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Neural activation in a septal area is related to intrinsic motivation for non-courtship singing in adult zebra finches

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Intrinsic motivation, which drives animals, including humans, to exhibit various voluntary behaviors, spontaneously originates within the brain without immediate external stimuli such as rewards or punishments. The zebra finch, a songbird, provides an ideal model for studying the neural substrates of intrinsic motivation because male birds spontaneously produce many renditions of non-courtship song ("undirected song") with a highly quantifiable structure for vocal practice. Here, we identified a brain area associated with intrinsic motivation for undirected singing through brain-wide mapping of neuronal activity using immediate early gene expression in birds with different levels of singing motivation. We found that birds with relatively high singing motivation exhibit increased expression levels of *Arc* mRNA in a septal area, the nucleus of the hippocampal commissure (NHpC), compared with birds with low singing motivation. Such high *Arc* mRNA expression was not observed after highly motivated birds decreased their singing motivation. These findings demonstrate that neuronal activity in the NHpC is associated with the degree of singing motivation, marking a crucial initial step in understanding the neural circuitry regulating intrinsic motivation for spontaneous singing behavior in songbirds.

Keywords Zebra Finch, Song, Motivation, Immediate early gene, Arc, Songbird

Animals, including humans, voluntarily perform a variety of behaviors without receiving immediate external rewards or punishments. For example, children voluntarily play with toys and friends and acquire a variety of knowledge and skills. Adults also voluntarily engage in sports, creative activities, and social interactions. These voluntary behaviors are driven by intrinsic motivation, which arises spontaneously in the brain, although the same behaviors can be driven by extrinsic motivation when external rewards or punishments are provided[1–4]. Unlike extrinsically motivated behaviors, intrinsically motivated behaviors do not result in immediate increases in fitness or changes in the homeostatic state of the animal. However, intrinsically driven behaviors allow animals to learn new motor skills and knowledge, leading to the development and maintenance of physical and cognitive function, and ultimately increasing the animal's fitness[1–4]. Moreover, intrinsically motivated behaviors bring us a sense of pleasure and satisfaction, while decreased intrinsic motivation is closely linked to mental disorders, including depression[1–4]. Thus, intrinsic motivation is an essential element for individuals to live a fulfilling and enriched life, and understanding its neural mechanisms holds great significance for people worldwide.

Intrinsic motivation has been actively studied across various fields, including psychology, human behavioral science, and education[4,5]. However, the neural underpinnings of this phenomenon have not been extensively studied. One key challenge is that intrinsic motivation occurs without immediate external rewards or punishments (e.g., food or electric shocks), making it difficult for researchers to experimentally manipulate its level. Additionally, many intrinsically motivated behaviors observed in common experimental animals such as mice and rats are complex, variable, and infrequently produced. For instance, spontaneous "social play" behaviors observed in young rats are highly intricate and rely heavily on interactions with other individuals[6]. These behavioral characteristics have posed significant challenges to systematic studies of intrinsic motivation, leaving the underlying mechanisms unclear.

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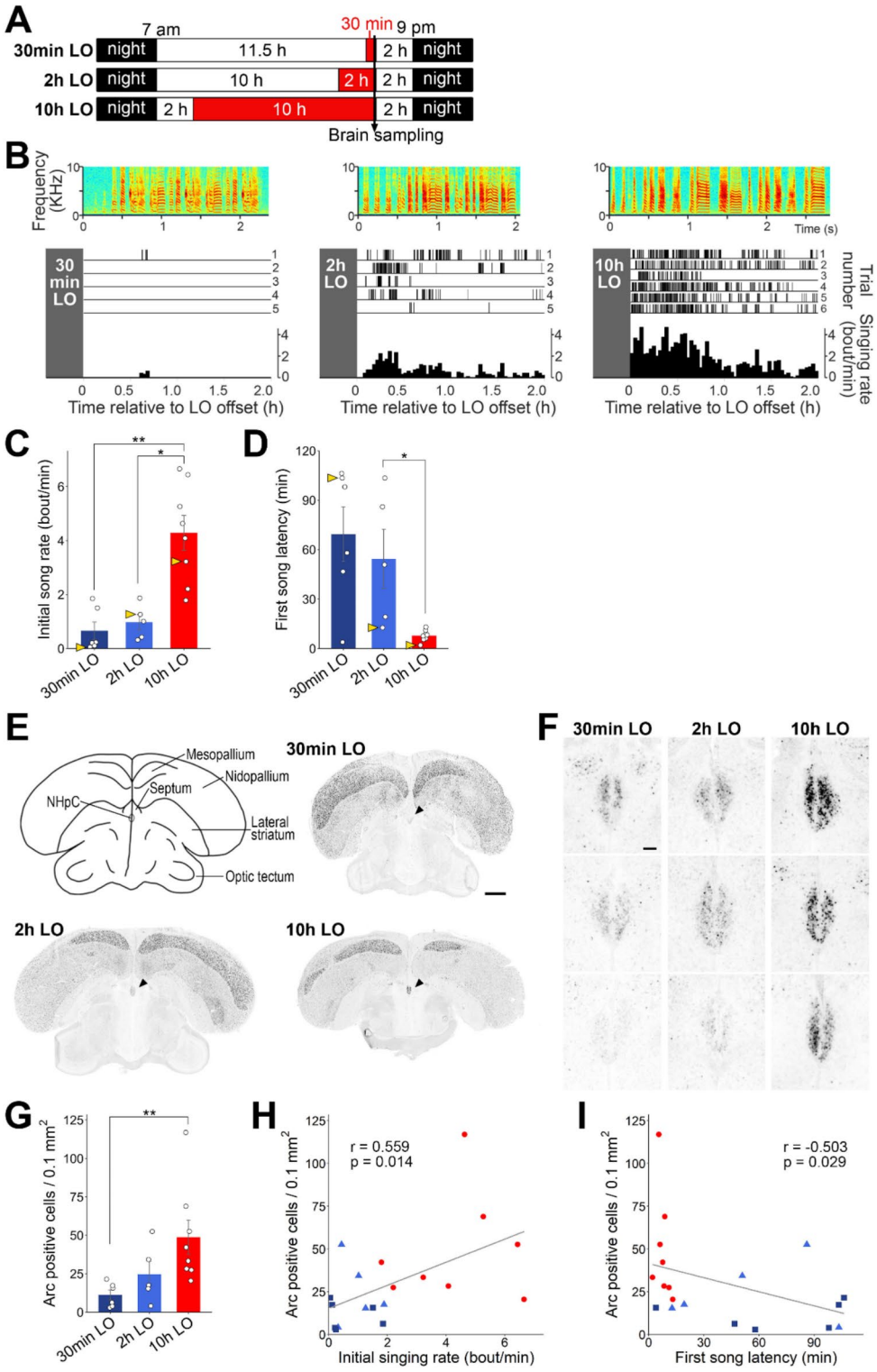


