Transgenic songbirds with suppressed or enhanced activity of CREB transcription factor

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Songbirds postnataally develop their skill to utter and to perceive a vocal signal for communication. How genetic and environmental influences act in concert to regulate the development of such skill is not fully understood. Here, we report the phenotype of transgenic songbirds with altered intrinsic activity of cAMP response element-binding protein (CREB) transcription factor. By viral vector-mediated modification of genomic DNA, we established germ line-transmitted lines of zebra finches, which exhibited enhanced or suppressed activity of CREB. Although intrinsically acquired vocalizations or their hearing ability were not affected, the transgenic birds showed reduced vocal learning quality of their own songs and impaired audio-memory formation against conspecific songs. These results thus demonstrate that appropriate activity of CREB is necessary for the postnatal acquisition of learned behavior in songbirds, and the CREB transgenic birds offer a unique opportunity to separately manipulate both genetic and environmental factors that impinge on the postnatal song learning.

The development of behavioral traits in animals is influenced both by intrinsic and extrinsic factors. The contributions of the genetic and environmental factors on the development of such behaviors have often attracted public interest, i.e., the “nature versus nurture” debate; however, tangible dissection of the magnitude of the contributions of such factors has been difficult. The songbird’s skill to vocalize and to perceive a bird-song, a vocal signal for intraspecies communication, is one of the prominent skills that require both genetic and environmental factors for the development (1, 2). During postnatal developmental periods of zebra finch (Taeniopygia guttata), juvenile birds hear songs of their conspecifics and store this information in their brain to acquire the knowledge to utter and to perceive vocal signals (3–5). Inadequate auditory experience during the postnatal development results in abnormalities of their songs (3, 6–8), which often results in a reduced communication or mating performance (9–11). On the other hand, even the songs of birds reared in acoustic isolation contain species-specific syllable elements (7, 12), and such birds prefer the songs of conspecifics over those of different songbird species (13, 14). Moreover, it has been reported that some characteristics of vocal traits are heritable (15, 16). Hence, both genetic and environmental influences are necessary for developing the functional neural network required for the proper song vocalization and perception in songbirds.

Neural activity-dependent gene transcription is one of the key mechanisms by which postnatal experience can affect the expression of genes in the neural systems (17, 18). Among the transcription factors that regulate the activity-dependent gene transcription in neurons, the cAMP response element-binding protein (CREB) is one of the most well studied (19–22). Various kinds of external stimuli induce phosphorylation of CREB, the modification of which is required for the transcription of target genes (19, 20). CREB functions as a molecular hub to regulate neuronal gene transcription depending on the neuronal activity (23) and is known to play a pivotal role in neuronal plasticity and memory formation in various species (24–27). In songbirds, however, a previous histological study has found that activation of CREB occurs in the brain regions responsible for vocalization and discrimination of songs in adult zebra finches after they hear songs (28), but its role in vocal learning or auditory discrimination of songs had not been analyzed.

Because the postnatal environment can be controlled experimentally, songbirds have been an ideal experimental animal to study how one’s ability develops according to postnatal experiences (1, 29–31). Furthermore, they are one of the rare species that exhibits imitation of vocal signals, a behavior that is thought to be important for the acquisition of languages in humans (32). Moreover, the skill to communicate with learned vocal patterns are culturally transmitted through social interaction (33, 34). These features make songbirds as a rare and promising experimental system to study not only the requirement of genetic and environmental factor, but also how social or cultural influence stimulates the individual development of a behavior. Nevertheless, the lack of efficient methods to manipulate the genome of songbirds has hampered the research needed to reveal the contribution of the genetic factor to the development of communicative ability. Recently, however, transgenic expression of exogenous genes became possible by the development of virus-mediated transgenic technology in songbirds (35). This technology has allowed us to study in detail the genetic and environmental influences on the song acquisition of zebra finches. Taking this approach, we have established germ line-transmitted lines of zebra finch that express mutant forms of CREB molecules. Here, we show that formation of auditory memories against conspecific songs and the acquisition of own song is impaired in these transgenic birds, although the basal hearing ability or the acoustic quality of intrinsic vocalizations are not compromised.

Significance

In highly socialized animals such as humans or songbirds, individuals postnatally develop their skills to communicate with conspecifics under the social influence. Both genetic and environmental influences play a crucial role in the development of such abilities, but dissection of the influences has been difficult, because genetic manipulation of avian species is still a challenging issue. In this study, we applied transgenic technology to songbirds along with an experimental song-training paradigm to separately manipulate both genes and social environment, and found that appropriate activity of cAMP response element-binding protein (CREB) is necessary for the postnatal song learning in songbirds.

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altered. These birds demonstrate that appropriate activity of CREB is necessary for song learning and offer an opportunity to separately manipulate both genetic and environmental factor influence in the acquisition of postnatally learned behaviors.

Results

Generation of the Transgenic Zebra Finches with Modified CREB Activity. By regulating the expression of multiple genes, transcription factors that show neural activity-dependent gene transcription, including CREB, can alter the proteomic landscape depending on environmental influences (24, 36). To substantiate the link between CREB-mediated gene transcription and the quality of the postnatally acquired communicative ability of songbirds, we manipulated the activity of CREB in zebra finches by transgenically expressing mutant CREB molecules. Introduction of transgenes was performed by injecting lentiviral vectors bearing the transgene into the early embryo in fertilized zebra finch eggs (35) (SI Materials and Methods). Exogenously expressed mutant CREBI genes harboring the amino acid substitution affect the activity of CREB-mediated gene transcription by forming heterodimer with the endogenous CREB molecule (37). In the present study, we tried to express these mutant zebra finch CREBI under the control of a human SYNT (synapsin I) promoter, which restricts the transgene expression to neurons (38). Transgenic lines expressing the phosphorylation-deficient form of CREB (DN) (S119A, equivalent to mouse CREB-S133A) and the constitutively active form of CREB (Actv) (Y120F, equivalent to mouse CREB-Y134F) were established (Fig. 1 and Fig. S1). Germ line transmission and the expression of the transgenes (EGFP-CREB) in their offspring were observed in 20 out of 1,473 virus-injected eggs. By crossing these 20 founder birds (11 for DN and 9 for Actv) with wild-type (WT) birds, a total of 116 DN and 103 Actv transgenic G1 offspring were obtained. Integration of the transgene into genome (Fig. 1B and Fig. S1B), and brain expression of transgenes were observed in the G1 offspring (Fig. 1C and Fig. S1A). As observed in the transgenic quails (38), the expression of transgene was observed throughout the NeuN-expressing cells (Fig. 1D). These birds express exogenous CREB mutants in addition to the endogenous CREB (Fig. 1E).

