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THE CHICKEN IMMEDIATE-EARLY GENE *ZENK* IS EXPRESSED IN THE MEDIO-ROSTRAL NEOSTRIATUM/HYPERSTRIATUM VENTRALE, A BRAIN REGION INVOLVED IN ACOUSTIC IMPRINTING, AND IS UP-REGULATED AFTER EXPOSURE TO AN AUDITORY STIMULUS

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Abstract—The immediate-early gene *zenk* (an acronym for the avian orthologue of the mammalian genes *zif-268*, *egr-1*, *ngfi-a* and *krox-24*) has been extensively employed, in studies on oscine birds, as a marker of neuronal activity to reveal forebrain structures that are involved in the memory processes associated with the acquisition, perception and production of song. Audition-induced expression of this gene, in brain, has also recently been reported for the domestic chicken (*Gallus gallus domesticus*) and the Japanese quail (*Coturnix coturnix japonica*). Whilst the anatomical distribution of *zenk* expression was described for the quail, corresponding data for the chicken were not reported. We have, therefore, used *in situ* hybridisation to localise the mRNA that encodes the product of the *zenk* gene (which we call ZENK) within the brain of the 1-day-old chick. We demonstrate that this transcript is present in a number of forebrain structures including the medio-rostral neostriatum/hyperstriatum ventrale (MNH), a region that has been strongly implicated in auditory imprinting (which is a form of recognition memory), and Field L, the avian analog of the mammalian auditory cortex. Because of this pattern of gene expression, we have compared the level of the ZENK mRNA in chicks that have been subjected to a 30-min acoustic imprinting paradigm and in untrained controls. Our results reveal a significant increase ($P \leq 0.05$) in the level of the ZENK mRNA in MNH and Field L, and in the two forebrain hemispheres; no increase was seen in the ectostriatum, which is a visual projection area.

The data obtained implicate the immediate-early gene, *zenk*, in auditory imprinting, which is an established model of juvenile learning. In addition, our results indicate that the ZENK mRNA may be used as a molecular marker for MNH, a region that is difficult to anatomically and histochemically delineate.

Key words: activity-dependent gene, Field L, *Gallus gallus domesticus*, *in situ* hybridisation, juvenile learning, molecular marker.

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Abbreviations: Cb, cerebellum; EMBL, European Molecular Biology Laboratory; IEG, immediate-early gene; IMHV, intermediate and medial part of the hyperstriatum ventrale; MNH, medio-rostral neostriatum/hyperstriatum ventrale; Ndc, dorso-caudal neostriatum; PBS, phosphate-buffered saline; TeO, tectum opticum.

Juvenile emotionally modulated learning events are a prerequisite for the establishment and maintenance of functional neuronal connectivities in the developing brain. Disturbances to these learning processes, for example, as a result of parental deprivation or traumatic experience, can interfere with normal brain development, which can cause enduring behavioural and cognitive impairment (Poeggel et al., 2003). One of the best known and widely investigated juvenile learning events is filial imprinting in precocious birds (Lorenz, 1935; Hess, 1959). Imprinting is a learning paradigm in which young animals develop a preference for the first imprinting-relevant stimuli they are exposed to. In the chicken (*Gallus gallus domesticus*), different forebrain areas have been strongly implicated in imprinting on either a visual or an auditory stimulus. Whereas the intermediate and medial part of the hyperstriatum ventrale (IMHV) seems to be predominantly involved in visual imprinting paradigms (see Horn, 1998, 2004), the medio-rostral neostriatum/hyperstriatum ventrale (MNH) and the reciprocally connected dorso-caudal neostriatum (Ndc) seem to be particularly important in auditory imprinting (Maier and Scheich, 1983, 1987; Wallhäusser and Scheich, 1987; Bock et al., 1996, 1997; Metzger et al., 1998; Braun et al., 1999). One of the most prominent characteristics of auditory imprinting is a stimulus-induced selective reduction of dendritic spines on defined neuronal subpopulations within the MNH and Ndc (Scheich, 1987; Wallhäusser and Scheich, 1987; Bock and Braun, 1999a,b), and there is convincing evidence that the activation of the *N*-methyl-D-aspartate receptor is critically involved in this process (Bock and Braun, 1999a). Interestingly, a single 30-min exposure to an acoustic imprinting stimulus is sufficient to induce the selective reduction of dendritic spines in the MNH (Bock and Braun, 1998). However, the cellular and, in particular, the molecular mechanisms that trigger the synaptic changes that are observed during this juvenile learning process are still unclear. What is evident is that early learning in the chicken involves two temporally distinct rounds of gene expression, only the second of which is sensitive to protein synthesis inhibitors (Freeman et al., 1995; Rose, 2000).

Immediate-early genes (IEGs) are defined as genes that are rapidly (within a few minutes) and transiently induced in response to a variety of biochemical, electrical, pharmacological and/or physiological stimuli (for reviews, see Chaudhuri, 1997; Herdegen and Leah, 1998; Tischmeyer and Grimm, 1999); this initiation of transcription

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occurs in the absence of *de novo* protein synthesis. Some IEGs encode transcription factors, which mediate cascades of cellular events by activating so-called late-response genes. Since IEGs can be induced by a number of different stimuli, they can be used as endogenous markers of neuronal activity and, consequently, to map functional activity within the vertebrate brain (Chaudhuri, 1997; Herdegen and Leah, 1998; Tischmeyer and Grimm, 1999). For example, experiments on canaries and zebra finches have demonstrated that the level of the mRNA transcribed from the *zenk* gene (which is an acronym for the avian orthologue of the mammalian genes: *zif-268*, *egr-1*, *ngfi-a* and *krox-24*; Milbrandt, 1987; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988; Mello et al., 1992) is strongly augmented in certain structures within the auditory telencephalon during the acquisition of song by juvenile birds (Jin and Clayton, 1997) and the perception of song by adults (Mello et al., 1992; Mello and Clayton, 1994; Jarvis et al., 1995). In juvenile birds, this increase has been shown to correlate with the number of song elements that are learnt (Bolhuis et al., 2000). IEGs are also known to play a major role in the consolidation of long-term memories (Tischmeyer and Grimm, 1999; Davis et al., 2003).

