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Sexual Experience Modulates Neuronal Activity in Male Japanese Quail

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Abstract

After an initial increase, repeated exposure to a particular stimulus or familiarity with an event results in lower immediate early gene expression levels in relevant brain structures. We predicted that similar effects would occur in Japanese quail after repeated sexual experience within brain areas involved in sexual behavior, namely, the medial preoptic nucleus (POM), the bed nucleus of stria terminalis (BST), and the nucleus taeniae of the amygdala (TnA), an avian homolog of medial amygdala. High experience subjects copulated with a female once on each of 16 consecutive days, whereas low experience subjects were allowed to copulate either once or twice. Control subjects were never exposed to a female. High experience subjects were faster to initiate sexual interaction, performed more cloacal contacts, and completed each cloacal contact faster than low experience subjects. Low experience subjects showed an increase in *egr-1* (ZENK) expression, an immediate early gene product used as marker of neural activation in birds, in the areas of interest. In contrast, in high experience animals, *egr-1* expression in the POM, BST and the periaqueductal gray (PAG) was not different than the level of expression in unmated controls. These results show that experience modulates the level of immediate early gene expression in the case of sexual behavior. Our results also indicate that immediate early gene expression in specific brain areas is not necessarily related to behavioral output, but depends on the behavioral history of the subjects.

Keywords

c-fos; learning; conditioning; mating; sexual behavior; plasticity; hippocampus; nidopallium; substantia grisea centralis; FOS

Introduction

In a variety of behavioral paradigms, initial exposure to a stimulus results in enhanced neural activity within relevant neural sites, as evidenced by increased expression of immediate early gene (IEG) precursor mRNAs or protein products. However, repeated exposures to the same stimulus or behavioral training results in lowered IEG expression. This phenomenon has been found with a variety of behavioral paradigms and stimulus types, including stress-inducing stimuli (Girotti, et al., 2006; Kollack-Walker et al. 1999; Stamp and Herbert 1999; Ryabinin, et al., 1999), spatial learning (Guzowski et al. 2001; Struthers et al., 2005), taste stimuli (Montag-Sallaz et al., 1999), visual stimuli (Zhu et al., 1995), and song learning (Mello and Clayton, 1994; Mello et al., 1995).

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However, the attenuation of IEG expression has not been examined with exposure to stimuli or behaviors (such as sexual activity) that involve complex sensory stimulation and motor coordination. Experience is an integral part of a fully developed and functioning sexual behavior system. Two closely related lines of research point out that, with experience, organisms become more adept and successful in sexual encounters, and that the sexual behavior becomes more robust. First, experience results in profound changes in sexual behavior and these changes lead to more successful copulations (Domjan, 1992; Pfaus et al., 2001; Woodson, 2002). Through repeated sexual interactions with a female conspecific, male rats increase the efficiency of their sexual behaviors. They become faster to initiate sexual contact, and ejaculate faster with fewer mounts and intromissions than do their inexperienced counterparts (Dewsbury, 1969; Larsson, 1959). In addition, sexually experienced animals are less prone to disruptive effects of experimental manipulations. For example, whereas bilateral olfactory bulb lesions cause longer intromission and ejaculation latencies in naïve rats, the negative effect of lesions is limited to longer ejaculation latencies in sexually experienced rats (Bermant and Taylor, 1969). Similar observations have been reported after removal of the vomeronasal organ in hamsters and rats (Meredith, 1986; Saito & Moltz, 1986) and with pharmacological manipulations of neurotransmitter systems such as nitric oxide synthase (Benelli et al., 1995) or castration (Costantini et al., 2007).

