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Songbirds have emerged as powerful experimental models for the study of auditory processing of complex natural communication signals. Intact hearing is necessary for several behaviors in developing and adult animals including vocal learning, territorial defense, mate selection and individual recognition. These behaviors are thought to require the processing, discrimination and memorization of songs. Although much is known about the brain circuits that participate in sensorimotor (auditory-vocal) integration, especially the "song-control" system, less is known about the anatomical and functional organization of central auditory pathways. Here we discuss findings associated with a telencephalic auditory area known as the caudomedial nidopallium (NCM). NCM has attracted significant interest as it exhibits functional properties that may support higher order auditory functions such as stimulus discrimination and the formation of auditory memories. NCM neurons are vigorously driven by auditory stimuli. Interestingly, these responses are selective to conspecific, relative to heterospecific songs and artificial stimuli. In addition, forms of experience-dependent plasticity occur in NCM and are song-specific. Finally, recent experiments employing high-throughput quantitative proteomics suggest that complex protein regulatory pathways are engaged in NCM as a result of auditory experience. These molecular cascades are likely central to experience-associated plasticity of NCM circuitry and may be part of a network of calcium-driven molecular events that support the formation of auditory memory traces.

§1. Introduction

Language is transmitted culturally; in other words, it is a *learned behavior*. A requirement for spoken language is a behavior known as *vocal learning* — the ability to learn vocalizations through imitation, or based on an auditory model, as opposed to instinct. This remarkable behavioral trait has been found in just three groups of mammals (humans, cetaceans [whales and dolphins] and some species of bats) and in three avian groups (songbirds, parrots and hummingbirds).[1] No other animal groups, including non-human primates and rodents, are known to exhibit vocal learning behavior, but instead display innate, species-specific vocalizations.

Of all vocal learning groups, songbirds are the most extensively used model. This is in part due to the well defined period and set of processes that underlie the development of this behavior, ease of use and availability and, in some species, relatively simple and reliable learned communication signals (songs), which provide a highly quantifiable natural signal. As a result of extensive use as models, the anatomical and functional organization of the songbird brain circuits that enable vocal learning are relatively well understood.

In songbirds, vocal learning involves two critical stages: 1) a sensory phase, when

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vocal learners must listen to and memorize the vocalizations of an adult tutor — the memorized tutor model's song or songs are known as the "template memory"; 2) a later sensorimotor phase, when young birds hear their own vocalizations and use the template memory to "calibrate" their vocal output through sensorimotor feedback. In the early stages of the sensorimotor phase, when juvenile animals begin to spontaneously vocalize, their songs are highly abnormal and unstructured in their spectral and temporal features, relative to the songs of their tutors. However, with practice that takes place over the course of weeks or months, the structure of these vocalizations increasingly resembles the properties of the tutor song and, by the end of the critical period for vocal learning, the young birds' songs are remarkably similar to those of their tutors. For many species, such as the zebra finch, the bird's song is then "crystallized" and does not change thereafter (zebra finches learn one song each).

§2. The song control system

A set of interconnected brain areas, commonly referred to as the song-control system, or the song-control nuclei, enable the learning and production of vocalizations.[1)−8)] This system encompasses two brain circuits: 1) a posterior forebrain pathway (PFP), or vocal-motor pathway, that controls production of learned vocalizations, and 2) an anterior forebrain pathway (AFP) that is required for the learning and maintenance of the bird's own song or songs (Fig. 1A).[8)−13)] The AFP consists of topographically organized projections reminiscent of cortico-basal ganglia-thalamic loops found in the mammalian brain, that appear to play a role in the acquisition of motor skills that require fine sequential sensorimotor integration.[4),14)−22)]

In juveniles, the acquisition and development of songs (vocal learning behavior) relies directly on intact auditory processing. Deafening and interference with appropriate auditory feedback prevents song learning.[1),23)−26)] In addition, complete or selective impairment of auditory feedback in adult songbirds leads to a gradual deterioration of learned song structure,[25),27),28)] a phenomenon that is also observed in human speech following hearing impairment.[29)−32)] These findings indicate that intact hearing is required for both the acquisition and maintenance of the structure of learned communication signals.

