VENTRAL PALLIDUM SELF-STIMULATION INDUCES
STIMULUS DEPENDENT INCREASE IN C-FOS EXPRESSION
IN REWARD-RELATED BRAIN REGIONS

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Abstract—Neuronal expression of Fos, the protein product of the immediate early gene c-fos has been used as a high resolution metabolic marker for mapping polysynaptic pathways in the brain. We used Fos immunohistochemistry to reveal neuronal activation following self-stimulation of the ventral pallidum. Four groups of rats were allowed to self-stimulate for 30 min with 0.4 s trains of cathodal rectangular pulses of constant intensity (0.4 mA) and duration (0.1 ms). Each group was assigned a different pulse frequency, (3, 17, 24 and 50 pulses/stimulation train), which was preselected from within each animal’s rate–frequency function. The subjects that were assigned three pulses failed to self-stimulate and were considered as controls. The subjects that were assigned 17 pulses self-stimulated at half-maximal rate, whereas those that were assigned 24 and 50 pulses self-stimulated at maximal rates. The animals were sacrificed 90 min after the self-stimulation session and their brains were processed for Fos-like immunoreactivity. Fos-like immunoreactivity was found to increase as a function of pulse frequency in several brain regions known to be involved in drug and/or brain stimulation reward (medial prefrontal cortex, lateral septum, nucleus accumbens, lateral hypothalamus and ventral tegmental area), whereas it was not affected in structures devoid of such involvement (substantia nigra reticulata and dorsolateral striatum). The level of Fos expression induced by trains of 50 pulses was considerably higher than that produced by 24 pulses although both frequencies supported the same (maximal) self-stimulation rate.

This finding indicates that Fos expression correlated with reward magnitude (known to increase between these frequencies), not with bar-pressing rate, thus suggesting the presence of a reward-specific effect. The finding of a frequency-dependent Fos expression in a behavioural paradigm can be considered analogous to a pharmacological dose–response curve and, as such, our results may open new avenues for the use of Fos immunohistochemistry in quantitative neurobehavioural studies.

Key words: ventral pallidum, self-stimulation behaviour, C-fos immunohistochemistry, limbic forebrain, VTA, reward.

The ventral pallidum (VP) is interconnected with motor and limbic structures and may be considered as an interface between motivational and effector neural signals. This structure shares reciprocal connections with the ventral tegmental area (VTA) and the nucleus accumbens (NAC),19,25 two areas involved in brain stimulation and drug reward, and sends fibres to other reward implicated regions such as the prefrontal cortex, the lateral septum, the amygdala, the lateral hypothalamus, the habenula, and the pedunculopontine and raphe nuclei.19 The VP contributes to the regulation of drug self-administration,21 drug17,20 and sucrose-induced27 place preference, and modulates hypothalamic self-stimulation (SS).23,24 Furthermore, a recent study using moveable electrodes30 showed that electrical activation of nearly all VP sites maintains vigorous SS. Characterization of VP SS neurons using the double-pulse stimulation technique32 showed that post-stimulation recovery time differs substantially between VP SS sites: For some sites, partial neural recovery was apparent as short as 0.6 ms following the first conditioning pulse and was completed 1 ms later whereas, for other sites recovery started later and failed to get completed before 5 ms. Short and long refractory period sites were rather dispersed than topographically arranged within the VP. In addition, step-like recovery was noted for several VP sites.

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Abbreviations: DA, dopamine; DL-STR, dorsolateral striatum; HF, high frequency; LSI, intermediate aspect of the lateral septum; MF, moderate frequency; MF/B, medial forebrain bundle; mPFC, medial prefrontal cortex; NAC, nucleus accumbens; PBS, phosphate-buffered saline; PHF, passive high frequency; PLH, posterior lateral hypothalamus; SNR, substantia nigra reticulata; SS, self-stimulation; VHF, very high frequency; VLF, very low frequency; VP, ventral pallidum; VTA, ventral tegmental area.
sites, an indication that the electrode may have activated sets of neurons with non-overlapping refractory periods. The fastest recovery estimates were similar to those found for non-monoaminergic medial forebrain bundle (MFB) SS neurons whereas, the slowest overlapped with those found for medial prefrontal cortex (mPFC) SS sites and those estimates known for dopaminergic (DAergic) fibres.