G1 offspring of neither genotype showed any significant difference in their body weight compared with that of the WT control birds (Fig. S2A). Also, their brain morphology looked unchanged, and the size of their song nuclei (HVC, Area X, robust nucleus of the archicerebellum) did not show any significant difference (Fig. S2 B and C). We next asked whether these birds show any difference in their hearing ability or behavioral reactions to stimuli, by observing the reactions against the increasing volume of sounds (Fig. S3). When the change in behavioral reaction was analyzed by calculating the frequency of vocalization [“calls,” a short vocalization different from “songs” (39)], birds of all genotypes equally discriminated the increased volume of white noise between 45 and 47 dB (Fig. S3B). Notably, we found that, whereas WT and DN birds suppressed calling behaviors in response to the volume change of 45 to 47 dB, Actv birds tended to increase them (Fig. S3C). These observations indicate that, although the basal hearing ability needed to discriminate the sounds was unchanged between the transgenic birds, Actv birds tend to show a differential reaction in response to stimuli compared with WT and DN birds.

Through maintaining the transgenic lines, we noticed that, although the transgenic birds look normal, they tend to die abruptly. We next analyzed the difference of survival ratios among the genotypes. Being kept in our rearing condition, in which all birds were separated from the parents after the fledge and were kept in a soundproof chamber through ~30–140 d post hatch (dph), G1 offspring of both genotypes lived significantly shorter compared with WT birds (Fig. S4A). After they become sexually mature, we tried to obtain G2 offspring by crossing G1-TgN to WT birds. However, we found that the fertilization rates of G1-TgN were remarkably lower in both sexes (Fig. S4B). Even for the pairs that succeeded to leave G2 offspring, more days were needed to obtain chicks compared with WT (Fig. S4C). Because we were unable to obtain sufficient numbers of G2 offspring, further behavioral analysis was performed using the male G1 offspring from multiple transgenic lines.

Transgenic Zebra Finches Showed Altered Intrinsic Activity of CREB. The activity of CREB-mediated gene transcription was assayed by a lentivirus-based transcription reporter construct (Fig. 2A). In this construct, a constitutive human phosphoglycerate kinase 1 (PGK1) promoter expresses an infection reference gene [flag-tagged Histone-2B (H2B-flag)], whereas in the other direction, a minimal promoter expresses a reporter gene [turboGFP (tbGFP)]. The expression of the reporter gene is influenced by the CREB binding sequence (CRE), inserted just upstream of the minimal promoter in LV-CREB-reporter constructs. By normalizing the quantified number of the transcribed reporter mRNAs using those of internal infection reference, these constructs can reliably reflect the activity of CREB irrespective of the transfected number or the genome-inserted locus of the reporter constructs (Fig. 2B). Injecting these lentivirus-based transcription reporter into the brains of transgenic birds, we compared the intrinsic activity of CREB between WT, DN, and Actv birds. CREB-mediated gene transcription was significantly suppressed in DN birds and augmented in Actv birds, compared with WT birds (Fig. 2C). Quantitative RT-PCR analysis of the relative expression of endogenous genes in G1 offspring further revealed the effect of CREB-mediated gene transcription in these transgenic finches. As observed with the transgenic mice expressing mutant CREBs (23), the expression of many genes was increased or decreased in DN and Actv birds compared with WT birds (Fig. S5). Specifically, in both DN and Actv birds, the expression levels of genes of which human homologs have CREs in
the promoter region (CRE+ genes) were signiﬁcantly changed, compared with those genes without CRE (CRE– genes) (Fig. 2D and Fig. S5). Collectively, these results demonstrate that CREB-mediated gene transcription was misregulated in both DN- and Actv-CREB-expressing transgenic ﬁnches.

**Deficit of Memory Formation in Zebra Finch with Mutated CREB.** CREB plays a pivotal role in neuronal activity-dependent gene regulation and neural plasticity in various species (24–26, 40). For example, disrupting CREB function is known to suppress the formation of long-term memory in fear-conditioned mice (41). To assess whether the transgenic manipulation of CREB activity has any effect on the memory formation in songbird, we analyzed the process of associative auditory memory formation in WT and the transgenic ﬁnches. To this end, a standard classical auditory conditioning paradigm was used to assess formation of memory after training. For using the classical fear memory conditioning in zebra ﬁnches, we developed an auditory song-conditioning test for songbirds (Fig. S4; SI Materials and Methods). In this test, a subject ﬁnch was isolated in a soundproof chamber, and ﬁve songs of zebra ﬁnches, recorded from five different individuals unfamiliar to any of the subjects, were played through a speaker in a random order; after one particular song (conditioned sound stimulus (CS)), calls of a crow were presented [unconditioned stimulus (US)], whereas the other songs [control song stimulus (Cont)] were followed only by an interval of silence. The presentation of a crow’s call caused freezing behavior (conditioned response), which was reﬂected in a signiﬁcant decrease in their behavior (such as calling) throughout the training blocks (TBs) (Fig. 3B). These freezing behaviors to the crow’s call seemed to be intrinsic, because the zebra ﬁnches used in this study had never heard such calls before. At the fourth training block (TB4), WT birds began to decrease call behavior in response to CS, indicating that they began to associate the appearance of US with CS (Fig. 3C). By contrast, DN birds did not show any signiﬁcant difference in their change in behavioral responses to CS compared with those to the control songs (DN; TB1, P = 0.21; TB4, P = 0.67; Student’s paired t test; Fig. 3C). Actv birds did not show signiﬁcant change in behavior either (Actv; TB1, P = 0.45; TB4, P = 0.60; Fig. 3C). At the training blocks on the next day, the WT birds showed the conditioned response even at the beginning session (TB5), indicating that the memory was retained to the next day. In contrast, the DN birds failed to show conditioned responses, even at the ﬁnal training block (DN; TB5, P = 0.22; TB8, P = 0.92; Fig. 3C); and the Actv birds showed conditioned responses only at the ﬁnal block (Actv; TB5, P = 0.12; TB8, P < 0.001; Fig. 3C). Similar results were obtained when the presented song stimulus was changed to another set of songs, to exclude the possibility that such behaviors were speciﬁc to a particular song (Fig. S6A and B). The decrease in the learning in DN and Actv-TgN birds was not due to motor defects or impaired auditory perception, because signiﬁcant behavioral responses to US were observed for each genotype in every training block (Fig. 3B), indicating that the memory formation associating CS with US was speciﬁcally impaired in DN and Actv-TgN birds.