Although songbirds rely on hearing to generate the auditory memories that are used as templates for the normal development of vocal behavior,[1),23),33)−35)] the role of the song-control system in relation to the auditory processing of songs is unclear. In anesthetized male songbirds, auditory-driven responses have been documented for all nuclei that constitute the song-control system, and have been shown to exhibit selectivity to the bird's own song.[36)−39)] This selectivity emerges during the critical period for vocal learning.[40)−41)] These findings have fundamentally contributed to our understanding of how auditory information shapes neuronal activity within the AFP and PFP. However, such hearing-evoked responses in the nuclei of the song-control system are primarily seen only under anesthesia or during sleep. In awake animals, hearing-driven electrophysiological responses are substantially reduced or even absent;[42)−44)] (but see 45)–46)), suggesting that the contributions of the song-
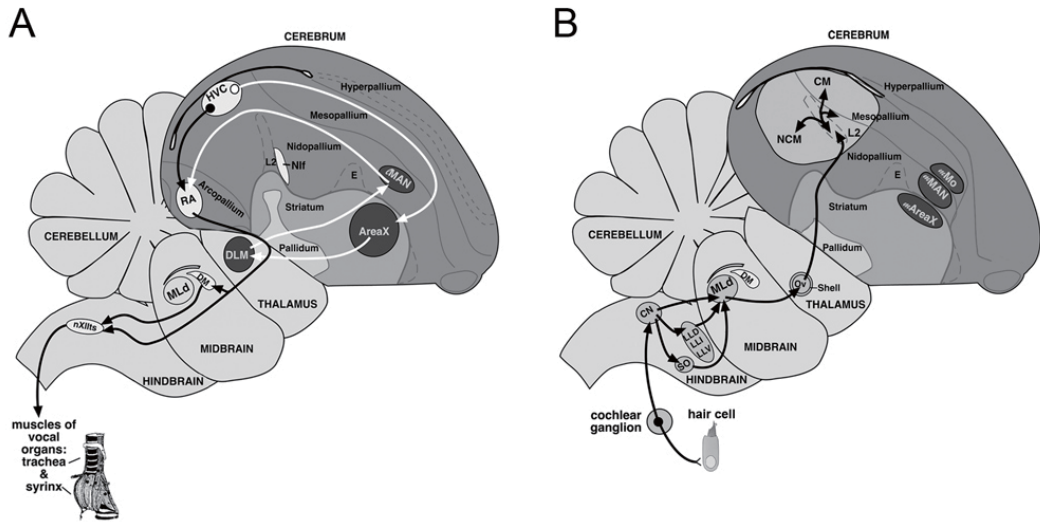
Fig. 1. Schematic representation of parasagittal sections through a zebra finch brain detailing the connectivity of the main stations of song-control system (A) and of the ascending auditory pathway (B). For clarity, only the main nuclei and projections are shown in each diagram. A) Projection systems participating in the posterior forebrain pathway are indicated by black arrows, while projections that compose the anterior forebrain pathway are detailed by white arrows. B) The focus of the present study, nucleus NCM, receives input from the thalamo-recipient layer Field L2, and has reciprocal connectivity with the caudal mesopallium (CM). Anatomical abbreviations not mentioned above or in text: CN, cochlear nuclei; DM, dorsal medial mesencephalic nucleus; E, entopallium; LL, lateral lemniscal nuclei; MLd, dorsal lateral mesencephalic nucleus; N, nidopallium; NIf, interface nucleus; Ov, ovoidalis; SO, superior olive.

control system to the perceptual processing of songs may be limited. Moreover, the auditory processing and discrimination of songs plays a central role in the biology of female songbirds. Auditory processing of songs by females is regularly necessary for mate selection and individual recognition, yet the female song-control system is often either nonexistent or largely atrophied.[47],[48] Nevertheless, auditory processing of songs by females is often necessary for mate selection and individual recognition.

§3. Auditory circuits and auditory processing

In part because both male and female songbirds exhibit behaviors that require them to hear, discriminate and form memories of songs that they are exposed to throughout life, a significant effort in the field has been directed at understanding the anatomical and functional organization of auditory circuits outside of the traditional song control circuit. It is thought that part of this auditory circuitry may play a central role in the perceptual processing of songs required for the auditory discrimination that forms the basis of auditory memory formation (discussed below). Irrespective of sex, songbirds are equipped with a set of ascending and descending auditory pathways that have structural and functional relationships resembling the general organizational principles of the mammalian brain.[16],[50],[51] These pro-

