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C-fos down-regulation inhibits testosterone-dependent male sexual behavior and the associated learning

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Abstract

Environmental stimulation results in an increased expression of transcription factors called immediate early genes (IEG) in specific neuronal populations. In male Japanese quail, copulation with a female increases the expression of the IEGs *zenk* and *c-fos* in the medial preoptic nucleus (POM), a key nucleus controlling male sexual behavior. The functional significance of this increased IEG expression that follows performance of copulatory behavior is unknown. We addressed this question by repeatedly quantifying the performance of appetitive (learned social proximity response) and consummatory (actual copulation) sexual behavior in castrated, testosterone-treated males that received daily intracerebroventricular injection of an antisense oligodeoxynucleotide targeting *c-fos* or control vehicle. Daily antisense injections significantly inhibited expression of copulatory behavior as well as acquisition of the learned social proximity response. A strong reduction of the proximity response was still observed in antisense-treated birds that copulated with a female, ruling out the indirect effect of the absence of interactions with females on the learning process. After a two-day interruption of behavioral testing but not of antisense injections, birds were submitted to a final copulatory test that confirmed the behavioral inhibition in antisense-injected birds. Brains were collected 90 min after the behavioral testing for quantification of *c-fos* immunoreactive cells. A significant reduction of the number of *c-fos*-positive cells in POM but not in other brain regions was observed following antisense injection. Together, data suggest that *c-fos* expression in POM modulates copulatory behavior and sexual learning in male quail.

Keywords

Sexual learning; *c-fos* antisense; Japanese quail; medial preoptic nucleus; appetitive sexual behavior

INTRODUCTION

Immediate early genes (IEGs) form a group of genes that are rapidly and transiently transcribed in response to cellular, and particularly neuronal, activation. Due to their rapid and transient upregulation following exposure to external stimuli (Graybiel *et al.*, 1990; Fowler *et al.*, 2011), IEGs have proven particularly useful to identify the neural networks activated by specific events (see for reviews: (Hoffman *et al.*, 1993; Hoffman *et al.*, 1995;

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Clayton, 2000)). In particular, in numerous species, the performance of male sexual behavior significantly increases the expression of c-fos and zenk notably in the medial preoptic area, as well as in defined subregions of the amygdala and of the stria terminalis (see reviews in (Coolen *et al.*, 1996; Newman *et al.*, 1997; Pfaus & Heeb, 1997; Hull *et al.*, 2002); in quail: (Meddle *et al.*, 1997; Charlier *et al.*, 2005b; Taziaux *et al.*, 2006).

The role of IEG expression in the brain has been investigated in mice with targeted disruption of the gene of interest or with the use of antisense oligonucleotide techniques in rats. For example, numerous studies have identified important deficits in memory formation and in the underlying neural plasticity in the hippocampus following depletion of c-fos expression (Abraham *et al.*, 1991; Rose, 1991; Brennan *et al.*, 1992; He *et al.*, 2002; Countryman *et al.*, 2005; Yasoshima *et al.*, 2006). The function and importance of immediate early gene upregulation in other brain regions such as the hypothalamus after the performance of male or female sexual behavior is, however, less clear. The absence of c-fos expression in constitutive c-fos^{-/-} knock-out mice leads to a significant alteration of male sexual behavior, including clear deficiencies in the initiation of mounting an estrous female, mount frequencies, mount latencies and mount ratios (Baum *et al.*, 1994). It should also be noted that the upregulation of IEG transcription in the MPOA is induced by, and therefore follows, the expression of sexual behavior. This suggests that the proteins derived from these genes cannot control in a direct manner the manifestation of the behavior. Studies in quail have demonstrated that sexual experience can modulate IEG expression associated with copulation (Can *et al.*, 2007). Males who had copulated repetitively over several days did not exhibit an enhanced induction of zenk (Can *et al.*, 2007).

The aim of the present study was to investigate the function of c-fos expression in the brain in relation to the control of male sexual behavior in Japanese quail. In this species, expression of male-typical copulatory behavior induces a dense expression of c-fos in a number of brain nuclei including most prominently the medial preoptic nucleus (POM) (Meddle *et al.*, 1997; Charlier *et al.*, 2005b; Taziaux *et al.*, 2006). This nucleus is a key brain region involved in the control of male sexual behavior. Bilateral lesions of the nucleus completely block male sexual behavior while stereotaxic testosterone implants placed within the boundaries of the POM fully restore the testosterone-dependent sexual behavior in castrated males (Balthazart & Surlemont, 1990; Balthazart & Ball, 2007).

MATERIAL AND METHODS

A total of forty-one adult male Japanese quail (*Coturnix japonica*), obtained from our breeding colony were used in the present experiment and were single-housed after castration (see below). Stimulus females were housed similarly in large breeder cages with up to six hens per cage. Birds were kept under conditions simulating their breeding conditions under a long day photoperiod (16 h light and 8 h dark per day), with temperature kept at 21°C and food and water available *ad libitum* throughout their life. All experimental procedures were in agreement with the Belgian laws on “Protection and Welfare of Animals” and on the “Protection of experimental Animals”. The protocols were approved by the Ethics Committee for the Use of Animals at the University of Liège.

Cloning and sequencing

Total brain RNA from a male Japanese quail was isolated by TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. RNA was reverse transcribed using M-MLV and random hexamer primers (both from Promega, Madison, USA). We designed several pairs of primers based on the homology between *c-fos* nucleotide sequences from chicken, human, mouse and rat to clone the quail *c-fos* ATG region. One set of primers (Fw: CGGACACAGGGACGCAGC and

Rv:ACGGGTAGTAGGTGAGGC) allowed the amplification by PCR of a fragment of the expected size of around 200 bp. PCR products were cloned in pCR 2.1-TOPO by the TA cloning® method (Invitrogen) and sequenced in both directions. Sequencing was done by GIGA sequencing facility and confirmed the exact length of 191 bp of the fragment containing the ATG region.

Behavioral pretest and c-fos knockdown

Male Japanese quail were castrated at four weeks of age as previously described (Schumacher & Balthazart, 1984). Four weeks after castration they were implanted with a chronic injection cannula (Bilaney Consultants, UK) reaching the third ventricle at the level of the medial preoptic area to allow the central administration of modified oligodeoxynucleotide (LNA, see below) (coordinates of the cannula tip were 5.10 mm anterior, 2.35 mm above, and 0.00 mm lateral to the center of the interaural axis). A small drop of cerebrospinal fluid leaked out of the injection cannula at the time of implantation and before each injection of LNA when the dummy insert was removed, confirming the location of the tip of the cannula inside the ventricle. Animals were then allowed to recover for 2 weeks. At the age of 10 weeks, all subjects received two 20-mm long SilasticTM implants (Dow Corning nbr 602-205, 1.02 mm, i.d.; 2.16 mm, o.d.) filled with crystalline testosterone (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). One day before the first LNA injection, i.e. 7 days after the testosterone implantation, all subjects were pre-tested for appetitive sexual behavior to confirm the effectiveness of the testosterone treatment. During these tests, we only quantified the frequency of rhythmic cloacal sphincter movements (RCSM), a measure of male motivation (Ball & Balthazart, 2010), and did not test for copulatory performance because we wanted the subjects to be sexually naïve before the initiation of treatments with the c-fos antisense.