**Fig. 2.** Transgenic zebra ﬁnches show misregulated CREB-mediated gene transcription. (A) Schematics of the lentivirus (LV)-based transcription reporter constructs. A constitutive human phosphoglycerate kinase (PGK1) promoter expresses an infection reference gene [flag-tagged Histone-2B (H2B-flag)]. In the other direction, a minimal promoter expresses a reporter gene [turbGFP fused to PEST sequence (tbGFP-PEST)], whose expression is inﬂuenced by the presence of CREB binding sequence (CRE), in the LV-CREB-reporter. (B) HEK293T cells transfected either with LV-CREB-reporter or LV-Control-reporter at multiplicities of infection (MOIs) of 1, 0.1, and 0.01, and were treated with vehicle (0.1% DMSO) or 100 μM forskolin to stimulate the cAMP-dependent activation of CREB. Each reporter activity was quantiﬁed by dividing the amount of tbGFP-PEST by the amount of H2B-flag, both of which were quantiﬁed by quantitative RT-PCR. Stimulus-dependent changes in CREB activity were calculated by dividing the reporter activity of LV-CREB-reporter by those of LV-Control-reporter, *P < 0.0001 against each vehicle-treated cell. Bar graph shows mean ± SEM; n = 4 independent experiments. (C) Activity of CREB-mediated gene transcription in transgenic birds. LV-CREB-reporter and LV-Control-reporter were injected into the striatum and the reporter activities were quantiﬁed for each subject. WT, n = 10; DN, n = 6; Actv, n = 5 birds. Bar graphs indicate mean ± SEM. *P < 0.05, Dunnett’s post hoc test. (D) Quantitative RT-PCR analysis of endogenous RNA collected from WT and transgenic birds (n = 11 birds for each genotype). Unpaired t test; bar graph shows mean ± SEM of the absolute log2 value of relative amount of expression against WT, comparing gene with (n = 47) and without (n = 32) CREs. See Fig. S5 and Table S1 for details.
Although both DN and Actv birds displayed difficulties of song memory formation, we noticed a trend that DN and Actv birds react differently to US. Although presentation of US caused a significant reduction in call behavior (Fig. 3B) to DN and Actv birds, Actv birds showed significantly more of calling, suggesting that they are behaviorally more active after the presentation of US compared with WT and DN-TgN birds (Fig. 5C; WT, P < 0.0016; DN, P < 0.014; Tukey's post hoc analysis; two-way ANOVA, F_{(2,288)} = 5.27, genotype factor, P < 0.0058). Together, these data show that transgenic manipulation of the CREB transcriptional activity altered the memory formation in adult birds, consistent with a reported role of CREB in memory formation in other animal models (24–26).

At the beginning of the fourth training block (TB4), when the behavioral association between CS and US was observed, phosphorylation of CREB at serine 119 (equivalent to serine 133 in mouse CREB), which activates its transcriptional activity (42), was detected in the brains of auditory conditioned WT birds. We observed pCREB signal in various brain regions. One of the brain regions that showed differential phosphorylation of CREB was the basal ganglia including Area X, the nucleus essential for song learning (43, 44) (Fig. S7A). The conditioning, we did not observe the singing of the subjects; this phosphorylation was similarly observed when the subjects were auditory conditioned in a dark chamber, indicating that such phosphorylation was not caused by singing (45) (Fig. S7B). These signals may be caused by perception of auditory stimuli (28), or by motor behavior of subjects such as vocalization of calls (46), or by neuromodulators such as dopamine (47). This phosphorylation was suppressed by injection of STO609 (20 μM), a selective inhibitor of calmodulin-dependent protein kinase kinase (CaMKK), which is known to suppress the activity-dependent phosphorylation of CREB through suppressing the activity of calcium calmodulin-dependent protein kinase IV (48), into the basal ganglia before the trainings (Fig. S7C).

The basal ganglia is known to play an important role in the learning of sequential motor behavior or in selecting the action in classical conditioning (49), not only in rodents, but also in avian species (50, 51). We therefore investigate the role of CREB activation in basal ganglia to the auditory conditioning formation. We injected vehicle or STO609 bilaterally into the basal ganglia of WT birds before the auditory conditioning task, and analyzed the effect of these manipulations on the memory formation (Fig. S7D–F). The injected drug seems to spread to parts of Area X and the surrounding striatum (Fig. S7C; SI Materials and Methods). The injection of vehicle or STO609 did not affect the freezing response against the presentation of the unconditioned stimulus in the next day sessions (Fig. S7E). However, injection of STO609 before the conditioning training abolished the conditioned response in the next day (Fig. S7F; STO609; TB5, P = 0.12; TB8, P = 0.41, Student’s paired t test), whereas the vehicle-treated birds showed normal conditioned responses (Fig. S7F; Vehicle). Thus, the pharmacological method to suppress CREB activation in a local brain structure reproduced the results of CREB-transgenic animals, indicating that CREB was involved in the formation of the conditioned response in this experiment.