jections convey information from the songbird cochlea through a series of midbrain and thalamic nuclei (Fig. 1B).[16),52)−55)] Thalamic projections then reach the telencephalon through the brain area Field L.[16),56)] Field L neurons primarily project to two auditory areas, the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM). Based on anatomical criteria, it has been proposed that the thalamo-recipient Field L may be the songbird analogue of layer IV of the mammalian primary auditory cortex (A1), while NCM and CMM, which have substantial reciprocal connectivity, may be analogous to the supragranular layers of A1.[16),55),56)] Although it is currently not clear what specific roles each telencephalic auditory station may play in the perceptual processing of songs, a growing body of evidence suggests that they may be involved in the processing needed for auditory discrimination and the formation of auditory memories, which are requirements for vocal learning (for reviews, see 56)–58); see also 59)–61)). Most research efforts in the ascending auditory pathway have focused on NCM, the auditory area that will be the focus of this review (Fig. 1B).

§4. NCM: Processing and discrimination of songs

NCM has been the most extensively studied forebrain auditory area in songbirds. Increasing interest in NCM has resulted from a series of findings that suggest that this structure is specialized for the auditory processing of vocal communication signals in songbirds and that it may be involved in both auditory discrimination and the formation of auditory memories (for reviews, see 56), 62) and 63)).

Experiments carried out with activity-dependent markers, such as the immediate early genes (IEGs) *zenk, c-fos* and *arc*, have revealed a robust activation of NCM as a result of auditory stimulation in awake songbirds.[56),61),64),65)] Electrophysiological studies have also revealed vigorous activation of NCM neurons as a result of auditory stimulation in awake animals. Interestingly, NCM neurons appear to be tuned to auditory stimuli that are more complex than those that trigger responses in earlier stations of the auditory pathway,[66)−69)] a finding that suggests that NCM neurons may be a hierarchically higher station in the ascending auditory pathway, and involved in the processing of complex communication signals. Gene expression and electrophysiological approaches support this hypothesis. In canaries and zebra finches, playbacks of species-specific (conspecific) songs activate a larger number of neurons in NCM, relative to other-species (heterospecific) songs, as revealed by the expression of the IEGs *zenk* and *arc* (Fig. 2).[64),65)] Likewise, extracellular electrophysiological activity obtained from the NCM of awake animals revealed that conspecific auditory stimuli (including conspecific songs, male and female calls and the bird's own song) often trigger more vigorous responses than a series of heterospecific songs and artificial stimuli that included pure tones.[68),70)] Importantly, these vigorous responses to auditory stimuli are obtained in the awake songbird, and appear to differ markedly from the auditory activity detected in stations of the song-control system. Together these results suggest that NCM neurons are more heavily recruited, and are selective to species-specific natural communication signals, providing evidence that NCM is involved in the auditory processing of such signals.
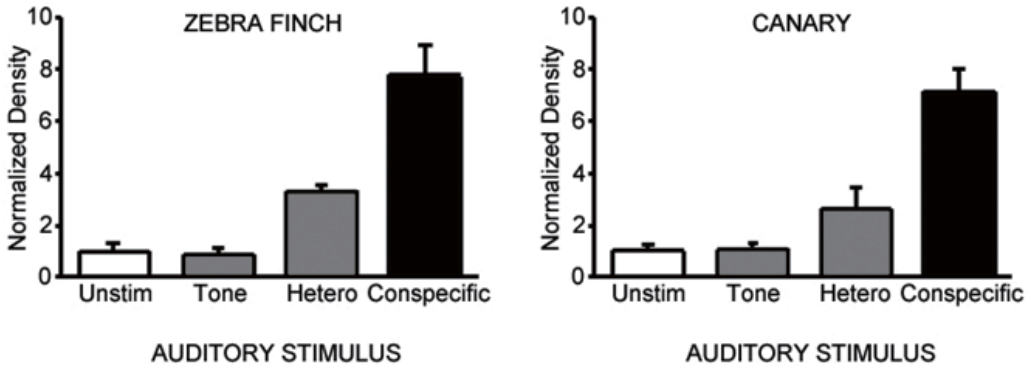
Fig. 2. Relative levels of ZENK induction in NCM of birds stimulated with either conspecific or heterospecific (Hetero) songs, tone bursts (Tone), or unstimulated controls (Unstim). Note that regardless of species, gene expression levels are highest in NCM after conspecific song stimulation. ZENK expression levels were quantified by densitometric measurements of in-situ hybridization autoradiograms. Graphs illustrate the mean $\pm$ S.E. of the normalized optical densities in NCM. Adapted, with author's permission, from Mello et al. (1992); PNAS 89: 6818-6822.
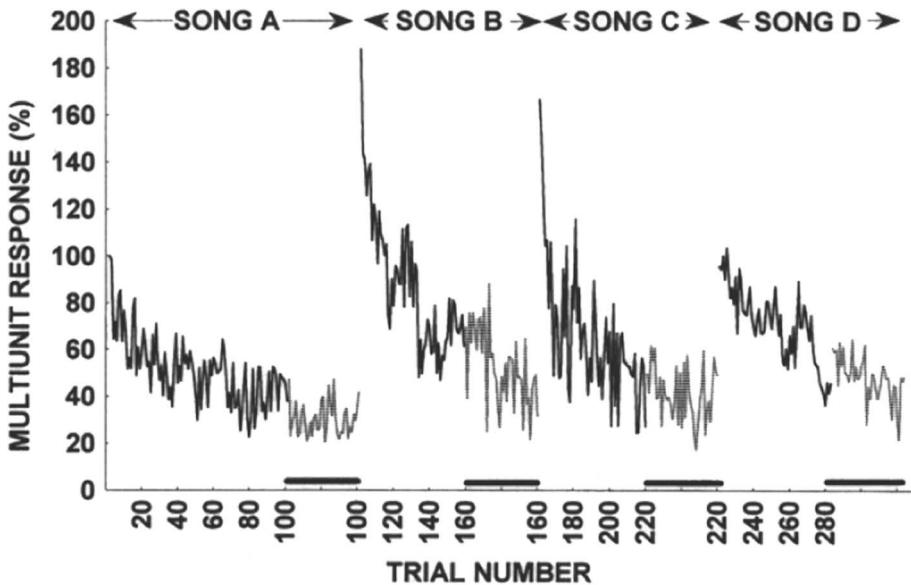

