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ORIGINAL PAPER

Seasonal changes in intrinsic electrophysiological activity of song control neurons in wild song sparrows

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Abstract Song behavior and its underlying neural substrate can change seasonally in adult songbirds. To test whether environmental cues induce seasonal changes in electrophysiological characteristics of song control neurons, we measured in vitro intrinsic neuronal activity in the song control nucleus RA of adult male song sparrows (*Melospiza melodia)* in both the fall non-breeding and spring breeding seasons. We found that RA neurons in spring-captured birds show a more than threefold increase in spontaneous firing rate compared to those from fall-captured birds. We conclude that environmental cues are sufficient to induce seasonal changes in electrophysiological properties of song control neurons, and that changes in these properties may underlie seasonal changes in song behavior.

Keywords Spontaneous activity · Song sparrow · Electrophysiology · Songbird · Seasonal plasticity

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Abbreviations

13:11	13 h light:11 h dark
ACSF	Artificial cerebrospinal fluid
AFP	Anterior forebrain pathway
DLM	The medial portion of the dorsolateral nucleus of the anterior thalamus
HVC	Used as a proper name
LD + T	Long-day photoperiod (20 h light:4 h dark) and a systemic testosterone implant
LMAN	The lateral magnocellular nucleus of anterior nidopallium
RA	The robust nucleus of the arcopallium
RIA	Radioimmunoassay
SD	Short-day photoperiod (8 h light:16 h dark)
SEM	Standard error of the mean
T	Testosterone
WC	White-crowned sparrow
X	Area X, a subdivision of the medial striatum

Introduction

The arrival of the breeding season typically induces changes in reproductive-related behaviors (Wingfield and Kenagy 1991; Tramontin and Brenowitz 2000). The neural substrates that control reproductive behavior often undergo a functional reorganization, facilitating enhanced performance (Tramontin and Brenowitz 2000; Brenowitz 2004). These phenomena are observed in all vertebrate taxa, but one of the best-studied and most pronounced examples occurs in singing behavior and the song-control system in adult songbirds (Fig. 1a, Nottebohm 1981; Tramontin and Brenowitz 2000; Ball et al. 2004; Brenowitz 2004).

Song is a learned vocalization used to attract mates, defend territories, and recognize mates or territorial