The above findings may indicate that the VP represents the origin of, or the termination for, various fibre systems involved in SS. Finally, VP SS was found to be responsive to systemic manipulations of dopaminergic neurotransmission, opening the possibility that VP reward signals are relayed by mesolimbic-frontal DA neurons.

The findings reported above highlight the need for further characterization of VP’s reward signals. One research strategy would be the identification of areas that project to, or receive projections from, the VP. Application of this strategy is already obvious in studies which have successfully identified the brain regions that get activated following SS of MFB sites. In these studies the 2-deoxyglucose autoradiography, or the cytochrome oxidase staining or the neuronal activation is quantitatively determined through optical density measurements. In more recently developed immunohistochemistry methods the evaluation of neuronal activation is based on the number of cells that get activated. Relevant measurements may be considered as reflecting higher precision and as such may be more appropriate for parametric studies. Indeed Sagar et al. and Draganow and Faull suggested that c-fos expression and Fos immunohistochemistry may be used as a high resolution, cellular metabolic marker for mapping polysynaptic pathways in the brain. c-fos is a member of a small group of genes called “immediate early genes” and is rapidly expressed in neurons in response to a variety of physiological and pathological challenges such as noxious stimulation, activation of various receptors, administration of several psychoactive drugs, and kindling (for a review, see Ref. 22). Within this context, we have recently shown that burst stimulation of the MFB, which increases DA release in target areas, selectively increases Fos-like immunoreactivity in the limbic forebrain of the rat. These data underline the significance of the temporal organization of the action potentials of the midbrain DA neurons in conveying information to their target areas.

The present study was designed to evaluate the dynamic consequences of VP SS by determining Fos-like immunoreactivity in various brain regions of four groups of animals that were allowed to self-stimulate with different pulse frequency values. In order to cover the entire range of SS performance, the frequency values were selected from within the function relating SS rate to pulse frequency. Thus, the experimental design is analogous to the pharmacological dose–response protocol. Immunostaining for Fos, the proteins encoded by the nuclear proto-oncogene c-fos was used as a marker for neuronal activity in the brain regions examined.

**EXPERIMENTAL PROCEDURES**

Subjects and surgery

Twenty male Sprague-Dawley rats (Charles River, Italy), weighing 300–350 g at the time of surgery, were used. Before surgery, they were housed in groups of three and had free access to food and water in a temperature and humidity-controlled room, under a 12 h light/12 h dark cycle. All subjects were anaesthetized with ketamine hydrochloride (100 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) prior to stereotaxic surgery. Atropine sulfate (0.6 mg/kg, i.m.) was injected to reduce bronchial secretions. A moveable monopolar stimulating electrode (Model SME-01, Kinetrods, Ottawa, Canada) made of a plastic guiding base and a 0.25 mm diameter moveable stainless-steel wire, which was insulated except for its conically shaped tip, was stereotaxically implanted unilaterally into the VP. With the skull held horizontal, the stereotaxic coordinates were 0.3 mm posterior to bregma, 2.5 mm lateral to the midline and 7.8 mm below the skull surface. The anodal current returned through a flexible stainless-steel wire that was wrapped around five skull screws and soldered to a miniature Amphenol plug. The electrode assembly was chronically fixed to the skull with dental acrylic cement.

Efforts were made to minimize animal suffering. Animal care and the procedures used were in accordance with NIH public document 85-23 (1985).