IEGs, such as c-*fos* and c-*jun*, are well known to be involved in memory formation in avian species (Anokhin et al., 1991; McCabe and Horn, 1994; Freeman and Rose, 1999; Tischmeyer and Grimm, 1999). In addition, as mentioned above, *zenk* is induced in several sensory brain areas of songbirds during the learning of song (Jarvis et al., 1995; Jin and Clayton, 1997). In contrast, comparatively little is known about the induction of *zenk* gene expression in the domestic chicken. A recent report (Long et al., 2002) has demonstrated that ZENK mRNA is slightly elevated in many brain regions of the 1-day-old chick and the 1-day-old Japanese quail (*Coturnix coturnix japonica*) after a 30-min exposure to conspecific (i.e. from same species) but not heterospecific (i.e. from different species) maternal calls. Surprisingly, although these authors described the neuroanatomical distribution of the ZENK mRNA in the quail, using *in situ* hybridisation, no such data were reported for the chicken. We have, therefore, determined the distribution of the ZENK transcript in the 1-day-old chick brain, and have compared the level of this mRNA (using quantitative *in situ* hybridisation) in brains from birds that have been subjected to a short acoustic training paradigm and untrained controls.

EXPERIMENTAL PROCEDURES

Animals

For studies on the distribution of the ZENK transcript in brain, we obtained 1-day-old chicks from a local supplier (Lohmann Tierzucht, Cuxhaven, Germany). These were decapitated and the brains removed, and frozen over liquid nitrogen. For the imprinting experiments, eggs of White Leghorn chickens were obtained from a local hatchery (Horstmann, Nienburg, Germany) and incubated individually at 37.5±0.3 °C in acoustically isolated boxes. After hatching, the chicks were individually reared in these boxes at 28–30 °C until the beginning of the experiment. The boxes were illuminated by diffuse light, and the birds were subjected to a continuous white noise, to avoid visual and acoustic deprivation, respectively. These well-controlled rearing conditions were chosen in order to exclude external sensory stimuli, which could lead to incidental imprinting, and to ensure the novelty of the stimulus they were subjected to during the training session. All efforts were made to minimise the number of animals used and their suffering. All animals were treated according to ethical principles defined by the German Animal Welfare Act. The experimental protocols were approved by a review committee of the state of Saxony-Anhalt, Germany.

Auditory imprinting

The behavioural training (imprinting) was conducted in a V-shaped arena, equipped with a goal box at the end of each wing. Six hours to 12 h after hatching, individual chicks were placed in the middle of the arena and the imprinting stimulus (a hen-like rhythmic, frequency-modulated, tone-pulse with an average frequency of 400 Hz; for details, see Bredenkötter and Braun, 1997) was presented from one wing, where an immobile mother surrogate (hen decoy) was placed. The decoy was visible to the chick and acted as an emotional reward. The chicks were allowed to approach the imprinting stimulus (tone-pulse) for a maximum of 5 min, and were then continuously exposed to the acoustic stimulus in the presence of the mother surrogate. The total stimulation time was 15 min. This procedure was repeated from the opposite wing of the arena, resulting in an overall stimulation time of 30 min. Directly after the behavioural training (i.e. 30 min after the stimulus onset), the chicks were decapitated, and the brains removed and rapidly frozen at −80 °C. Untrained chicks served as controls.

In situ hybridisation

To determine the distribution of the ZENK mRNA in the 1-day-old chick brain, *in situ* hybridisation was performed using a radiolabelled 45-base oligonucleotide probe: 5′-AATTATTGGTAACCCCTGGAGATGGGAAGGAAGTGGCCACCTGGG-3′. This is complementary to a sequence (nucleotides 467–511: European Molecular Biology Laboratory (EMBL) accession number AF026082) that encodes part of the carboxy-terminus of the chicken ZENK protein (Long and Salbaum, 1998). Since the carboxy-terminus of ZENK is longer than those of related sequences (i.e. other EGR family members; O'Donovan et al., 1999), the antisense oligonucleotide is specific for the ZENK mRNA. For each brain, 10 μm coronal sections were prepared using a cryostat (Jung CM 3000; Leica, Bensheim, Germany) and thaw-mounted onto 3-aminopropyltriethoxysilane (Sigma, Deisenhofen, Germany)-coated slides. They were then fixed in 2% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 130 mM sodium chloride, 7 mM disodium hydrogen orthophosphate, 3 mM sodium dihydrogen orthophosphate) for 5 min at 4 °C, washed in PBS for 2×5 min, and dehydrated in an ascending ethanol series. To directly compare the levels of the ZENK transcript in brains from acoustically imprinted chicks ($n=7$) and untrained controls ($n=7$), fixed sections from pairs of brains (coded so that the experimenter was blind to experimental group) were hybridised and washed together, and then exposed to the same sheet of Kodak BioMax MR X-ray film (Integra Biosciences, Fernwald, Germany) at room temperature for 7 days. The labelling of the transcript-specific oligonucleotide, with [α^{35}S]dATP (1250 Ci/mmol; NEN/PerkinElmer, Boston, USA), and the *in situ* hybridisation and wash conditions, have been described previously (Wisden et al., 1991; Harvey et al., 1998). Note that control hybridisations, which contained, in addition, a 200-fold excess of the same unlabelled oligonucleotide, did not yield any specific autoradiographic signal.