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Comparison of the locomotor activating effects of bicuculline infusions into the preoptic area and ventral pallidum

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Abstract

Ambulatory locomotion in the rodent is robustly activated by unilateral infusions into the basal forebrain of type A gamma-aminobutyric acid ($GABA_A$) receptor antagonists, such as bicuculline and picrotoxin. The present study was carried out to better localize the neuroanatomical substrate(s) underlying this effect. To accomplish this, differences in total locomotion accumulated during a 20 minute test period following bicuculline versus saline infusions in male Sprague-Dawley rats were calculated, rank ordered and mapped on a diagram of basal forebrain transposed from immunoprocessed sections. The most robust locomotor activation was elicited by bicuculline infusions clustered in rostral parts of the preoptic area. Unilateral infusions of bicuculline into the ventral pallidum produced an unanticipatedly diminutive activation of locomotion, which led us to evaluate bilateral ventral pallidal infusions, and these also produced only a small activation of locomotion, and, interestingly, a non-significant trend toward suppression of rearing. Subjects with bicuculline infused bilaterally into the ventral pallidum also exhibited persistent bouts of abnormal movements. Bicuculline infused unilaterally into other forebrain structures, including the bed nucleus of stria terminalis, caudate-putamen, globus pallidus, sublenticular extended amygdala and sublenticular substantia innominata, did not produce significant locomotor activation. Our data identify the rostral preoptic area as the main substrate for the locomotor activating effects of basal forebrain bicuculline infusions. In contrast, slight activation of locomotion and no effect on rearing accompanied unilateral and bilateral ventral pallidal infusions. Implications of these findings for forebrain processing of reward are discussed.

Keywords

lateral preoptic area; ventral pallidum; substantia innominata; dopamine; reward; $GABA_A$ receptor antagonist

Introduction

It was shown years ago that ambulatory locomotion in the rodent is robustly activated by unilateral infusions of picrotoxin, a type A gamma-aminobutyric acid ($GABA_A$) receptor antagonist, into the basal forebrain (Mogenson and Nielsen, 1983; Mogenson et al., 1985). Whereas much of the ensuing literature attributed this and related effects of various GABAergic, glutamatergic and peptide receptor ligands to actions in the lateral preoptic area, ‘subpallidal region’ and substantia innominata (Mogenson et al., 1983;1985; 1993; Shreve and Uretsky, 1988; 1989; 1991; Mogenson and Yang, 1991), other influential papers

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(Austin and Kalivas, 1989; 1990; 1991; Kalivas et al., 1991; Johnson et al., 1996) appear to have led to a consensus view (see, e.g., Willens et al., 1992; Gong et al., 1999; Johnson and Napier, 2000; Chen et al., 2001; June et al., 2003; Hubert et al., 2010) that the effective structure is the then relatively recently described ventral pallidum (Heimer et al., 1972; Heimer and Wilson, 1975; Switzer et al., 1982). Ventral pallidum (VP) since has been implicated in reward mechanisms (Johnson et al., 1996; Tindell et al., 2004; 2005; 2006), control of population activity of midbrain dopaminergic neurons (Floresco et al., 2003) and transmission to midbrain dopaminergic neurons of signals originating in the hippocampus (Lodge and Grace, 2006). Almost exclusively bilateral drug infusions have been used to elicit locomotion from the VP, such that subsequent to the earlier reports of Mogenson and his colleagues (Mogenson and Nielsen, 1983; Mogenson et al., 1985) the literature seems to convey that forebrain-elicited locomotor activation requires bilateral infusions.