**Impaired Vocal Learning in Transgenic Zebra Finches.** Next, we analyzed how the genetic manipulation of CREB activity affects song development, which requires social learning during the postnatal period. Postnatal song acquisition was assayed by use of our song-training paradigm, which allows us to compare the accuracy with which song of a tutor (used repeatedly to different juveniles) can be copied by individuals of different genetic backgrounds (Fig. 4A; SI Materials and Methods). We found that the relative qualities of acquired songs (52) between the transgenic birds and WT birds, tutored by a common male tutor, were strongly affected by the genotype (Fig. 4B–F and Fig. S8A). Birds with DN-CREB expression developed songs with severely reduced similarity scores compared with WT controls [Fig. 4C; one-way ANOVA: F_{(2,85)} = 4.10, P < 0.0004; DN, P < 0.0008, Dunnett’s post hoc test]. On the other hand, birds with Actv-CREB expression developed songs that showed no significant difference compared with those of WT birds (Fig. 4C; Actv, P = 0.87). Both WT and Actv birds, but not the DN birds (P = 0.11), showed an increase in song quality during development (between 60 and 140 dph) (Fig. 4D). Song similarity analysis based on the similarity of an entire motif (52), a stereotyped temporal sequence of syllables, also yielded a similar result (Fig. 4E and F and Fig. S8B). In contrast to songs, call is known to be acquired mainly intrinsically (15, 39), although some feature of calls are modified postnatally through learning (53). Notably, we did not observe any significant genotype effect on the similarity of tutee’s calls against the tutor’s calls (Fig. 5A and B and Fig. S8C; Kruskal–Wallis test, P = 0.50), nor the acoustic quality of calls (Fig. 5C). These results indicate that postnatally acquired behavior was specifically affected in the mutant CREB-transgenic birds. Collectively, the findings demonstrate that genetic manipulation of intrinsic factor, the activity of CREB, differentially affected the postnatal song development even within shared environmental conditions.

**Discussion**

The transgenic technology has been applied to a wide variety of animal species to study the effect of genetic involvement on animal behaviors and development. However, because the early embryogenesis of avian species has specific features different...
from other animals, generating transgenic avian species is still challenging (38, 54). Recently, transgenic lines of zebra finch that express GFP was generated by injecting lentiviral vectors into the early embryos (35). Using this approach, we obtained several lines of zebra finches expressing mutated CREBs. In addition to the existing methods, the new transgenic technology in songbirds will be a strong tool to study how specific genes influence the acquisition of behaviors. For example, by generating transgenic zebra finches with suppressed CREB activity, we showed that birds expressing DN-CREB develop songs with poor copying quality of tutor’s songs (Fig. 4). Transgenic manipulation of the genome allows a uniform expression of transgenes in the entire population of cells involved in executing or learning certain behaviors. In a certain situation, this method is better than other methods such as local injection of pharmacological reagents or viral vectors into the brain, which are unable to control the extent of diffusion or the efficiency of transfection among the cell population. For example, the transgenic strategy seems to be particularly advantageous in the present study because manipulations of the activity of CREB in a subset of the neuronal population has been shown to lead compensation of the disturbed function by the surrounding population that is not affected by the treatment (55). One caveat that should be mentioned about our present study is the transgenic strategy used in this study results in the expression of transgenes in a wide population of neurons throughout development, owing to the activity of the synapsin promoter used to express the transgene. Another concern is the possibility of disrupting the expression of endogenous genes especially near the integration locus of transgenes, which may cause some difference of phenotypes among the transgenic lines. Further technical refinements using transgenic strategies already applied in other animals, such as the application of cell type-specific promoters or inducible promoters, or knock-in of transgene into a specific locus, may specify the neuronal circuitries or the time window of plasticity involved in the development of the postnatally learned behaviors in songbirds.

Although the effect on the intrinsic phenotypes still needs to be analyzed, the results presented here indicate that activation of the CREB signaling pathway is essential for the proper song learning in postnatal periods. In other animal models, CREB has been known to function as a positive regulator of memory formation, depending on the expression level of proteins or the testing context (40, 57–59). In our hearing threshold analysis (Fig. S3), although Actv birds showed normal auditory ability to discriminate the change of sound volume, we noted that the reaction to the auditory signal presentation was different; i.e., whereas WT and DN birds showed a reduction in their number of calling behavior, Actv birds showed a significant increase in it (Fig. S3C). Similarly, Actv birds were more active in their response to the US in the auditory conditioning test (Fig. S6C). The difference in the reaction to sound stimuli may be partly attributable to the disturbed formation of conditioned responses in the Actv-CREB birds.

Although the efficiency is still low, the transgenic technology has opened the door to perform molecular genetic studies on songbirds (35). Additional technologies, such as gene knockout or conditional expression of transgenes, will clarify the contribution of genes and environments, as well as how these are intermingled, to the development of the sophisticated ability for song acquisition in songbirds. Because we can manipulate both genetic and environmental factors, as shown in this study, songbirds may provide valuable knowledge as to how environments affect the development or disorders of an animal’s behaviors.

Materials and Methods

Also see SI Materials and Methods for detailed descriptions.

Animal Care and Treatment. All animal experiments were performed with the approval of The Animal Care and Use Committee of Kyoto University. To generate transgenic zebra finches, freshly laid eggs were collected from nests, and lentiviral vectors were microinjected around the central portion of the embryos as described earlier (35). Germ line transmission of transgene was analyzed by performing PCR-mediated genotyping of the offspring that were produced by crossing the virus-injected bird with WT birds (G0 generation). Transgene expression was further checked by RT-PCR and by immunostaining of brain sections.

Song Similarity Analysis and Behavioral Analysis. At each developmental time point, birds were isolated in a soundproof chamber; and their vocalizations were recorded with a microphone. The songs and calls were analyzed with Sound Analysis Pro-2011 (SAP) (32), using the similarity batch mode. Behavioral analysis was done as described previously (34). Only male birds were used for the behavioral analysis. For the auditory conditioning, birds were isolated in a soundproof chamber and their responses against song presentation were video recorded. For normalization of the call behavior to the C5, the number of call responses during the 1-min period before the CS presentation was subtracted from the number of call responses of the 1-min period after (and during) the C5, and divided by the sum of the values before and after. For the US, the number of call behaviors during the 1-min period before the CS presentation was subtracted from the number of call responses of the 1-min period after (and during) the US, and divided by the sum of the values. Statistical analysis was performed using the paired t test on raw values before and after the stimulus presentation (without normalization).
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Supporting Information

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**SI Materials and Methods**

