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SEXUAL BEHAVIOR ACTIVATES THE EXPRESSION OF THE IMMEDIATE EARLY GENES *C-FOS* AND *ZENK* (*EGR-1*) IN CATECHOLAMINERGIC NEURONS OF MALE JAPANESE QUAIL

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Abstract—We analyzed the expression of the immediate early genes *c-fos* and *Zenk* (*egr-1*) in the brain of male quail that were gonadally intact (I) or castrated and treated (CX+T) or not (CX) with testosterone and had been exposed for 60 min either to a sexually mature female (F), or to an empty arena (EA) or were left in their home cage (HC). Alternate sections in the brains collected 90 min after the start of behavioral interactions were stained by immunocytochemistry for the proteins FOS or ZENK alone or in association with tyrosine hydroxylase (TH), a marker of catecholaminergic neurons. *C-fos* and *Zenk* expression was statistically increased in six brain areas of sexually active birds (I+F, CX+T+F) compared with controls (CX+F, CX+T+EA, CX+T+HC), i.e. the preoptic area, bed nucleus striae terminalis, arcopallium, nucleus intercollicularis, periaqueductal gray and the ventral tegmental area. Interestingly, *c-fos* and *Zenk* expression was high in the nucleus intercollicularis, a midbrain vocal control nucleus, of I+F and CX+T+F birds that displayed copulatory behavior but emitted few crows but not in the nucleus intercollicularis of CX+T+EA birds that crowed frequently. Increases in *c-fos* expression were observed in TH-immunoreactive cells in the periaqueductal gray and ventral tegmental area, but not in the substantia nigra, of I+F and CX+T+F birds indicating the activation of dopaminergic neurons during sexual behavior. Together, these data confirm the implication of the steroid-sensitive preoptic area and bed nucleus striae terminalis in the control of copulation and support the notion that dopamine is involved in its control. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: preoptic area, dopamine, immediate early genes, sexual behavior, ventral tegmental area, mesencephalic central gray.

The study of neural circuits regulating specific behaviors has been greatly facilitated in the past 15 years by the use of immediate early gene (IEG) induction techniques to identify cell groups that express such genes in association with the behavioral or physiological response of interest (e.g. Hoffman et al., 1993). The method has been particularly useful for the study of the neural control of reproductive behaviors such as male-typical and female-typical sexual behaviors (e.g. Baum and Everitt, 1992; Pfaus et al., 1993; Veening and Coolen, 1998) as well as maternal behavior (e.g. Numan and Numan, 1995; Lonstein et al., 2000). An initial wave of studies utilized single-label studies in which IEG induction was investigated in animals with close attention to the exact stimuli eliciting the response based on the use of carefully constructed control groups (Robertson et al., 1991; Baum and Everitt, 1992; Veening and Coolen, 1998). Another type of study combined the localization of IEG expression with the identification of defined cell types based either on the chemical phenotype or the hodological properties of the cell expressing the IEG (Asmus and Newman, 1994; Coolen et al., 1998; Gréco et al., 1998b, 1999). This second type of study has been particularly useful for the identification of functional circuits related to sexual behavior. For example, the IEG *c-fos* is expressed in several sites in the brain and spinal cord in association with male copulatory behavior (Robertson et al., 1991; Baum and Everitt, 1992; Veening and Coolen, 1998). Double-label studies have shown that many cell types that express steroid receptors for hormones that activate sexual behavior also express *c-fos* specifically in association with sexual responses (Wood and Newman, 1993, 1995; Gréco et al., 1998a,b, 1999). By combining *c-fos* expression induced by ejaculation with cells immunoreactive for the peptides galanin or cholecystokinin in order to identify cell types in the spinal cord that project to the thalamus, Truitt et al. (Truitt and Coolen, 2002; Truitt et al., 2003) were able to identify cells that convey ascending sensory information about ejaculation and to demonstrate that these cells were present in male but not female rats. Thus studies about IEG induction in cells of defined chemical phenotype and connectivity are particularly useful.

A well-known feature of the neural control of male sexual responses is that telencephalic and diencephalic brain areas that regulate male sexual behavior are modulated by ascending inputs from brainstem catecholamine groups (Hull, 1995; Hull et al., 2002). Although there is a long tradition of pharmacological studies that have established the importance of catecholamine transmitters such as norepinephrine and dopamine in the regulation of these

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Abbreviations: AM, medial arcopallium; ANOVA, analysis of variance; BSTM, nucleus striae terminalis medialis; CCM, cloacal contact movement; CX, gonadectomized; I, sham-operated and left with intact testes; ICo, nucleus intercollicularis; IEG, immediate early gene; ir, immunoreactive; LoC, locus ceruleus; M, mount; MA, mount attempt; MLd, nucleus mesencephalicus lateralis, pars dorsalis; MPOA, medial preoptic area; NG, neck grab; nPGi, nucleus paragigantocellularis; PAG, periaqueductal gray; PAP, peroxidase–antiperoxidase; PBST, 0.01 M PBS containing 0.1% Triton X-100; PLSD, protected least significant difference; POA, preoptic area; POM, medial preoptic nucleus; SCv, subceruleus ventrale; SN, substantia nigra, pars compacta; T, testosterone; TH, tyrosine hydroxylase; VTA, ventral tegmental area; *Zenk*, an acronym for *zif-268 egr-1 ngfi-a krox-24*.