To address this issue, we used the zebra finch (*Taeniopygia guttata*), a songbird, to investigate the neural substrates of intrinsic motivation for voluntary behavior. Male zebra finches spontaneously produce hundreds of stereotyped songs every day in a solo context (referred to as "undirected singing") partly for vocal practice[7–10]. Undirected singing is produced in the absence of immediate external rewards, such as food or copulation, and thus appears to be driven by intrinsic motivation that is spontaneously generated in the brain. Therefore, undirected singing provides an ideal model system for studying the neural substrates of intrinsic motivation for voluntary motor behavior. However, in contrast to the neural mechanisms by which birds develop and maintain song structure through undirected singing, the mechanisms of intrinsic motivation driving undirected singing remain largely unexplored. Here, as a first step in understanding the neural mechanisms of intrinsic motivation for undirected singing, we explored the neural circuitry involved in regulating intrinsic singing motivation using

◀**Fig. 1**. Suppression of undirected singing by turning off ambient light increases both singing motivation and *Arc* expression in the NHpC. (**A**) Experimental schedules of LO periods (red areas) of three different durations (30 min, 2 h, and 10 h) and timing of brain sampling. (**B**) Representative singing activity following 30 min, 2 h, and 10 h LOs (left, middle, and right panels, respectively) recorded from three different birds. For each bird, a spectrogram of a song bout (top), raster plot of song bouts (middle), and corresponding histogram of singing rate (bottom; bin size of 2 min) are shown. Note that singing activity starts sooner and at higher rates as the LO duration increases. (**C**–**D**) The initial singing rates (**C**) and first song latencies (**D**) after 30 min LO, 2 h LO, and 10 h LO in the bird used for IEG expression analysis (for initial singing rates, 10 h LO vs. 30 min LO, $^{**}p = 0.008$; 10 h LO vs. 2 h LO, $^{*}p = 0.014$; for first song latencies, 10 h LO vs. 30 min LO, $p = 0.072$; 10 h LO vs. 2 h LO, $^{*}p = 0.014$; Steel-Dwass test). Circles and bars represent the data of individual birds and the mean ± SEM across birds, respectively; yellow arrowheads indicate the data obtained from the birds shown in Fig. 1B. (E) Schematic of the zebra finch brain (in coronal view) illustrating the NHpC and major brain regions (upper left panel), and typical examples of *Arc* mRNA expression in the three LO conditions. Arrowheads indicate the NHpC. Scale bar = 1.0 mm. (**F**) Higher magnification images of *Arc* expression around the NHpC (three birds in each LO condition). Scale bar = 100 µm. (**G**) *Arc*-positive cell density in the NHpC under 30 min LO, 2 h LO, and 10 h LO conditions in the birds shown in Fig. 1C & D. Dots and bars represent the data of individual birds and the mean ± SEM across birds, respectively. *Arc*-positive cell density in the NHpC was significantly greater after 10 h LO compared to after 30 min LO ($^{*}p = 0.008$; Steel-Dwass test). (H) *Arc*-positive cell density (number of cells per 0.1 mm^2) in the NHpC plotted against the initial singing rate for individual birds. Red, 10 h LO birds; light blue triangles, 2 h LO birds; dark blue squares, 30 min LO birds. (**I**) *Arc*-positive cell density in the NHpC plotted against the first song latencies. The conventions are the same as in (**H**).

brain-wide mapping of neuronal activation combined with a behavioral paradigm to enhance intrinsic singing motivation recently established in our laboratory[11].

