

# ***c-fos* expression as a tool to search for the neurobiological base of the sexual behaviour of males**

**Michał Biały<sup>1</sup> and Leszek Kaczmarek<sup>2</sup>**

<sup>1</sup>Department of Physiology, Medical Academy of Warsaw, 26/28 Krakowskie Przedmieście St., 00-325 Warsaw; and <sup>2</sup>Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland, e-mail: leszek@nencki.gov.pl

**Abstract.** Elevated expression of *c-fos* mRNA and/or its protein (c-Fos) has been repeatedly observed following activation of brain cells with various stimuli. As a consequence it has been proposed that elevated *c-fos* expression may serve as a marker of neuronal activation reflecting a predisposal to long-term changes in cell functioning (neuronal plasticity). In studies on copulatory behaviour of male animals, elevated Fos immunoreactivity (related most probably to c-Fos) has been used by many researchers as a tool to reveal functional neuroanatomy of the behaviour. They found that brain regions such as medial preoptic area (MPOA), bed nucleus of stria terminalis (BNST), medial amygdala (MeAm) and central tegmental field (CTF) are always activated in a copulation-dependent manner in various species (rats and hamsters were the most often studied in this regard). This paper reviews the data gathered in this field, and discusses them in the context of a role of elevated Fos expression in acquisition vs. performance of the sexual behaviour.

Review

<sup>2</sup>To whom correspondence should be addressed

**Key words:** immediate early genes, copulatory behaviour, neuronal mapping, learning and memory, neuronal plasticity, rat, hamster, mouse

## INTRODUCTION

### c-Fos as a component of AP-1 transcription factor

Two protooncogene families, *fos* and *jun*, have received special attention within the neurophysiological community during the last decade, as it has been well documented that these components of the transcription factor AP-1 (activator protein 1) are expressed in brain cells in various physiological and pathological situations (for review see Hughes and Dragunow 1995). Findings that a plethora of insults may evoke rapid changes in gene expression have opened new avenues of research on genomic responses in neurones.

So far there are four cloned *fos* genes: *c-fos*, *fos-B*, *fra-1* and *fra-2*, which encode four proteins designated as: c-Fos, Fos-B, Fra-1, Fra-2, and there are three *jun* genes: *c-jun*, *jun B* and *jun D*, coding respectively for: c-Jun, Jun B, and Jun D (Morgan and Curran 1991). Various combinations of Jun proteins, either alone or together with one of the Fos proteins, form functional dimers, recognizing a specific DNA regulatory element with a consensus sequence TGAC/GTCA. Upon DNA binding, AP-1 may influence the transcription rate of its target genes. Since the basal level of c-Fos is low and increases dramatically after various kinds of cell stimulation, it is believed that it may be involved in the transcription factor whose main function is to reorganize cellular response to various environmental cues, termed cell activation (Kaczmarek and Kaminska 1989).

### *c-fos* and neuronal activity

The observation that elevated *c-fos* expression is a consequence of multiple kinds of neuronal stimulation has led to the proposal that it may serve as a useful marker of brain regions activated following physiological stimulation (Morgan and Curran 1991, Hughes and Dragunow 1995). The specific value of this marker is underlined by the fact that, contrary to 2-deoxyglucose or electrophysiological

mapping of metabolically and/or electrically active cells, *c-fos* expression apparently marks neurones undergoing long-term changes in their functioning, including plasticity processes, requiring gene expression (Goelet et al. 1986, Kaczmarek and Nikolajew 1990, Robertson and Dragunow 1990, Sheng and Greenberg 1990, Rose 1991, Morgan and Curran 1991, Kaczmarek 1992, 1993a, 1993b, 1994, 1995).

### Methods used to study *c-fos* expression in the brain

Before describing studies on *c-fos* expression in the brain we would like first to present a short, critical assessment of the methods used in this regard. *c-fos* expression in the brain could be analysed on various levels, including transcription activity (run-on transcription assay), mRNA abundance (Northern blot, dot blot, *in situ* hybridization) and finally the protein content (Western blot, immunocytochemistry). Since for mapping purposes single cell resolution is preferable if not an absolute prerequisite, c-Fos immunocytochemistry has been found to be the most useful. However, application of this approach has been complicated by the fact that close homologies among Fos proteins make obtaining of antibodies specific for a single antigen difficult, and often, purposely, an obscure term – Fos-li (Fos-like immunoreactivity) – has been used. Moreover, results obtained with this method are difficult to quantify. Western (immunoblotting) method may help to define the antigen recognized by the antibody, and to provide quantitative data, but extensive post-translational modifications of the c-Fos make the task quite challenging. To obtain unequivocal identification of *c-fos* expression, the studies of the gene product are much more appropriate. Run-on transcription is technically difficult and requires a lot of material, and apparently has never been used in this regard. On the other hand, *in situ* hybridization and Northern blot were employed repeatedly. *In situ* hybridization, however, suffers from poor single cell level resolution and does not solve all the drawbacks described for immunocytochemistry. Finally, Northern blot gives the most unequivocal

identification of the *c-fos* mRNA, as well as providing quantitative data, however, it is not a good tool to investigate gene expression at the sub-structure, not to mention single cell, level.