The medial preoptic nucleus (POM) is a key neural site involved in the control of male sexual behavior in vertebrate species and is interconnected to an extensive network of brain areas as shown by tract tracing studies (Absil et al., 2001; Absil et al., 2002a; Balthazart et al., 1994; Balthazart and Absil, 1997; Carere et al., 2007; Coolen, et al., 1998). This nucleus controls male sexual behavior in a variety of species, as evidenced in studies using electrical stimulation, cell recordings, lesions, hormone implants, local pharmacological manipulations (for a review see, Dominguez and Hull, 2005). Furthermore, the performance of male sexual behavior is associated with enhanced immediate early gene (IEG) expression such as c-Fos in this nucleus in a variety of mammalian species (Baum and Everitt, 1992; Coolen et al., 1996; Fernandez-Fewell and Meredith, 1994; Heeb and Yahr, 1996; Robertson et al. 1991).

In Japanese quail (*Coturnix japonica*), male sexual behavior is controlled by a network of brain areas including the POM, the bed nucleus of stria terminalis (BST), the nucleus taeniae of the amygdala (TnA, the avian equivalent of the medial amygdala) and the periaqueductal gray (PAG, also known as substantia grisea centralis in avian species). In this species, lesions of the POM completely block consummatory male sexual behavior and greatly reduce appetitive sexual behaviors (Balthazart et al., 1998), and stereotaxic implantation of testosterone into the POM is sufficient to restore sexual behavior in castrated male quail (Balthazart and Surlemont, 1990). In contrast, lesions in the BST disrupt only consummatory sexual responses to some degree in male quail (Balthazart et al., 1998), but, in male rats, lesions in this region cause deficits in both consummatory and appetitive behaviors (Liu et al., 1997). On the other hand, in male quail, lesions of the TnA increase consummatory sexual behavior over normal levels but do not affect appetitive responses, and therefore the TnA is thought to be involved in sexual satiety (Absil et al., 2002b, also see Thompson et al., 1998, for other findings). The PAG, is a relay area bridging the POM and motor aspects of sexual behavior, and, both in male Japanese quail and rat, receives extensive projections from the POM (Absil et al., 2001; Carere, 2007; Rizvi et al. 1992). Lesions of the PAG prevent penile responses normally induced in intact subjects by electrical stimulation of the POM in male rats (Marson, 2004). Experimentally-induced activation of the POM induces FOS expression in this area (Rizvi et al., 1996). Similarly, in male quail, copulation appears to stimulate FOS expression in the PAG, even though results are not always significant (Charlier et al., 2005; Meddle et al., 1997; Taziaux et al., 2006).

While the performance of male sexual behavior has been associated with enhanced expression of IEG products in areas controlling sexual behavior in male quail, such as FOS or egr-1 protein, as it is the case in mammalian species (Charlier et al. 2005; Meddle et al. 1997; Taziaux et al. 2006), these observations were reported either after subjects' first encounter with a female (Meddle et al. 1997), or in subjects with very limited sexual experience (Charlier et al., 2005; Taziaux et al., 2006). It is unclear whether repeated sexual activity would continue to produce enhanced IEG expression within the POM and associated areas with increasingly higher sexual experience. Accumulating experimental evidence indicates that increases in IEG expression often coincide with biologically significant events, such as novel experiences (Clayton; 2000). We hypothesized that elevated IEG expression, which has been observed after initial sexual encounters, is a result of the novelty of the sexual experience. If this is true, then IEG expression should be lower in brain areas related to sexual behavior in male Japanese quail given repeated sexual experience compared to subjects copulating for the first or second time. This prediction was evaluated in the present study by assessing IEG expression following the 1st, 2nd, or 16th copulatory episode in independent groups of Japanese quail. IEG expression was assessed through immunocytochemistry to egr-1, a commonly used marker of neural activity in birds also known as ZENK (Mello et al., 1992).

Materials and Methods

Subjects

Thirty-six sexually naïve, gonadally-intact male Japanese quail (*Coturnix japonica*) from the breeding colony maintained at the University of Texas at Austin served as experimental subjects. After hatching, chicks were kept in mixed-sex colony cages. Once 30 days old, as secondary sex characteristics became distinguishable, but well before sexual maturity was reached, male subjects were removed from the colony cages and placed in individual wire-mesh home cages for the rest of the experiment. Female quail were always kept in a separate room while not serving as copulation partners. Male subjects were distributed in home cages in such a way that they could not see other experimental quail copulating with females. All subjects and female copulation partners were between 12-14 weeks old at the beginning of the experiment. During all phases of the experiment, the animals were exposed to long days (16L: 8D; lights on at 6:00). Food and water were freely available in home cages. All experimental procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee and carried out in an AALAC-accredited facility.