The frequency of RCSM was quantified in a glass aquarium (40×20×25 cm) adjacent to another similar aquarium containing a female (Balthazart *et al.*, 1998; Seiwert & Adkins-Regan, 1998; Cornil *et al.*, 2004). The glass wall facing the experimenter was obstructed by piece of opaque cardboard to prevent the subject from being distracted by the presence of the experimenter. A mirror was placed at a 45° angle under the cage to allow the experimenter to observe the cloacal area of the male. A sliding opaque panel was initially inserted between the two aquaria to avoid visual contact between the male and female during 2.5 min. After this time, the sliding opaque panel was raised and the male had visual access to the female during the next 2.5 min but could not physically interact with her. The number of RCSM was recorded separately during the two 2.5 min-periods when the male had or not visual access to the female. The low basal RCSM frequency displayed by the male in the absence of visual contact (average: 10.8 contractions/2.5 min) was then subtracted from the RCSM frequency recorded when viewing the female to obtain a measure of the female-induced RCSM. The males were then randomly distributed into 3 groups based on their performance during this appetitive sexual behavior pretest.

Starting one day after this pretest, birds were then injected daily with 2 µg of c-fos antisense (AS) or scrambled (SC) LNA, or vehicle as controls (see Fig. 1). We used chimer oligodeoxynucleotide consisting of deoxyribonucleic acid (DNA) and locked nucleic acid (LNA) (Charlier *et al.*, 2005a; Charlier *et al.*, 2006; Niessen *et al.*, 2011) purchased from Eurogentec S.A. (Seraing, Belgium). The sequence for the 19-mer antisense spanning the putative start codon of the quail *c-fos* was 5 TGGTAcatcatgtcgGCTG 3 (the monomers are represented by uppercase letters and DNA monomers by lowercase letters), and the scrambled control oligodeoxynucleotide was 5 GTTGaactcatgcgCGGT 3. The acronym LNA will be used throughout the text to refer to the modified antisense. LNA were dissolved in sterile saline (2 µg/µl in NaCl 0.9%). A basic local alignment search tool (BLAST) search was performed (<http://www.ncbi.nlm.nih.gov/BLAST/>) to confirm that

both antisense and scrambled sequences were not complementary to any other known sequence. Two hours after the second injection, birds were pretested to check for gross behavioral effects of the LNA. They were introduced in an empty test arena in which we measured the general activity by counting the number of times that birds entered the three thirds of the arena and their preening frequency. We did not detect any major qualitative effect of the injection (AS, SC, or vehicle) on the general activity nor on the frequency of preening. Throughout the experiment, the birds treated with antisense LNA also consumed food at a normal rate, as noted by the fact that no difference in body mass was observed at the end of the experiment.

Behavior tests

The animals were tested every day, 2 hours after the central injection of LNA or its controls, for appetitive and consummatory behavior in presence of a sexually mature female starting on the third day of the injection protocol. We used the learned social proximity response to evaluate the appetitive behavior (Domjan & Hall, 1986a; b; Balthazart *et al.*, 1995; Balthazart *et al.*, 1998) in which a male will stand in front of a window providing visual access to a female after this situation has been repeatedly paired with physical access to a female and the possibility to freely interact and copulate with her. This response (presence at the window) is not expressed by sexually naïve males and is only acquired after the male has copulated with the female in the experimental chamber. Copulations in another arena do not promote expression of the proximity response. Its development across behavior tests can thus be considered as a form of sexual learning (Domjan & Hall, 1986a; b).

The proximity response was acquired in a two-compartment test cage, with a large compartment for the experimental male subject ($90 \times 90 \times 50$ cm), and a smaller cage for stimulus females ($20 \times 26 \times 24$ cm), centered on a lateral wall of the main compartment. The two compartments were separated by a vertically sliding door that could be remotely controlled. A small “window” (vertical slit, 1 [wide] $\times$ 15 [high] cm) was located in the middle of this door and provided the male with restricted visual access to the female. When the window was open, the male present in the main chamber could only see the female located in the lateral chamber if he was positioned in front of and facing the window.

Each behavioral test lasted for a total of 10 min. The male was first introduced in the main chamber, and the stimulus female was placed in the adjacent smaller cage. During the first 5 min, the male had visual access to the female (through the open window) but could not physically interact with her. The male’s behavior was video recorded and then the total time during which the male was looking through the window was manually recorded from these videos by an observer blind to treatments thus providing a score ranging from 0 to 300 s (within a 5 min test). These data provided a measure of the appetitive sexual behavior of the male (see (Balthazart *et al.*, 1995; Balthazart *et al.*, 1998) for more detail on these procedures). The sexual nature of these appetitive responses is indicated by the facts that (a) they are exhibited at high rates in response to a female stimulus, (b) they are markedly decreased if the target cage is empty (Balthazart *et al.*, 1998) or contains a male (they are not simply social) (Domjan & Hall, 1986a), and (c) they decrease if no physical access to the female is provided, so that copulation cannot take place (Riters & Balthazart, 1998).

At the end of this period, the door separating the two compartments was lifted, and the male was allowed to freely interact with the female for 5 min. During that time, the frequency and latency of the first occurrence of male sexual behaviors were directly recorded. The frequency of the following behavior patterns was systematically noted: neck-grabs (NG), mount attempts (MA), mounts (M), and cloacal contact movements (CCM; see (Adkins & Adler, 1972; Hutchison, 1978) for a detailed description). These data provided a measure of the consummatory sexual behavior of the birds. Because the analysis of these four behaviors

produced similar conclusions, only data relative to mount attempts and to the end of the copulatory sequence (CCM) will be presented here to avoid redundancies. This experimental protocol lasted during 4 consecutive days (injection days 3–6). After this 4 day-period of behavioral acquisition, animals were still injected with antisense or their controls but not tested for a 2 days recovering period (injection days 7–8) and injected and submitted to a final test on injection day 9. Two hours after the last behavioral test, the subjects were killed by decapitation, brains were processed for histology (acrolein fixation) and stored at -80°C . All subjects were, at that time, checked for the presence of the SilasticTM implants and completeness of castration. All numbers of subjects presented below represent males that were in the desired endocrine condition. Seven animals were discarded due to injury, testes regrowth or died during the experiment. This experiment included a final total of 34 male Japanese quail. 13 males were injected daily with the antisense LNA (AS Group), 11 were injected daily with the scrambled LNA (SC) group and other 10 were injected daily with saline solution.