During the process of formulating our recent report on robust locomotor activations elicited by unilateral infusions of bicuculline, another $GABA_A$ receptor antagonist, into the preoptic area (Reynolds et al., 2006) we noticed that a number of early studies also mapped their effective infusion sites more prominently to the preoptic area than ventral pallidum (Mogenson and Nielsen, 1983; Shreve and Uretsky, 1988; 1989; 1991; Willens et al., 1992). Consequently, we did the present study in order to revisit the localization of basal forebrain site(s) most effective in producing locomotor activation.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 225–250g were used in accordance with policy mandated in the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (http://grants.nih.gov/grants/olaw/references/phspol.htm) provided by the U.S. Department of Health and Human Services. The rats were housed on a 12-hour light-dark cycle in groups of two to four until surgeries were done, after which all were singly housed. Access to food and water was provided *ad libitum.*

Immunohistochemistry

Rats were anesthetized with a 1.6 ml/kg i.p. injection of a solution containing ketamine (100 mg/ml), xylazine (20 mg/ml) and saline (0.9%) at a volume ratio of 9:7:4, resulting in final doses of ketamine and xylazine of 72 and 11.2 mg/kg, respectively. Under deep anesthesia, the rats then were killed by vascular perfusion with a solution of 0.1 M Sorenson's phosphate buffer (SPB, pH 7.4) containing 4.0% paraformaldehyde and 2.5% sucrose, preceded by a brief rinse with 0.01 M SPB-buffered saline containing 0.5% procaine and 2.5% sucrose. The brains were removed immediately and stored for at least four hours in the fixative solution, and then transferred to SPB and, after at least an hour, were sunk in SPB containing 30% sucrose. Then, frozen sections at 50 μm were taken with a sliding microtome. The sections were stored at −20° C in a solution of 0.1 M SPB containing 30% ethylene glycol and 30% sucrose pending further processing.

Sections were retrieved from cryoprotectant, rinsed in SPB and treated for 15 minutes in 1% sodium borohydride after which they were subjected to additional multiple rinses in SPB to remove all traces of sodium borohydride. The sections were then immersed at room temperature with agitation into SPB containing 0.1% triton X-100 (SPB/triton) and antibodies against parvalbumin (PV) or nitric oxide synthase (NOS), which in previoius studies done in our laboratory were revealed as excellent markers of basal forebrain neuroanatomical organization (Zahm et al., 2003; 2011; 2013). The following morning the sections were rinsed in SPB and immersed for 1.5 hours in SPB/triton containing

biotinylated anti-mouse antibodies made in horse (Vector, Burlingame, CA, used at a dilution of 1:200), again rinsed, and then placed for 1–2 hours in SPB/triton containing avidin-D/biotinylated horseradish peroxidase complex (ABC, Vector, used at a dilution of 1:200). After further rinses, the sections were reacted in one of two ways. For preparations in which the intent was to reveal only PV (n=5) or NOS (n=5) immunoreactivity, DAB chromogen was generated with the aid of a coupled glucose oxidase reaction (Itoh et al., 1979). Briefly, sections were placed in a solution of 0.05 M SPB containing DAB (0.05%), β-D-glucose (0.2%), NH_4Cl (0.04%), and 0.0004% glucose oxidase (Sigma) for 20–40 minutes after which they were rinsed in distilled water and mounted in serial order on glass slides. Then the DAB reaction product was intensified by placing the slides sequentially through 0.005% aqueous OsO_4 and aqueous thiocarbohydrazide (0.1 mg/100ml H_2O) followed by a second immersion in the osmium solution, with brief rinses between. Sections reacted with PV antibodies and intended to undergo further processing to reveal immunoreactivity against calbindin-D 28 kD (CB, n=3), another good marker of basal forebrain organization (Zahm, 1999), were instead reacted in a solution containing 15 mg of DAB dissolved in 42 ml of distilled water to which was added 8 ml of 5% aqueous nickel ammonium sulfate, 50 ml of 0.05 M Tris-HCl buffer (pH 8.0) and 20 μl of 3% H_2O_2. After ten minutes, these sections were rinsed and then placed overnight in SPB/triton containing mouse anti-CB antibodies. The following morning they were rinsed in SPB/triton and immersed in SPB containing unconjugated biotin (Sigma, at a concentration of 0.01%) for 1 hour in order to block unreacted biotin sites on avidin already present in the sections. After further rinsing in SPB, the sections were again immersed for 1–2 hours at room temperature in SPB containing biotinylated anti-mouse IgGs made in horse (Vector, used at 1:200) and, after further rinsing, in SPB containing ABC reagents (Vector, used as described above). The sections were again rinsed three times in SPB prior to being reacted with DAB using the coupled glucose oxidase method as described above, after which they were rinsed and mounted in serial order on glass slides. Mounted sections were coverslipped with Permount (Fisher, St. Louis, MO).