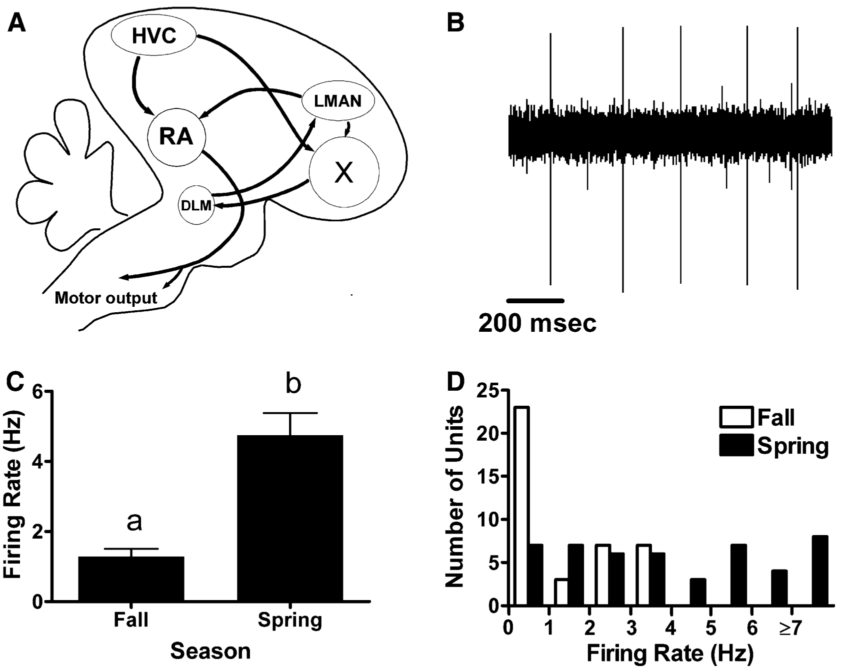

Fig. 1 **A** Simplified schematic of the avian song control system. HVC and RA are part of the descending motor circuit necessary for song production. RA projects to the brainstem motoneurons that control the muscles involved with respiration and the sound production organ, the syrinx. HVC, X, DLM, and LMAN comprise the anterior forebrain pathway (*AFP*), which is necessary for song learning and plasticity. *HVC* used as a proper name, *DLM* the medial portion of the dorsolateral nucleus of the anterior thalamus, *LMAN* the lateral magnocellular nucleus of anterior nidopallium, *RA* the robust nucleus of the arcopallium, *X* Area X, a subdivision of the medial striatum. The nomenclature used here follows Reiner et al. (2004). **B** Representative extracellular recording of a single unit. **C** Average spontaneous firing rates in fall and spring (mean ± SEM). *Different letters* above the bars denote significant differences. **D** Histogram of firing rate distributions in fall and spring

neighbors (reviewed by Marler and Slabbekoorn 2004). During the breeding season, song production, stereotypy and duration increase (Tramontin and Brenowitz 2000; Ball et al. 2004; Brenowitz 2004). Accompanying these behavioral changes, several song nuclei expand, some through incorporation of new neurons and some through the growth of pre-existing neurons. These morphological and behavioral changes are mediated by steroid hormones and can be mimicked in captive birds by manipulating photoperiod and plasma testosterone (*T*) concentration (Nottebohm 1981; Tramontin and Brenowitz 2000; Ball et al. 2004; Brenowitz 2004). Only recently have seasonal changes in electrophysiological properties begun to be studied in avians. Research has generally focused on either how auditory stimuli are differentially processed between seasons in wild-caught birds or seasonal-like conditions in laboratory-housed birds (Lucas et al. 2002; Del Negro and Edeline 2002; Del Negro et al. 2005; Lucas et al. 2007), or on how seasonal-like changes in the electrophysiological properties of song control nuclei might contribute to creating more stereotyped song (Park et al. 2005). Specifically, one change recently discovered is that the spontaneous activity of neurons in the pre-motor song-control nucleus RA (Fig. 1a; robust nucleus of the arcopallium; nomenclature used here follows Reiner et al. 2004) increases when adult male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) are exposed to breeding-season photoperiod and systemic *T* levels (Park et al. 2005).

To test whether environmental cues alone induce similar changes in neural activity in wild birds, we studied adult male song sparrows (*Melospiza melodia)* in the fall non-breeding season and in the spring breeding season. Song sparrows are closely related to the white-crowned sparrows used in laboratory studies (Carson and Spicer 2003). Seasonal changes in song sparrow song behavior, song nucleus structure, and endocrinology parallel those observed in laboratory-manipulated white-crowned sparrows (Wingfield and Hahn 1994; Smith et al. 1997a; Tramontin and Brenowitz 1999; Tramontin et al. 2001; Brenowitz 2004; Perfito et al. 2004; Thompson and Brenowitz 2005; Soma 2006). Moreover, song sparrows in western Washington state remain on their territories all year round, enabling study of the same population across seasons (Wingfield and Hahn 1994). In both species, song structure becomes more stereotyped, and plasma *T* levels, song nucleus volumes, and RA soma areas increase compared to non-breeding condition birds (Smith et al. 1997a; Tramontin et al. 2001; Brenowitz 2004). Both species are age-limited song learners (Marler and Tamura 1964; Marler and Peters 1987; Nordby et al. 2002).

After capturing a song sparrow in the field, we quickly returned to the laboratory, prepared brain slices and measured the intrinsic spontaneous activity of RA neurons. Firing rates of RA neurons from birds caught in the spring were higher than in the fall. This is the first demonstration of seasonal change in any electrophysiological property in the song system of a non-captive bird. Comparison of seasonal differences in song sparrow RA spontaneous activity to those measured in wild-caught and captive individuals of other species in comparable physiological states reveal similar changes in firing rates, suggesting common underlying ionic and endocrine mechanisms.