**Animal Care and Treatment.** All experiments conformed to the guidelines of the National Institutes of Health (NIH) experimental procedures and were approved by the Animal Care and Use Committee of Kyoto University. The founder birds of our aviary were obtained from a local pet trader (Asada Chou Trading Company) and maintained by breeding in Kyoto University. Zebra finches used in this study were raised in our aviary and kept in rooms with a 14-h light, 10-h dark period. Food and water were given ad libitum throughout the rearing and the analysis. For the normal rearing, juvenile birds were kept with their parents in a mating cage (33 × 33 × 42 cm, visually concealed from other cages by white paper screens) until 60 dph and afterward kept with birds of similar age. For the rearing with experimentally controlled environments, juvenile birds just after the fledge state (25-32 dph, after they became able to feed themselves independently) were removed from their home cage and kept in a soundproof chamber until 140 dph, together with four to six age-matched birds of mixed genotypes and sexes. In the soundproof chamber, their cage was placed adjacent to a cage of a mature male bird (tutor) between which they could interact visually and auditorily. At 60 and 90 or 140 dph, male birds were isolated in another soundproof chamber alone; and their songs (undirected songs) were recorded for 2–3 d as described earlier (1). After the recording at 140 dph, these chamber-reared birds were moved to the aviary and kept in cages with normally reared birds with mixed genotypes. Chamber-reared birds were kept in the aviary for more than 4 wk before being subjected to the auditory conditioning test or the hearing threshold analysis. For song development analysis, all of the birds available at the time of the experiment were used and reported. Data from birds that died before 140 dph were excluded from analyses except for the survival curve analysis. In our tutoring condition, we did not observe a significant effect of cross-learning between the juveniles tutored together; WT birds reared only with WT birds, WT birds reared together with DN birds, and WT birds reared together with Actv birds (syllable similarity score at 140 dph; WT only, 77.8%, n = 16; WT with DN, 82.5%, n = 8, WT with Actv, 75.8%, n = 7; P > 0.5 in each pairwise comparison, Tukey’s post hoc test). To obtain samples for histological and molecular biological studies, subjects were randomly selected from the available similar-aged birds of each genotype.

**Generation of Transgenic Zebra Finch.** To generate transgenic zebra finches, freshly laid eggs were collected from nests, and lentiviral vectors were microinjected around the central portion of the embryos as described earlier (2). The expression construct EGFP-zfCREB encodes a fusion of EGFP and zebra finch CREB (3) under the control of human synapsin promoter, −575 to −98 bp from the transcription start site of human SYN1 (4). Although SYN1 gene is not present in avian genome (5), being regulated by the endogenous gene transcription molecular system, this promoter is known to drive transgene expression specifically to all neurons, as previously tested in the in vivo and in vitro transfection experiments using viral vectors and also in transgenic studies (4, 6). A woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was added to enhance expression. Because the overexpression of WT CREB may function as an activator of CREB (7), we created two lines of transgenic birds having repressed (S119A) and enhanced (Y120F) function of CREB. Full-length CREB were PCR amplified from zebra finch cDNA by a primer set, 5′-GGTACCGAAGATGACCATTGGATCGAGGCA-3′ and 5′-GATCGCAATCTGATTGTGCGAATTA-3′. Mutant CREB gene was created by the primer set of 5′-GGATCGACCCGCCCTACAGAACA and 5′-GATCTTAATCTGATTTTGTTTGCGAGTA-3′ for CREB(S119A)-C-term, 5′-GTCGACCGTCCTCTCCGAGAAGA-3′ and 5′-GATCTTAATCTGATTTTGTTTGCGAGTA-3′ for CREB(Y120F)-C-term, each of which is concatenated with the zfCREB-N-term region, which was amplified with the primer set 5′-GGAGGTGCAGCTGGAGACAGATG-3′ and 5′-GGAGGTGCAGCTGGAGACAGATG-3′. All constructs were confirmed by sequencing. Viral titers [infectious units (ifu)] were determined by quantifying the genome-integrated transgene by a quantitative PCR analysis of the genomes, 2 d after transfecting the virus into HEK293T cells. A total of 150–300 nL of viral solution (titer, 5.0 × 10^11 ifu·mL^-1) was injected by a glass pipette, at four to six sites per embryo through a small hole in the egg shell. Special care was taken to keep the opening in the egg shell membrane as small as possible. After infection, virus-injected eggs were sealed with adhesive film and incubated at 37.5 °C in a humidified incubator until they hatched. We obtained hatched chicks from 10% to 25% of virus-injected eggs, depending on the transgene constructs. Hatched chicks were moved into the nests of foster parents that had similar aged chicks, and reared until 60 dph. Germ line transmission of transgene in each virus-injected bird was analyzed by performing PCR-mediated genotyping of the offspring that were produced by crossing with WT birds (G1 generation). Transgene expression was further checked by RT-PCR and by immunostaining of brain sections of offspring for screening of transgenic lines expressing the transgenes. Only the lines showing the expression of transgenes in their brain were selected for further expansion of the colony. Germ line transmission and the expression of the transgenes (EGFP-CREB) in offspring was observed in 20 out of 1,473 virus-injected eggs. By crossing these 20 founder birds (11 for DN and 9 for Actv) with WT birds, a total of 116 DN and 103 Actv G1 offspring were obtained and used in this study. Because the reproductive efficiency of G1 offspring was low, we used G1 offspring from multiple lines for the behavioral and histological analysis in this study.

**PCR and Reverse Transcription.** For genotyping, genomic DNA from each bird was purified from blood. PCR-based genotyping was conducted with the following primer sets: EGFP, 5′-TCAA-GATCCGCCCAACACATC-3′ and 5′-TCTGTGGTGGTCTTTGTC-3′; GAPDH, 5′-AGTGAAGGCTGCTGCTGATG-3′ and 5′-TCGATCAAAGGTGGAGGAAGA-3′; WPRE, 5′-CCGTTGTCAGGCACGTG-3′ and 5′-AGCTGACAGGTGGGCCCAT-3′. For reverse transcription, total RNA was isolated from the entire telencephalon of the right hemisphere from each bird (60 dph) using TRizol reagent (Life Technologies) and reverse transcribed with a PrimeScript RT reagent kit with gDNA-eraser (Takara Bio) according to the manufacturer’s instruction. For the lentivirus-based transfection reporter, a gene-specific primer mix targeted at WPRE (5′-CCACATAGCTAAAGGGACAC-3′; 5′-TGGTTGCTGTTCCTTATAGA-3′) and exogenous PEST sequence (5′-TGGTTGCTGTTCCTTATAGA-3′) and exogenous PEST sequence (5′-GCGACGACGAGGCTGCTGCTTATAGA-3′) was used in the reverse transcription that was performed at 42°C.