## FOS AND SEXUAL BEHAVIOUR

### Copulatory behaviour of male rats and hamsters

Studies involving *c-fos* mapping have been carried out mostly on two species of experimental animals: rats (*Rattus norvegicus*) and Syrian golden hamsters (*Mesocricetus auratus*). Very briefly, sexual behaviour can be described as consisting of two phases: courtship and copulation. During courtship, cues from the female are perceived by males and evoke sexual arousal (Madlafousek and Hlinak 1983). Dewsbury (1972) described 16 different patterns of copulation in mammals dependent on: lock, thrusting, multiple intromission or multiple ejaculation. In general, rats and hamsters share a similar copulatory pattern, with neither copulatory lock nor thrusting, but with multiple intromissions to achieve ejaculation and with multiple ejaculations during a copulatory session. In rats and hamsters three kinds of copulatory behaviour can be observed: (1) mounting (copulation without erection of penis), (2) intromission (copulation with very fast erection and insertion of penis to vagina during a very short time period) and (3) ejaculation (copulatory pattern with insertion of penis to vagina and with seminal emission). Usually under laboratory conditions, males are habituated to the copulatory arena for a few minutes and a receptive female is then introduced. Different copulatory parameters are taken as measures of sexual behaviour: (1) mount latency; i.e., time from introduction of female to first copulation (mount or intromission), (2) intromission latency; time from introduce of female to first intromission, (3) ejaculation latency, i.e., time from first intromission to ejaculation, (4) intromission frequency, i.e., number of intromission needed to achieve ejaculation, (5) hit ratio, i.e., number of intromissions divided by number of in-

tromissions plus number of mountings, (6) postejaculatory interval, i.e., time from ejaculation to first intromission of next copulatory series, (7) number of ejaculations, etc. (Larsson 1956, Sachs and Barfield 1976, Sachs and Meisel 1988).

The design of experiments employed by various investigators has usually been as follows. The males (either sexually naive, or with different levels of sexual experience) are exposed to ovariectomized, hormonally prepared (through injections of estradiol benzoate and progesterone, two days and four-six hours before sessions, respectively) receptive females (one or more per training/test session). The length of the session is either fixed with respect to time (e.g., 0.5 h) or defined by a behavioural criterion such as number of ejaculations. Other parameters of the male's behaviour have been scored for only occasionally. Since *c-fos* expression has been postulated to correlate with learning processes (Kaczmarek 1993a,b) and acquisition of sexual proficiency may involve learning (Beach 1947, Larsson 1959, Carr et al. 1965, Dewsbury 1969, Domjan 1992, Biały and Beck 1993, Matuszczyk and Larsson 1994, Vega-Matuszczyk et al. 1994, Biały 1995, Pfaus and Wilkins 1995), the exact nature of the training/test session seems to be of utmost importance in interpreting the results, whether the observed changes in *c-fos* expression can be taken as an index of neuronal plasticity in various brain structures, accompanying the acquisition of the copulatory reaction, or simply reflect functional anatomy of performance of sexual behaviour.

### Functional neuroanatomy of male sexual behaviour revealed by c-Fos mapping

#### RATS

Robertson et al. (1991) pretrained rats for six sessions (30 min each, with a single female), and then treated them with an additional session lasting for 60 min and involving an exposure to up to four females, changed consecutively. Just after the last session the rats were sacrificed, and Fos-li revealed heavy labelling of the medial preoptic area

(MPOA), bed nucleus of stria terminalis (BNST), nucleus accumbens (NAc) and piriform cortex (PCx), which was not evident in control animals pretrained similarly to the experimental ones, but not exposed to females during the test day.

Baum and Everitt (1992) pretrained male rats with receptive females until they ejaculated during one test. These sexually experienced animals were tested under various experimental paradigms: single or double ejaculation(s), one or five intromission(s), and various controls: no access to female due to her location in the arena in a special cage, and exposure to a female with taped vagina. In addition they used as controls males who failed to mount even with free access to females and sexually naive male rats who spent 1 h in an empty arena. The authors found in ejaculating animals elevated c-Fos expression in MPOA, BNST, medial amygdala (MeAm) and central tegmental field (CTF). More restricted c-Fos increase was noted in MPOA, BNST and MeAm in animals either with intromission(s) only or with ejaculation(s), as well as animals not mounting the available female. In all structures studied, the number of Fos-li cells did not increase in males mounting females with taped vagina. On the contrary, only rats ejaculating and displaying 5 intromissions were characterized by elevated Fos-li in CTF.

In their second experiment Baum and Everitt (1992) tested sexually experienced rats with unilateral electrothermic lesions of the olfactory peduncle. Two experimental paradigms were used: males ejaculating once and rats achieving about 55 mounts, and a few intromissions, but without ejaculation, due to lidocaine-anaesthetized penis. Such a treatment led to a significant reduction in Fos-li in the ipsilateral (to the lesioned side) piriform cortex in both ejaculating and non-ejaculating rats, and in unilateral MeAm but only in the non-ejaculating males. In the BNST, MPOA and CTF no differences between ipsi- and contralateral sides were found. Males copulating to ejaculation showed significantly more Fos-li in the CTF than non-ejaculating ones.