Methods

Animals

All procedures used in this study were approved by the Institutional Animal Care and Use Committee at the University of Washington. We collected five adult male song sparrows (*Melospiza melodia*) using mist nets at field sites in Washington State during the autumn non-breeding season (early October 2005, late September and early October 2006), and five more during the spring breeding season (late March 2006), as in Smith et al. (1995) and Tramontin et al. (2001). We transported the birds to the laboratory and created acute brain slices from the birds within 12 h of capture. We used the in vitro acute brain slice preparation to ensure that the electrical activity recorded was intrinsic to the neuron. After sacrifice, we visually confirmed the presence and state of growth of the testes to ensure that the birds were male and that the testes were regressed in the fall and large in the spring.

Electrophysiology

Preparation of brain slices

Methods for preparing slices have been described elsewhere (Park et al. 2005). Briefly, each animal was anesthetized with isoflurane, euthanized by decapitation and the brain rapidly dissected into ice-cold, oxygenated artificial cerebral spinal fluid (ACSF), containing (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 16.2 $NaHCO_3$, 11 D-glucose, and 10 HEPES, osmolarity adjusted to 310–320 mOsm with sucrose. Parasagittal brain slices (300 µm thick) were prepared using a Vibratome, and slices were stored at room temperature submerged in bubbled ACSF in which HEPES was replaced with equiosmolar $NaHCO_3$. All chemicals were obtained from Sigma–Aldrich (St Louis, MO, USA).

Electrophysiological recording

Recording methods have been described elsewhere (Park et al. 2005; Solis and Perkel 2006). Recordings were carried out at least 60 min after slices were collected. For recording, a slice was submerged in a small chamber perfused with ACSF maintained at 30°C and containing 150 µM picrotoxin (Sigma) and 1 mM kynurenic acid (Sigma). Single-unit extracellular recordings were obtained from neurons within a region that could be reliably identified as RA using trans-illumination. Only well-isolated spikes with high signal-to-noise ratios were studied (all waveforms had a signal-to-noise ratio of ≥ 3). Recording electrodes were made from pulled borosilicate glass pipettes (WPI, Sarasota, FL, USA) with tips broken to a resistance of 3–6 MΩ and filled with 0.9% NaCl. Extracellular potentials were amplified 10× using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) and amplified a further 100× using a Brownlee model 410 amplifier (Brownlee Precision, Santa Clara, CA, USA). The filtered signals (low-pass filtered at 3kHz) were then digitized at 20kHz with a Digidata 1322A (Axon Instruments) and stored on a PC using pCLAMP 9 (Axon instruments).

Data analysis

Data analysis methods have been more fully described elsewhere (Park et al. 2005; Solis and Perkel 2006). Briefly, spontaneous spike trains were analyzed off-line using a custom written program in IGOR (Wavemetrics, Lake Oswego, OR, USA) by Michele M. Solis and David J. Perkel, and with pCLAMP 9 (Axon Instruments). To ensure that spike-events were single units, we analyzed the spike amplitude and waveform. Only well-isolated spikes with high signal-to-noise ratios were studied (Fig. 1b). The spontaneous activity was observed for at least five minutes and the firing rate was obtained by dividing the number of spikes observed by the duration of the recording.

Brain histology and morphometry

At the end of the recording, we fixed each slice overnight in 4% paraformaldehyde solution in 0.1 M PB at 4°C. The slices were then briefly washed with 0.1 M PB, cryoprotected in 30% sucrose in 0.1 M PB, and re-sectioned to a thickness of 50 µm using a freezing microtome. We mounted sections on slides and stained them with cresyl violet. We then measured the area of 100 somata in RA by tracing their diameters, using a random systematic sampling method (Tramontin et al. 1998). Neurons were distinguished from glia by having one round nucleolus, a well-defined nuclear envelope, nongranular cytoplasm, and/or an obvious axon hillock (Goldman and Nottebohm 1983; Smith et al. 1995, 1997a, b; Tramontin et al. 1998).

Hormone assay

We collected blood from wing veins into a heparinized microhematocrit tube and stored it on ice until centrifugation. Blood was successfully obtained from all birds at sacrifice and from some birds in the field; we did not collect enough plasma from two spring birds to accurately test *T* concentration. Plasma was harvested and stored at −20°C for subsequent steroid radioimmunoassay (RIA). To measure circulating *T*, we followed the RIA protocol of Tramontin et al. (2001), using a Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA). The minimum detectable plasma *T* concentration was 0.2 ng/ml. Birds with undetectable T levels were treated as having concentrations of 0.2 ng/ml for statistical analysis. We note that circulating steroid hormone levels are not necessarily identical to those in the brain parenchyma, due to local neurosteroid synthesis (Schlinger and London 2006).