Quantitative real-time PCR (qPCR) was performed with a StepOnePlus system (Applied Biosystems) with SYBR Premix ExTaqII (Takara Bio). All qPCR was conducted at 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles of 95 °C for 15 s and 63.5 °C for 1 min, except for the qPCR of mRNA for the
lentivirus-based transcription reporter, which was conducted at 95 °C for 2 min, and then 49 cycles of 95 °C for 5 s and 65.0 °C for 30 s. The specificity of the reaction was verified by melting curve analysis and gel electrophoresis. Primer sets used for the qPCR are listed in Table S1.

For calculations of relative expression values, each value was divided by the mean value of WT. For calculating how each gene expression has changed in the transgenic DN-CREB or Actv-CREB birds, log₂ of the expression change against WT was calculated for each gene. The expression change shown in Fig. 2D indicates the absolute value of the log₂ of relative expression values:

\[
\text{Expression change to } WT = \log_2 \left( \frac{V_{WT}}{V_{WT}} \right)
\]

where \(V_{WT}\) is a mean expression value of WT and \(V_{TgN}\) is a mean expression value of either DN-CREB or Actv-CREB birds. Because the currently reported zebra finch genome often contains unsequenced regions particularly in promoter regions, and the information about the transcription initiation sites is limited, we performed transcription binding site analysis on the putative human homolog of such genes, estimated by the Unigene cluster (2013/03/01; www.ncbi.nlm.nih.gov/unigene). The presence of cAMP-response elements in their promoters was queried in the CREB target database (8).

To analyze the integration loci of the transgenes, genomic DNA from each transgenic line was purified from brain using Wizard SV Genomic DNA Purification System (Promega). Purified genomic DNAs were digested with EcoRI. The digested products were self-ligated and conducted with a PCR using a primer set against exogenous 3′-LTR sequence (9) (5′-AGTA-GTTGTTGCGCCGTCGT-3′ and 5′-TAGGCTTAAGCAGTG-3′). The PCR products were cloned into cloning vectors and sequenced. Sites of transgene integration were mapped by BLAT (10) using zebra finch genome assembly (WashU taeGut324/taeGut2).

Lentivirus-Based Transcription Reporter. For the lentivirus-mediated reporter assay, reporter genes were each expressed from bidirectional promoters (minimal promoter and PGK1 promoter), insulated by a FII insulator. A constitutive human PGK1 promoter expressed 2× FLAG-tagged Histone-2B for the infection reference. As a transcription factor-reporter control, destabilized nuclear GFP (green fluorescent protein fused with NLS and PEST sequences) was expressed under the control of a minimal promoter (from pGL4.29; Promega). For the CREB-reporter, CRE sequence (from pGL4.29; Promega) was inserted before the minimal promoter. The expression of each reporter mRNA was quantified by qPCR. The reporter activity was calculated by dividing the reporter mRNA (CREB-reporter or Control-reporter) by the reference mRNA (Flag-tagged histone). For the in vivo reporter activity analysis, the lentivirus particle (titer, 2.0 × 10^11 TCID50/mL) was injected into the striatum (0.3 μL at four sites; distance from the Y sinus: lateral, ±1,400 μm; rostral, 5,500 μm; depth, 2,750 and 2,250 μm at beak angle 65° and 70°) in adult (180–360 dph) males under ketamine/xylazine anesthesia. Each bird was injected with LV-CREB-reporter and LV-Control-reporter in the alternative hemisphere. After 2 wk of keeping the subjects in soundproof chambers together with mixed genotype, birds were euthanized, and the total RNA was collected from each hemisphere and quantified by qPCR. For the in vitro reporter activity analysis, HEK293T cells infected with LV-CREB-reporter or LV-Control-reporter lentivirus were stimulated with forskolin (100 μM; Wako Pure Chemical Industries) or vehicle (0.1% DMSO), 1 wk after the transfection. The mRNA was collected 14 h after the stimulation and quantified by qPCR.

Behavioral Analysis. Behavioral analysis was done as described previously (1). Only male birds were used for the behavioral analysis. All behavioral analysis were done from 10:00 AM to 6:00 PM. For hearing threshold analysis, adult birds were isolated in a soundproof chamber for a day. In the next day, the birds were presented with increasing step of white-noise volume (45, 47, 65, and 71 dB; 30 s each) played through a speaker, and the behavioral reaction were video recorded. Call responses were counted off-line by an experimenter who was blind to the genotype of the subject. To compare the behavioral reaction among relatively silent and active individuals, normalization of behavioral reaction number was used. For normalization of the call behavior, the absolute values of the number compared with that for the silent period were divided by the call number in the silent period (30 s before the presentation of 45-dB noise).

For the auditory conditioning, five zebra finch undirected songs were selected from our zebra finch song corpus. Because the songs selected in this study were recorded >3 y before the birth of histological Analysis. Immunostaining was performed on free-floating cryosections (40 μm) of tissue perfused with 3% (wt/vol) paraformaldehyde solution as described earlier (1). Rabbit anti-phosphorylated CREB (S133; Abgent; AP3077a; 1:1,000), anti-GFP (Life Technologies; A6455; 1:800), and mouse anti-NeuN (EMD Millipore; MAB377; 1:750) were used. All sections were imaged with a fluorescence microscope (BX-9000; Keyence). Images were acquired with a 10× objective and joined to show the whole section images. Immunostained signals were quantified using Image J (NIH) as described previously (1). For the quantification of nuclear volumes, every third section (50 μm) from the perfusion-fixed hemisphere from male birds was collected and subjected to immunostaining with NeuN antibody and 4,6-diamidino-2-phenylindole (DAPI) (Dojindo Molecular Technologies) staining, and was imaged using a 4× objective. Nuclei were outlined manually according to the stained image by an experimenter blind to the genotype, and the area was measured by using Bioanalyzer II (Keyence).