Experimental Procedures

Subjects were randomly assigned to four groups (Groups 0, 1, 2, and 16). Subjects in the high sexual experience group (Group 16) (n=8) were given the opportunity to copulate with a female conspecific for a period of 10 minutes per day for 16 consecutive days in their home cages (25 × 27 × 31 cm). For Group 16, female copulation partners were rotated among the experimental subjects systematically so that each individual female copulated with each male twice throughout the experiment, once every 8th trial. For Group 2, two different female copulation partners were used for each subject. All subjects were monitored for the presence of sexual behavior in their first encounter with a female, and performed at least one cloacal contact in the observed trials.

A wood block (10 × 7 × 2 cm) was placed in the home cages of subjects in the low sexual experience groups for the first 14 (Group 2) (n=9) or 15 days (Group 1) (n=9) of the experiment for 10 minutes, as a control for non-specific disruption effects of introducing a female. The no-experience (baseline) group (Group 0) (n=10) received a wood block instead of a female for 10 minutes per day throughout the experiment for 16 days. On the 16th day of the experiment, subjects Groups 1, 2, and 16 copulated with a female prior to being sacrificed,

while Group 0 subjects again received a wood block. The last trial was videotaped for later behavioral observations. No cell count data were collected for one subject from Group 1 and another one from Group 2 due to tissue damage; nevertheless, behavioral data from these subjects were included in the statistical analyses.

Behavioral Observations

Four behavioral variables were quantified from video recordings: latency of the male to grab the neck of female, cloacal contact frequency (Wilson and Bermant, 1972), interaction duration with the female, and interaction duration/cloacal contact frequency ratio. Interaction duration was defined as the total amount of time that the male was in close contact with the female, including the time when the male chased the female with or without physical contact. In order to assess subjects' efficiency in performing copulatory behaviors, another measure was calculated by dividing interaction duration by the number cloacal contacts for each subject. This measure shows the average time it takes for subjects to successfully perform each cloacal contact during interaction with the female. No sexual behavioral data was collected for Group 0, since they never copulated during the experiment.

Tissue Preparation and Immunocytochemistry

All subjects were quickly decapitated 90 minutes after the beginning of the last trial. Brains were rapidly removed from the skull and fixed by immersion in 5% acrolein in 0.1 M KPBS buffer (pH. 7.2) for 3 hours at room temperature (Castelino and Ball, 2005). Then, brains were rinsed in 0.1 M KPBS buffer and placed first in 15% sucrose/KPBS solution overnight, and then 30% sucrose/KPBS solution until sunk. After that, brains were embedded in 8% gelatin molds until blocks solidified overnight and then frozen on dry ice and stored at -80°C (Deviche et al. 2000). Brains were sectioned with a cryostat at -15°C into 40- μm thick coronal sections distributed into three series and stored in cryoprotectant at -20°C until use (Watson et al., 1986).