Apparatus and procedure for self-stimulation

One week after surgery, the subjects were trained to self-stimulate by pressing a lever in a box made of transparent acrylic. Each bar-press triggered a constant-current generator that delivered a 0.4 s train of cathodal rectangular pulses of constant intensity and duration (0.4 mA and 0.1 ms, respectively) and variable frequency. The pulse frequency, i.e. the number of pulses within a train, was progressively increased up to 50/stimulation train until the subject showed vigorous SS. Following sufficient training with the selected frequency (five daily sessions of 40 min duration), the function relating bar-pressing rate/min to the pulse frequency was determined for each animal separately. A rate–frequency function was obtained from a series of 60 s SS trials. Two consecutive trials were separated by a 30 s period during which the stimulation was not available. Between trials, the pulse frequency was varied in ascending then in descending order twice, in order to cover the entire range of SS performance. Before each trial, the subject was primed with three stimulation trains using the pulse frequency that was destined for that trial. The rate–frequency function was replicated once daily, until the curve reached stability criterion (i.e. three consecutive sessions showing a lateral shift smaller than 0.05 log units of pulse frequency).

Following completion of this phase, the animals were randomly assigned in five groups of four. Four of these groups were allowed to self-stimulate for 30 min, each group with a different pulse frequency which was selected from each animal’s rate–frequency function described above (see also Fig. 2). For the first group, the pulse frequency, taken from the floor of the sigmoidal rate–frequency function, supported no SS (very low frequency (VLF) group). The second group was allowed to self-stimulate with the pulse frequency known to support half-maximal rate of SS (moderate frequency (MF) group). The third group was allowed to self-stimulate with the lowest frequency found to support maximum rate, that is, the frequency corresponding to the shoulder of the sigmoidal rate–frequency curve (high
frequency (HF) group). The fourth group was allowed to self-stimulate with a frequency approximately twice the value of HF (very high frequency VHF group). Finally, a fifth group received HF stimulation passively (PHF group). For this group, the stimulation trains were delivered automatically at the same average rate that was self-administered by these animals during the determination of their rate–frequency function.

**Fos immunohistochemistry**

Fos immunohistochemistry was performed as described by Robertson and Fibiger. Ninety minutes after completion of the SS session, the animals were deeply anaesthetized with sodium pentothal (100 mg/kg, i.p.) and transcardially perfused with saline (200 ml) followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Subsequently, the brains were removed, postfixed for 2 h in paraformaldehyde and placed in 30% sucrose in PBS for seven days. The brains were then cut in 30-µm coronal sections on a microtome.

Sections were washed in PBS (2 × 15 min) and placed in 0.3% H₂O₂ in PBS and then washed in PBS (3 × 20 min) before being incubated for 48 h in PBS containing 0.3% Triton X, 0.02% Na azide, 2% normal rabbit antibody and Fos primary antibody diluted 1:2500 (sheep polyclonal antibody, Cambridge Research Biochemicals OA-11-824). The sections were then washed in PBS (3 × 20 min), and incubated for 1 h with a biotinylated rabbit anti-sheep secondary antibody (Vector Laboratories) diluted 1:200. After washing in PBS (3 × 20 min) and then being incubated for 1 h in PBS containing avidin–biotinylated–horseradish peroxidase complex (1:100, Vector Laboratories), the sections were washed again in PBS and then rinsed for 10 min in 0.2 M Na acetate buffer. The reaction was visualized using a glucose oxidase 3,3-diaminobenzidine–nickel method terminated by washing in PBS. Sections were mounted on gelatin-coated slides, dehydrated and coverslipped for microscopic observation.

**Quantification of Fos-positive cells**

The number of Fos-positive nuclei was counted within a 500 × 500 µm grid placed over the mPFC, the NAC, the dorsolateral striatum (DL-STR), the intermediate aspect of the lateral septum (LSI), the posterior lateral hypothalamus (PLH), the VTA and the substantia nigra reticulata (SNR) at 250 × magnification. Cells were counted by two observers bilaterally in three slices from each region in all brains. The brain drawings shown in Fig. 3 illustrate the placement of the grid that was used to count the number of immunoreactive nuclei.