Statistics

Fall firing rates measured by pooling cells across all birds did not approximate a normal distribution, so we tested statistical significance using a Mann–Whitney *U* test. The nonparametric Kolmogorov–Smirnov two-sample test (K–S test) was used for comparison of the cumulative frequency distributions between spring and fall firing rates. An alpha level of 0.05 was used. To compare firing rates between different species and manipulations (Fig. 3), we used a non-parametric Kruskal–Wallis ANOVA followed by Dunn's Multiple Comparison test. If firing rates are instead analyzed by calculating a mean firing rate for each individual bird, the resulting distributions pass a normality test, and we therefore used a *t* test. Soma area distributions were normally distributed, and we used a *t* test to determine significance. We compared plasma *T* concentrations using an ANOVA and a Tukey's Multiple Comparison test.

Results

We recorded 40 single units from five male song sparrows captured in the fall, and 48 single units from five male song sparrows captured in the spring. RA units were spontaneously active in vitro (Fig. 1B), as previously described in zebra finches (Mooney 1992; Spiro et al. 1999; Solis and Perkel 2006), and white-crowned sparrows (Park et al. 2005). To ensure that the spontaneous activity was an intrinsic property of recorded neurons, the bathing solution contained the glutamate receptor antagonist kynurenic acid (1 mM) and the GABA_A receptor antagonist picrotoxin (150 μM). Additionally, the in vitro slice preparation removed the cell bodies of many excitatory afferents to RA.

RA neuron firing rates changed seasonally; neurons discharged at low rates in the fall, and considerably faster in the spring (Fig. 1C, D; Table 1; $P < 0.0001$, $U = 354.0$, 40 fall units, 48 spring units). A cumulative distribution analysis further demonstrated that spring firing rates were higher compared to the fall (Fig. 2A; $P < 0.001$, $D = 0.5$, 40 fall units, 48 spring units). If seasonal changes are compared by calculating the mean firing rate for each bird, spring firing rates continue to be significantly higher than those in the fall (Fig. 2B, $P < 0.0005$, $t = 6.505$, $df = 8$).

Fall *T* levels and RA neuronal soma sizes were low, and spring *T* levels and soma sizes were significantly higher (Table 1; $P < 0.05$ for both; $t = 2.374$ $df = 8$ for soma area; $F = 7.186$ for *T* levels), replicating previous work (Smith et al. 1997a; Tramontin et al. 2001). Spring plasma *T* levels measured at the time of sacrifice were significantly lower than those measured at capture (Table 1; $P < 0.05$), but both sets of measurements are in the physiological range of spring-captured birds (Smith et al. 1997a; Tramontin et al. 2001; Soma et al. 2004).

Discussion

We found that RA spontaneous activity was high during the spring breeding season, and low during the fall non-breeding season. These changes in firing rates were temporally correlated with previously reported differences in song

Table 1 Values are mean ± SEM; in the "Number" column, values not in parentheses are total number of units recorded, and values in parentheses are numbers of animals

Season	Number	Spontaneous firing rate (Hz)	Mean soma size (μm^2)	Plasma *T* at sacrifice (ng/ml)	Plasma *T* at capture (ng/ml)
Fall	40(5)	1.3 ± 0.2^a	82.8 ± 7.4^a	0.20 ± 0.07^a	N/A
Spring	48(5)	4.7 ± 0.6^b	103.9 ± 4.9^b	0.94 ± 0.28^b	8.35 ± 4.0

Within columns, values with different superscripts differed significantly from each other. In the spring, plasma *T* levels at capture differ significantly from levels at sacrifice, but both sets of measurements were in the physiological range of spring-captured birds (Smith et al. 1997a; Tramontin et al. 2001). We note that the minimum detectable plasma *T* level was 0.2 ng/ml (see "Methods"), and that in both this and previous studies the plasma *T* levels of wild birds captured in the fall were near or below the *T* detection limit (Smith et al. 1997; Tramontin et al. 2001; Soma et al. 2004)