Immunohistochemistry

The brains were cut in the coronal plane using a cryostat starting at the level of the tractus septopallio-mesencephalicus and collected in four series of 30 μm -thick free floating sections. One of these series was then stained by immunohistochemistry for the c-fos protein by techniques previously described and validated for quail (D'Hondt *et al.*, 1999). Briefly, sections were incubated for 15 min in 0.1% sodium borohydride in PBS and washed in 0.1M phosphate buffer pH 7.2. Endogenous peroxidase activity was blocked by incubating the sections for 20 min 0.6% hydrogen peroxide, the non-specific antibody binding sites were blocked with 5% goat normal serum, and the sections were incubated with the primary c-fos antibody at 4°C (1/8000 rabbit polyclonal anti- c-fos antibody raised against an avian FOS protein sequence (Fujiwara *et al.*, 1987) prepared and validated for use in quail brain by D'Hondt and collaborators (D'Hondt *et al.*, 1999). Sections were then left for 2 h in secondary biotinylated goat anti-rabbit antibody (1/400, Dako A/S, Goslstrup, Denmark) and finally incubated in ABC Vectastain elite Kit PK-6100 (Vector Laboratories) for 1h30. All reagents were in Phosphate-buffered saline 0.05 M containing 0.1% triton X-100 (PBST) and several rinses were performed between each step. The peroxidase enzymatic activity was then visualized with 3, 3' diaminobenzidine tetrahydrochloride (DAB, 2%), 0.012% hydrogen peroxide in PBS. Reaction was terminated by several rinses in PBS and the sections were mounted in an aqueous gelatin medium and coverslipped.

Image analysis

Image acquisitions were performed by an observer blind to the treatment group using a ccd camera (Model CFW-1612C, Scion Corporation, MD, USA) attached to an Olympus microscope and connected to a MacIntosh computer (Software: ImageJ 64, Wayne Rasband, NIH, Bethesda, MD, USA). C-fos expression was quantified in four brain areas, illustrated in Figure 2. In each area, the number of c-fos-immunoreactive (c-fos-ir) cells was manually counted in one entire field that was placed in a standardized manner based on predefined anatomical landmarks in the sections (e.g. edge of ventricles or prominent fiber tracts). Brain structures were identified based on the atlas of the chicken brain (Kuenzel & Masson, 1988) but the nomenclature used in the present paper is based on the revised nomenclature for the avian brain (Reiner *et al.*, 2004).

Medial preoptic nucleus (POM)—The quantification field (0.636 mm^2) was placed in the corner formed by the ventral edge of the anterior commissure (CA) and the lateral edge of the third ventricle at the level where the CA reaches its largest extension (corresponding to plate A8.2 in the chicken atlas (Kuenzel & Masson, 1988) (see Fig. 2A). The image was taken with a 10 x objective. The entire computer field was then divided into two equal parts,

of 0.318 mm² each, by a standardized line parallel to the third ventricle thus dividing the POM in a median part (bordering the third ventricle) and a lateral part. The number of c-fos-positive cells was counted separately in the median (POM med) and lateral part (POM lat) and the results are presented separately for the median and lateral parts and their sum.

Bed nucleus of the stria terminalis (BNST)—c-fos expression in the BNST was measured at two separate rostro-caudal levels. In the section located at the largest extension of the anterior commissure (CA; Fig. 2B), the quantification field (0.158 mm²; 20 x magnification) was placed just dorsal to the commissure at a position where the field was centered on the most ventral extension of the overlying lateral ventricle. In the section immediately caudal to this one where the BNST has acquired a V-shape structure (Fig. 2C), two quantification fields (0.158 mm²; 20 x magnification) were used: the first one was centered, like in the previous section, on the most ventral extension of the lateral ventricle while the second one was located one field length more medial and one field length more ventral.

Lateral septal nucleus (SL)—The quantification field (0.158 mm²) covered entirely the bulge of the lateral septal nucleus into the lateral ventricle, at the level where the anterior commissure reaches its largest extension (Fig. 2B). The picture was taken at a 20 x magnification.

Nucleus taeniae of the amygdala (TnA)—The same section used for analysis the lateral septum was analyzed (see Fig. 2B). The quantification field (0.158 mm²) was aligned with the ventral surface of the brain and dorsally with the ramification of the CA. The picture was taken at 20 x magnification.

Periaqueductal grey (PAG)—The section analyzed presented the largest medio-lateral extension of the posterior commissure and corresponds approximately to plate A4.8 in the chicken brain atlas (Kuenzel & Masson, 1988). The nucleus was previously known as substantia grisea centralis or GCt. The quantification field (0.158 mm²) was delimited by the corner formed by the ventral edge of the posterior commissure (CP) and the lateral edge of the aqueductus mesencephali (see Fig. 2D). The picture was taken with a 20 x magnification.

Hippocampus—c-fos expression was finally quantified at three separate rostro-caudal levels corresponding to the largest extension of the anterior commissure (CA; Fig. 2B) and to levels that are 2 sections rostral (Fig. 2A) or one section caudal to this one (Fig. 2C). Two quantification fields (0.158 mm² each) were placed in each section. The most ventral one was placed as ventrally as possible to allow it to be fully contained in the hippocampal tissue between the inter-hemispheric separation and the lateral ventricle, the second one was one field more dorsal and moved laterally until it touched the lateral ventricle.

Statistical analyses

No significant difference was observed between the two control groups (scrambled LNA and vehicle) and there was no interaction between repeated testing and type of control injection for all morphological (body mass, cloacal gland size, numbers of FOS-ir cells) and behavioral (NG, MA, M, CCM, Time looking through Window) variables that were analyzed, thus confirming the absence of non-specific effects of LNA injections. To increase the power of statistical analyses, results from these two groups were thus pooled as a single control group (CTRL) consisting of 21 animals for subsequent analyses.

Behavioral data collected during the acquisition phase of the social proximity response were analyzed by mixed two-way ANOVA with one independent factor (experimental groups) and one repeated factor (the successive behavioral tests), followed when appropriate by *post hoc* Bonferroni tests. The frequencies of behavior recorded during the final test and histology results were analyzed using bilateral Student's *t*-test. Percentages of birds displaying a behavior were compared by the bilateral Fisher exact probability test. Differences were considered significant for $p < 0.05$.