In the third experiment sexually experienced animals copulated to one ejaculation after unilateral

quinolinic acid-lesion of the MPOA (Baum and Everitt 1992). This procedure prevented an increase in Fos-li in ipsilateral MPOA and, surprisingly, in ipsilateral BNST as well.

The last experiment was conducted with sexually experienced rats with a unilateral lesion in either MeAm or CTF or both of these structures combined. The animals copulated to 5 intromissions. The lesions in either MeAm or CTF obviously abolished c-Fos expression in the damaged structures. However, injections of quinolinic acid into both MeAm and CTF blocked an increase in Fos-li in ipsilateral MPOA and BNST too.

Veening et al. (1994) identified an area of the CTF, labelled by Fos -li as a consequence of copulatory behaviour, to be limited to the parvocellular part of the nucleus subparafascicularis. Interestingly, the same brain region was marked by c-Fos antibodies also after aggressive behaviour (Veening et al. 1994). An anatomical study involving Fos-li and retrograde tracing revealed that many Fos immunoreactive neurones, observed after sexual training, in both MeAm (posterodorsal nucleus of amygdala) and in the parvocellular part of the nucleus subparafascicularis were projecting to MPOA. Increased c-Fos immunostaining as a consequence of ejaculatory behaviour was also found in MeAm and BNST (Veening et al. 1994, Coolen et al. 1995).

Wersinger et al. (1993) pretrained male rats with estrous females until the males achieved ejaculation(s) during three sessions. After that, the males were gonadectomized and given seven daily s.c. injections of 5 µg of estradiol benzoate and one day after last injection also of 500 µg of progesterone four hours before a test session. The purpose of the hormonal treatment was to compare Fos-li in males' *versus* females' brains. Experimental animals copulated to one ejaculation. Control rats were placed into an empty testing arena. In the second experiment some animals received bilateral pelvic nerve transection and some were sham operated. Unpaired rats were also used for control. All experimental animals copulated to one ejaculation. Mating induced an increase in the number of Fos immunoreactive cells in animals of both sexes in

MPOA, BNST, MeAm, CTF and also in ventromedial hypothalamus (VMH). Pelvic nerve transection in male rats decreased Fos-li in CTF when compared to sham operated animals. The authors analysed such behavioural parameters as number of mounts, number of intromissions and hit ratio (HR – number of intromissions/number of mounts + number of intromissions), and found significant decrease in HR in pelvic nerve transected males.

Humm et al. (1995) investigated effects of prenatal stress on Fos-li in MPOA after initiation of copulation in male rats. Prenatal stress is known to modify sexual behaviour *via* modification of sexual differentiation. Prenatal stress procedure was conducted starting at 15 days of pregnancy, and involved heat, light, and restraint. In the control group females were not stressed during the pregnancy. For the c-Fos study, the adult male (about 100 days old) was acclimated to a copulatory aquarium for 24 h and then the receptive female was introduced. After the first mount the animals were separated, but the male had access to the olfactory, visual, auditory cues from the female. The authors found less Fos-li in MPOA in prenatally stressed males when compared to the non-stressed ones. On the other hand, the animals did not differ significantly in mount latencies.

Rizvi et al. (1996) found in anaesthetized male rats that intensive stimulation of MPOA provoked an increase in Fos-li in midbrain periaqueductal gray (PAG). Using retrograde labelling of PAG neurones the authors also showed that Fos-positive neurones project to the rostroventral medulla. The authors suggest that MPOA exerts an effect on PAG and rostroventral medulla and this projection plays a functional role in regulating neuroendocrine, motor and autonomic responses during sexual behaviour.

## HAMSTERS

Kollack and Newman (1992) studied sexually naive Syrian hamsters exposed to three females, changed consecutively, during a single mating test lasting 55 min. Control males remained in their

home cages. The authors detected Fos-li in both control and trained animals in 20 subregions and subnuclei including MPOA, BNST, amygdala, and also piriform cortex, paraventricular nucleus of hypothalamus (PVN), lateral septum and NAc. After the session of intensive copulatory behaviour, Fos-li significantly increased in posteromedial BNST (pmBNST), magnocellular medial preoptic nucleus (mag MPN), caudal part of medial preoptic nucleus (cMPN), rostral part of MPN, PVN and posterointermediate BNST. At the level of the amygdala, mating induced Fos-li in the caudal part of posterodorsal MeAm, amygdalohippocampal area, rostral part of posterodorsal MeAm, anterodorsal MeAm, anteroventral MeAm, posteromedial cortical nucleus, and posterolateral cortical nucleus. In the caudal part of posterodorsal MeAm and pmBNST, characteristic dense Fos-li regions were observed. In each of these structures, Fos-li cells formed two distinct clusters of neurones. Parfitt and Newman (1995) suggested that activation of Fos-li cells clusters in pdMeAm was associated with onset of sexual satiety.