Song Similarity Analysis. At each developmental time point, birds were isolated in a soundproof chamber; and their songs (undirected songs) were recorded with a microphone (ECM8000; Behringer), digitized at 44.1 kHz, and digitally filtered at 0.7–14.5 kHz. The songs were analyzed with Sound Analysis Pro-2011 (11) (SAP2011, version 2011.103), using the similarity batch mode with the default setting except for the “minimal duration of similarity selection,” which was set at 51 ms. Song similarities were calculated by two methods: syllable-based calculation and motif-based calculation. For the former, syllables, longer than 100 ms in duration and distinct from each other, were selected manually from each tutor’s song and used as the template syllables. Each template syllable was compared with every syllable in the tutee’s song, and the score of syllables that showed the highest value was adopted; then scores of multiple template syllables were averaged. For the motif-based analysis, a whole motif (four to six syllables) was used as a motif template. For each tutee’s song, the whole-motif comparison was performed against the syllable or the motif templates, without defining the target syllable or motif; and similarity scores (percentage of imitation) were calculated. Ten bouts of songs, randomly selected from the recorded song corpus, were analyzed and averaged for each condition. For the acoustic comparison of calls, 10 bouts of long calls were collected from the recorded corpus and used as call templates. For the calculation of the similarity score of calls, each of 10 call-templates of a tutee’s call (recorded in isolation at 140 dph) was scored against each 10 call-template of the tutor’s and the values were averaged. Acoustic features of tutee’s calls were calculated by SAP2011 using the 10 call-templates and averaged.
the subjects, each subject bird had never been exposed to exactly the same songs before the conditioning experiments. For the presentation of the control song stimulus, two bouts of songs were played for 10 s followed by 2 min, 50 s of silence. For the presentation of the conditioned song stimulus (CS), two bouts of songs were played for 10 s followed by 50 s of silence and then two bouts of a crow’s call [unconditioned stimulus (US)]. The crow’s call was used because our initial study revealed that they can reliably elicit freezing behavior in the subject birds even after repeated presentation. After the presentation of US, 6 min of silence followed before the presentation of the next songs. For one training block (TB), each of five song stimuli (including the CS) was randomly ordered and played a total of three times, and the numbers of call responses were averaged for each song stimulus. Total 59.9 s of five conspecific songs and 12.5 s of crow’s call was presented in one training block. For the experiment with another set of songs, the result of which is shown in Fig. S6, 27.9 s of five conspecific songs and 3.20 s of crow’s calls was presented in one training block. Each TB was separated with 20–30 min of silent intervals. A total of eight training blocks were performed over 2 successive days. The first and the last block of each day were video recorded and designated as TB1, TB4 (day 1) and TB5, TB8 (day 2). The orderings of songs were randomly determined, and the experimenter was blind to which songs had been played. Call responses were counted off-line by an experimenter who was blind to the genotype of the subject. To compare the behavioral reaction among relatively silent and active phase of subjects, normalization of behavioral reaction number was used. For normalization of the call behavior to the CS, the number of call responses during the 1-min period before the CS presentation was subtracted from the number of call responses of the 1-min period after (and during) the CS, and divided by the sum of the values before and after. For the US, the number of calls during the 1-min period before the CS presentation was subtracted from the number of call responses of the 1-min period after (and during) the US, and divided by the sum of the values. Statistical analysis was performed using the paired t test on raw values before and after the stimulus presentation (without normalization).

For the histological analysis of CREB phosphorylation during the auditory conditioning, WT male birds were auditory conditioned as described above. After three rounds of training blocks, before the start of TB4, 230–260 min after the beginning of the conditioning, the subjects were anesthetized and immediately perfused with fixative. Before the birds were killed, the subject had been repeatedly exposed to 108 song bouts of five conspecific songs. The experimental time course of the conditioning experiment overlaps with some of the previous reports that observed habituation to the repeatedly presented stimuli in respect to the neuronal spiking (12), the expression of immediately early genes (13), and the activation of MAPK-signaling (14). During the conditioning, we did not restrict the subject nor omit the subject executing certain behavior from analysis.

For the behavioral analysis of drug-injected birds, drugs were dissolved in PBS and stereotaxically injected bilaterally into the striatum (0.35 μL at four sites; distance from the Y sinus: lateral, ±1,400 μm; rostral, 5,500 μm; depth, 2,750 and 2,250 μm at beak angle 65° and 70°) of adult (180–360 dph) males under ketamine/xylazine anesthesia. For the identification of the injection site, Alexa 488-conjugated cholera toxin B (Invitrogen) was injected along with the drug. STO609 was purchased from EMD Millipore and used at 20 μM in final 0.4% DMSO. After the recovery (~2 h) from anesthesia, birds were conducted with auditory conditioning sessions. To identify the extent of diffusion of STO609 (Mw, 374.35) in the drug injection experiments, we injected DAPI (Mw, 277.32) similarly to WT birds and histologically estimated the area of drug diffusion. We estimated that drugs diffuse within a radius of 673.8 ± 58.7 μm (mean ± SD; n = 6) from the injection locus during the 4- to 6-h period. We cannot rule out the possibility that the area outside the drug-injected striatum may have contributed to some of the effect in the auditory conditioning.

**Statistical Analysis.** All experiments were performed with a minimum of three independent biological replicates. The n values in this study represent biological replicates. Sample sizes were chosen according to standard practice in the field and to previous analysis (1). Significance level of P = 0.05 was used to reject the null hypothesis. Statistical analysis was performed using PRISM 6.03 software (GraphPad).