RESULTS

Partial cloning and sequencing quail *c-fos* mRNA

The published quail *c-fos* mRNA (NCBI access code: AF364329) did not include the sequence upstream of the ATG start codon, required to design an efficient LNA-antisense probe. Forward primers complementary to the 5' untranslated *c-fos* sequence were designed based on the nucleotide sequences from chicken (NCBI access code: NM_205508). One set of primers allowed the amplification by PCR of a fragment of the expected size of 191bp containing the ATG start-codon. The sequence upstream of the ATG (92 bp) showed 99% identity with the chicken sequence (see Figure 3). The sequence used to prepare the antisense LNA was however fully homologous to the corresponding chicken sequence.

Appetitive sexual behavior

During the first test for social proximity response, both CTRL and AS subjects spent little to no time in front of the window (see Fig. 3). Starting on the second test, the CTRL group demonstrated a progressive and significant increase in the time spent looking through the window while there was no change in response in AS group (Fig. 4). Analysis of these data by mixed-design two-way ANOVA revealed a significant effect of repeated testing ($F_{3,32}=8.802$, $p<0.0001$), treatment ($F_{1,32}=10.43$, $p=0.0029$) and a significant interaction ($F_{3,32}=5.447$, $p=0.0017$). Bonferroni post-hoc tests indicated that the two groups were statistically different on tests 3 and 4. After the four-day acquisition phase, animals were allowed to rest for 2 days before the final test. On this final test day, CTRL animals spent a significant longer time looking through the window in comparison to AS animals (DF=32, $t=2.581$, $p=0.0146$).

Consummatory sexual behavior

After each test for social proximity response, the door separating the two compartments was opened allowing the male to freely interact with the female. The latencies to start the copulatory sequence were significantly longer in animals treated with the *c-fos* antisense compared to their controls. The mixed two-way ANOVA revealed a significant effect of repeated *c-fos* antisense injections on the latencies of NG ($F_{1,32}=5.524$, $p=0.0251$), MA ($F_{1,32}=4.889$, $p=0.0343$) and M ($F_{1,32}=4.289$, $p=0.0465$), and a strong tendency to longer latencies for CCM ($F_{1,32}=3.751$, $p=0.0617$). There was no effect on these latencies of repeated testing and no interaction between these two factors ($p>0.1$). During the final test, latencies of all aspects of the copulatory sequence were significantly longer in the antisense-treated group than in controls (NG : DF=32, $t=3.524$, $p=0.0013$, MA: DF=32, $t=3.514$, $p=0.0013$, M: DF=32, $t=3.685$, $p=0.0008$ and CCM: DF=32, $t=2.789$, $p=0.0088$).

Analysis of the behavioral frequencies of these behaviors by mixed design two-way ANOVA revealed a significant inhibitory effect of repeated *c-fos* antisense injections on NG ($F_{1,32}=4.202$, $p=0.0486$) and M ($F_{1,32}=6.170$, $p=0.0184$), and a strong tendency to inhibition in MA ($F_{1,32}=3.798$, $p=0.0601$) and CCM ($F_{1,32}=4.012$, $p=0.0537$) (see Fig. 5). There was no effect of repeated testing and no interaction ($p>0.1$). During the final test, frequencies of all behaviors in the copulatory sequence were significantly inhibited in the antisense-treated

group for all aspects of the copulatory sequence (NG: $DF=32$, $t=2.797$, $p=0.0087$, MA: $DF=32$, $t=2.674$, $p=0.0117$, M: $DF=32$, $t=2.966$, $p=0.0057$ and CCM: $DF=32$, $t=2.882$, $p=0.007$).

The relative numbers of subjects that performed copulatory behaviors was also significantly different between CTRL and AS birds both during the entire experiment (MA: 18/21 in CTRL vs. 7/13 in AS group, $p<0.022$; CCM: 18/21 in CTRL vs. 3/13 in AS group, $p<0.001$ by Fisher exact probability tests) and during the last test performed just before brain collection (MA: 15/21 in CTRL vs. 2/13 in AS group, $p<0.004$; CCM: 11/21 in CTRL vs. 1/13 in AS group, $p<0.011$ by Fisher exact probability tests).

Interactions between appetitive and consummatory sexual behavior

Previous studies clearly showed that the social proximity response is a learned process in which only animals that interacted sexually with a female subsequently spend large amounts of time in front of the window (Domjan & Hall, 1986a; b; Balthazart *et al.*, 1995). This therefore raised the possibility that the lack of acquisition of the social proximity response in the AS groups might be due to a lack of interaction with the female and not to a direct consequence of reduced c-fos expression. The acquisition of the learned social proximity response was therefore analyzed again, but this time only for animals that performed at least one mount attempt during the first 3 behavioral tests. This included 16 CTRL (76,19%) and 6 AS (46,15%) subjects. Again, during test 1, both CTRL and AS subjects spent little time looking through the window (see Fig. 6). The CTRL group then demonstrated a progressive increase in the time spent looking from the second test onward, while there was no change in response in the AS group. Analysis by mixed-design two-way ANOVA revealed a significant effect of repeated testing ($F_{3,20}=7.475$, $p=0.0002$), treatment ($F_{1,20}=6.387$, $p=0.02$) and a significant interaction ($F_{3,20}=2.976$, $p=0.0386$). Bonferroni post-hoc tests indicated that the two groups were significantly different during test 4. During the final test, CTRL animals showed a tendency to spend more time looking through the window in comparison to AS animals, but difference was not fully significant ($DF=20$, $t=1.745$, $p=0.0963$).

The significant interaction obviously resulted from the differential time-course of the behavior in the two groups: a steady increase in the CTRL and absence of change in the AS group. This was confirmed by one-way ANOVA analyzing changes in the look response as a function of repeated testing in these two groups (CTRL: $F_{3,60}=12.820$ $p<0.0001$; AS: $F_{3,36}=0.587$, $p=0.6273$).

This difference was apparently not due only to the fact that AS birds showed a reduced copulatory activity because if the same analysis was repeated focusing only on the 6 AS males that showed mount attempts during the first 3 tests, no change across tests in the time they spent looking through the window could be detected ($F_{3,15}=0.961$, $p=0.4365$). The same was true for the analysis of the time spent at the window by the 3 males that actually performed CCM during the first 3 behavioral tests ($F_{3,6}=0.665$, $p=0.6035$).

These data suggested that the absence of the social proximity response was not an immediate result of the lack of sexual motivation during the “learning phase” since the few males that did attempt to mount the female in the initial tests were apparently not acquiring this response. It remained however, possible that the response had been acquired but was not displayed (“latent learning”) because sexual motivation had vanished and was no longer present on the final test day. Based on available data and since there is often a substantial degree of inter-individual variability in the learned social proximity response, it is however impossible to evaluate this idea due to the low number of active birds in the AS group (2 birds only performed MA and only one performed CCM during the last test).