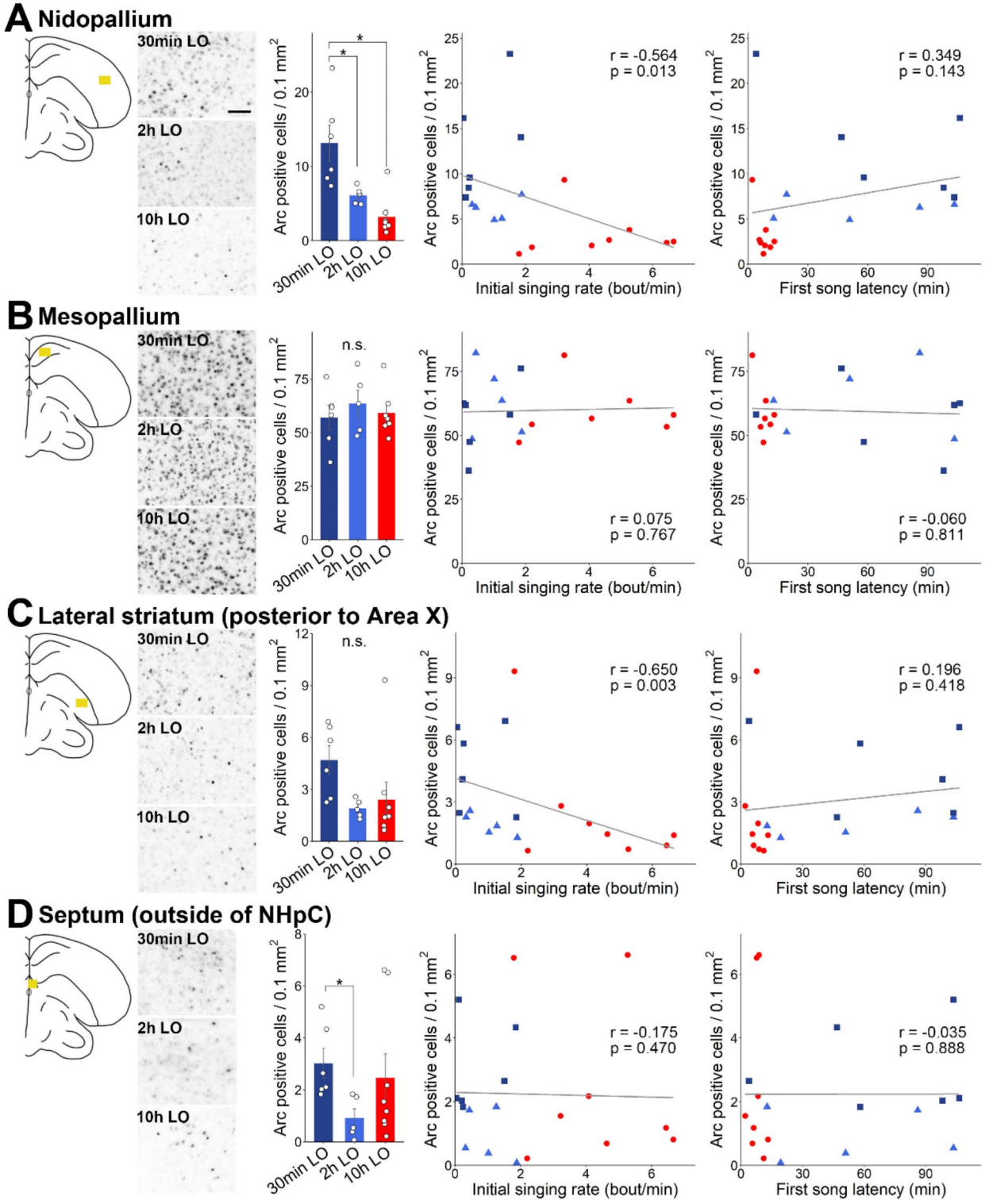
Results

Our previous study demonstrated that temporary suppression of spontaneous undirected singing in adult zebra finches by turning off ambient light increases intrinsic motivation for undirected singing, depending on the duration of singing suppression[11]. Specifically, longer singing suppression results in higher levels of singing motivation, as quantified by the singing rate and latency to the first song following the singing suppression period. Using this behavioral paradigm, we prepared three groups of adult birds with varying levels of singing motivation and examined which brain areas were activated in correlation with these motivation levels. To induce relatively low, intermediate, and high levels of singing motivation, spontaneous undirected singing was suppressed by lights-out (LO) in the sound recording chamber for 30 min, 2 h, and 10 h, respectively, according to the schedule shown in Fig. 1A. In each group, one of these LOs was repeated 5–6 times (once per day), alternating with LOs of different duration (see Methods for more details), and the singing motivation enhancement by the LO of interest was quantified by the initial singing rate and the first song latency, as reported in our previous study[11]. Consistent with the results in our previous study[11], we observed that intrinsic motivation for undirected singing was enhanced by the LO manipulation depending on LO duration (Fig. 1B–D; $n = 6$, 5, and 8 birds for 30 min LO, 2 h LO, and 10 h LO, respectively): As the LO duration increased, the initial singing rate increased (Fig. 1C; 30 min LO vs. 10 h LO, $p = 0.008$; 2 h LO vs. 10 h LO, $p = 0.014$; 30 min LO vs. 2 h LO, $p = 0.407$; Steel-Dwass test) and the first song latency decreased (Fig. 1D; 30 min LO vs. 10 h LO, $p = 0.072$; 2 h LO vs. 10 h LO, $p = 0.014$; 30 min LO vs. 2 h LO, $p = 0.847$).

