:2000 Abcam Egr-1 2-day incubation, (F) 1:5000 Abcam Egr-1 1-day incubation, (G) 1:5000 Abcam Egr-1 2-day incubation, (H) 1:1000 Proteintech Egr-1 55117-1-AP 1-day incubation, (I) 1:1000 Proteintech Egr-1 2-day incubation, (J) 1:500 Proteintech Egr-1 1-day incubation, (K) 1:1000 Abcam c-Fos ab209794 2-day incubation, (L) 1:500 Abcam c-Fos 2-day incubation.

Treatment	Song 1	Song 2	Average Song	Silence	Scaled Song	Scaled Silence
A	26.8	20.2	23.5	42.1	0.50	0.90
B	-	-	-	-	-	-
C	-	-	-	-	-	-
D	45.0	49.3	47.2*	15.7	1.00	0.33
E	20.8	17.7	19.2	10.5	0.41	0.22
F	40.1	32.7	36.4	10.5	0.77	0.22
G	17.9	17.7	17.8	9.1	0.38	0.19
H	27.1	0	13.5	6.0	0.29	0.13
I	22.6	0	11.3	1.6	0.24	0.03
J	20.9	3.3	12.1	1.0	0.26	0.02
K	23.8	29.8	26.8	20.0	0.57	0.42
L	14.9	5.1	10.0	16.3	0.21	0.35

(*) indicates value used to scale counts.

incubated for 1-day in 10% normal goat serum and 1-day in primary antibody. After the incubation in normal goat serum, sections were transferred into their assigned primary antibody treatment group suspended in 0.3% 0.1 M PBS/Triton X-100 mix. The primary antibody mixture was calculated for 12 wells (4 wells $\times$ 3 trays for each treatment). In treatments with a concentration of 1:1000 (groups A, B, C, H, I, and K), 3.8 μ l of primary antibody was added to 3.8ml 0.3% 0.1 M PBS/Triton X-100 mix. Treatments with a concentration of 1:2000 (groups D and E) had 1.9 μ l primary antibody added to 3.8 ml 0.3% 0.1 M PBS/Triton X-100 mix. Treatments with a concentration of 1:5000 (groups F and G) had 0.76 μ l primary antibody added to 3.8 ml 0.3% 0.1 M PBS/

Triton X-100 mix. Treatments with a concentration of 1:500 (groups J and L) had 7.6 μ l primary antibody added to 3.8 ml 0.3% 0.1 M PBS/Triton X-100 mix. For each treatment, tissue in one well of a song treatment bird did not receive the primary antibody, instead incubating in 0.3% 0.1 M PBS/Triton X-100 mix as a negative control.

Sections were then washed three times in 0.1% 0.1 M PBS/Triton X-100 mix before being incubated in the secondary 1:250 biotinylated goat-anti-rabbit antibody (30 μ l antibody in 7.5 ml 0.3% 0.1M PBS/Triton X-100 per tray; Vector Labs, Burlington, ON, Canada) for 1 h. After three more washes in 0.1% 0.1 M PBS/Triton X-100 mix, sections were incubated for 1 h in avidin-biotin horseradish peroxidase (18.75 μ l 'A' and 18.75 μ l 'B' in 7.5ml 0.3% 0.1 M PBS/Triton X-100 per tray; ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada), followed by three washes in 0.1% 0.1 M PBS/Triton X-100 mix. Sections were then processed with 3,3'-diaminobenzidine tetrachloride (1 tab of DAB plus 1 tab of UREA dissolved in 15 ml of dH₂O per 2 trays; Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) for 2 minutes, or until tissue was deemed too dark to visualize labeling of ZENK or c-Fos positive cells, followed by three washes with 0.1 M PBS to remove any excess visualizing agents.

2.6. Histology part 2

Brains were sectioned sagittally from the midline, and 40 μ m sections were collected and stored in PBS. Sections were stored in 24 well trays, with two sections per well, two trays per brain. All sections were run using a similar immunohistochemistry protocol as in section 2.5, but only for the treatment Group 6 (1:5000 Abcam Egr-1 ab133695 1-day incubation) with one full tray per bird. Sections were first washed twice in 0.1 M PBS for a minimum of 5 min, transferred to a 0.5% H₂O₂ solution (135 μ l of 30% H₂O₂ in 7.5 ml dH₂O per tray) and incubated for 15 minutes. Three 5 min washes in 0.1 M PBS followed with an incubation in 10% normal goat serum (0.835 ml of NGS in 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray). Sections were incubated for 1-day in 10% normal goat serum and 1-day in primary 1:5000 Abcam Egr-1 ab133695 (1.52 μ l primary added to 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray).