Histological analysis

All subjects were killed 90 minutes after their last behavioral test for brain analysis. No difference in body weight (CTRL: 199.2±4.8 g; AS: 198.4±5.9 g, $p>0.9$) and cloacal gland area (CTRL: 264.4±7.1 mm²; AS: 255.4±12.5 mm²; $p>0.5$) was observed at the end of experiment, indicating a lack of systemic effect of c-fos antisense injection and of side effect of the local reduction of c-fos expression in the brain.

The localized effect of antisense injections on c-fos expression was confirmed by immunohistochemistry (Fig. 7). C-fos expression was significantly reduced in the entire POM (DF=31, $t=2.147$, $p=0.0397$). Interestingly, this inhibition was more important in the medial part of the nucleus, directly adjacent to the third ventricle (DF=31, $t=2.324$, $p=0.0269$, Fig. 6A), while this decrease did not reach full statistical significance in the lateral part (DF=31, $t=1.896$, $p=0.0673$, Fig. 6B), suggesting that antisense had a limited penetration capacity and only reached effective concentrations in the tissue immediately adjacent to the third ventricle.

To test further whether the decrease in c-fos expression was due to the antisense injections and not a consequence of the absence of copulatory behavior (which is known to enhance expression of this IEG in POM and BNST), we also compared the number of c-fos-ir cells in the medial POM of birds in the antisense and control group as a function of whether they had or had not copulated (expressed CCM) during the final test. The corresponding two-way ANOVA did not detect any effect of the sexual activity ($F_{1,30}=0.205$, $p=0.6536$) and no interaction of this factor with the treatment effect ($F_{1,30}=1.22$, $p=0.2781$). The treatment effect was in the analysis showing a trend toward significance ($F_{1,30}=3.033$, $p=0.0918$). It must be noted however that the power of this analysis was very limited since only one male in the antisense group copulated during this last test. The same analysis based this time on the presence/absence of MA during the last test (2 AS birds active here) similarly failed to identify a connection between number of c-fos positive cells in POM and sexual activity (MA present vs. Absent: $F_{1,30}=0.382$, $p=0.5411$; Treatment effect: $F_{1,30}=2.224$, $p=0.1463$; Interaction: $F_{1,30}=0.1918$, $p=0.56646$). The antisense injected birds that were sexually active thus seemed to have similar low numbers of positive cells in POM as AS birds that were sexually inactive (see Fig. 8 for these quantitative results and representative photomicrographs).

C-fos induction in the BNST was not inhibited in the antisense treated birds as compared to their controls (Fig. 7C). This was true for counts performed at the CA (DF=30, $t=1.177$, $p=0.2483$) and at the CA+1 (DF=29, $t=1.207$, $p=0.2373$) levels as well as in their total (DF=29, $t=1.164$, $p=0.2539$).

No inhibition of c-fos expression was observed in nuclei also located along ventricles but more distant from the injection site, such as the lateral septum (SL, DF=31, $t=0.8715$, $p=0.3902$) or the periaqueductal grey (PAG, DF=31, $t=1.589$, $p=0.1223$; Fig. 7D and F).

We also investigated another nucleus usually activated after expression of sexual behavior, the nucleus taeniae of the amygdala, and did not find any difference in c-fos immunoreactive signal (TnA, DF=31, $t=0.7057$, $p=0.4856$; Fig. 7E),

Analysis of c-fos expression in the hippocampus similarly failed to identify any major decrease following antisense treatment. A two-way ANOVA analyzing the total of the measures (sum of counts in the dorsal and ventral fields) collected at the three rostro-caudal levels (repeated measure) identified no effect of the treatment ($F_{1,21}=0.140$, $p=0.7116$), no effect of the location in the rostro-caudal axis ($F_{2,42}=2.068$, $p=0.1392$) and no interaction between these factors ($F_{2,42}=0.355$, $p=0.7030$; Fig. 7G).

DISCUSSION

We demonstrate here that blockade of c-fos expression with the use of antisense oligonucleotides results in a strong inhibition of testosterone-dependent male sexual behaviors in Japanese quail. Both appetitive and consummatory aspects of the behaviors were significantly affected. This inhibition of behavior was associated with a local absence of c-fos induction in the POM (adjacent to the injection site) but other brain regions relevant to the control of sexual behavior did not show a reduction of this immediate early gene expression. Additionally, the down regulation of c-fos expression completely blocked the acquisition of the learned social proximity response suggesting that c-fos is also implicated in sexual learning.

The immunocytochemical quantification of the protein products of immediate early genes such as c-fos or egr-1 has been extensively used to identify brain regions that are activated during sexual behavior. In most species investigated to date, the medial preoptic area in males consistently shows an enhanced c-fos expression following sexual interactions with a female (Robertson *et al.*, 1991; Baum & Everitt, 1992; Ball *et al.*, 1997; Meddle *et al.*, 1997; Coolen *et al.*, 1998; Veening *et al.*, 1998; Tlemcani *et al.*, 2000; Charlier *et al.*, 2005b; Taziaux *et al.*, 2006). (Reviews in (Coolen *et al.*, 1996; Newman *et al.*, 1997; Pfau & Heeb, 1997; Hull *et al.*, 2002)). The mechanistic role played by this upregulation has not been investigated and in general the function of immediate early gene expression occurring after, but not before, the performance of sexual behavior is somewhat enigmatic, especially in quail that usually copulate within seconds after introduction of a female in their cage provided they are sexually experienced and motivated.

The role of immediate early gene expression though has been extensively studied in the hippocampus, amygdala and cortex (for review: (Papa *et al.*, 1995; Zhu & Herbert, 1997; Cammarota *et al.*, 2000; Vann *et al.*, 2000; Guzowski, 2002) and it was hypothesized that the transient expression of IEGs after numerous behavioral tasks contributes to the modification of synaptic networks associated with learning and memory, an interpretation largely supported by experiments down-regulating IEG expression by antisense administration (Grimm *et al.*, 1997; He *et al.*, 2002; Countryman *et al.*, 2005; Yasoshima *et al.*, 2006; Doron & Rosenblum, 2010; Katche *et al.*, 2010).

To our knowledge, the present study is the first to examine the role of an acute inhibition of c-fos expression specifically in the preoptic area in relation to the control of male sexual behavior. Results suggest that the c-fos upregulation induced following performance of male sexual behavior plays a role in the development and stabilization of behavioral changes associated with repeated expression of these behaviors. The importance of c-fos expression in POM and its potential association with sexual learning therefore would extend the role of the IEG that had been originally implicated in more well-known learning paradigms largely based on hippocampal activity. A few aspects of these results deserve however additional discussion.

Consummatory behavior

Can and colleagues (Can *et al.*, 2007) reported an absence of upregulation of the immediate early gene Zenk in the POM of male quail following repeated sexual interactions (after 16 consecutive days of copulation) while naïve animals had a high expression level after their first copulation (Can *et al.*, 2007). Similar results were obtained for c-fos expression: experienced male quail showed significantly fewer c-fos-ir cells in POM after copulating with a female as compared to first-time copulators (Holloway *et al.*, 2009).