Fig. 3. Electrophysiological recordings (multi-unit activity) obtained from NCM in response to the sequential presentation of four different conspecific songs. Note that repeated presentations of the same song lead to a rapid and significant decrease in the responsiveness of NCM activity for each of the songs (inter-song interval 11-12 sec). Once responses are habituated to a given song and re-tested later (grey dotted lines, dark bars on x axis), electrophysiological activity remains decreased in a song-specific manner even after training with the other songs. Adapted, with author's permission, from Chew et al. (1995); PNAS 92: 3406-3410. Copyright © 1995 by The National Academy of Sciences of the United States of America, all rights reserved.
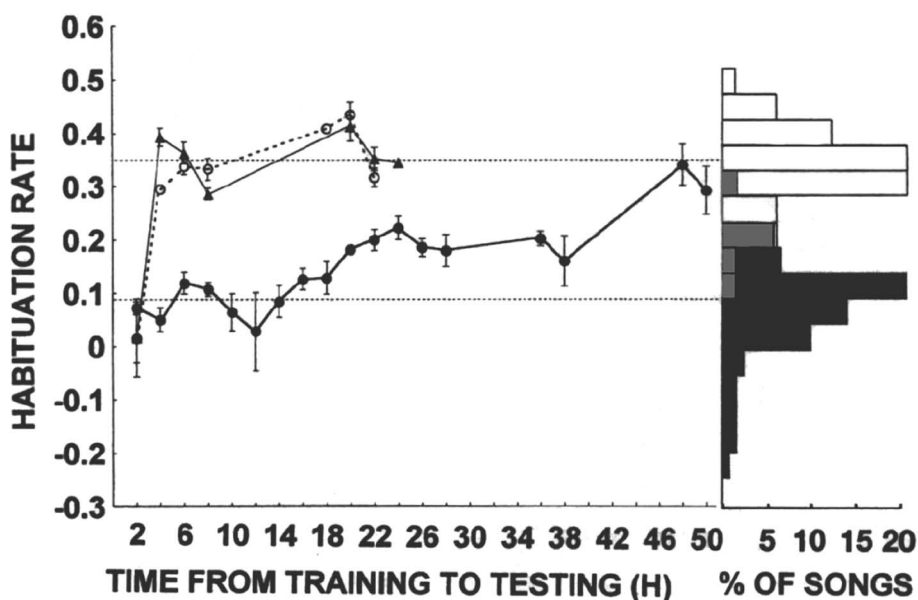
Fig. 4. Habituation of NCM responses are long-lasting, especially for conspecific songs. Plotted
are habituation rates (± S.E.) for conspecific (●), heterospecific songs (○) and human speech
(▲) at various delays from training to testing. The histograms show the percentage frequency
distributions of habituation rates for novel songs (open bars) and familiar songs tested after ≤
10 hours (solid bars). The small area of overlap of these distributions is represented by shaded
bars. The dashed horizontal lines indicate the mean habituation rates for novel (upper line) and
familiar (lower line) songs. Adapted, with author's permission, from Chew et al. (1995); PNAS
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In zebra finches and canaries, NCM neurons undergo a significant decrease in
electrophysiological responsiveness when introduced to repeated presentations of the
same auditory stimulus.[67),68),71)] This "habituation" of NCM's electrophysiological
responses is *song-specific*, since presentations of novel auditory stimuli restore strong
electrophysiological responsiveness (Fig. 3).[67),68)] In addition, habituation of NCM
neurons is *long-lasting*, especially in response to conspecific song stimuli, as reduced
responses to conspecific songs persist for approximately 40 hours (reduced responses
to heterospecific songs only persist for about 5 hours) (Fig. 4).[67)] Importantly, song-
specific habituation does not appear to occur in other auditory stations within the
auditory telencephalon, raising the possibility that this neuronal property is intrinsic
to NCM's circuitry. NCM ehabituationf provides an experience dependent mecha-
nism that likely involves comparative analysis and selective modulation of responses
to renditions of multiple songs, over the recent history of auditory stimulus presen-
tation. Furthermore, the ability to differentially respond to familiar versus novel
songs suggests that NCM neurons are capable of retaining memory traces of the
songs that they have been exposed to, implicating NCM as a structure involved in
the formation of auditory memories of behaviorally-relevant stimuli.