In another paper, Kollack-Walker and Newman (1995) compared Fos-li in different brain subnuclei in Syrian hamsters after copulatory or aggressive (agonistic) behaviour. Sexually naive males were taken from their home cages and moved to a dimly lighted table. Then they were returned to the home cages, and immediately afterwards they were given 10 min access to either a receptive female or to an intruder male, or they were left alone as a control. For a measure of the copulatory behaviour the number of ejaculations was counted. All mated males achieved a minimum of one (average 2.5) ejaculation. The authors found that in the posteroventral part of pmBNST, the caudal MPN, and the MPOA Fos-li was selectively increased after copulation. Both the aggressive and sexual behaviour significantly elevated Fos-li in many regions including: the anterodorsal MeAm, anteroventral MeAm, pd MeAm, caudal part of pd MeAm, amygdalohippocampal area, posteromedial cortical nuclei of amygdala, lateral septum, anteromedial BNST, posterointermediate BNST, anterodorsal level of

pmBNST, ventral premammillary nucleus of hypothalamus, PVN and ventromedial hypothalamus. In two of these structures, the caudal part of pdMeAm and anterodorsal pmBNST, significantly more Fos-li cells were found after copulatory than aggressive behaviour. The authors also observed that aggressive behaviour specifically increases Fos-li in midbrain, in the dorsal periaqueductal gray. In conclusion it was suggested that the increase in Fos-li in both aggressive and copulatory behaviour reflects a general state of arousal. However, these results also showed some neuronal circuits specifically activated during sexual behaviour.

Fiber et al. (1993) investigated the role of chemosensory cues from females (vaginal secretions) on Fos-li in the brains of male Syrian golden hamsters. The authors exposed the males to golden hamster vaginal secretions. As a control an exposure to either a clean cotton swab, Djungarian hamsters (*Phodopus sungorus*) vaginal secretions or peanut butter was used. Unfortunately, the authors provided no information about the sexual experience of the animals used in the experiment. The authors found a significant increase in the number of Fos-li cells in the accessory olfactory bulb granule cell layer and main olfactory bulb granule cells layer following the exposure to vaginal secretions coming from both species of hamsters, and in pmBNST, posterior subdivision of MeAm and mgnMPN after exposure to golden hamster vaginal secretions only.

In the experiments of Fernandez-Fewell and Meredith (1994) with golden hamsters, one group of sexually naive male (intact animals) - after 1 min of habituation - copulated for 45 min with two or three naturally cycling, behaviourally receptive females, changed consecutively. Another group of males was exposed only to females' vaginal secretions. As a control the authors used animals placed for 90 min in a clean box and with a cage lid opened at least twice providing a mildly arousing stimulus. In addition, other hamsters with either bilateral or unilateral removal of vomeronasal organs were either exposed to female vaginal secretions or mated, or left untouched as a control. Mating resulted in a significant increase in Fos-li in the acces-

sory olfactory bulb, MeAm, rostral pmBNST and MPOA. In the caudal part of pmBNST the number of Fos immunoreactive neurones increased in both intact and lesioned hamsters following mating as well as in males that did not mate but there were exposed to receptive females. In MeAm and the rostral part of pmBNST the authors found a characteristic pattern of activation with two clusters of nuclei. In the MPOA Fos-li increased only after copulation. Exposure to vaginal secretions activated Fos-li in accessory olfactory bulb, MeAm and caudal part of pmBNST. Males exposed to the same situation after unilaterally lesion of vomeronasal organ showed significantly higher Fos expression in ipsilateral side in accessory olfactory bulb and MeAm, but not in BNST and in MPOA.

#### OTHER SPECIES

Besides work on rats and hamsters, single studies dealt with mice, gerbils, ferrets, quail and macaques as well.

Baum et al. (1994) analysed 4 male mice for a single session lasting until one ejaculation. One hour later the animals were sacrificed and their brains processed for Fos immunocytochemistry. The authors found a striking increase in Fos expression - when compared to unmated controls - in MPOA, posterodorsal preoptic nucleus, BNST, MeAm, CTF, and rostro-lateral portion of the PVN.

Heeb and Yahr (1993) studied *c-fos* expression in the sexually dimorphic area of the gerbil (*Meriones unguiculatus*) hypothalamus as well as related areas during sexual activity. The males achieved either ejaculation or intromissions or mountings or they were only investigating the female. The authors found that mating induced Fos-li in sexually experienced males in several areas including sexually dimorphic area, NAc, caudomedial BNST, pdMeAm, ventral premammillary nucleus, PVN, central tegmental area and retrorubral field. Ejaculation produced the most pronounced labelling for Fos-li, and investigation of the female the least. The males that intromitted and/or ejaculated had Fos-li in ventral pallidum, and

only after ejaculation Fos-li in posterodorsal preoptic nucleus.