Tissue sections were labeled for *egr-1* immunocytochemistry according to a previous protocol (Castelino and Ball, 2005). Sections were first rinsed in 0.05M TBS buffer (ph. 7.6) and then treated with 1% sodium borohydride for 10 minutes. Then, sections were placed for 25 minutes in a solution containing 1 % hydrogen peroxide to block endogenous peroxidase activity, 20% normal goat serum to prevent non-specific binding, and 0.3% Triton X-100 to increase permeability of section in TBS. After that, sections were incubated in *egr-1* antibody (rabbit polyclonal antibody, 1/1000 dilution; 200 $\mu\text{g}/\text{ml}$, sc-189, recognizing the C-terminus of human *egr-1*, (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.05M TBS buffer with 0.3 % Triton X-100 containing 2% NGS for 48 hours at 4°C . After rinsing, the sections were incubated in the secondary antibody (biotinylated goat anti-rabbit IgG; Vector Labs., Burlingame, CA) for 45 minutes. Tissue sections then were rinsed and placed in the tertiary (Vectastain ABC Elite Kit, Vector Labs. Burlingame, CA) for another 45 minutes. After the final rinsing, sections were incubated for 5 minutes in diaminobenzidine (DAB, Sigma-Aldrich, 0.5 mg/ml) in a 0.05M TBS buffer solution containing 0.05% hydrogen peroxide. Sections then were mounted on gelatin coated slides and coverslipped with permount.

Cell counts

Sections labeled by immunocytochemistry were digitized with a Coho CCD camera connected to a Nikon Eclipse E600 light microscope and coupled with a Macintosh computer. Immunoreactive nuclei in sample frames from these brain areas were digitized under 20x objective, and these images were quantified by ImageJ software bundle originally developed by NIH (Rasband, 2006). Samples for quantification (0.322×0.243 mm for all areas except hippocampus, for hippocampus size of quantification fields were 0.153×0.115 mm) were taken from the center of each selected area. For each area quantified, the density histogram

was adjusted with the gain to a standard value to normalize background levels. Cell counts were performed automatically by using the ImageJ software after standardized grey-level thresholding based on the density histogram (Shipley et al., 1989; David et al., 2004). Counts were corrected for double counting. All quantifications were made by an experimenter blind to group assignments.

Six brain areas, the POM, BST, TnA, PAG, Hp (hippocampus) and N (nidopallium) were selected for quantification (Foidart et al., 1994). These areas were identified using the chicken brain atlas (Kuenzel and Masson, 1988) and the quail brain atlas (Bayle et al., 1974). To locate the exact positions of quantified fields in these structures, the guidelines and methods detailed by Taziaux et al. (2006) were followed, for the most part, as explained below.

Briefly, for the POM, the section corresponding to Frame 8.2 in the chicken brain atlas (Kuenzel and Masson, 1988) and three more sections immediately rostral to this level were used for quantification. The BST was quantified at two different levels. For the rostral part, quantification was made on the section corresponding to Frame 8.2 in the chicken brain atlas (Kuenzel and Masson, 1988). For the caudal part, the section corresponding to Frame 7.6 was used and two quantification were sampled (Taziaux et al., 2006). Quantification for TnA was conducted on the same two sections that used for the BST (namely Frame 8.2 and 7.6). For the PAG, the section corresponding to Frame 4.6 was used as described in Charlier et al. (2005). For the Hp, three different levels corresponding to Frame 8.2, 7.6, and 6.4 of the chicken brain atlas (Kuenzel and Masson, 1988) were sampled, and at each level two quantification fields were quantified in a fashion described in Taziaux et al. (2007). For the N, samples were taken from sections corresponding to Frame 8.2 and 7.6. This area was located approximately 2.3 mm lateral to the intersection of the lateral ventricles and the interhemispherical fissure. All quantifications were made with a 20x objective on the microscope.

In all cases, samples were taken from both hemispheres of the brain. The measurements from different levels and hemispheres were averaged for each brain area. The individual averages were then compared between groups.

Data Analysis

All variables collected during this study were compared independently through one-way ANOVAs, followed by Tukey-Kramer multiple comparisons post-hoc tests. Statistical decisions were based on two-tailed tests and statistical significance level was selected as 0.05.