Primary antibodies and specificity controls

Omission of primary antibodies produced tissue lacking specific immunoreactivity. Where the antigen was available to us, we did preabsorbtion controls (see below under *anti-NOS*). For anti-CB and anti-PV, we relied on information provided by the vendors regarding primary antibody specificity (see below). Patterns of immunostaining generated by these antibodies in our hands are consistent with those published in the literature (e.g., for anti CB and anti-PV - Celio and Heizmann, 1981, Gerfen et al., 1985, Cowan et al., 1990; for anti-NOS - Rodrigo et al., 1994). Additional information on the characterization of the antibodies and specificity controls is given below.

anti-CB—This is a monoclonal antibody raised against purified bovine kidney calbindin-D 28 kD and isolated from mouse ascites fluid (Sigma Chemical Company, St. Louis, MO, cat. # C9848) The vendor states that [1] the antibody does not react with other members of the EF-hand family such as calbindin-D 9K, calretinin, myosin light chain, parvalbumin, S-100a, S-100b, S100A2 (S100L) and S100A6 (calcyclin); [2] species cross-reactivity was observed with human, bovine, goat, sheep, porcine, rabbit, dog, cat, guinea-pig, rat and mouse; [3] a weaker reactivity was observed with chicken CB.

anti-NOS—This is a polyclonal antibody raised in rabbit against amino acids 251–270 of nitric oxide synthase (GDNDRVFNDLWGKDNVPVILC) conjugated to keyole limpet cyanin (Sigma, cat. # N7155). NOS immunoreactivity was abolished in our hands by preabsorption with the cognate peptide (10 μg/ml).

anti-PV—This is a monoclonal antibody raised against purified carp muscle parvalbumin and isolated from mouse ascites fluid (Sigma, clone PA-235, cat. # P3088). The vendor states that [1] the antibody is immunospecific for parvalbumin as determined by indirect immunoperoxidase staining and immunoblotting; [2] the antibody reacts specifically with parvalbumin of cultured nerve cells and tissue originating from human, monkey, rat, mouse, chicken and fish; [3] it specifically stains the ^{45}Ca-binding spot of parvalbumin (M.W. 12,000, pI of 4.9) by immunobinding.

Maps and photomicrographs

Basal forebrain territories were photographed (Fig. 1A–D) and mapped (Fig. 1E and F) according to patterns of immunohistochemical staining in representative frontal sections. Mapping was done under brightfield optics (Olympus BX51) with the aid of the Neurolucida dedicated hardware-software platform (MFB Bioscience, Williston, VT). Digital micrographs were captured using a Nikon Optiphot microscope and Q Imaging Fast 1394 digital camera and adjusted for brightness and contrast with Adobe Photoshop (CS2) software. The plates were constructed using Adobe Illustrator (CS2) software.