After obtaining the behavioral data, we sampled the birds' brains immediately before the end of the LO period of interest and conducted brain-wide mapping of neuronal activity using in situ hybridization for three immediate early genes (IEGs), *c-fos*, *Egr1* (*zenk*), and *Arc*, to search for brain regions where neurons were activated in parallel with intrinsic singing motivation. As a first step in identifying such brain regions, we visually inspected the hybridized brain sections and compared the overall expression levels of these IEGs between the 30 min LO and 10 h LO conditions in a wide range of the nidopallium, arcopallium, mesopallium, subpallium, thalamus, and midbrain. As a result, we found that, among these IEGs and brain regions, only *Arc* expression in a septal area, the nucleus of the hippocampal commissure (NHpC), was clearly higher in the 10 h LO condition than in the 30 min LO condition (Fig. S1 & Table S1–3). Given these results, we performed more quantitative analyses of *Arc* expression in the NHpC and several major brain regions to examine the relationships between neuronal activity and singing motivation.

We compared the density of *Arc* mRNA-expressing cells (number of cells per 0.1 mm^2) in the NHpC between the 30 min, 2 h and 10 h LO conditions and found that it monotonically increased with LO duration (Fig. 1E–G). Given the LO duration-dependent enhancement of singing motivation, these results reveal a striking parallel between neuronal activity in the NHpC and the levels of singing motivation. Indeed, *Arc*-positive cell density in the NHpC was positively correlated with initial singing rates ($r = 0.559$, $p = 0.014$, Spearman's rank correlation; Fig. 1H) and negatively correlated with first song latencies ($r = -0.503$, $p = 0.029$; Fig. 1I). Thus, neuronal activity in the NHpC changes in parallel with the degree of singing motivation, supporting the idea that the NHpC is a part of the neural circuits regulating the level of intrinsic motivation for undirected singing.

Consistent with the results of our visual inspection of brain sections, increases in *Arc*-positive cell density in parallel with the levels of singing motivation, as seen in the NHpC, were not observed in other major brain regions. In the nidopallium (Fig. 2A, left), the LO-dependent trend of *Arc* expression levels was opposite to that observed in the NHpC, with a greater density of *Arc*-positive cells in birds with shorter LO durations (Fig. 2A, bar plot). These higher *Arc* expression levels in the shorter LO conditions appear to reflect the longer light



exposure of the birds and the resulting greater amount of their non-song movement (e.g., hopping) prior to brain sampling, based on a previous study showing movement-induced expression of other IEGs[12]. Consistent with these results, *Arc*-positive cell density was negatively correlated with the initial singing rate (Fig. 2A, left scatter plot) and tended to be positively correlated with the first song latency (Fig. 2A, right scatter plot), in sharp contrast with the results in the NHpC (Fig. 1E–I). In the mesopallium, lateral striatum (posterior to Area X), and septum (outside the NHpC), we found no clear trends of *Arc* expression levels in relation to LO duration (Fig. 2B–D, bar plot). Additionally, no significant correlations were observed between *Arc* expression levels and initial singing rate or first song latency (Fig. 2B–D, scatter plots), except the negative correlation between *Arc* expression levels and the initial singing rate in the lateral striatum (Fig. 2C, left scatter plot), as seen in the nidopallium.

Fig. 2. *Arc* expression levels in other brain regions do not increase as singing motivation increases. (**A**–**D**) Brain regions where *Arc*-positive cell density was examined: the nidopallium (**A**), mesopallium (**B**), lateral striatum (posterior to Area X) (**C**), and septum (outside of the NHpC) (**D**). In each row, a schematic diagram of a coronal section is shown in the leftmost panel. The yellow box in the diagram indicates the area where *Arc*-positive cell density was measured. Images next to the diagram show typical examples of *Arc* expression signals under the three LO conditions obtained in the yellow box (scale bar = 100 μm). The bar plots in the middle *Arc*-positive cell density under the three LO conditions. The scatter plots show *Arc*-positive cell density plotted against the initial singing rate (left) and first song latency (right); conventions are the same as in Fig. 1G–I. In the nidopallium (**A**), *Arc*-positive cell density was significantly greater after 30 min LO compared to after 2 h LO and 10 h LO (10 h LO vs. 30 min LO, $^{*}p = 0.012$; 10 h LO vs. 2 h LO, $p = 0.071$; 30 min LO vs. 2 h LO, $^{*}p = 0.028$; Steel-Dwass test). In the septum (**D**), *Arc*-positive cell density was significantly greater after 30 min LO than after 2 h LO ($^{*}p = 0.028$). In addition, there were significant negative correlations between *Arc*-positive cell density and the initial singing rates in the nidopallium (**A**) and lateral striatum (**C**).