**Statistical analysis**

Between group differences in the number of Fos-positive nuclei within specified regions were evaluated using two-way ANOVA with independent variables the stimulation frequency and the side of the brain. For post hoc comparisons the Scheffé test was used.

**RESULTS**

Electrode tips placement was examined in 15 subjects. In Fig. 1 the various ventral pallidal sites reached by the electrode tips are represented. Figure 2 shows the mean rate of bar-press as a function of frequency values that were assigned to the various groups of subjects. All subjects of the VLF group were assigned three pulses, that is, a value much lower than that required to sustain minimal SS. For the MF group, the pulse number ranged from 16 to 18, depending on the subject (mean=17). Three subjects of the HF group received 25 pulses whereas one subject received 22 pulses (mean=24.25). All subjects of group VHF received 50 pulses, that is,
approximately twice the value required for maximal SS. One animal self-stimulating at VHF showed some signs of seizure activity. In view, however, of the finding that neither the distribution nor the number of \textit{c-fos} expressing cells differed between this particular animal and the other animals in the same group, the results of this animal were included in data analysis. Finally, for the animals of the passive group, the pulse number varied from 25 to 32/stimulation train (mean=30.25).

Fos-like immunoreactivity was quantified in several brain areas which were preselected on the basis of their involvement in reward and of their connections with the VP. Basal Fos-like immunoreactivity was noted in all regions examined, with numbers of immunoreactive nuclei varying from a few (NAC) to around 20 (VTA).

Figure 3 illustrates brain regions and Fig. 4 shows the number of Fos-like immunoreactive nuclei for the brain regions illustrated in Fig. 3. Significant stimulation frequency-dependent increases in the number of Fos-positive nuclei were revealed in the mPFC ($F_{4,15}$:9.38; $P<0.001$), NAC ($F_{4,15}$:25.32; $P<0.001$), LSI ($F_{4,15}$:14.39; $P<0.001$), PLH ($F_{4,15}$:7.44; $P<0.01$) and VTA ($F_{4,15}$:7.19; $P<0.01$). In contrast no increase in the number of Fos-positive nuclei was noticed within the DL-STR and SNR.

Significant brain side-dependent increases in the number of Fos-positive nuclei were also indicated in
Fig. 4. Mean ± S.E.M. number of Fos-like immunoreactive nuclei counted in various brain regions following self-administered stimulation of the VP (solid bars, stimulated hemisphere; open bars, unstimulated hemisphere). Asterisks point to a significant difference between the HF, the VHF or the PHF and the VLF group, and crosses point to a significant difference between ipsilateral and contralateral hemispheres, at probability levels of 0.05–0.001.
the NAC ($F_{1,15}:14.98; P<0.001$), the LSI ($F_{1,15}:12.41; P<0.01$), the PLH ($F_{1,12}:56.33; P<0.001$) and the VTA ($F_{1,12}:56.33; P<0.001$).

Statistical analysis yielded significant interaction (stimulation frequency × brain side) effect for the NAC ($F_{4,15}:3.10; P<0.05$), the LSI ($F_{4,15}:4.3; P<0.01$), the PLH ($F_{4,10}:11.18; P<0.001$) and the VTA ($F_{4,12}:12.09; P<0.001$). Post hoc comparisons demonstrated that VHF-stimulated subjects exhibited significantly greater number of Fos-positive nuclei than either VLF-, MF- or HF-stimulated animals in the ipsilateral to the stimulation mPFC ($P<0.01$), NAC ($P<0.001$), LSI ($P<0.001$), PLH ($P<0.001$), VTA ($P<0.001$) and in the contralateral to the stimulation mPFC ($P<0.01$) and NAC ($P<0.001$).

The PHF-stimulated animals exhibited significantly higher number of Fos-positive nuclei than the VLF-, MF- and HF-stimulated rats in the ipsilateral to the stimulation NAC ($P<0.01$) and the VTA ($P<0.001$).