Similar to the electrophysiological data discussed above, repeated presentations

of the same song drive a significant reduction in the number of song-responsive neurons that express the IEG *zenk* in NCM.[51),72)] Subsequent presentation of a novel song has also been shown to reinstate high IEG expression levels.[72)] While such a song-specific decrease in *zenk* expression levels is analogous to the electrophysiological habituation described above, it is currently unclear whether or not these two phenomena are tightly correlated. Irrespective of a causal relationship between the long-term maintenance of electrophysiological habituation of song-evoked responses, and the decrease in the number of zenk-positive cells following successive presentations of the same song, it is clear that NCM neurons have mechanisms of experience-dependent plasticity, and may support auditory discrimination and the formation of auditory memories associated with song stimuli.

§5. Molecular mechanisms underlying experience-dependent plasticity in NCM: Implications for memory formation

Although song-specific decreases in electrophysiological responses occur rapidly, on a scale of seconds, the long-term maintenance of habituated responses depends on *de-novo* protein synthesis.[67)] These findings suggest that a network of protein regulatory events is necessary for the long-term maintenance of cellular traces of songs that NCM neurons have been exposed to. This notion is consistent with experience-dependent alterations in the expression levels of the IEG *zenk* following habituation-inducing protocols, as described above.[72)] The current knowledge on the identity of molecules impacted by sensory experience in NCM is, however, extremely limited. To date, in addition to the findings detailed above for the modulation of *zenk* expression, only a handful of proteins are known to be regulated by song in NCM. These include the IEGs *arc, c-fos* and *c-jun*,[51),56),62),64),65),73)] the extracellular-signal regulated kinase (ERK),[74)] that has been shown to be phosphorylated as a result of auditory stimulation, and the protease caspase-3 and its endogenous inhibitor BIRC4.[75)] Studies focusing on these proteins have substantially furthered our understanding of the anatomical and functional organization of NCM, and its response to birdsong auditory stimulation. However, these proteins are likely only a part of a large, complex protein regulatory network that remains to be elucidated. Uncovering this network and its processes will be central to understand how the physiology of NCM neurons is affected by sensory experience, and may provide key insights into the specific roles of NCM in the auditory and perceptual processing of natural communication signals and, likely, the formation of auditory memories.