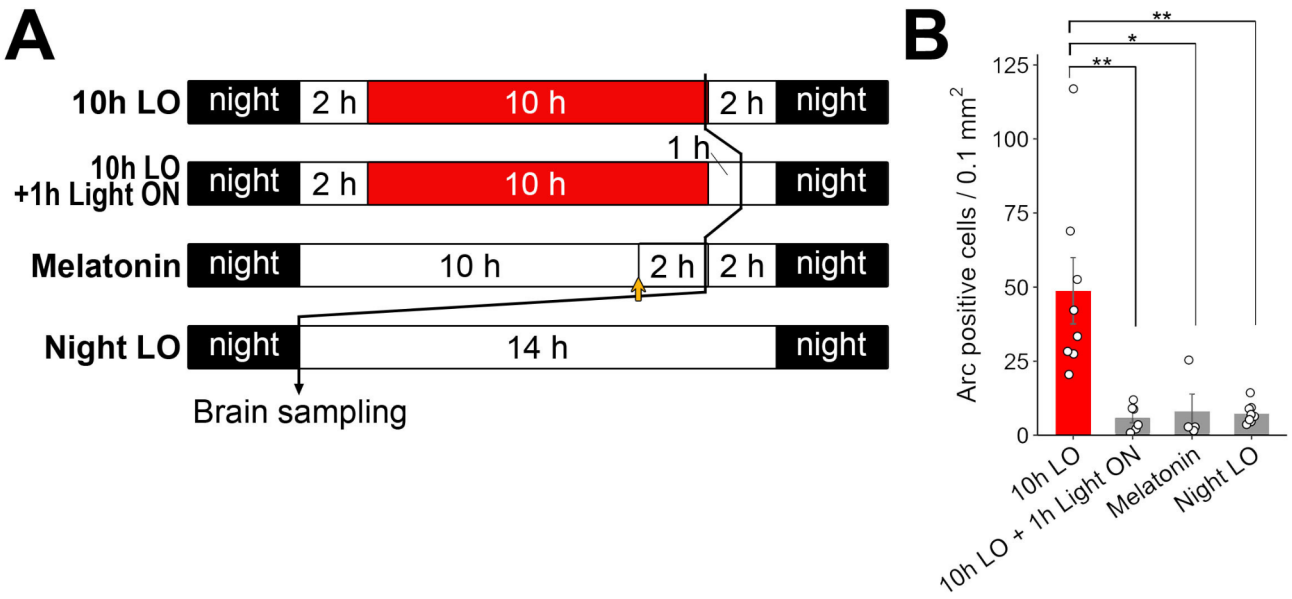


Fig. 3. Comparison of *Arc* mRNA expression in the NHpC between 10 h LO and control experimental conditions. (**A**) Experimental timelines of LO periods (red areas) and of control manipulations (see Methods), and timing of brain sampling. The yellow arrow indicates melatonin administration. (**B**) *Arc*-positive cell density in the NHpC under the conditions of 10 h LO ($n = 8$ birds), 10 h LO + 1 h Light ON ($n = 6$ birds), melatonin ($n = 4$ birds), and night LO ($n = 8$ birds). The conventions are the same as those in Fig. 1G. *Arc*-positive cell density was significantly greater under the 10 h LO condition than under the 10 h LO + 1 h Light ON condition ($^{**}p = 0.005$; Steel test), the melatonin-administered condition ($^{*}p = 0.030$), and the night LO condition ($^{**}p = 0.002$).

Our previous study showed that birds subjected to 10 h LO begin to sing at high rates immediately after the LO period ends, and then gradually decrease their singing rates over ~ 1 h[11] (also see Fig. 1B, right). Since singing rates reflect the level of singing motivation, these results suggest that singing motivation enhanced by 10 h LO decreases over a similar time course. Thus, if the increased *Arc* expression in the NHpC observed in the 10 h LO birds is associated with increased singing motivation, *Arc* expression is predicted to be lower in birds that were allowed to sing for 1 h in the light condition following the 10 h LO period (Fig. 3A, second row). Consistent with this prediction, *Arc*-positive cell density in the NHpC was significantly smaller in the 10 h LO + 1 h Light ON birds ($n = 6$) than in the 10 h LO birds (Fig. 3B, 10 h LO vs. 10 h LO + 1 h Light ON: $p = 0.005$, Steel test). These results provide further evidence supporting that the NHpC neuronal activity is associated with intrinsic motivation for undirected singing.

Additionally, we investigated the possibility that prolonged LO periods may lower the arousal level of birds or induce sleep, potentially resulting in increased *Arc* expression in the NHpC. To test this, we systemically administered melatonin to induce sleep 2 h before sampling the brains and examined *Arc* expression in the NHpC (Fig. 3A, third row). We found that *Arc*-positive cell density was significantly smaller in the melatonin-administered birds ($n = 4$) compared to the 10 h LO birds, and similar to the level of the 30 min LO birds (Figs. 1G and 3B, 10 h LO vs. melatonin: $p = 0.030$). These results suggest that the increased *Arc* expression levels in the NHpC of 10 h LO birds were not caused by sleepiness or low arousal levels.

Because LO manipulation removes the light input to the birds, it is possible that LO-dependent *Arc* expression in the NHpC is caused by the darkness itself of the LO period independently of intrinsic singing motivation.