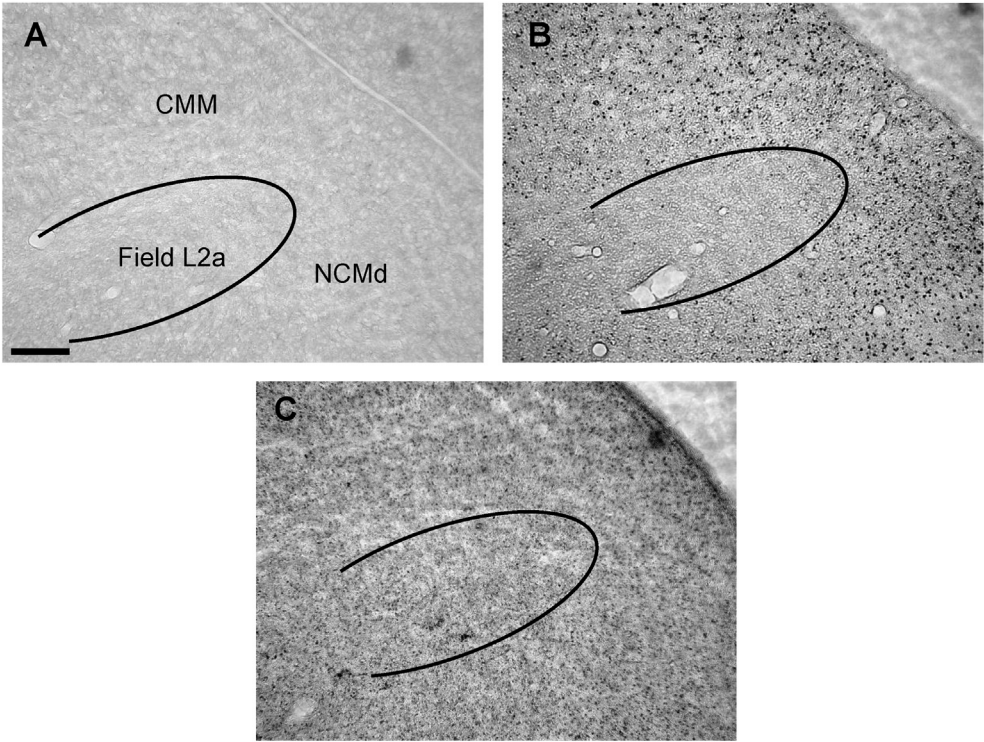


Figure 2. Examples of no labeling, specific labeling, and non-specific labeling. IEG labeled cells in the CMM, NCMD, and Field L2a at a 10X magnification. A) No labeling in any area in a bird who heard silence and treated with 1:5000 Abcam Egr-1 1 day incubation. B) Specific labeling in CMM and NCMD, with no labeling in Field L2a as expected, in a bird who heard songs and treated with 1:5000 Abcam Egr-1 1 day incubation. C) Non-specific labeling in Field L2a, in a bird who heard songs and treated with 1:1000 Proteintech Egr-1 2 day incubation. Scale bar = 160 μ m, same for all images.