In the Japanese quail (*Conturnix japonica*) copulation induced Fos-li in the preoptic area, NAc and in the nucleus intercollicularis. Only a small percentage of Fos-li neurones were double labelled for Fos and either aromatase or testosterone (Balthazart et al. 1995).

In male ferrets (Lambert et al. 1992) mating significantly increased Fos-li in MeAm in mated vs. unpaired animals. The authors also found with double-labelling methods, that only a few neurones immunoreactive to LHRH were co-labelled with Fos-li.

In the macaques, Fos-li was detected in several structures important for sexual behaviour such as MPOA, PVN, ventromedial and arcuate nuclei of hypothalamus and MeAm in both mated and unmated males. No significant differences in Fos-li after copulation were found (Zumpe et al. 1995).

### **Hormones, neurotransmitters, neuropeptides and c-Fos expression in the copulating brain**

In addition to functional neuroanatomy of copulatory behaviour, its pharmacology was also investigated with c-Fos immunoreactivity as a tool.

Baum and Wersinger (1993) pretrained male rats twice with a receptive female until ejaculation. After that, males were castrated and during seven days (up to the testing day) received either testosterone propionate (T), dihydrotestosterone (DHTP), estradiol benzoate (EB) or oil vehicle. In addition, one group of intact animals not treated with the hormones was used. The experimental animals copulated until they achieved 8 intromissions. Additional controls were provided by castrated males receiving either EB or oil vehicle, and placed in empty arena for 15 min. Copulation resulted in an increase in the number of Fos-li neurones in MPOA, BNST, MeAm and CTF. No differences after mating were found between intact and hormonally-treated rats. The authors suggest that the increase in Fos-li in MPOA, BNST, MeAm and CTF observed after mating is hormone-independent.

Wood and Newman (1993) used double-labelling techniques to study coincidence between presence of androgen receptors and increased Fos-li in neurones after copulation in golden hamsters. The authors tested males with a receptive female during 30 min. Hamsters achieved a minimum of two ejaculations. The control males were placed for 30 min in an empty cage. After mating, Fos-li increased in several subnuclei of the amygdala (the posteromedial cortical nuclei, the posteroventral MeAm, the pdMeAm and the caudal part of the pdMeAm), in the BNST (the postero-intermediate BNST, the pmBNST), in the MPOA (the rostral MPN, caudal MPN and the magnocellular MPN) and the lateral septum. In the amygdala the authors found that 25-48% of Fos-li neurones contained androgen receptors, but no significant differences between subnuclei were found in this regard. In the postero-intermediate BNST and the pmBNST as well as in the caudal and the rostral MPN nearly 50% of Fos-li neurones contained androgen receptors. The greatest colocalization (70%) was observed in the magnocellular MPN.

Asmus and Newman (1994) using double-labelling methods for c-Fos and tyrosine hydroxylase containing neurones, reported high coincidences between both markers in MeAm, paraventricular hypothalamic nucleus (PVN) and nucleus of solitary tract (NST). The authors tested sexually naive golden hamsters for 10 min with sexually receptive females. All but one animal ejaculated. The handled controls were placed into a clean cage near a dim light for 10 min. Unhandled controls (animals staying in a home cage) were also used. In the posterior MeAm both controls and mated animals showed that a high percentage (from 55-67%) of Fos-immunoreactive neurones contained tyrosine hydroxylase. In the PVN and the NST a higher coincidence between tyrosine-hydroxylase containing neurones and Fos-li was detected after handling or mating, so it corresponds to high levels of arousal.

Witt and Insel (1994) investigated in rats Fos-li in PVN in neurones containing oxytocin. They pretrained males with sexually receptive female for 20 min during 3-4 weeks, 2-3 times per week. The

males were allocated to one of the three groups: (1) paired with an estrous female in a novel arena and copulating to 15 intromissions (approximately 18 mounts) but without ejaculation, (2) paired with an estrous female in a novel arena and achieved one ejaculation, and (3) for control males exposed to a novel arena with access to females odors. All observations were confined to a 15 min maximal testing period. Sexual behaviour induced elevated Fos-li in anterior parvocellular subdivision, medial parvocellular area, dorsal and medial parvocellular regions, posterior magnocellular area and lateral parvocellular region. They also found using double-labelling technique, that in lateral parvocellular subdivision located in the most caudal aspects of PVN, 31% of the Fos-li cells occurred in neurones containing oxytocin in ejaculated males, and 29% in those displaying only intromissions. This was in contrast to controls (exposed to a novel environment) where no double-labelled neurones containing Fos-li and oxytocin neurones were found.