In particular, birds are known to have photosensitive neurons in the deep brain regions that detect seasonal changes in photoperiod (see Guh et al. 2019[13] for review), and those neurons may mediate LO-dependent *Arc* expression in the NHpC. Alternatively, or in addition, the prolonged inactivity of the birds caused by the long LO periods could contribute to increased *Arc* expression. To test these possibilities, we sampled the brains of the birds immediately before the end of the LO period at night (10 h LO for 2 birds and 13 h LO for 6 birds) and examined *Arc* expression levels in the NHpC (Fig. 3A, bottom row). During the night LO periods, birds do not appear to greatly increase their intrinsic singing motivation unlike during 10 h LO periods[10,14], but both LO periods deprive light input to the birds. Thus, if *Arc* expression in the NHpC is related to singing motivation but not to darkness itself, *Arc* expression levels in the night LO condition would not be as high as those in the 10 h LO condition. Consistent with this prediction, *Arc*-positive cell density in the night LO condition was significantly smaller than in the 10 h LO condition and similar to that in the 30 min LO condition (Figs. 1G and 3B, 10 h LO vs. night LO: $p = 0.002$). These results provide evidence that the increased *Arc* expression in the 10 h LO condition is not related to the darkness itself of the LOs.

Previous studies have shown that the NHpC neurons respond to food deprivation stress with increased expression of the corticotropin-releasing hormone mRNA[15]. Because birds may not have as easy access to food or water under dark conditions as they do under light conditions, it is possible that 10 h LO put the birds in a fasting state, increasing *Arc* expression in the NHpC due to food deprivation stress rather than increased intrinsic singing motivation. However, we found evidence that refutes this possibility. When we fasted birds for 10 h by removing food and water from their cages, they stopped producing undirected singing within a few hours after the start of fasting and did not produce any songs even after the 10 h fasting period (Fig. S2; $n = 3$ birds). These results sharply contrast with immediate and intense singing consistently observed following 10 h LO (Fig. 1B–D and Fig. S2; see also Kim et al. 2021[11]), indicating that 10 h LO does not cause substantial food deprivation stress in the birds. Thus, it is unlikely that 10 h LO increased *Arc* expression in the NHpC mainly through food deprivation stress, strengthening the conclusion that neuronal activity in the NHpC is associated with intrinsic motivation for undirected singing.

Discussion

In the current study, we demonstrated that neuronal activity in a septum nucleus the NHpC of adult zebra finches changes in parallel with the level of intrinsic motivation for undirected singing. These findings provide evidence for the involvement of the NHpC in regulating the intrinsic singing motivation. To our knowledge, this is the first study in songbirds to identify a brain region in which neuronal activity is associated with intrinsic singing motivation. Therefore, our results represent an important initial step in understanding the neural substrates of intrinsic singing motivation and lay the groundwork for more detailed future studies.

Previous studies have demonstrated that NHpC neurons are activated in response to various stressors including food deprivation and acute restraint[15–19]. Given these findings, it is possible that the increased *Arc* expression in the NHpC under 10 h LO conditions is caused not only by increased singing motivation but also by concurrent stressors. However, the contribution of such stressors is unlikely to be substantial based on the following reasons: while 10 h LO induces intense singing immediately after the LO period, 10 h-food/water deprivation results in virtually no singing behavior after the manipulation (Fig. S2), indicating that the 10 h LO manipulation does not cause significant food/water deprivation stress; blood corticosterone levels in LO conditions are not significantly different from those in light conditions[20]; in contrast with acute (~1 h) restraint stress, chronic restraint stress does not greatly activate NHpC neurons in birds[17]. Thus, we conclude that heightened *Arc* expression in the NHpC under 10 h LO conditions is mainly attributable to increased singing motivation rather than to stress-related factors caused by LO manipulation.

Undirected singing in zebra finches and similar non-courtship singing in other songbird species, both driven by intrinsic motivation, have been suggested to be tightly associated with internal rewards (a positive emotional state)[21,22]. In European starlings, the medial preoptic nucleus (POM) has been shown to play a critical role in the process of this singing-associated reward[22,23]. Notably, POM has axonal projections to the NHpC, although to our knowledge they have only been studied in non-passerine birds[24,25]. Since singing-associated rewards likely enhance intrinsic motivation for subsequent song production, the POM-to-NHpC projections may mediate the reward-motivation interactions during successive undirected singing. Although the NHpC has also been shown to have neuronal connections with other brain areas in non-passerine birds[26–29], its connections with the song system in songbirds remain unexplored. Further research should include the identification of neural circuits that connect the NHpC with the song system, as well as the examination of the functional roles of those circuits in regulating intrinsic motivation for undirected singing.

Previous studies in rodents have shown that motivation for social play peaks following a 24-hour period of isolation[6]. Moreover, the number of c-fos positive cells in the habenula increases after the 24-hour isolation period and decreases following social play[30]. These findings parallel our results in zebra finches that intrinsic motivation for undirected singing is enhanced by long-term singing suppression and that *Arc* expression in the NHpC changes in accordance with the levels of singing motivation. Unlike rodent social play behavior, undirected singing in zebra finches is a highly stereotyped and quantifiable behavior that is frequently produced without social interactions with other individuals and is generated by discrete and well-identified neural circuits. Given these advantages, the present study highlights the importance of songbirds as an animal model for investigating the neural mechanisms of intrinsic motivation for voluntary motor behavior, opening a new avenue of research in this field.

As voluntary behaviors driven by intrinsic motivation bring about a sense of satisfaction and happiness in humans, their decline is linked to mental disorders such as depression[1–4]. Moreover, intrinsic motivation declines in the face of stress, aging, and external rewards, leading to decreased learning motivation and productivity. Consequently, a deeper understanding of the mechanisms by which animals regulate intrinsic motivation holds

promise for developing treatments and psychological therapies to address these motivation-related challenges in the future.