To shed light on the identities of the proteins that belong to this network, and to understand how they are dynamically regulated as a function of auditory experience in NCM, we initiated efforts employing unbiased, large-scale quantitative proteomics screenings.[63)] Our approach of choice was two-dimensional differential in-gel electrophoresis (2D-DIGE)-based proteomics, coupled with extensive data analyses for protein quantification and tandem mass spectrometry. This approach offers several advantages over other high-throughput screening methodologies. For example, 2D-DIGE-based proteomics is unbiased, allowing for the screening of virtually the complete NCM proteome for both known and unknown proteins. In addition,
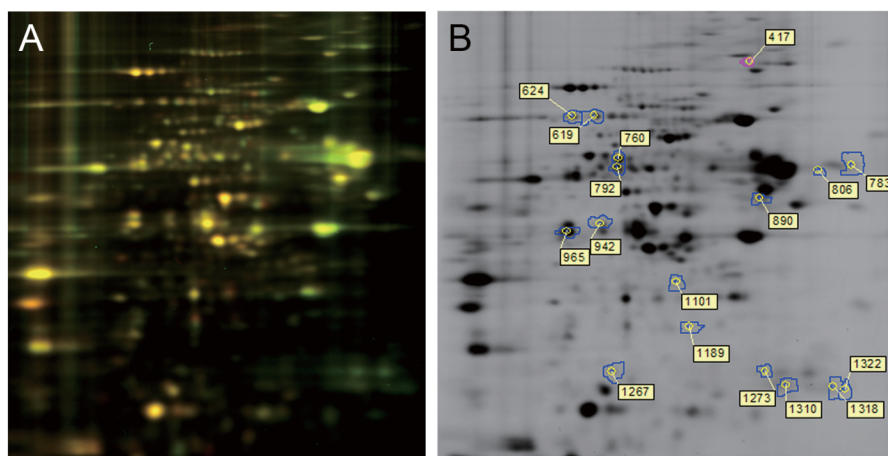
Fig. 5. A) Representative 2D-DIGE gel illustrating fractionated proteins from NCM in a comparison between controls and animals that experienced 3 hr of conspecific song stimulation. Control samples were labeled with the fluorophore Cy3 (green) while experimental samples were labeled with Cy5 (red). Internal control samples were labeled with Cy2 (blue, not shown). B) Coomassie blue-stained gel illustrating differentially-regulated spots in the 3 hr condition, as revealed by quantitative and statistical analyses with DeCyder software. All differentially-regulated spots underwent protein fingerprinting by mass-spectrometry.

post-translational modifications such as phosphorylation and oxidation are readily detectable with this method. Importantly, protein detection is highly reliable, typically yielding an extremely low number of false-positive candidates ($<5\%$). Finally, 2D-DIGE proteomics is highly quantitative, allowing for measurements of the strength of the activation and deactivation of molecules or molecular cascades of interest.[63]

In initial experiments, we have focused on high-abundance proteins, and screened approximately 8000 proteins in four groups, which accounts for an estimated 5–10% of the NCM proteome. These efforts were centered on uncovering proteins regulated by conspecific song stimulation, in order to study how they are dynamically regulated as a result of repetitions of the auditory stimuli, a paradigm that triggers substantial song-specific habituation in NCM.