Interestingly a very different effect has been reported based on experiments in rats testing the effects of D1 dopaminergic receptors on the induction of c-fos expression after sexual interactions: c-fos induction in the medial preoptic area was shown to be increased in experienced (7 tests in 5 days) as compared to naïve males (Lumley & Hull, 1999). It is important to note that these experienced rats had higher intromission frequencies compared to naïve subjects so that the larger induction of c-fos may be secondarily related to this increased behavior performance. The authors also note that the higher c-fos expression in experienced rats might reflect conditioning to olfactory cues originating from the females since previous work showed that these cues alone are able to induced c-fos expression (Baum & Everitt, 1992). In contrast quail are not expected to rely on olfactory cues in their social behavior (but see (Balthazart & Taziaux, 2009)) and the experienced quail displayed increased sexual behavior as compared to naïve birds but did not exhibit an increase in zenk expression in association with these increased sexual interaction frequencies (Can *et al.*, 2007). The decrease in c-fos expression observed after repeated testing in these birds thus cannot be a secondary consequence of changes in behavioral performance.

Another study reported that the experience-induced facilitation of sexual motivation and performance are mediated by an accumulation of deltaFosB in the rat nucleus accumbens but not, however, in the medial preoptic area (Pitchers *et al.*, 2010). Since c-fos is a downstream gene repressed by deltaFosB, the accumulation of delatFosB in the accumbens was associated with an inhibition of c-fos expression. These results are in agreement with the results reported in the studies of experience and IEG expression in quail (Can *et al.*, 2007) even if they concern different brain areas.

Based on these studies in quail and rodents, it was thus hypothesized that IEG over-expression in quail was required to improve sexual performance and, once optimized performance was acquired, further enhanced immediate early gene expression was no longer required (Can *et al.*, 2007).

In line with this hypothesis, we demonstrate here that the inhibition of c-fos expression in the AS group significantly reduced the expression of copulatory behavior in male quail, as shown by a significantly longer latency of the male to interact sexually with the female and by the lower frequency of copulatory behaviors. However, it must be noted that sexual behavior in the AS group was already inhibited on Test 1. These subjects had been injected with the antisense LNA for three days before experiencing sexual interaction with a female. Furthermore, we did not observe an obvious increase in copulatory performance (decreases in latency and/or increases in frequency) in control animals across tests throughout the experiment contrary to what was observed previously (Can *et al.*, 2007), possibly because birds were subjected here to only 4 successive tests as opposed to 16 tests in this published experiment. In any case, it is impossible to explain the differences in copulatory behavior observed here to a defect in learning processes per se and other mechanisms need to be invoked. The c-fos antisense inhibited copulatory behavior in a general manner in this study even before birds had acquired any sexual experience.

The MPOA in rodents or POM in quail constitutively expresses very low levels of c-fos in the absence of sexual interactions. One should thus assume that this basal expression is nevertheless functionally significant and promotes the expression of genes that play a substantial role in the activation of male sexual behavior. It should be noted that birds had been already treated with exogenous testosterone for 2 weeks when behavior testing was initiated. Several studies have previously demonstrated that administration of testosterone (or of its metabolite estradiol) increases c-fos expression the brain including the MPOA in the absence of any other stimulation (Insel, 1990; Dimeo & Wood, 2006; Nagypal & Wood, 2007) and a similar increase after estradiol treatment has been observed in peripheral tissues

(rat arteries: (Eyster *et al.*, 2007). Treatment with sex steroids is known to induce the expression of a variety of genes that ultimately result in changes in neural activity that will support expression of sexual behavior. It is extremely likely that expression of some of these genes is under the control of IEG such as c-fos and therefore conceivable that the blockade of basal c-fos expression results in an inhibition of male sexual behavior, independently of any interaction with sexual learning and indeed with c-fos induction by sexual interactions.

Accordingly, previous work in mice with targeted disruptions of the c-fos gene also indicated that the complete deletion of c-fos strongly reduces male sexual behavior (latencies and frequencies of behavior and percentage of active subjects were affected) (Baum *et al.*, 1994). One of the interpretations proposed by Baum and his colleagues suggested that the inhibition they observed resulted from developmental problems induced by the c-fos deletion because their c-fos^{-/-} males also exhibited a reduction in body size, a diminished stress response, lymphocytopenia and abnormal gametogenesis. However, our inhibition of c-fos expression was here limited in time and space and its effects on behavior cannot be explained by these sorts of longer term effects. The present data clearly demonstrate that c-fos expression is required in the MPOA, during adulthood, for the normal performance of copulatory behavior.

It is likely that this effect of c-fos is mediated through the direct control of the expression of testosterone (estradiol)-dependent gene(s) but alternative mechanisms are also possible. C-fos inhibition could for example interfere with the circadian control of general activity (Baum & Everitt, 1992) and in this way indirectly affect sexual behavior. Alternatively, a reduction of c-fos expression could affect the responses to a novel environment, which would also indirectly modify copulatory behavior since it was tested in an arena that was not familiar to the birds before the experiment. Additional work is necessary to discriminate between these mutually non-exclusive possibilities.

Behavioral and neuroanatomical specificity of c-fos down regulation

The most parsimonious view of our data is that the infusion of the LNA into the third ventricle knocked down c-fos expression in the POM and this blocked the expression of sexual behavior and acquisition of the learned social proximity response. This interpretation suggests that c-fos expression associated with sexual behavior is essential for behavior performance. Another interpretation is that the LNA manipulation blocked expression of c-fos or of an associated protein elsewhere in the brain and this inhibition blocked the occurrence of copulatory behavior. The lack of behavioral expression then could result in the decrease of c-fos expression in the POM. This second scenario seems less likely as will be reviewed now.

First a decrease in number of c-fos-ir cells in the AS group was indeed observed only in the POM whereas all other nuclei that were more distant from the site of antisense LNA injections (BNST, SL, PAG, TnA, Hip) were not significantly affected. This includes the BNST where a very consistent increase in c-fos expression following expression of copulatory behavior has been observed in male quail (Meddle *et al.*, 1997; Charlier *et al.*, 2005b; Taziaux *et al.*, 2006). This small range of action is in agreement with previous studies reporting a limited spread and cellular uptake of fluoresceine-coupled ODN (Ogawa *et al.*, 1994; Grzanna *et al.*, 1998; Sommer *et al.*, 1998; Zalachoras *et al.*, 2013). Furthermore, the inhibition of c-fos expression was clearly more prominent in the medial part of the POM, close to the ventricle, than in the lateral part of this nucleus where the inhibition actually did not reach full statistical significance. Finally, the two-way ANOVAs analyzing c-fos expression in the POM of the two experimental groups as a function of whether birds had or had not shown sexual activity during the last tests before brain collection detected no significant effect of sexual activity. In particular, the few birds treated

with antisense LNA that still displayed sexual behaviors had a very low number of c-fos positive cells in the medial POM (Figure 8) thus suggesting, even if the number of active subjects in the AS group is very low, that the decrease in c-fos expression relates to the AS injection rather than to the absence of sexual behavior.