### Functional role of c-Fos in sexual behaviour?

With the advent of techniques for homologous recombination in mice it became possible to create animals devoid of the *c-fos* gene (*c-fos* null mice, Johnson et al. 1992, Wang et al. 1992). Using the animals obtained by Johnson et al. (1992), Baum et al. (1994) investigated copulatory behaviour in male mice. The authors performed two experiments. During the first one males who were experienced – through several occasions of overnight exposure to estrous females were tested individually for 2 h with one or more estrous female(s) during the light phase of the light/dark cycle. The *c-fos* null mutants showed a clear deficiency in mounts (both number of mountings was decreased as well as their latency was increased), although this did not reach statistical significance, whereas the number of mounts per minute was on the order of two levels of magnitude lower in the mutants, and this difference was statistically significant. The mutants were unable to ejaculate during test. Interestingly,

the heterozygotes also did not ejaculate during the test, contrary to wild type homozygotes, 40% of which were found to ejaculate. After completion of these observations, each male was left overnight with an estrous female, and the next morning the presence of a seminal plug in the female's vagina was noted in the case of all wild type homozygotes as well as heterozygotes, but in 4 out of 6 *c-fos* null mutants.

In the second experiment the authors employed males that ejaculated in the first experiment as well as additional animals found to deposit a seminal plug during a night with an estrous female. This time the tests were conducted during the night. Males received 1-3 tests, and data from those males observed in 2-3 tests were averaged and these values together with values from males receiving a single test were used in the calculation of overall group means. The authors found that ano-genital investigation tended to be more pronounced in *c-fos* mutants than in both heterozygotes and wild type animals, although this tendency did not reach statistical significance. As in the first experiment, mutant homozygotes were deficient in mounting. However, no clear differences were noted for ejaculation and intromission characteristics.

These results raise a number of interesting issues. However, their interpretation is difficult since *c-fos* mutants are clearly disabled, being of much smaller weight, and suffering from serious developmental abnormalities, in particular related to osteogenesis. Unless other, similarly impaired mutants, such as osteopetrotic mutants (where the abnormality is of a different molecular nature), are investigated in parallel, no credible conclusions can be drawn.

### *c-fos* expression during sexual learning

Since the studies described above did not aim at distinguishing and comparing acquisition *versus* performance of the copulatory reaction, we decided to investigate the problem in more detail. In our experiments (Bialy et al. 1992), three groups of rats were consistently used: animals exposed for the



first time to the female, animals well trained to perform the copulatory task (7-8 sessions up to single ejaculation) and animals at intermediate stages of learning of the reaction (3-5 sessions up to single ejaculation each). Northern blot technique was used to provide an unambiguous tool to detect *c-fos* mRNA. Since in preliminary experiments clear induction of *c-fos* mRNA was observed in the parieto-occipital cortex, this brain region, encompassing various sensory cortices, was used for further studies. Surprisingly, no increase in *c-fos* mRNA level was noted after single training session. On the contrary, several-fold increase was observed after sessions 3, 4 and 5. Then low *c-fos* mRNA level was again observed after sessions 7 and 8. During the training three parameters of copulatory behaviour were measured, namely, latencies of mount, intromission and ejaculation. The first two shortened dramatically after the first and the second session. To the contrary, latency of ejaculation decreased only after 3-4 training sessions. In additional experiments (Biały et al., in preparation) we have found that blocking of NMDA receptors with either 0.1 mg/kg of MK-801, or 1 mg/kg CGP40116 prevented shortening of ejaculation latency but not mount and intromission latencies. Interestingly, MK-801 completely blocked increase of *c-fos* mRNA level during the fourth training session.

## CONCLUSIONS - THE QUESTIONS LINGER

Despite quite a number of studies dealing with *c-fos* and sexual behaviour of males, only a limited number of conclusions could be drawn. Definitely, there is an increase of Fos expression (most repeatedly shown on a protein level) related to sexual behaviour of males belonging to various rodent species. Most probably it is c-Fos that is activated, however, no proof has been provided in this regard. Such proof would be, e.g., immunoblotting analysis detecting c-Fos antigen, and/or wider employment of gene probes for combined *in situ* and Northern hybridization. The observed Fos expression is most easily seen in a group of subcortical structures im-

plicated previously in sexual behaviour, such as MPOA, MeAm, BNST, CTF, which are also known to be anatomically linked. Interestingly, in both MeAm and BNST two subregions could be delineated with the aid of Fos studies to correlate with intensive ejaculatory behaviour. Much less is said about other brain regions such as hippocampus and neocortex. Very little is known about pharmacology of the increase of *c-fos* expression. Similarly we still do not know how to answer the most intriguing question, i.e., function of the phenomenon. Finally, it is not evident whether this presumed elevation of *c-fos* expression is related to performance of copulatory behaviour, or to its acquisition. This distinction is of utmost importance since an overall unspecific arousal - obviously particularly strongly expressed during first sexual encounters - may contribute significantly to the observed phenomena, and obscure specific effects of sexual behaviour. Future studies based on rigorous, well defined training/testing procedures and involving developments in molecular biology, permitting functional investigations of transcription factors, should help us to address these issues in more detail.

## ACKNOWLEDGEMENT

This work was supported by grant KBN No 6 P 207 015 04.