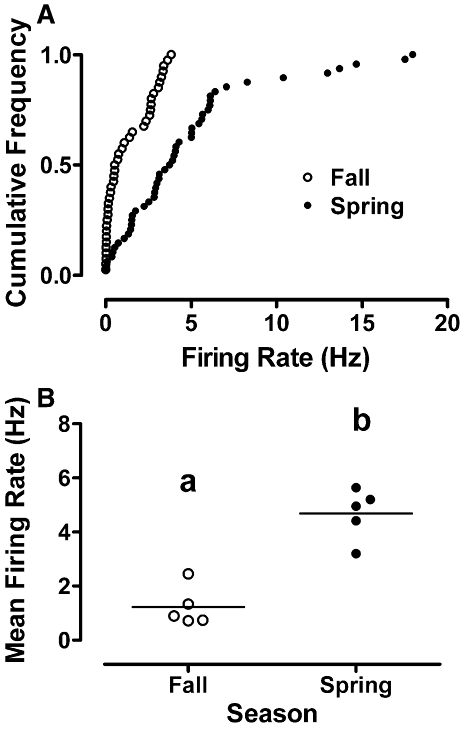

Fig. 2 RA firing rates are faster in the spring than in the fall. **A** Cumulative frequency distributions of firing rates recorded in fall and spring captured birds (see text for statistical significance). **B** Firing rates analyzed by calculating a mean firing rate for each bird, instead of pooling cells from all birds as in all other analyses. *Horizontal lines* indicates mean firing rate of all birds caught in that particular season. *Different letters* denote significant differences

stereotypy, plasma *T* concentration, and song nucleus volume and neuronal attributes (Smith et al. 1997a; Tramontin et al. 2001; Soma et al. 2004; Thompson and Brenowitz 2005). This is the first demonstration of seasonal change in any electrophysiological property in the song system of a non-captive bird.

To determine the generality of our results we compared the firing patterns observed here to those found in wild-caught and captive individuals of other species in comparable physiological states. We generally find the same pattern: birds in non-breeding condition have slow spontaneous firing rates, while birds in breeding condition have higher spontaneous firing rates (Fig. 3). Spontaneous firing rates from song sparrows and a spotted towhee (*Pipilo maculates;* unpublished data, JM) captured in the wild in fall and Gambel's white-crowned sparrows exposed in the laboratory to nonbreeding conditions (data from Park et al. 2005) were slow. Spring-caught song sparrows, laboratory-manipulated white-crowned sparrows exposed to breeding photoperiod and hormonal conditions, and laboratory zebra finches (*Taeniopygia guttata*; data from Solis and Perkel 2006) in breeding condition all had significantly faster spontaneous firing rates than non-breeding condition birds ($P < 0.01$, KW = 104.4, sample numbers noted in Fig. 3). These similar changes in RA spontaneous firing rate seen in both wild and laboratory-manipulated birds suggest common underlying endocrine and ionic mechanisms, and illustrate the complementary nature of comparative laboratory and field studies (Brenowitz and Beecher 2005). Though laboratory environments provide greater control of relevant variables compared to the field environment experienced by wild animals, it is necessary to demonstrate that the phenomenon studied in the laboratory mimics that found in the wild (e.g., Smith 1996; Fusani et al. 2005). This is especially important for a phenomenon like seasonal plasticity, since environmental (Wingfield and Kenagy 1991; Moore et al. 2004; Perfito et al. 2004; Perfito et al. 2005) and social cues (Wingfield and Kenagy 1991; Tramontin et al. 1999; Sartor and Ball 2005) can be difficult to reproduce in the laboratory (Wingfield and Kenagy 1991; Fusani et al. 2005). Thus, this study's finding that changes in spontaneous firing rates occurs in wild-caught song sparrows validates and extends the laboratory manipulations and species used to study mechanisms and functional consequences of seasonal plasticity of the adult song system (Ball et al. 2004; Brenowitz 2004; Park et al. 2005).