Placement of guide cannulae

Rats were deeply anesthetized as described above and placed in a Kopf stereotaxic instrument. An incision was made to expose the skull in which a small burr hole was drilled in line with the intended injection site. Guide cannulae, consisting of 13 mm lengths of 22 gauge stainless steel tubing (PlasticsOne, Roanoke, VA) were implanted in the openings. Unilateral guide cannulae aimed at the preoptic area (n=48) or other basal forebrain structures, including the bed nucleus of stria terminalis (BST, n=6), sublenticular extended amygdala (SLEA, n=7), caudate-putamen (CPu, n=5), sublenticular substantia innominata (SLSI, n=5) and globus pallidus (GP, n=5), were implanted at an angle of 20° in the mediolateral plane so as to avoid the lateral ventricle. Unilateral guide cannulae aimed at the ventral pallidum (n=13) were implanted mainly vertically. Another group of rats was implanted with bilateral guide cannulae aimed vertically at the ventral pallidum on both sides of the midline (n=30). A final group of rats was implanted with a single vertical guide cannula targeting the left lateral ventricle (n=3). All guide cannulae were set to a depth 2 mm short of the center of the intended target. Cannulae were secured with dental cement anchored to stainless steel screws turned into the skull. Stainless steel wire obturators were inserted into the guide cannulae to maintain patency. The incisions were closed with cyanoacrylate glue and the rats were kept warm until they awakened. A solution containing lidocaine was infused into the incisions before they were closed. The rats were given an i.p. injection of Rimadyl (carprophen, 10 mg/kg) before they awoke and on each of the next three days.

Behavioral testing

Three days after cannulae implantations and at least 20 minutes prior to testing, the rats were brought to the behavioral studies room, where locomotion was measured in standard activity monitoring chambers, each comprising a square, white floor space (43.2 × 43.2 cm) bounded on the sides by clear plexiglass walls 30.5 cm in height (Med Associates, St. Albans, VT). Fixtures containing horizontal rows of 16 point-source infrared illuminators and detectors spaced at 2.54 cm were bracketed to the walls on opposite sides of the chambers at heights of 2.54 and 12.7 cm from the floor. The lower of these comprised two sets of illuminators and detectors mounted on adjacent walls at a right angle to each other so as to form a grid of 16 × 16 intersecting beams for measurement of horizontal (forward) locomotion, whereas the upper consisted of a single illuminator/detector pair, which detected vertical locomotion (rearing). Beam breaks were evaluated with the aid of Activity Monitor software (Med

Associates). The monitoring chambers were housed inside dimly illuminated sound attenuating chambers with exhaust fans running (Med Associates). Prior to being placed in the activity monitoring chambers, the rats were removed from the home cages, the obturators were removed and injector cannulae connected by a small length of polyethylene tubing (PE 20 0.38/1.09, Stoelting, Wood Dale, IL, cat. # 51155) to the Luer needle of a 1.0 μl Hamilton syringe were inserted into the guide cannulae. To mitigate the resistance of brain tissue at the injector tip to the flow of infusate and thus facilitate its uniform spread into the targeted area, a mock injector extending 2 mm past the ends of the guide cannulae was inserted briefly and then removed after which the actual injection cannula was inserted and advanced 1.5 mm past the ends of the guide cannulae. On all subsequent trials only the injector cannula was inserted and always advanced 1.5 mm past the guide cannula. The infusate was propelled by a syringe pump (Harvard Apparatus, Holliston, MA, model '11' plus) calibrated to eject solution at a rate of 0.25 μl/min.

The infusions were done mainly using groups of six rats of which each was first infused with saline for 1 minute at a rate of 0.25 μl/minute. One minute after the infusions were completed, the rats were placed in the locomotor apparatus and beam breaks were monitored for a period of 20 minutes, after which the rats were returned to their home cages. After about a half hour rest, the rats were again infused, this time with 0.25 μl of saline containing 1(S),9(R)-(−)-bicuculline methbromide (Sigma, St. Louis, MO) prepared at a dilution of 1 mg bicuculline / 3 ml saline, which resulted in the delivery of 67 ng (0.18 nmol) of bicuculline. After 1 minute, the rats were placed in the activity chambers and monitored for 20 minutes as before. The concentration of bicuculline infused in the present study produced the most robust locomotor activation in our previous investigation (Reynolds et al., 2006).