It should also be noted that the alternative hypothesis (c-fos inhibition decreased behavior and this secondarily inhibited c-fos expression in the POM) would imply that the antisense LNA decreased sexual behavior by a mechanism independent of the c-fos inhibition and there is at present no identified mechanism that could mediate such an inhibition. In addition, the BLASTN search using the AS sequence suggested that the LNA could only hybridize to c-fos mRNA, as no other significant complementary sequences around the start region are, to our knowledge, present in quail or other vertebrate.

This conclusion however then begs the question as to why we did not observe here a clear effect of the performance of copulatory behavior on c-fos expression in the POM and BNST. It is important to remember in this context that the present experiment was clearly not designed to demonstrate such an effect. All birds were allowed in this study to interact with a female (even if some did not exhibit sexual behavior) before brain collection and there was thus no control group exposed to no stimulation from females. It is conceivable that just being with a female activates a number of sensory circuits eventually converging to the BNST and POM and that this activation was not or only partly blocked by the antisense injections.

Acquisition of the learned social proximity response a measure of appetitive sexual behavior

Importantly, we also observed that the down-regulation of c-fos induction completely blocked the acquisition of the learned social proximity response, a measure of appetitive sexual behavior (Ball & Balthazart, 2010). Previous work demonstrated that this response is a form of associative learning that is closely linked to the performance of copulatory behavior. This response is only acquired by males after they have expressed (during a previous test) sexual behaviors towards a female in the same test arena (Domjan & Hall, 1986a; Balthazart *et al.*, 1995). Because the inhibition of c-fos expression in this experiment significantly reduced, and in some subjects completely eliminated, male copulatory behavior, it could be argued that the reduction of learned social proximity response was not due to a blockade of sexual learning but was rather caused by the lack of reinforcement normally provided by copulation but the analysis of the social proximity response that excluded subjects that had shown no sexual activity at all during the first 3 behavior tests did not support this interpretation: the social proximity response did not increase in the 3 AS males who were able to copulate (perform CCM) during the first 3 acquisition tests.

As discussed in the introduction, the few experiments that have tested the functional significance of c-fos expression in the brain have all related expression of this gene to different forms of hippocampus-dependent learning.. We argue in the previous section of this discussion that the behavioral inhibition observed in our study is caused by the blockade of c-fos induction in the medial POM (no significant blockade elsewhere). How could an inhibition of sexual learning be mediated by the MPOA, a brain area that is not obviously related to learning processes?

It should first be noted that steroid action in the MPOA causes a suite of cellular and hodological changes that precedes, and in all probability mediates, the expression of sexual behavior. In the quail POM, it was for example shown that, treatment of castrated males with testosterone increases the general volume of the nucleus (Panzica *et al.*, 1991), the size of its neurons (Panzica *et al.*, 1991), their sub-cellular organization (Panzica *et al.*, 1995;

Castagna *et al.*, 1999) and multiple aspects of their neurochemical organization and functioning (Panzica *et al.*, 1996; Aste *et al.*, 1997; Aste *et al.*, 1998).

It is conceivable that the acquisition of the social proximity response is based on additional changes in connectivity and activity that take place in the POM or in other nuclei under the influence of activity in the POM in response to sexual interactions (Balthazart *et al.*, 2001). C-fos inhibition could well block these changes in POM connectivity or block some aspect of neuronal activity in POM that mediate in a transynaptic manner changes in other brain regions that support acquisition of the social proximity response. The brain circuitry controlling sexual behavior exhibits extensive modifications as a result of experience. For example, previous studies have shown that sexually experienced male rats have higher levels of nitric oxide synthase (nNOS) in the MPOA as compared to first time copulators or completely naive animals, regardless of whether or not the subjects mated prior to brain collection (Dominguez *et al.*, 2006). In male rats, copulatory experience increases cellular metabolic capacity, as measured by cytochrome oxidase activity, in the MPOA, bed nucleus of the stria terminalis and medial amygdala (Sakata *et al.*, 2002). C-fos induction following copulatory behavior has been shown to be dependent at least in part on the activation of D1 dopaminergic receptors (Lumley & Hull, 1999). It is also conceivable that conversely c-fos expression modulates the expression of D1 receptors that are known to be implicated in the regulation of copulatory behavior in rats (Hull *et al.*, 2002; Hull *et al.*, 2007) and quail (Balthazart *et al.*, 1997; Castagna *et al.*, 1997) and in quail specifically of the expression of the learned social proximity response (Balthazart *et al.*, 1997; Castagna *et al.*, 1997). These neurochemical features are potentially direct target genes for c-fos action (see for example the presence of AP1 sites on nNOS promoter (Xie *et al.*, 2000).

The inhibition of c-fos induction could also reduce the motivation of the male to interact sexually with a female thereby preventing the development of the link between copulation and social proximity response. This could happen via interference with the genomic regulation of aromatase expression by testosterone and its metabolite estradiol (Balthazart & Foidart, 1993; Balthazart *et al.*, 2004). Aromatase, the enzyme that catalyzes the transformation of testosterone into estradiol is a key protein in the activation of both appetitive and consummatory aspects of male sexual behavior (Balthazart *et al.*, 2004). The control of aromatase transcription is very complex and relies on several tissue-specific promoters and untranslated first exons (Simpson *et al.*, 1994; Simpson *et al.*, 2002). The brain-specific aromatase promoter I.f, does not contain estrogen response elements but includes two AP-1 responsive elements. Yilmaz and colleagues demonstrated that aromatase expression in the N42 hypothalamic cell line relies on the interaction of AP-1 and liganded ER (although this does not seem to be true in another cell lines such as N38) (Yilmaz *et al.*, 2009). C-fos could thus regulate aromatase transcription in the brain and in this way modulate sexual motivation. However, a potential problem with this mechanism arises from previous immunohistochemical studies indicating that c-fos colocalizes with only a very small number of aromatase positive neurons in the quail POM (Foidart *et al.*, 1999) and that there is also little colocalization between ER and aromatase (Balthazart *et al.*, 1991). One should therefore assume that either these colocalizations were underestimated in these earlier studies or that the few cells where colocalizations take place are key nodes in the circuit(s) regulating sexual motivation and consummatory sexual behavior.