Why do firing rates, soma sizes and nucleus volumes change between the breeding and non-breeding seasons? One hypothesis is that decreasing firing rates and soma sizes reduce the energetic demands imposed by the song control nuclei outside the breeding season, a time of year when song is not used for mate attraction and the use of song for territorial defense is relaxed or absent. Maintaining the song nuclei in their fully grown state with high spontaneous activity in at least RA increases the metabolic demands imposed by the song system (Wennstrom et al. 2001). Much of the energetic cost imposed by the brain is due to processes that enable spiking activity (Ames 2000; Attwell and Laughlin 2001; Lennie 2003). Decreasing spontaneous activity of RA neurons outside the breeding season would decrease these energetic costs. The consequent decrease in song stereotypy, duration, and rate at this time of year can be tolerated due to the absence of mating and reduced or absent territoriality. This hypothesis predicts that spontaneous activity in RA is in some way functionally related to song production, and thus song should change along with spontaneous activity, whether seasonal or developmental. This prediction is supported by the present study, where changes in spontaneous activity temporally correlate with known changes in song stereotypy and RA soma size (Smith et al. 1997a; Tramontin et al. 2001), and studies of seasonal-like plasticity in the laboratory, where hormone-induced changes in spontaneous activity temporally correlate with changes in song stereotypy and

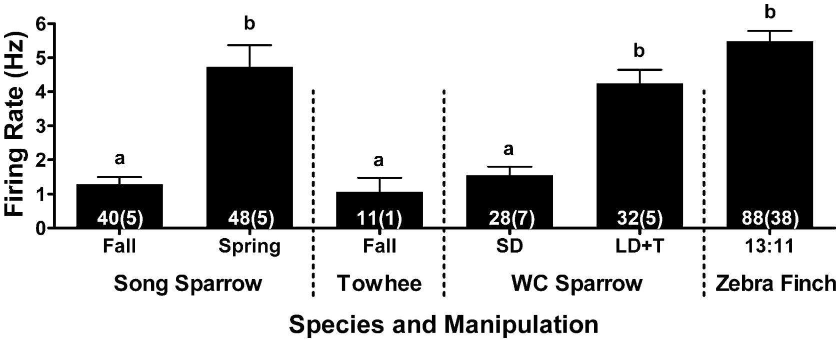

Fig. 3 Absolute RA firing rates were similar across species and manipulations. *Numbers* within the bars indicate number of recorded units and, in parentheses, number of animals. *Different letters* above the bars denote significant differences. See text for data sources. *WC*: white-crowned. *SD*: short-day photoperiod for at least 10 weeks (8 h light:16 h dark). *LD* + *T*:long-day photoperiod (20:4) and a systemic testosterone implant for 3 weeks. 13:11:lab zebra finches kept on 13 h light:11 h dark

RA soma size in white-crowned sparrows (Smith et al. 1995; Tramontin et al. 2000; Park et al. 2005). Similar relationships between spontaneous activity and song characteristics are observed in the developing zebra finch, where spontaneous activity (Adret and Margolish 2002), soma size (Bottjer et al. 1986; Konishi and Akutagawa 1985, 1990; Adret and Margolish 2002), and song stereotypy (Adret and Margolish 2002) all increase with age. In adult canaries (*Serinus canarius)*, similar seasonal or hormonally-induced changes in song (Nottebohm et al. 1986; Nottebohm et al. 1987) and anatomy (Nottebohm 1981; Canady et al. 1988) have been reported. Spontaneous activity in RA, however, has not been investigated in canaries. Though spontaneous activity is only one of many electrophysiological properties of a neuron, it has been shown in other systems to be directly related to a neuron's intrinsic excitability (Häusser et al. 2004; Gittis and du Lac 2006), and can influence how a neuron responds to synaptic input (Häusser et al. 2004; Loewenstein et al. 2005). Thus, recording spontaneous activity is a useful tool for studying neuronal excitability (Häusser et al. 2004; Gittis and du Lac 2006), but further work in seasonally-breeding birds is needed to relate spontaneous activity to other electrophysiological and morphological properties in RA, and to further delineate its functional relevance to song production. Finally, seasonal changes in song control neuron electrophysiology are likely not limited to either spontaneous activity or the nucleus RA, and this will be explored in future studies.

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