## REFERENCES

- Asmus S.E., Newman S.W. (1994) Colocalization of tyrosine hydroxylase and Fos in the male Syrian hamster brain following different states of arousal. *J. Neurobiol.* 25: 156-168.
- Balthazart J., Meddle S.L., Wingfield C., Ramenofsky M., Foidart A., Follett B.K. (1995) Copulation activates *c-fos* expression in neurons of the quail preoptic area that do not normally contain aromatase. *Soc. Neurosci. Abstr.* 21: p. 701.
- Baum M.J., Brown J.J., Kica E., Rubin B.S., Johnson R.S., Papaioannou V.E. (1994) Effect of null mutation of the *c-fos* proto-oncogene on sexual behavior of male mice. *Biol. Reprod.* 50: 1040-1048.
- Baum M.J., Everitt B.J. (1992) Increased expression of *c-fos* in the medial preoptic area after mating in male rats: role

- of afferent inputs from the medial amygdala and midbrain central tegmental field. *Neuroscience* 50: 627-646.
- Baum M.J., Wersinger S.R. (1993) Equivalent levels of mating-induced neural *c-fos* immunoreactivity in castrated male rats given androgen, estrogen, or no steroid replacement. *Biol. Reprod.* 48: 1341-1347.
- Beach F.A. (1947) Evolutionary changes in the physiological control of mating behavior in mammals. *Psychol. Rev.* 54: 297-315.
- Biały M. (1995) Neurobiological basis of acquisition of sexual experience by male rats. Ph.D. thesis. Medical Academy of Warsaw, Warsaw, 134 p.
- Biały M., Beck J. (1993) The influence of vibrissae removal on copulatory behaviour in male rats. *Acta Neurobiol. Exp.* 53: 415-419.
- Biały M., Nikolaev E., Beck J., Kaczmarek L. (1992) Delayed *c-fos* expression in sensory cortex following sexual learning in male rats. *Mol. Brain Res.* 14: 352-356.
- Carr W.J., Loeb L.S., Dissinger M.E. (1965) Responses of rats to sex odors. *J. Comp. Physiol. Psychol.* 59: 370-377.
- Coolen L.M., Oliver B., Veening J.G. (1995) Fos immunoreactivity in the male rat brain, following 5-HT<sub>1A</sub> agonist 8-OH-DPAT-induced ejaculation: possible role for oxytocin. *Soc. Neurosci. Abstr.* 21: 701.
- Dewsbury D.A. (1969) Copulatory behaviour of rats (*Rattus norvegicus*) as a function of prior copulatory experience. *Anim. Behav.* 17: 217-223.
- Dewsbury D.A. (1972) Patterns of copulatory behavior in male mammals. *Q. Rev. Biol.* 190: 947-954.
- Domjan M. (1992) Adult learning and mate choice: possibilities and experimental evidence. *Am. Zool.* 32: 48-61.
- Fernandez-Fewell G.D., Meredith M. (1994) *c-fos* expression in vomeronasal pathways of mated or pheromone-stimulated male golden hamsters: contributions from vomeronasal sensory input and expression related to mating performance. *J. Neurosci.* 14: 3643-3654.
- Fiber J.M., Adames P., Swann J.M. (1993) Pheromones induce *c-fos* in limbic area regulating male hamster mating behavior. *NeuroReport* 4: 871-874.
- Goelet P., Castelluci V.F., Schacher S., Kandel E. R. (1986) The long and short of long term memory: a molecular framework. *Nature* 322: 419-423.
- Heeb M.M., Yahr P. (1993) C-Fos in the sexually dimorphic area (SDA) of the gerbil hypothalamus, and related areas, during sexual activity. *Soc. Neurosci. Abstr.* 19: 1019.
- Hughes P., Dragunow M. (1995) Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. *Pharmacol. Rev.* 47: 133-178.
- Humm J.L., Lambert K.G., Kinsley C.H. (1995) Paucity of *c-fos* expression in the medial preoptic area of prenatally stressed male rats following exposure to sexually receptive females. *Brain Res. Bull.* 37: 363-368.
- Johnson R.S., Spiegelman B.M., Papaioannou V. (1992) Pleiotropic effects of a null mutation of the *c-fos* proto-oncogene. *Cell* 71: 577-586.
- Kaczmarek L. (1992) Expression of *c-fos* and other genes encoding transcription factors in long-term potentiation. *Behav. Neural Biol.* 57: 263-266.
- Kaczmarek L. (1993a) Glutamate receptor-driven gene expression in learning. *Acta Neurobiol. Exp.* 53: 187-196.
- Kaczmarek L. (1993b) Molecular biology of vertebrate learning: is *c-fos* new beginning? *J. Neurosci. Res.* 34: 377-381.
- Kaczmarek L. (1994) Glutamate-evoked gene expression in brain cells - focus on transcription factors. *Amino Acids* 7: 245-254.
- Kaczmarek L. (1995) Towards understanding of the role of transcription factors in learning processes. *Acta Biochim. Pol.* 42: 221-226.
- Kaczmarek L., Kaminska B. (1989) Molecular biology of cell activation. *Exp. Cell Res.* 183: 24-35.
- Kaczmarek L., Nikolajew E. (1990) *c-fos* protooncogene expression and neuronal plasticity. *Acta Neurobiol. Exp.* 50: 173-179.
- Kollack S.S., Newman S.W. (1992) Mating behavior induces selective expression of Fos protein within the chemosensory pathways of the male Syrian hamster brain. *Neurosci. Lett.* 143: 223-228.
- Kollack-Walker S., Newman S.W. (1995) Mating and agonistic behavior produce different patterns of Fos immunolabeling in the male Syrian hamster brain. *Neuroscience* 66: 721-736.
- Lambert G.M., Rubin B.S., Baum M.J. (1992) Sex difference in the effect of mating on *c-fos* expression in luteinizing hormone-releasing hormone neurons of the ferret forebrain. *Endocrinology* 131: 1473-1480.
- Larsson K. (1956) Conditioning and sexual behavior in the male albino rat. *Acta Psychol. Gothoburgensia* 1: 1-269.
- Larsson K. (1959) Experience and maturation in the development of sexual behaviour in the male puberty rat. *Behaviour* 14: 101-107.
- Madlafousek J., Hlinak Z. (1983) Importance of females precopulatory behaviour for the primary initiation of males copulatory behaviour in laboratory rat. *Behaviour* 86: 237-249.
- Matuszczyk J.V., Larsson K. (1994) Experience modulates the influence of gonadal hormones on sexual orientation of male rats. *Physiol. Behav.* 55: 527-531.
- Morgan J.I., Curran T. (1991) Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annu. Rev. Neurosci.* 14: 421-451.
- Parfitt D.B., Newman S.W. (1995) Specific neuronal activation in the medial amygdala after mating is correlated with the onset of sexual satiety. *Soc. Neurosci. Abstr.* 21: p. 702.