Conclusion

The present experiment demonstrates that the down-regulation of c-fos expression by repeated intracerebroventricular injections of antisense oligodeoxynucleotides inhibits the expression of male copulatory behavior in castrated male quail treated with exogenous testosterone and also, in a largely independent manner, blocks the acquisition of the learned

social proximity response, a measure of appetitive male sexual behavior. Effects seem to be mediated by a decrease in c-fos expression in the POM since this is the only location where a blockade of the copulation-induced c-fos up-regulation was detected. These data thus suggest that the up-regulation of c-fos expression that is normally observed AFTER the expression of sexual behavior plays a role in sexual learning and is involved in the modifications of sexual behaviors that take place when animals acquire sexual experience. C-fos expression in the context of male sexual behavior would thus have a function very similar to what is observed in the context of learning experiments. Additional studies are needed to confirm this interpretation and to identify the specific mechanisms implicated in this inhibition of sexual learning.

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Figure 1.

Experimental design: Animals were castrated 4 weeks post-hatching and implanted with a stereotaxic injection-cannulae at 8 weeks post-hatching. After a 2 weeks recovery period, animals were subcutaneously implanted with a testosterone (T)-filled SilasticTM capsule. Starting seven days after the testosterone implantation animals were daily ICV injected with either 2 μ g of antisense (AS), 2 μ g of scrambled oligodeoxynucleotides (SC) or vehicle. On the second day of ICV injections animals were pretested for gross behavioral effects of the treatment in an empty test arena. On third, fourth, fifth and sixth days of ICV injections animals were tested for appetitive and consummatory sexual behavior. Animals were allowed to rest on seventh and eighth day of ICV injections. On the ninth day of ICV injections animals were tested one last time for appetitive and consummatory sexual behavior and killed for brain collection 90 minutes after the behavioral tests.

Figure 2.

Schematic drawings of coronal sections through the quail brain illustrating the areas where c-fos expression was quantified (dark areas). Panels A to D represent coronal sections through the quail brain presented in a rostral to caudal order. Aq: aqueductus mesencephali; BNST, bed nucleus of the stria terminalis; CA, commissura anterior; Cb, cerebellum; CO: chiasma opticum; CP, commissura posterior; CPa: commissural pallii; FPL, fasciculus prosencephali lateralis; GLv: nucleus geniculatus lateralis, pars ventralis; mPOA: medial preoptic area; Hp, hippocampus; LaM, lamina mesopallialis; M, mesopallium; N, nidopallium; PAG, substantia grisea centralis; SL, nucleus septalis lateralis; SM, nucleus septalis medialis; TeO: tectum opticum; TnA, nucleus taeniae of the amygdala; TSM: tractus septopallio-mesencephalicus; VL, ventriculus lateralis; VLT: nucleus ventro-lateralis thalami; VT, ventriculus tecti mesencephali.

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c-fos Quail   -97 CCGACACAGGGACGCAGCCGGCCCCCTCTTTGCGTTCGCCGCGAGGCGCTCCGCTATAGCACCGCAAGGCCCGCACGG -18
c-fos Chicken -84 CGGACACAGGGACGCAGCCGGCCCCCTCTTCGCCTTCTCCCGCAGGGCGCTCCGCTA--GCACCGCACGGCCC----- -15
*****
c-fos Quail   -17 CCACGCAAA CAGCCGACATGATGTACCA GGGCTTCGCCGGGGAGTACGAGGCGCCCTCCTCCCGCTGCAGCAGCGCT +59
c-fos Chicken -14 ---CGCAAA CAGCCGACATGATGTACCA GGGCTTCGCTGGGGAGTACGAGGCGCCCTCCTCCCGCTGCAGCAGCGCT +59
*****
c-fos Quail   +60 TCCCCGGCCGGGGACAGCCTCACCTACTACCCGT +92
c-fos Chicken +60 TCCCCGGCCGGGGACAGCCTCACCTACTACCCGT +92
*****

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Figure 3.

Alignment of the sequence obtained from the Japanese quail with the chicken c-fos (NM_205508). The start codon ATG is in bold. The sequence upstream of the ATG (92 nucleotides) showed 99% identity with the chicken sequence. The sequence in the box is targeted by the antisense LNA is fully identical to the corresponding chicken sequence

Figure 4.

Inhibitory effect of c-fos down-regulation by daily AS LNA injections in the third ventricle at the level of the POM on one measure of the appetitive male sexual behavior (learned social proximity response) in castrated male quail treated with exogenous testosterone. Mean ($\pm$ SEM) time that the subjects stood looking through the window is presented. The line graphs summarize the social proximity response during the acquisition phase, while the bar graphs represent the social proximity response during the final test. *= $p < 0.05$, ***= $p < 0.001$ versus CTRL by Bonferroni post-hoc or t-test.

Figure 5. Inhibitory effect of c-fos down-regulation by daily AS LNA injections in the third ventricle at the level of the POM on two measures of the male consummatory sexual behavior, the frequencies of Mount Attempts (MA; A) and Cloacal Contact Movements (CCM; B) in castrated male quail treated with exogenous testosterone. Mean ($\pm$ SEM) number of MA or CCM produced by the subjects of the AS or CTRL group are presented. The line graphs summarize the behavior frequencies during the acquisition phase, while the bar graphs represent the frequencies during the final test. *= $p < 0.05$ and **= $p < 0.01$ versus CTRL by Bonferroni post-hoc or t-test.

Looking through window MA positive (Test 1-3)

Figure 6.

Inhibitory effect of c-fos down-regulation by daily AS LNA injections in the third ventricle at the level of the POM on one measure of the appetitive male sexual behavior (learned social proximity response) in castrated male quail treated with exogenous testosterone. The results concern exclusively subjects that showed at least one MA during the first three days of behavioral testing. Mean ($\pm$ SEM) time the subjects stood looking through the window is presented. The line graphs summarize the social proximity response during the acquisition phase, while the bar graphs represent the social proximity response during the final test.

**= $p < 0.01$ versus CTRL by Bonferroni post-hoc test.

Figure 7.

Quantification (means $\pm$ SEM) of FOS-ir cells in the brain of CTRL and AS-treated subjects. Brains were collected 90 min following a test trial consisting of 5 min social proximity response testing and 5 min of consummatory sexual behavior testing where subjects were able to freely interact with a sexually mature female. *= $p < 0.05$ versus CTRL by t-test.

Figure 8.

A. Quantification (means $\pm$ SEM) of FOS-ir cells in the medial part of the POM of CTRL and AS-treated subjects as a function of whether they did or did not display cloacal contact movements (CCM) or mount attempts (MA) during the final tests before brain collection. **B.** Representative photomicrographs illustrating FOS-ir cells in the POM of the 4 sub-groups of birds as defined in panel A. Magnification bar= 500 μ m; AC: anterior commissure, the * indicates the position of the third ventricle.