Results

Behavior

Major behavioral differences were observed between low experience and high experience subjects (Figure 2). High sexual experience subjects were faster to initiate copulation than low experience subjects, as measured by the timing of the first neck grab. An ANOVA revealed that there was an overall significant effect of group [$F(2, 23)=6.39, p<0.01$]. Tukey-Kramer multiple comparisons post-hoc test showed that both Group 2 ($p<0.05$) and Group 16 ($p<0.05$) were faster to initiate copulation than Group 1. There was also a significant overall effect of group in number of cloacal contacts [$F(2, 23)=3.61, p<0.05$]. High experience subjects (Group 16) performed more cloacal contacts as compared to low experience subjects (Group 1) ($p<0.05$), but there was no statistically significant difference between Group 2 and Group 1. The group differences in interaction duration were not significant [$F(2, 23)=1.5, p>0.05$]. However, there was an overall effect of groups in the measure of efficiency (interaction duration/number of cloacal contacts ratio) [$F(2, 23)=5.48, p<0.05$]. Post-hoc comparison

showed that Group 16 subjects completed cloacal contacts on average almost 4 times faster than Group 1 ($p < 0.01$).

Cell Counts

The number of egr-1-immunoreactive nuclei varied between groups within the POM, from a minimum in animals without sexual experience to a maximum in animals after 1 or 2 copulation sessions, and back to a minimum after 16 parings (Figure 3). An analysis of variance showed a significant overall effect of group [$F(3, 30) = 3.91$, $p < 0.05$]. Post-hoc tests showed that number of egr-1-immunoreactive nuclei in Group 1 was significantly higher than Group 0 ($p < 0.05$) and Group 16 ($p < 0.05$). However, neither Group 2, nor Group 16 were significantly different from Group 0.

In the BST, egr-1 immunoreactivity showed similar patterns of differences between groups. There was a significant overall group effect [$F(3, 29) = 6.64$, $p < 0.01$], and post-hoc tests revealed that whereas Group 1 and Group 2 had significantly higher egr-1 activity in this region than Group 0 ($p < 0.01$), there was no statistically significant difference between Group 16 and Group 0.

In the TnA, there was also a significant overall group effect [$F(3, 30) = 14.2$, $p < 0.001$]. Individual comparisons showed that each of Groups 1, 2, and 16 had higher egr-1 immunoreactivity than the baseline, Group 0 ($p < 0.001$, $p < 0.001$, and $p < 0.01$ respectively).

In the PAG, there was an overall group effect [$F(3, 28) = 4.382$, $p < 0.05$]. Post-hoc tests showed that number of egr-1 immunoreactive cells in Group 1 was higher than Group 0 ($p < 0.01$), but none of the other pairwise comparisons was statistically significant.

Finally, there was no statistically significant overall group effect in either the Hp [$F(3, 30) = 2.56$, $p > 0.05$], nor the N [$F = 0.271$, $p > 0.05$].

Discussion

Repeated opportunities to copulate with females substantially altered the sexual behavior of the male Japanese quail. Subjects with extensive sexual experience (Group 16) initiated sexual interaction considerably faster than first time copulators (Group 1) and made more cloacal contacts. In addition, high experience subjects spent far less time completing each cloacal contact compared to first time copulators with no previous sexual experience. This increase in efficiency can be considered a type of motor learning (Pfaus et al., 2001; Woodson, 2002). Changes in sexual behavior that result from experience can be interpreted as a manifestation of learning instrumental contingencies between correct motor responses and rewarding aspects of the successful completion of copulation (Crawford et al., 1993; Kagan, 1955; Pfaus et al., 2001; Woodson, 2002). Consistent with our results, instrumental conditioning has been shown to induce IEG activity initially but this effect declines with increasing levels of training (Bertaina-Anglade et al., 2000; Hernandez et al., 2006; Kelly and Deadwyler, 2003). It should be noted that instrumental learning does not have to be experimentally controlled to modify sexual behavior, and instrumental conditioning is not necessary for male quail to respond to females (Domjan et al. 1992). In the absence of explicit instrumental contingencies, sexual experience increases responding to species specific stimuli provided by potential mates (Domjan, 1992), such as visual features of females for male Japanese quail (Domjan and Hall, 1986; Nash et al., 1989) and male guppies (Magurran and Ramnarine, 2004), and olfactory cues from estrous females for male rats (Lydell and Doty, 1972).