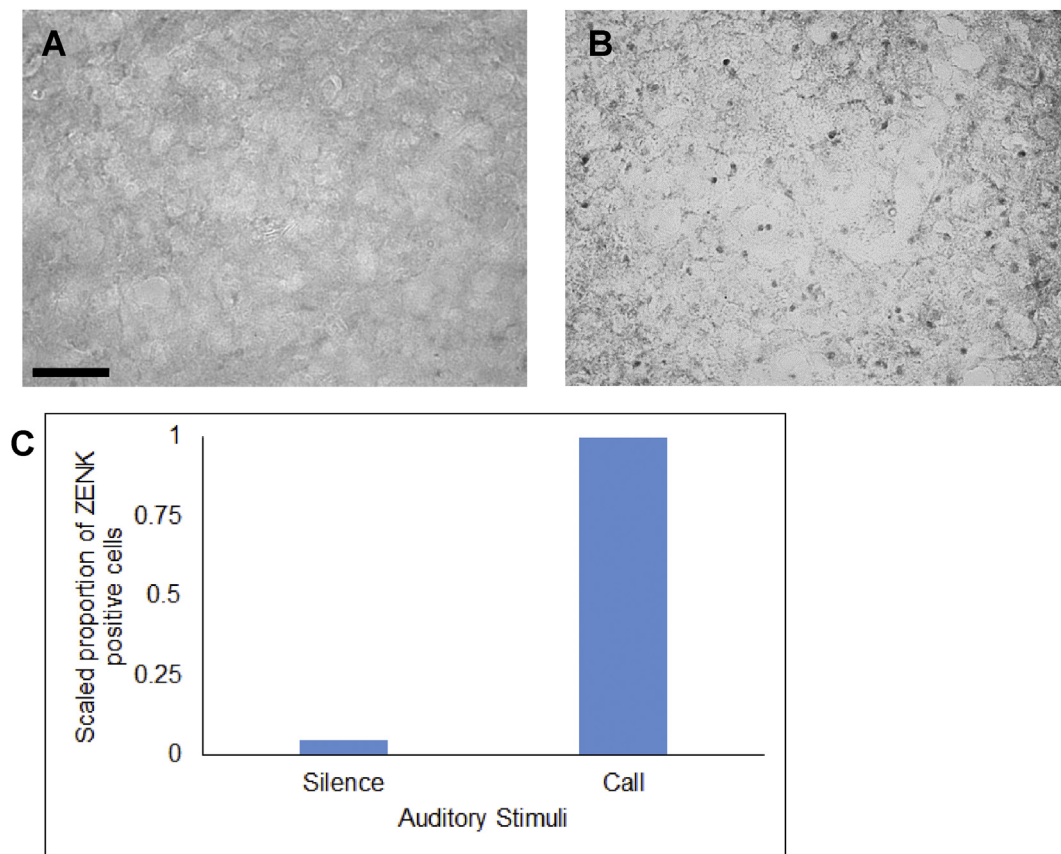


Figure 3. ZENK positive cells in response to call in black-capped chickadees. ZENK labeling in the CMM of the subject in the (A) silence condition and (B) conspecific call condition (C) Scaled proportion (all counts scaled to highest count) of ZENK positive cells across all three auditory areas CMM, NCMd, and NCMv using Abcam monoclonal Egr-1 ab133695 primary antibody. Scale bar = 50 μ m, same for all images.

Sections were then washed three times in 0.1% 0.1 M PBS/Triton X-100 mix before being incubated in the secondary 1:250 biotinylated goat-anti-rabbit antibody (30 μ l antibody in 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray; Vector Labs, Burlington, ON, Canada) for 1 h. After three more washes in 0.1% 0.1 M PBS/Triton X-100 mix, sections were incubated in avidin-biotin horseradish peroxidase (18.75 μ l 'A' and 18.75 μ l 'B' in 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray; ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada) for 1 h, followed by three washes in 0.1% 0.1 M PBS/Triton X-100 mix. Sections were then processed with 3,3'-diaminobenzidine tetrachloride (DAB; 1 tab of DAB plus 1 tab of UREA dissolved in 15 ml of dH₂O per 2 trays; Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) for 1–2 minutes until desired darkness to visualize labeling of ZENK, followed by three washes with 0.1 M PBS to remove any excess visualizing agents.

2.7. Imaging

Eight sections, four per hemisphere, were mounted for each treatment separately on a microscope slide and coverslipped. Three neuroanatomical regions (CMM, NCMd, and NCMv) were subsequently imaged using a Leica microscope (DM5500B; Wetzlar, Germany) to quantify ZENK labeled cells. Four images of each region of interest were captured per hemisphere for a total of 24 images per subject. Images were obtained using a 40 x oil immersion objective lens, a Retiga Exi camera (Qimaging, Surrey, BC, Canada), and Openlab 5.1 on a Macintosh OS X (Version 10.4.11). Overlap in the dorsal and ventral regions of the NCM was carefully avoided by imaging the dorsal most and ventral most regions. ImageJ version 1.46v was used to quantify immunopositive ZENK or c-Fos cells. Using the 'Analyze Particles' functions, neuron size was defined as being between 9.07–27.21 μ m², with a circularity between 0.4–1.00.

Counts were scaled to the highest value to view the proportion of labeled cells between the treatment groups.

3. Results and discussion

Due to Santa Cruz polyclonal Egr-1 no longer being produced and recent issues with non-specific labeling in newer batches, the need to replace this antibody is critically important. Previous research (e.g., Mello et al., 1992; Avey et al., 2014) demonstrated that Santa Cruz polyclonal Egr-1 labeled more ZENK positive cells in birds exposed to conspecific song compared to birds exposed to silence; however, the current study (Group A) found the reverse (Figure 1M). This is not the first instance that the validity or reliability of Santa Cruz polyclonal Egr-1 has been questioned. Recently, researchers have generated multiple reports of Santa Cruz polyclonal Egr-1 produced after 2015 not reliably labelling ZENK positive cells in songbird auditory nuclei (unpublished observations).