Materials and methods

Subjects

The subjects were 40 adult male zebra finches (97–169 days post-hatch [dph]). Birds were bred in our colony at the Korea Brain Research Institute. They were raised with their parents and siblings until ~60 dph and then housed with their siblings and/or other male conspecifics until the start of the experiments. The study was reviewed and approved by the Animal Care and Use Committee at the Korea Brain Research Institute. All procedures reported in this study were carried out in compliance with the ARRIVE guidelines[31].

Song recording and singing suppression

Birds were housed individually in sound-attenuating chambers (MC-050, Muromachi Kikai, or custom-made chambers) on a 14:10-h light: dark cycle. Songs were recorded using a microphone (PRO35, Audio-Technica) positioned 1–5 cm above the cage (20 × 20 × 20 cm) and a custom-made recording program (tRec, R. O. Tachibana, 2020). The output from the microphone was amplified by a mixer (402-VLZ4, Mackie) and digitized via an audio interface (Octa-Capture UA-1010, Roland) at 44.1-kHz (16-bit). Recorded data were down-sampled to a sampling rate of 32-kHz. Recording was triggered if the program detected 4 or 5 consecutive sound notes, each of which was defined based on sound amplitude, duration, and intervening gap duration (those consecutive sound notes were included in the recorded file). Recording ended if a silent period lasted longer than 0.5 s (i.e., each song file contains a single "song bout" that is separated from other bouts by > 0.5-s silent periods). Songs were recorded throughout the day, and all song recordings were of undirected song (i.e., no female was present).

After birds were acclimated to the sound-attenuating chambers and started singing sufficient amounts of undirected songs (> 300 song bouts per day), spontaneous undirected singing was suppressed daily by turning off the light in the sound-attenuating chambers using a digital timer for 30 min, 2 h, and/or 10 h according to the schedules shown in Fig. 1A (single lights-out [LO] period per day). Individual birds received both relatively long (10 h) and short (30 min–2 h) LOs 5–6 times alternately to prevent habituation to the same LO and to exclude the birds that did not exhibit clear enhancement of intrinsic singing motivation by the long LO (Fig. S3). More specifically, the birds to be examined for IEG expression in the 30 min LO condition received 30 min LOs alternated with 10 h LOs (Fig. S3A); the birds to be examined for IEG expression in the 2 h LO condition received 2 h LOs alternated with 10 h LOs (Fig. S3B); the birds to be examined for IEG expression in the 10 h LO condition received 10 h LOs alternated with shorter LOs (30 min–2 h LO; Fig. S3C). At the end of the last LO of interest (e.g., 10 h LO for the birds to be examined for IEG expression in the 10 h LO condition), the birds were decapitated and the brains were sampled (see below for more detail). We excluded from the IEG expression analysis three birds that did not exhibit clear enhancement of singing motivation by 10 h LO relative to shorter LO, quantified as the first song latency (the mean + SD of the latency for 10 h LO was not smaller than the mean of the latency for shorter LO; see below for the definition of the first song latency).

Song analysis

Singing behaviors following individual LO periods were quantified using two measures, the first song latency and initial singing rate[11]. The first song latency was measured as the time interval from the offset of an LO period to the onset of the first song recorded. We visually inspected the spectrograms of the sound files recorded after the LO period to identify the first file that included at least one song motif. If the bird produced no songs during the post-LO period (2 h duration, see Fig. 1A), the first song latency for the LO period was assigned a time of 2 h (120 min). The first song latency was calculated for each LO period and then averaged across all LO periods of the same duration. The initial singing rate was measured as the mean singing rate over a 30 min period starting at the onset of the first song produced after an LO period (the timing of the 30 min period varied across trials, depending on the first song latencies). To measure singing rates, we screened all sound files recorded during the periods of interest to exclude non-song files using a previously reported semi-automated method[11]. Briefly, we sorted song files (files that included at least one full song motif) and other sound files by focusing on the temporal structure of two acoustic features: sound amplitude and Weiner entropy. We compared the trajectories of these features between a canonical song motif and all sound files recorded by calculating the cross-correlation function. Sound files that had relatively low correlation coefficients in both the amplitude envelope and entropy trajectory were then excluded from the analysis. If the bird produced no songs during the post-LO period, the initial singing rate was assigned zero. The initial singing rate was calculated for each LO period and then averaged across all LO periods of the same duration.

Brain tissue collection and sectioning

After collecting the song data, we sampled the brains of the birds immediately before the end of the LO period of interest (before the light was turned on). Birds were deeply anesthetized with isoflurane (Abbott Laboratories) (for < 10 s) and euthanized by decapitation. Immediately afterwards, brains were removed, embedded in Tissue-Tek OCT compound (Sakura Fintechnical), frozen, and stored at −80 °C until use. Serial frontal sections of 18-µm thickness were cut throughout the brain using a cryostat.