We found a significant increase in egr-1 immunoreactivity in first time copulators (Group 1) compared to control subjects in four brain areas studied, the medial preoptic area, the bed

nucleus of stria terminalis, the nucleus taeniae of the amygdala, and the periaqueductal gray. These results are consistent with previous findings in male Japanese quail (Charlier et al., 2005; Meddle et al., 1997; Taziaux et al., 2006). However, more importantly, in our experiment, *egr-1* expression in the POM, BST, and PAG in Group 16 were not different than Group 0 levels, even though Group 16 subjects copulated in the last trial. These results show that elevated IEG activity in certain parts of sexual behavior circuitry is not an inevitable consequence of the performance of sexual behavior. Rather, it is modulated by sexual experience.

In the hippocampus, we did not find a statistically significant change in *egr-1* expression ($p=0.0735$). However, the pattern of expression was somewhat similar to that of the POM. This suggests that there is a possibility that IEG activity in the hippocampus may also be modulated by experience. Experimental evidence supports this idea. For example, it has been previously shown that FOS expression in hippocampi of male zebra finches increased dramatically after they were exposed to, and courted, a female conspecific compared to those subjects that were continuously kept in the aviary and, therefore, presumably already had courting experience (Sadananda and Bischof, 2004). In addition, the lack of any significant effect in *egr-1* expression in the nidopallium shows that changes in *egr-1* expression are specific to the sexual areas.

A previous study in male rats also tested the effects of repeated exposure to sexual stimulation on IEG expression (Lumley and Hull, 1999) and obtained results contradictory to ours. In this study, male rats with sexual experience showed more FOS immunoreactivity in the POM than male rats copulating for the first time. However, in this study sexual experience was limited to eight trials distributed over a 40-day period and IEG activity was measured with FOS expression. In addition, more than one third of subjects were excluded from the first-time copulating group due to lack of sexual behavior. Each of these factors, or a combination of them, may have been responsible for the difference between our findings and those of Lumley and Hull (1999).

Additional empirical evidence consistent with the hypothesis that repeated sexual interactions leads to levels of IEG expression similar to baseline levels is provided by previous experiments that did not find a difference in IEG expressions between copulated and control subjects. Typically, these studies tested subjects that had considerable sexual experience prior to the test trial. For example, researchers did not find an increase in FOS immunoreactivity in any of the brain areas of male cynomolgus monkeys (*Macaca fascicularis*) as a result of mating with a female conspecific (Michael et al., 1999). In another study, no significant difference was found in FOS immunoreactivity in the POM of male Japanese quail that copulated with a female conspecific and home-caged control subjects (Tlemcani et al., 2000). This finding contrasts with the results obtained by Meddle et al. (1997), who reported a robust increase in IEG expression in quail. The contrasting findings were probably due to differences in the sexual experience of the subjects. In the study by Tlemcani et al. (2000), experimental subjects were given 3 copulation pretests and 12 training trials of sexual experience with a female prior to the test trial as a part of a training protocol, whereas in Meddle et al. (1997) the subjects were sexually naïve first time copulators.

In addition to these IEG experiments, another study with male quail found no difference in 2-deoxyglucose uptake in the preoptic area except a modest increase in the rostral part between copulated and home cage controls (Dermon et al., 1999). This finding was puzzling since previous research indicated that the caudal part of this area plays a more important role in activation of sexual behavior (Panzica et al. 1996) and also shows high expression of FOS as a result of copulation (Meddle et al., 1997). Interestingly, in this study, the experimental design was virtually identical to that of Tlemcani et al. (2000) and therefore copulated subjects also

had extensive sexual experience prior to the test trial. This pattern of decline in metabolic activity fits well with findings of another 2-deoxyglucose study with male zebra finches in which researchers showed that engaging in courtship behavior increases 2-deoxyglucose uptake only if subjects have no prior courtship experience, while there is no change in experienced subjects as a result of courtship (Bischof and Herrmann, 1986). Consequently, the lack of a difference between copulated subjects and controls in all these studies in which subjects had extensive prior sexual experience is consistent with our findings.