Our quantitative proteomics screenings have revealed a significant number of proteins regulated by song in NCM (Fig. 5). These proteins were expressed in varied cellular loci including, but not limited to, the cytoplasm and pre-synaptic terminals.[63] Accordingly, these song-regulated proteins likely participate in a number of cellular biological functions such as neurotransmitter release, cell metabolism, and chaperone actions, among other functions. Surprisingly, however, a large number of the proteins detected with our proteomics screening appeared to converge on a single calcium-regulated biochemical cascade: the ERK (a.k.a., MAP Kinase) pathway (Fig. 6).[63] The ERK cascade has been repeatedly implicated in paradigms of learning and memory formation, and to significantly impact neuronal physiology in an experience-dependent manner.[76]−[78] The song-driven regulation of proteins potentially involved in the ERK pathway appears to converge on calcium signaling.
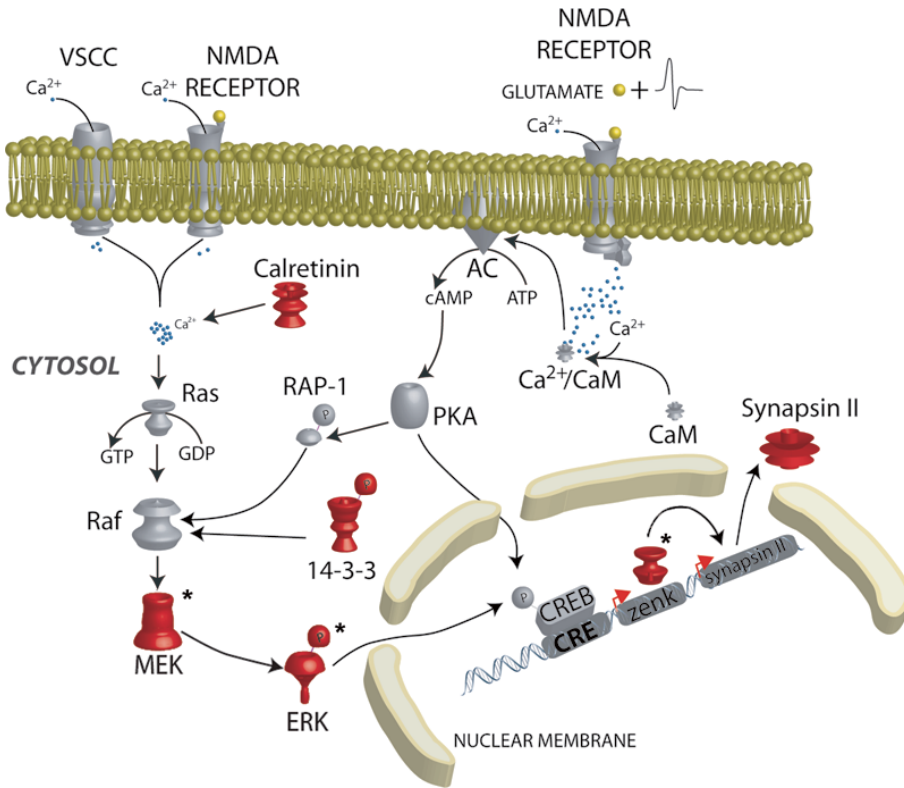
Fig. 6. Schematic representation of known interacting biochemical pathways of proteins detected by 2D-DIGE based proteomics screening, mapped onto previously known pathways. Proteins identified to be regulated by auditory stimulation by quantitative proteomics and in previous studies in NCM are color-coded in red, and identified without and with asterisks, respectively. Most of the interactions of the molecular signaling pathways shown here have been determined in non-songbird species, including the ZENK binding site in the synapsin II promoter.