Fold difference analysis and mapping of locomotion

In order to localize sites in the forebrain from which locomotor activation was most effectively elicited by bicuculline infusions, bicuculline-elicited responses were expressed in relation to those elicited by immediately preceding saline infusions (see preceding section) as fold differences [beam breaks/20 minutes following bicuculline infusion]/[beam breaks/ 20 minutes following saline infusion]. Fold differences were rank ordered as follows: 1 = 0–1.49 fold; 2 = 1.5–2.49 fold; 3 = 2.5–3.49 fold; 4 = > 3.5 fold; and the ranks were coded in symbols and mapped (see Results). Where more than one infusion was tested at a given site (see Table 1), the maximum locomotor response to bicucullline at that site was used to prepare the map.

Localization of infusions

The rats were deeply anesthetized and perfused precisely as described above. The perfused brains were removed and placed in the same fixative for at least four hours and then overnight at 4° C with agitation in distilled H_2O containing 30% sucrose. Five adjacent series of 50 μm thick sections were cut on a cryostat of which the first was thaw mounted on subbed glass slides and the rest were collected and stored in separate glass vials at −20° C in cryoprotectant consisting of 0.1 M SPB containing 30% sucrose and 30% ethylene glycol. The mounted sections were defatted in Coplin jars in 1:1 alcohol/chloroform for at least four hours, immersed in 0.1% cresyl violet solution for 5–10 minutes, rinsed quickly in distilled water, differentiated in 95% ethyl alcohol for 2–30 minutes, dehydrated in 100% alcohol 2×5 min, cleared in xylene 2×5 minutes and coverslipped with Permount. The ends of the infusion cannula tracts were identified by light microscopy with the 2x objective and plotted visually onto maps prepared by reference to the immunohistochemical preparations (see Figs. 1 and 2).

Results

Anatomy

Fig. 1A and B reveals that NOS immunoreactivity (-ir) is moderately to heavily expressed in extended amygdala structures, including the bed nucleus of stria terminalis (BST), interstitial nucleus of the posterior limb of the anterior commissure (IPAC) and sublenticular extended amygdala (SLEA). In contrast, NOS-ir (brown in Fig. 1A and B) is nearly absent in the ventral pallidum (VP) and has an intermediate level of expression in the lateral preoptic area (LPO). Material processed to exhibit PV-ir (black in Fig. 1C and D) reveals boundaries between the LPO, extended amygdala structures and ventral pallidum similar to those shown by NOS-ir. PV-ir neurons are numerous in striatopallidal structures, particularly VP (Fig. 1C), and, to a lesser degree, LPO. Fig. 1C and D also show that CB-ir (brown) is less heavily expressed in extended amygdala structures, as compared to striatopallidal. In view of the coherence of these several patterns of immunoreactivity, which is consistent with much literature cited in the Introduction, Materials and Methods and Discussion, they were used in the present study to aid in the generation of diagrams showing boundaries between basal forebrain structures, representative examples of which are shown in Figures 1E and F. Such diagrams were utilized to localize bicuculline infusion sites (Fig. 2).

Locomotor activation and localization of effective infusion sites

Unilateral infusions of bicuculline into the basal forebrain often were followed by some degree of activation of locomotion and rearing, but also often did not affect locomotion or, occasionally, diminished it, as compared to a preceding saline infusion. The variability of the effects of these infusions was such that infusions near each other frequently produced disparate effects on locomotion (Fig. 2), as did infusions into the same site in different sessions (Table 1). Rats exhibiting bicuculline-elicited locomotor activation had normal posture and perfectly coordinated movements even when, occasionally, they were so active as to escape the 12″ (30.5 cm) high plexiglass walls of the recording chamber. They never behaved as if perturbed or anxious or exhibited obvious fearfulness, as might be reflected in burrowing beneath the cage bedding or engaging in compulsive burying behavior, or aggressiveness, as might be reflected in piloerection and aggressive biting. Nor did they give the impression of ‘exploring’, insofar as the locomotion of rats responding to an initial bicuculline infusion was no different than that of rats well-habituated to the activity monitor that had received activating infusions in multiple test sessions.