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behaviors (Crowley and Zemlan, 1981; Bitran and Hull, 1987; Crowley et al., 1989; Hull, 1995) important questions remain concerning the specific catecholaminergic cells groups that are important for the control of reproductive behaviors. Because there is a complex set of catecholaminergic cell groups (defined by the expression of tyrosine hydroxylase; TH) with overlapping projections to the forebrain, it has been difficult to identify the specific role played by these different groups in the regulation of male-typical sexual behavior. The use of IEG induction associated with different aspects of male sexual behavior could be especially valuable in identifying catecholaminergic cell groups that are specifically involved in these behaviors. Studies co-localizing TH and the IEG, FOS in ferrets after mating have revealed TH cell groups such as the A2 group and the locus coeruleus that express fos in females in association with mating but no such expression was observed in males (Wersinger and Baum, 1997).

This study investigated in male Japanese quail (*Coturnix japonica*) the expression of the protein product of two IEGs, *c-fos* and *Zenk* (an acronym for *zif-268*; *egr-1*; *ngfi-a*; *krox-24*; Mello et al., 1992) associated with copulatory behaviors. In quail the hormonal control of male sexual behavior is relatively well understood (Balthazart and Ball, 1998a; Ball and Balthazart, 2002). Male sexual behavior is activated by a localized action of testosterone (T) in the brain, particularly the preoptic area (POA), and stereotaxic implantation of T into the POA restores male copulatory behavior in castrated males. Specifically, T acts on the sexually differentiated medial preoptic nucleus (POM) in activating male sexual behavior (Panzica et al., 1996; Balthazart et al., 1998a). Furthermore, T induces a large number of morphological and neurochemical modifications in this nucleus (Panzica et al., 1991; Balthazart et al., 1992; Aste et al., 1993, 1998a). In addition to the POA, several other brain regions such as the medial part of the bed nucleus striae terminalis (BSTM) and nucleus taeniae of the amygdala (and related areas of the medial arcopallium, AM) also play important roles in the control of male sexual behavior in quail as indicated by the behavioral effects of stereotaxic lesions of these structures (Balthazart et al., 1998a; Thompson et al., 1998; Absil et al., 2002) and by the changes in metabolic activity (as demonstrated by 2-deoxyglucose autoradiography; see Dermon et al., 1999) and in protein synthesis (as demonstrated by the expression of the IEG *c-fos* and *zenk*; see Ball et al., 1997; Meddle et al., 1997) observed after expression of the behavior. The homologous nuclei in mammalian species, such as rats (Baum and Everitt, 1992), Syrian hamsters (Newman et al., 1997) and gerbils (Heeb and Yahr, 1996), are also involved in the activation of sexual behavior. The identification of the circuitry mediating sexual behavior is however far from complete and, more specifically, the phenotype of the neurons activated during copulation is still largely unknown.

TH-immunoreactive (-ir) fibers and terminals have been observed in the close vicinity of aromatase-ir cells in the quail brain (Balthazart et al., 1998b). TH is the rate limiting enzyme in the synthesis of catecholamines (dopamine and norepinephrine) which play a major role in the control of various aspects of reproduction, including secretion of gonadotrophic hormones and the facilitation of male and female sexual behavior (Barraclough and Wise, 1982; Kalra and Kalra, 1983; Blackburn et al., 1992; Hull, 1995). The anatomical proximity of aromatase cells and TH-ir fibers suggests a functional relationship between steroids and catecholaminergic neurotransmission but how steroids and dopamine interact to control behavior remains unclear. The local production of estrogen could control dopamine/norepinephrine synthesis and release (Pasqualini et al., 1995) or alternatively, steroid action could be modulated by the catecholamines, through a change in either estrogen synthesis (Balthazart and Ball, 1998b) or steroid receptor availability (Blaustein and Olster, 1989; Blaustein et al., 1995). The present work was therefore undertaken with two major goals in mind: a) further identify the neuronal circuitry implicated in the control of male sexual behavior by mapping the expression of IEG after the expression of copulatory behavior and b) determine whether catecholaminergic neurons are specifically activated during copulation in male quail. Most previous studies that utilize IEG induction to map pathways in relation to male sexual responding in birds and mammals have utilized *c-fos* and no study has been completed on *egr-1* in relation to male sexual responses (although there has been some work in relation to female sexual responding or maternal behavior (Brennan et al., 1992; Polston and Erskine, 1995; Numan et al., 1998). We have published one short study previously on ZENK in birds (*egr-1*; Ball et al., 1997) so another valuable feature of our work is the characterization ZENK expression in association with male sexual responses.

EXPERIMENTAL PROCEDURES

Experiments described in this paper were carried out on Japanese quail (*C. japonica*) which were bought from a local breeder (C. Dujardin Farm, Liernu, Belgium) at the age of about 3 weeks. Throughout their life at the breeding colony and in the laboratory, birds were exposed to a photoperiod simulating long days (16 h light and 8 h dark per day) and had food and water available *ad libitum*. All experimental procedures were in agreement with the Belgian laws on “Protection and Welfare of Animals” and on the “Protection of Experimental Animals” and the International Guiding Principles for Biomedical Research involving Animals published by the Council for International Organizations of Medical Sciences. The protocols were approved by the Ethics Committee for the Use of Animals at the University of Liège. The number of subjects was kept to a minimum compatible with a sufficient statistical power of the experiment given the expected variance in the results. Care was taken throughout the experiment to minimize animal suffering as much as possible.

At about 4 weeks of age, a total of 41 male quail were either gonadectomized (CX) or sham-operated and left with intact testes (I) using surgical procedures that have been previously described in detail (Balthazart and Schumacher, 1984). Two weeks later, all subjects received two 20 mm long Silastic implants (Dow Corning no.602-252; 1.57 mm i.d.; 2.41 mm o.d.; Degania Bet, Israel) filled with crystalline T to restore physiological levels of the steroid (Balthazart et al., 1983) or left empty as control. At least 3 weeks later, the cloacal gland, an androgen-dependent structure (Sachs, 1967), was measured with calipers and all subjects were pre-