Fig. S1. Expression of transgenes in the individual lines of the transgenic finches. (A) Images of immunostained sagittal brain sections from WT (Left) and each three line of G1-transgenic DN-CREB (Middle) and Actv-CREB (Right) birds. Upper row, low-magnification images (scale bars, 500 μm); lower row, high-magnification image of nidopallium immunostained with EGFP (scale bars, 50 μm). Far right, anatomical profiles. A, arcopallium; H, hyperpallium; M, mesopallium; N, nidopallium; P, pallidum; S, striatum; T, thalamus. (B) Loci of chromosome integration (red or blue triangle) of transgene in each line identified by inverse PCR analysis (SI Materials and Methods). The expression of the nearest transcripts of each integration locus was analyzed by quantitative RT-PCR and normalized to the expression level of WT birds. Bar graphs indicate mean ± SEM.
Fig. S2. Basal phenotype of the transgenic finches. (A) Scatter plot of body weight. Data from male G1-transgenic birds are shown. Graphs at the right show box-and-whisker plots of the average body weight of three age groups: 1–180 d (Left), 181–360 d (Middle), and >360 d (Right). P values (as determined by Bonferroni–Dunn’s test) against WT in each age group are indicated. No significant differences were observed (Kruskal–Wallis test; 1–180 d, P = 0.23; 181–360 d, P = 0.28; >360 d, P = 0.31). (B) Images of immunostained sagittal brain sections from WT (Left) and G1-transgenic DN-CREB (Middle) and Actv-CREB (Right) birds. Upper row, NeuN; lower row, EGFP and DAPI staining. (Scale bars, 500 μm.) (C) Average volumes of hemispheric Area X (Left), HVC (Middle), and robust nucleus of the arcopallium (RA) (Right) from WT (n = 3), DN (n = 3), and Actv (n = 3) G1-TgN birds, aged 190–250 dph. Bar graph shows mean ± SD. P values (as determined by Dunnett’s test) against WT for each genotype are shown. No significant difference was observed for any nucleus.
Fig. S3. Hearing threshold was not affected in transgenic birds. (A) Schematic of the hearing threshold analysis. Adult male birds (>140 dph; WT, n = 14; DN, n = 14; Actv, n = 15) were presented with increasing volumes of white noise (random noise with uniform spectral density, 45 to 71 dB, 30 s each), and the number of call behaviors was counted. The background noise under these experimental conditions was ∼45 dB. (B) Normalized differences in call reaction number. The number of calls during each period was normalized to that in the silent period. *P < 0.05, Dunnett’s test compared with 45-dB data of each genotype. (C) Raw numbers for call behavior in each period (30 s). The birds that increased the call behavior in response to 47-dB sound: 0 of 14 in WT, 3 of 14 in DN-TgN, and 8 of 14 in Actv-TgN. A significant difference in the behavioral reaction was observed in Actv-CREB TgN birds. *P < 0.05, Tukey’s post hoc test [two-way ANOVA: genotype factor, F(2,240) = 12.14, P < 0.001]. Bar graphs indicate mean ± SEM.

Fig. S4. Reduced survival ratio and mating performance in the transgenic birds. (A) Survival ratio curve of G1 offspring and WT. Summarized data of both sexes are shown. All birds were reared under a song-training experimental paradigm from 30 to 140 dph, and afterward kept in an aviary with mixed genotype birds of similar age. Significant differences were observed between WT vs. DN, and WT vs. Actv (Mantel–Cox log-rank test). (B) Mating performance analysis. The total number of days in the mating cage and the number of occasions when offspring were produced by WT (41 pairs), virus-injected birds (birds hatched from the virus-injected egg, with and without germ line transmission confirmation, 25 pairs), and G1-TgN birds (15 pairs) are listed. Birds of both sexes were mated with WT birds. Significant differences were observed in WT vs. G1-TgN, and virus-injected vs. G1-TgN (P < 0.0001 and P < 0.0001, respectively; χ² test). (C) Number of days required to yield offspring. The total days in the mating cage were divided by the number of occasions of egg hatching. Only the pairs that succeeded to produce offspring are used for the calculation. One-way ANOVA, P < 0.0033. The P values calculated from Tukey’s post hoc analysis are shown. Boxes and whiskers show the respective median and 25th to 75th percentiles and 10th to 90th percentiles.
Fig. S5. Gene expression profiles of the transgenic finches. Quantitative real-time PCR analysis of mRNAs collected from brains of 60-dph birds (n = 11 birds for WT, DN, Actv). The bar graph shows the relative amount of expression normalized to the expression in WT birds. Genes are grouped according to whether the human homolog contains a CRE sequence in its promoter region (8). Bar graphs indicate mean ± SEM. *P < 0.05, Dunnett’s test.
Fig. S6. Deficits in auditory-memory formation in transgenic zebra finches. (A and B) Fear-conditioning test using a different set of songs used in the experiment shown in Fig. 3. Behavioral reactions against control song stimulus (Cont) (dotted lines) are shown along with the reactions against unconditioned stimulus (US) (solid lines; A) and conditioned song stimulus (CS) (solid lines; B). Changes in call behavior number after the presentation of stimuli (Cont, US, and CS) are normalized and are shown for each genotype (WT, Left; DN, Middle; Actv, Right). Mean ± SEM are shown. Asterisks indicate a significant difference in the call response before and after the presentation of each stimulus, *P < 0.05; Student’s paired t test; WT, n = 25; DN, n = 25; Actv, n = 25. (C) Actual number of call behaviors before (Silence, dotted lines; Left) and after the presentation of US (solid lines; Right).
Fig. S7. CREB activation in basal ganglia is involved in the formation of memory. (A) Immunostained sagittal brain sections showing signals of phosphorylated CREB (pCREB) (red) and NeuN (green). Adult birds were subjected to auditory conditioning (Auditory conditioned) or kept in silence (Control). WT birds were subjected to the auditory conditioning as shown in Fig. 3A, and at the beginning of TB4, subjects were killed. (B) A section from birds subjected to auditory conditioning in a dark chamber. Arrows indicate the Area X. (C) Sections from birds injected with vehicle or STO609 along with Alexa 488-conjugated cholera toxin B (tracer) and then auditory conditioned. Arrows indicate the injection sites of the drug or vehicle. (Scale bars, 500 μm.) (D) Schematics of the drug injection and auditory conditioning experiment. (E and F) Results of the auditory conditioning experiments. Behavioral reactions against control song stimulus (Cont) and unconditioned stimulus (US) (solid lines; E) or conditioned song stimulus (CS) (solid lines; F) are shown. Changes in call behavior number after the presentation of stimuli (Cont, US, and CS) are normalized and shown for each treatment (vehicle, Left; STO609, Right). Mean ± SEM are shown. Asterisks indicate the significant differences in call response before and after the stimulus presentation, $P < 0.05$; Student’s paired $t$ test. Vehicle, $n = 24$; STO609, $n = 24$. 

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**Fig. S8.** Song and call development in the individual lines of the transgenic finches. (A) Similarity score of tutee's songs at 140 dph, calculated from the similarity of each syllable. Tutees are grouped according to transgenic or WT lines. Each line is derived from common biological parents. (B) Similarity score of tutee's songs at 140 dph, calculated from the similarity of motif. (C) Similarity score of tutee's call at 140 dph. Bar graphs indicate mean ± SEM. The numbers of WT and transgenic birds analyzed are indicated in the bar graph in A.
Table S1. PCR primers used in this study

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PCR primer sets used in the quantitative PCR analysis of mRNAs.