In situ hybridization

Digoxigenin (DIG)-labeled riboprobe in situ hybridization was performed as described previously[32]. Total RNA was transcribed to cDNA using Superscript First-Strand Synthesis (Invitrogen, Carlsbad, CA, USA) with oligo (dT) primers. RT-PCR was performed using the following gene-specific primer pairs: 5′-AGCGGGTTTTGA CTCACCTA-3′ and 5′-TTTAGCTGGGCCGTTCATGT-3′ for *Arc* (1,199 bp); 5′-GCACCTACACCTCCACC

TTC-3′ and 5′-GTTGGAGATACCCAGCACCA-3′ for *c-fos* (510 bp); 5′-GGAGCCCGGCGCTCTCAT-3′ and 5′-GCCTTGCAACGTATGGTGTTG-3′ for *Egr1* (1,652 bp[33]). PCR products were examined on a 1.5% agarose gel and subcloned into the pGEM-T easy vector (Promega, Madison, WI, USA). To confirm that the target products were cloned, the sequences were validated using the Sanger method with BLASTN (DNA). It was assigned GenBank accession numbers XM_002186825.5 (*Arc*), XM_002200534.5 (*c-fos*), and NM_001080957.1 (*Egr1*). The resulting PCR fragment was used as the DNA template for SP6 or T7-primed in vitro transcription to synthesize digoxigenin (DIG)-labeled riboprobes with DIG-labeling mix (Roche Diagnostics).

Frozen sections were fixed in 4% paraformaldehyde/1× PBS, washed in 1× PBS, acetylated, washed in 2× and 0.1× SSPE, dehydrated in an ascending ethanol series, and then hybridized with DIG-labeled riboprobes in hybridization solution (50% formamide, 10% dextran, 1× Denhardt's solution, 1 mM EDTA [pH 8.0], 33 mM Tris-HCl [pH 8.0], 600 mM NaCl, 0.2 mg/mL yeast tRNA, 80 mM dithiothreitol, and 0.1% N-lauroylsarcosine) at 70 ∘C for 14 h. The slides were then washed as follows: 5× SSC solution at 65 ∘C for 30 min, formamide-I (4× SSC, 50% formamide, and 0.1% Tween 20) at 65 ∘C for 40 min, formamide-II solution (2× SSC, 50% formamide, and 0.1% Tween 20) at 65 ∘C for 40 min, 0.1× SSC at 65 ∘C for 15 min ×3 times, NTE buffer at RT for 20 min, and TNT buffer ×3 times. Hybridized probes were detected with anti-DIG HRP-conjugated antibody (1:1000; Roche Diagnostics). To visualize the signals, a chromogenic reaction with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was performed at RT for 16–18 h. The specificities of the probes were verified using sense-strand control probes as negative controls. No signals were detected in sections hybridized to the sense probes (Fig. S4).

Brain-wide analysis of IEG expression

Digital photographs of all brain sections on each slide glass were obtained semi-automatically using NanoZoomer XR (Hamamatsu Photonics). Expression levels of *c-fos*, *Egr1*, and *Arc* in brain sections were assessed in a wide range of the nidopallium, arcopallium, mesopallium, subpallium, thalamus, and midbrain. IEG-positive cell densities were visually estimated and expressed on a 3-level scale: −, +, and + + indicate virtually no, some, and many cells expressing the target IEG, respectively (Fig. S1 & Table S1–3). In each brain region, the expression levels were compared between the 30 min LO and 10 h LO conditions. Brain regions were identified based on previous studies[34–39].

Quantitative analysis of Arc expression

For quantitative analysis of *Arc* expression, we measured the density of *Arc* mRNA-expressing cells (number of cells per 0.1 mm^2) in the NHpC, nidopallium, mesopallium, lateral striatum (posterior to Area X), and septum (outside of the NHpC). For each brain region, consecutive brain sections (each 18-μm thick) containing the central part of the region were used (the number of sections per bird used for each region was as follows [mean ± SD]: NHpC, 10.49 ± 0.64; nidopallium, 7.5 ± 0.49; mesopallium, 10.79 ± 1.27; lateral striatum, 7.42 ± 0.34; septum outside of the NHpC, 7.31 ± 0.47). For the NHpC, all *Arc*-positive cells within the brain region were counted in each brain section to obtain the cell density, which was then averaged across the brain sections. For the other brain regions, the number of *Arc*-positive cells within a rectangular area located around the center of the brain regions (0.13–0.20 mm^2, the yellow boxes shown in Fig. 2, *left*) was counted to obtain the cell density, which was then averaged across the brain sections.

Melatonin administration

Melatonin (Sigma-Aldrich, #5250) dissolved in ethanol and PBS was injected subcutaneously in the breast region (12.5 μg/bird, ~ 1 mg/kg) at 2 h prior to brain sampling[8,40]. Subcutaneous injection was used instead of oral administration to ensure rapid and reliable responses to melatonin[41]. The dose was determined based on previous studies[40,41] and our pilot experiments, so that the melatonin administration induced sleep until brain sampling in all birds. Behavioral states were observed using video recordings.

Statistics Statistical tests were performed using R 4.4.1 (R Core Team and R Foundation for Statistical Computing) or MATLAB, R2015b (MathWorks). To examine the effect of LO with three different durations on *Arc* expression levels, we compared *Arc*-positive cell density across groups using a Steel-Dwass test. To analyze the relationship between *Arc*-positive cell density and the degree of singing motivation (first song latency and initial singing rate), we performed Spearman's rank correlation. To examine the effect of singing (1 h LO + 1 h Light ON) and that of melatonin administration on *Arc* expression levels, we compared *Arc*-positive cell density across groups using the Steel test. Differences were considered statistically significant at p values < 0.05.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

CM and SK designed the research; CM, SK, JOY, and YK conducted the experiments and data analyses; CM, YK, and SK wrote the paper.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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