Our results showed that *egr-1* expression in the nucleus taeniae of the amygdala (TnA) has a different pattern of activation than in the POM and the BST. We found that, unlike in the POM and BST, *egr-1* expression in the TnA increased as a result of copulation and was not differentially modulated by sexual experience. *Egr-1* activity in this region was higher in all copulated groups than non-copulated controls regardless of the level of sexual experience. The TnA is considered to be a homolog of mammalian medial amygdala (Cheng et al. 1999; Reiner et al., 2004; Roberts et al., 2002; Yamamoto et al., 2005). This area sends projections to the POM and the BST, among other brain sites (Canteras et al., 1995; Coolen and Wood, 1998) and is involved in control of sexual behavior (Absil et al., 2002b; Dominguez et al., 2001; Kondo, 1992; Newman, 1999; Thompson et al., 1998; Veening and Coolen, 1998). In particular, the medial amygdala acts as a relay station for transmission of sexually relevant chemosensory signals to the POM and the BST in male rodents (Baum and Everitt, 1992; Meredith, 1998) and this area shows IEG expression after mating (Heeb and Yahr, 1996; Wood and Newman, 1993).

The exact functional role of the TnA in avian species, which mainly rely on visual and auditory cues rather than olfactory stimuli for social interaction, needs to be better delineated (Absil et al., 2002b; Thompson et al., 1998). Experimental evidence from repeated social stress studies provides some insight into why IEG activity in the TnA is not modulated by repeated experience. A single social defeat induces *c-fos* expression in many brain sites, but after repeated social stress most of these areas show diminished IEG activity but the medial amygdala continues to express higher levels *c-fos* (Martinez et al., 1998). However this effect is limited to social stress and was not observed as a result of repeated restraint stress (Chen and Herbert, 1995). These parallels between repeated sexual experience and social stress suggest that differential IEG expression in the TnA may be related to its involvement in emotional aspects of sexual behavior.

Brain circuitry controlling the sexual behavior goes through extensive modifications as a result of experience. For example, researchers found that sexually experienced male rats had higher levels of nitric oxide synthase in the POM compared to first time copulators or completely naïve animals, regardless of whether or not the subjects mated prior to sacrificing (Dominguez et al., 2006). In male rats, extensive copulation experience increases cellular metabolic capacity, as measured by cytochrome oxidase activity, in the POM, BST, and the medial amygdala (Sakata et al., 2002). Cytochrome oxidase activity, unlike IEG expression, which is transient and occurs in response to acute stimulation, is closely associated with accumulated energy expenditure of neurons due to heightened activity (Sakata et al., 2005). Thus, cytochrome oxidase can be considered a marker of neuronal activity over a long period of time. Since, repeated sexual experience presumably causes higher total levels of neuronal activity in the POM, BST, and the medial amygdala, higher cytochrome oxidase activity in these areas is an expected outcome.

Even though it could be argued that changes in IEG expression in some brain areas is related to increases in plasma testosterone levels possibly occurring as a result of copulation, this interpretation is not valid in the case of male Japanese quail (Ball and Balthazart, 2001). While common in other species (Pinxten et al., 2003; Rundfeldt and Wingfield, 1985), this effect has

not been observed in male Japanese quail (Delville et al., 1984; Meddle et al., 1997). In addition, there is no evidence for an association between possibly enhanced testosterone levels and IEG expression after copulation in male quail. Gonadally intact and castrated animals treated with exogenous testosterone have similar levels of IEG expression after copulation (Charlier et al., 2005; Taziaux et al., 2006). Therefore, it is unlikely that IEG expression in the brain of quail after copulation reflects any changes in circulating levels of testosterone. The lack of increase in IEG expression in sexually experienced subjects in our study may have been the result of an overall decrease in neuronal activation because of habituation to sexual stimulation which presumably diminishes subjects' arousal in response to sexual stimuli or some long lasting changes in intracellular mechanisms which suppressed upregulation of IEG expression or rendered such increase unnecessary. Another possibility is that the effects of experience may be cumulative but transient, and the passage of time without additional experience may lessen its influence on IEG expression. Further studies should clarify this.