The HF group was significantly different from VLF ($P<0.001$) only in the PLH. Furthermore it was only in the PLH and in the VTA that significant differences between stimulated and unstimulated side in HF ($P<0.05$) and VHF ($P<0.001$) groups were indicated by post hoc comparisons.

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Fig. 5. mPFC. Photomicrographs illustrating Fos-immunoreactive cells following self-administered stimulation of the VP (A, VLF-ipsilateral; B, MF-ipsilateral; C, HF-ipsilateral; D, PHF-ipsilateral; E, VHF-ipsilateral; F, VHF-contralateral). Scale bar=50 µm.
Figures 5–9 are microphotographs from various brain regions illustrating differences in Fos immunoreactivity between the stimulation conditions.

**DISCUSSION**

Self-stimulation of the VP increased Fos-like immunoreactivity in a frequency-dependent manner in five regions involved in brain stimulation and/or drug reward, i.e. the mPFC, NAC, LSI, PLH and the VTA, but it failed to affect the number of Fos-positive nuclei in two regions devoid of such involvement, such as the DL-STR and the SNR. Studies using anterograde tracers have shown that all positive regions receive large numbers of efferents from the VP, whereas the negative regions are either poorly (SNR) or not at all innervated by this structure. Efferents to VP neurons for some of the areas tested have been also noticed. Since, to our knowledge, Fos induction via antidromic stimulation has not been dismissed, such an alternative should also be taken into consideration. In the present study, the highest density of immunoreactive nuclei was noted in the mPFC and the PLH, whereas the NAC and the VTA showed lower density.
The observation of a frequency-dependent increase in the number of immunoreactive nuclei represents a finding of appreciable methodological significance. It means that self-stimulation combined with Fos immunohistochemistry can be used in a manner analogous to the dose–response curve in pharmacology. Thus, the frequency-dependent induction of Fos, here observed for the first time, provides the basis for the use of Fos immunohistochemistry in quantitative (parametric) neurobehavioural studies.

An auxiliary but important finding of the present study was that the number of immunoreactive cells did not correlate with bar-pressing rate. As illustrated in Fig. 2, both HF and VHF lay along the asymptote of the rate–frequency function, indicating that they maintained identical SS rates. Nevertheless, VHF resulted in appreciably larger counts of immunoreactive nuclei than HF, in all positive regions. Self-stimulation studies involving various brain regions using a two-lever paradigm, revealed that SS-related reward does not saturate at the frequency that elicits maximal SS rate (the HF) but rather grows exponentially until this frequency value is at least doubled. If this phenomenon holds also for VP self-stimulation then, the present data would indicate that Fos immunoreactivity correlated well

Fig. 7. LSI. Photomicrographs illustrating Fos-immunoreactive cells following self-administered stimulation of the VP (A, VLF-ipsilateral; B, MF-ipsilateral; C, HF-ipsilateral; D, PHF-ipsilateral; E, VHF-ipsilateral; F, VHF-contralateral). Scale bar=50 µm.

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with reward magnitude, but not with bar-pressing behaviour.

As reported above, reward may vary rapidly between the asymptotic frequencies (HF and VHF) used in the present study. However, another variable represents the amount of activation in various reward-irrelevant neural elements that may lie within the electrode’s stimulation field. This is to say that the correlation between reward magnitude and Fos-like immunoreactivity may necessarily not be considered to reflect a causal relationship.