- Pfaus J.G., Wilkins M.F. (1995) A novel environment disrupts copulation in sexually naive but not experienced male rats: reversal with naloxone. *Physiol. Behav.* 57: 1045-1049.
- Rizvi T.A., Murphy A.Z., Ennis M., Behbehani M.M., Shipley M.T. (1996) Medial Preoptic area afferents to periaqueductal gray medullo-output neurons: a combined Fos and tract tracing study. *J. Neurosci.* 16: 333-344.
- Robertson H.A., Dragunow M. (1990) From synapse to genome: The role of immediate early genes in permanent alterations in the central nervous system. In: *Current aspects of the neurosciences* (Ed. N.N. Osborne). Vol. 2. Macmillan, New York, p. 143-157.
- Robertson G.S., Pfaus J.G., Atkinson L.J., Matsumura H., Phillips A.G., Fibiger H.C. (1991) Sexual behavior increases *c-fos* expression in the forebrain of the male rat. *Brain Res.* 564: 352-357.
- Rose S.P.R. (1991) How chicks make memories: The cellular cascade from *c-fos* to dendritic remodeling. *Trends Neurosci.* 14: 390-397.
- Sachs B.D., Barfield R.J. (1976) Functional analysis of masculine copulatory behavior in the rats. *Adv. Study Behav.* 7: 91-154.
- Sachs B.D., Meisel R.L. (1988) The physiology of male sexual behaviour. In: *The physiology of reproduction* (Eds. E. Knobil and J. Neill). Raven Press, New York, p. 1393-1485.
- Sheng M., Greenberg M.E. (1990) The regulation and function of *c-fos* and other immediately early genes in the nervous system. *Neuron* 4: 477-485.
- Veening J.G., Coolen J.M.M., De Boer S.F., Koolhaas J.M. (1994) Sexual and aggressive behavior in the male rat: a Fos-study combined with immunostaining and retrograde tracing. *Eur. J. Neurosci. (Suppl.)* 7: 173.
- Vega-Matuszczyk J., Appa R.S., Larsson K. (1994) Age-dependent variations in the sexual preference of male rats. *Physiol. Behav.* 55: 827-830.
- Wang Z-Q., Ovitt C., Grigoriadis A.E., Mohle-Steinlein U., Ruther U., Wagner E.R. (1992) Bone and haematopoietic defects in mice lacking *c-fos*. *Nature* 360: 741-745.
- Wersinger S.R., Baum M.J., Erskine M.S. (1993) Mating-induced Fos-like immunoreactivity in the rat forebrain: a sex comparison and a dimorphic effect of pelvic nerve transection. *J. Neuroendocrinol.* 5: 557-568.
- Witt D.M., Insel T.R. (1994) Increased Fos expression in oxytocin neurones following masculine sexual behavior. *J. Neuroendocrinol.* 6: 13-18.
- Wood R.I., Newman S.W. (1993) Mating activates androgen receptor-containing neurons in chemosensory pathways of the male Syrian hamster brain. *Brain Res.* 614, 65-77.
- Zumpe D., Clancy A.N., Michael R.P. (1995). *c-fos* expression in the brain of mated and unmated macaques. *Soc. Neurosci. Abstr.* 21: p. 702.

*Received 21 February 1996, accepted 1 March 1996*

*This paper is dedicated to Professor Stella Niemierko on the occasion of her 90th birthday, with esteem and admiration*