Taken together, the results of this study indicate that sexual experience causes major changes in sexual behavior and its neuronal circuitry, including lack of increase in IEG response to sexual interaction. This lack of increase in IEG expression is possibly related to lower biological significance of sexual interaction after repeated sexual experiences (Clayton, 2000). The IEG response to sexual behavior in a sense habituates over repeated interactions even though the sexual behaviors of experienced subjects are more efficient. Our findings identify a connection between learning and IEG expression in the sexual behavior system and indicate that the effect of sexual experience on IEG expression does not diminish in every brain area. Experience may differentially modulate brain areas involving different components of sexual behavior, depending on their functions in this behavior system.

In a broader sense, our results, along with findings in previous studies in other behavior systems, strongly suggest that, equating IEG expression with brain activity may not be valid in every situation. Since neuronal activity does not perfectly correlate with IEG expression all the time, interpretation of presence or lack of IEG expression should be made in the context of the behavioral history of the subjects and other experimental conditions (Pfaus and Heeb, 1997). Modifications in the brain as a result of experience are not limited to IEG expression, and the differences in *egr-1* levels that we observed here are a part of general changes in the brain. Future studies should focus on the functional significance of IEG expression in response to initial experiences in the context of the relationships of IEG with other experience-related changes in the brain and clarify the factors leading to lack of increase in IEG expression after repeated experiences both in the sexual behavior system and brain plasticity.

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Figure 1.

Schematic drawings showing the locations of brain areas selected for egr-1 immunoreactivity quantification, the medial preoptic area (POM), the bed nucleus of stria terminalis (BST), the nucleus taenia of the amygdala (TnA), the periaqueductal gray (PAG), the hippocampus (Hp) and the nidopallium (N).

Figure 2.

Behavioral comparisons (mean±S.E.M.) between subjects with different levels of sexual experience in the last copulation trial. Group names show the number of copulation trials that subjects in that group received prior to sacrificing. Group 0 is not shown since no behavioral data were collected from this group. Grab latency graph shows the time gap between the point when a female copulation partner is placed in the cage and the male's first neck grab. The cloacal contact frequency graph shows the average numbers of cloacal contacts performed during the last trial for each group. Interaction duration is the average time that subjects spent copulating with or chasing the female. Interaction duration/cloacal contact frequency ratio is calculated by dividing the interaction duration by the number of cloacal contacts performed, showing the average time that it took to complete one cloacal contact for each group of subjects.

Tukey-Kramer multiple comparisons test was used for post-hoc comparisons. “●” ($p < 0.05$) denotes a statistically significant difference compared to Group 1.

Figure 3. Photomicrographs showing *egr-1* immunoreactive nuclei in the POM at the level of 8.2 (Kuenzel and Masson, 1988). Sections are representative of their respective groups and selected from the subjects with the median number of immunoreactive (ir+) nuclei in each particular group. Scale bar= 100 μ m.

Figure 4.

Comparison of numbers of egr-1 immunoreactive (ir+) nuclei (mean±S.E.M.) within the medial preoptic area (POM), the bed nucleus of stria terminalis (BST), the nucleus taenia of amygdala (TnA), the periaqueductal gray (PAG), the hippocampus (Hp) and the nidopallium (N) between different sexual experience groups. Group names show number of copulation trials that subjects in that group received prior to sacrificing. Tukey-Kramer multiple comparisons test was used for post-hoc comparisons. “*” ($p<0.05$), “**” ($p<0.01$) and “***” ($p<0.001$) denote a statistically significant difference compared to Group 0. “•” ($p<0.05$) denotes a statistically significant difference compared to Group 1.