Unilateral SS of the VP led to increased Fos-like immunoreactivity in both hemispheres, although the effect in the unstimulated hemisphere was generally found to be lower. Among the brain regions that showed immunoreactivity ipsilaterally following VHF stimulation, the mPFC, LSI and NAC showed appreciable levels of contralateral immunoreactivity, whereas the PLH and VTA showed little or no change. To our knowledge, innervation of the contralateral mPFC, LSI and NAC by the VP has not been reported. If the excitability thresholds of VP and MFB SS neurons are similar, (as suggested by their refractory periods), the pulse parameters in the present study should excite SS neurons within a radius of 0.4–0.5 mm. This is to say that activation...
by some of our electrodes of extra-VP fibres that may project contralaterally cannot be excluded. Alternatively, contralateral Fos expression may have been induced polysynaptically via VTA fibres known to traverse frontally to the other hemisphere.\_1^,5\_8\_43

Passive HF stimulation elicited a somewhat higher Fos-like activity than that obtained from animals which self-stimulated at the same average rate using equivalent pulse frequency. This finding implies that variables other than strength and average rate of stimulation contributed to the magnitude of Fos-like immunoreactivity in the passively-stimulated group. One such variable is probably the polysynaptic activation of neural elements involved in stress and/or frustration. In principle, a stress reaction and/or frustration can be expected in animals that are unable to self-regulate the priming effect of the rewarding stimulation, i.e. the component of the stimulation that drives the animal to seek another stimulation. Relevant to this hypothesis is the fact that animals learn to switch-off forced rewarding stimulation if a lever is made available to them\_36\_ and that experimental stress induces measurable Fos-immunoreactivity.\_6\_10\_41

In conclusion, rewarding electrical stimulation of the VP increased Fos immunoreactivity in a
frequency-dependent manner in several brain structures known to participate in the regulation of drug and/or brain stimulation reward (PLH, NAC, LSI, VTA and mPFC), and failed to do so in structures devoid of such involvement (SNR and DL-STR). This finding, and the fact that Fos-immunoreactivity correlated with reward magnitude, but not with bar-pressing behaviour, suggests a reward-specific effect. In this regard, recent studies have shown that reward related behaviours such as sexual contact or cocaine-induced place preference as well as bar-pressing behaviour, suggests a reward-specific correlated with reward magnitude, but not with This finding, and the fact that Fos-immunoreactivity devoid of such involvement (SNR and DL-STR).

Limbic forebrain DAergic neurons have been repeatedly implicated in the mediation of reward-related processes. In this context of particular interest are the very recent data of Chergui et al. showing that burst firing of the MFB correlates with large dopamine overflow and c-fos induction in target limbic areas of the rat brain such as the NAC-shell and the lateral septum. More specifically, the role of DA in reward processes initiated in VP is supported by recent findings. For instance, morphine injections into the VP are associated with both place preference (Y. Anagnostakis and C. Spyraki, unpublished observations) and increased DAergic metabolic activity in the NAC. Also, VP SS is sensitive to pharmacologically-induced manipulations of DAergic neurotransmission. It should be noted, however, that neither for MFB SS nor for VP SS there is convincing evidence that dopaminergic neurons constitute a substantial component of the directly activated fibres mediating brain stimulation reward.

The data delimited various brain regions that get activated by rewarding VP SS. We are currently applying the behavioural version of the collision test between the VP and each of these regions in an aim to reveal which of these regions, if any, is linked to the VP with reward neurons.

**CONCLUSION**

This study provides evidence that VP SS is associated with increased Fos-like immunoreactivity in limbic areas, such as mPFC, LSI, NAC, PLH, and VTA, but not motor-related structures, such as DL-STR and SNR. The increase in c-fos expression in the above mentioned brain regions appeared to be frequency, but not bar-pressing rate dependent. Thus, it is suggested that the increase in c-fos expression induced by VP SS represent a reward-specific effect which is mediated by the well-known limbic reward-related circuit.

Acknowledgements—This work was supported by grants from the General Secretariat of Research and Technology of Greece (PENED - Nr 91ED665) to C.S.; the Medical Research Council of Sweden (projects 4747 and 11026) to G.G.N. and T.H.S.; the University of Crete and the Karolinska Institute. G.P was supported by a short-term fellowship from the European Science Foundation (European Neuroscience Programme). The excellent technical assistance of Mrs Anna Malmerfelt and Mrs Annika Olsson is gratefully acknowledged.

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