Accordingly, our proteomics screening revealed regulators of intracellular calcium levels, such as the calcium-binding protein calbindin, which was found to undergo a significant down-regulation as a result of song stimulation. This decrease in calbindin protein levels presumably translates into increased strength of calcium-driven biochemical and gene expression programs.[78),79)] Song auditory stimulation was also shown to impact the phosphorylation of ERK,[74)] and the MAPK adaptor protein 14-3-3 (a.k.a., protein kinase C inhibitor).[79)] Importantly, the expression of the IEG *zenk*, one of the best studied song-regulated molecular events in NCM, was shown to be dependent on calcium influx from NMDA receptor activation[78),80),81)] and on the activation of the ERK pathway.[65),74)] As indicated above, zenk encodes a transcription factor (ZENK) that is well positioned to regulate the expression of a large number of genes that contain the ZENK-binding consensus in their promoters. In accordance with this view, our proteomics screening revealed that auditory experience triggers a significant upregulation of synapsin II.[79)] Importantly, this protein has been shown to be directly regulated by ZENK *in-vitro*,[83)] and to be impacted by
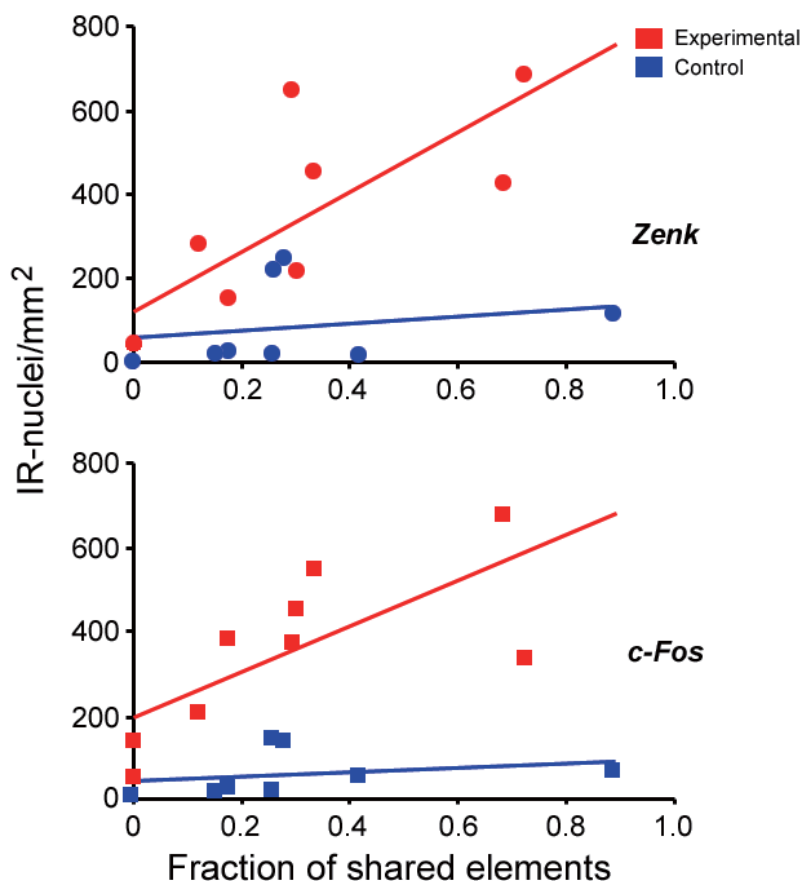
Fig. 7.   Graph sets illustrating the correlation between the number of neurons immunoreactive for the IEGs *zenk* (top) and *c-fos* (bottom) in NCM and the strength of song learning in individual zebra finches that were tutored and re-exposed to the tutor song (red). In control animals, that were tutored but not re-exposed to the tutor song, the correlation is not significant. Adapted, with author's permission, from Bolhuis et al. (2000); PNAS 97: 2282-2285. Copyright © 2000 by The National Academy of Sciences of the United States of America, all rights reserved.

alterations in calcium levels.[78),82)] Synapsin II appears to play a prominent role in the control of the readily releasable pool of neurotransmitter-containing vesicles in neurons.[78),82)] Together, we and others have utilized various strategies to investigate how the neuronal molecular machinery in NCM neurons is impacted by sensory experience. Although significant research efforts will be necessary to completely describe how the NCM proteome dynamically changes as a result of song stimulation, the findings obtained to date clearly implicate calcium signaling and the activation of the ERK pathway as key processes in the physiology of this auditory station. These experience-dependent molecular changes are thought to couple electrophysiological responses to the neuronal genomic machinery to generate adaptive responses in the physiology of NCM and, ultimately, impact behavior. This concept is supported by recent findings implicating NCM as a potential site involved in the formation of

auditory memories required for vocal learning. It was shown that the strength of song learning (the number of song elements a male copies from the song of a tutor) is positively correlated with neuronal activity in NCM, as evidenced by IEG expression (Fig. 7).[84],[85] Likewise, electrophysiological recordings obtained from adult zebra finches demonstrated that NCM neurons are selectively tuned to the song of a tutor heard early in life. Furthermore, the robustness of such selectivity is strongly correlated with the fidelity of vocal imitation.[86] Together, these findings suggest that activity within NCM appears to be more vigorous when memories associated with specific songs are more established or engrained, and that NCM may participate in the representation of memories associated with tutor songs. Consistent with this hypothesis, a recent study employing localized bilateral NCM lesions revealed a significant impairment in tutor song recognition, but not in the auditory processing and discrimination of calls, or song production.[61]

§6. Summary

Although an unambiguous role for NCM in auditory discrimination and memory formation remains to be determined, the data presented above clearly indicate that this auditory area is involved in auditory processing of behaviorally-relevant learned communication signals. NCM neurons are selectively tuned to conspecific songs and undergo long-term, song-specific plasticity that may be related to auditory discrimination and the formation of auditory memories. These processes appear to depend on a complex cascade of as yet poorly understood protein regulatory events, engaging calcium-dependent molecules that are modulated by sensory experience. Future research efforts will be focused at carefully determining the key components of these molecular cascades, and establishing how they modify the physiology of the NCM neurons that ultimately impact the behaviors that are dependent on auditory processing.

Acknowledgements

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