In Part 1 of the study, we found that some of the treatment groups resulted in specific ZENK labeling in the examined auditory nuclei. Abcam monoclonal EGR-1 ab133695 at a concentration of 1:1000 (Groups B and C) was found to be too concentrated. In Groups B and C the tissue darkened too much when visualized with DAB, rendering any ZENK positive cells unidentifiable and uncountable (Figure 1B and 1C). Abcam monoclonal Egr-1 ab133695 at a concentration of 1:2000 at 1- and 2-day incubation durations (Groups D and E) resulted in successfully labeled ZENK-positive cells (Table 1). At a concentration of 1:5000 at 1- and 2-day incubations (Groups F and G) Abcam monoclonal Egr-1 ab133695 was found to label ZENK positive cells in the auditory nuclei (CMM, NCMd, and NCMv) with less background staining than resulted from the same antibody when used at higher concentrations (Figure 1F &

1G). Proteintech polyclonal Egr-1 55117-1-AP was found to non-specifically label ZENK-positive cells at all concentrations and incubations due to labeling in the silent condition (Groups H, I, and J). Non-specific labeling was also identified in Field L2a, which is known not to express ZENK to song or call stimuli, blood vessels, and in the silence condition (Figure 2; Mello et al., 1992). Abcam c-Fos ab209794 at concentrations of 1:500 and 1:1000 with a 2-day incubation (Groups K and L; Figure 1K & 1L) also showed non-specific labelling in Field L2a and the silent condition. Based on these results, we concluded that Abcam monoclonal Egr-1 ab133695 used at a concentration of 1:5000 with an incubation of 1-day produced the optimal staining while maintaining the original protocol of our laboratory (Table 1). To confirm the reliability and generalizability of our findings, we conducted Part 2 of the experiment, using Abcam monoclonal Egr-1 ab133695 at a concentration of 1:5000 with a 1-day incubation in black-capped chickadees exposed to call playback.

In Part 2 of this study, we extended the findings of Part 1 using the protocol from Group F (1:5000 Abcam Egr-1 1 day incubation) on black-capped chickadees exposed to their own conspecific calls as the auditory stimuli. There was positive ZENK labeled cells in all three auditory areas, and no labeled cells in field L2, in the brain of the bird exposed to calls, and little to no labeling in the 3 auditory areas of the bird exposed to silence (Figure 3). These findings are congruent with previous findings using Santa Cruz polyclonal Egr-1 (e.g., Ribeiro et al., 1998; Avey et al., 2005; Gobes et al., 2009). Our results suggest that the use of Abcam Monoclonal Egr-1 ab133695 is a suitable replacement for Santa Cruz polyclonal Egr-1 as a primary antibody to mark ZENK positive cells in the songbird auditory nuclei.

An important factor to consider when selecting a new antibody to use is the specificity of the potential new antibody. In the current study we ran a negative control that consisted of one well not using the primary antibody on some sections, showing that the labeling was due to the primary, and not nonspecific labelling from the secondary. While we ourselves did not run a Western Blot, Abcam, the manufacturer of the antibody, did run a Western Blot using songbird cells, showing one major band associated with >99% of the signal (Abcam Scientific). In addition, the company provided more evidence of specificity by determining the dissociation constant (Kd) for the antibody as 10⁻¹¹ (Abcam Scientific). In addition, we also had positive controls in the current study in the case of running silence groups, which has previously been shown to have no to limited ZENK labeling, as well as examination of area L2a in the experimental groups (having heard songs or calls) which is also not expected to have labeled cells (Park and Clayton, 2002; Ribeiro et al., 1998). Given this information regarding previous tests of specificity and controls run in the current study, we believe we have provided sufficient current evidence supporting the effectiveness of Abcam Monoclonal Egr-1 ab133695 to be used as a primary antibody in songbirds.

4. Conclusion

Here, we tested three new antibodies which can be used for marking IEG expression in the songbird auditory nuclei. Our results demonstrate that Abcam monoclonal Egr-1 ab133695 is a suitable primary antibody replacement for Santa Cruz polyclonal Egr-1. We showed that Abcam monoclonal Egr-1 ab133695 at concentration 1:5000 at 1-day incubation best labeled ZENK positive cells in songbirds in response to both songs and calls.

Declarations

Author contribution statement

Erin Scully: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Juliana Montoya Sanchez: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Christopher Sturdy: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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