The most robust locomotor activations elicited by unilateral bicuculline infusions occupied a cluster centered over the rostral parts of the LPO and medial preoptic area (MPO), which, to denote this, hereafter will be referred to in this paper as LPO/MPO, despite their known structural and functional differences. Mann-Whitney rank sum tests revealed that the ranks (Fig. 2) and actual fold difference values (Table 1) for infusions into the LPO/MPO (n = 48) were significantly greater (P=<0.001 for ranks and fold difference values) than corresponding values acquired by combining infusion sites from all of the other evaluated forebrain structures (n = 48). Moreover, analysis using the Kruskal-Wallis one-way ANOVA on ranks with post hoc comparisons by Dunn’s test revealed that the ranks and actual fold difference values associated associated with bicuculline infusions into LPO/MPO were significantly greater (P = <0.001) than those associated with infusions into VP (n=13), extended amygdala (n = 13) and pooled remaining structures (e.g., CPu, GP, SLSI et al., n = 22). Due to the fact that more infusions were assessed in some cases than others, a possibility exists that sampling bias or cumulative effects of multiple infusions of bicuculline influence the comparison. However, the same statistical tests using only first infusions from each case (first of values listed in Tables 1 and 2) also revealed significantly

different fold difference values for LPO vs. all other tested structures (P=<0.001) and LPO vs. VP (p<0.003).

Unilateral infusions into the LPO/MPO and ventral pallidum

To strengthen the analysis, a comparison of total beam breaks elicited during the 20 minute test period by bicuculline and saline (Fig. 3) using paired t tests revealed that significantly greater locomotion was elicited by bicuculline than saline infusions into the LPO/MPO (n= 48, P=<0.001) and VP (n=13, P = <0.025). Additionally, a Kruskal-Wallis one-way ANOVA on ranks with post hoc comparisons by Dunn's test revealed that significantly greater locomotion was elicited by bicuculline in the LPO/MPO than VP (P = <0.001). Interestingly, rearing was significantly increased in association with unilateral bicuculline infusion in the LPO/MPO (P = <0.001), but not VP (Fig. 3B, see also 3D). Unilateral infusions of bicuculline into other forebrain structures, including the bed nucleus of stria terminalis, sublenticular extended amygdala and caudate-putamen did not increase locomotion relative to saline (Table 1).

Bilateral infusions into ventral pallidum

Bilateral infusions of bicuculline into the ventral pallidum produced moderate activation of horizontal locomotion (Fig. 3A and B) similar to that observed following unilateral VP infusions and, interestingly, a non-significant trend toward a reduction in rearing (Fig. 3D, see also Fig. 3C). Furthermore, rats that received bilateral infusions of bicuculline into the VP exhibited abundant abnormal movements consisting of repetitive bouts of compulsive rotatory locomotion, comprising one to several full rotations in the form of tight pivots around one of the hindlimbs (Table 2). When observed, pivots in one direction might be followed momentarily by a series of pivots in the same or opposite direction. Rats that exhibited pivoting movements also engaged in persistent, non-aggressive, gentle gnawing of accessible objects, such as, e.g., the handler's lab coat or finger or, during pivots, the subject's own tail. In two cases, rats with bilateral VP infusions of bicuculline compulsively grasped with the digits of both forepaws at buttons on the investigator's lab coat. In a single case, the rat adopted a gait abnormality in which the forepaws and hindpaws were not used alternately, but rather were engaged symmetrically, resulting in a hopping or 'galloping' forward progression. Abnormal movements were observed in 73% of cases in which infusion sites on both sides of the brain involved the VP (Table 2), but only one case of a total of 96 in which unilateral infusions were done and in only 2 cases with bilateral infusions of which only one substantially involved the VP. Series of bilateral infusions of bicuculline into the bed nucleus of stria terminalis (n=6) and caudate-putamen (n=6) done in a separate study (data not shown) produced no abnormal movements. Importantly, if one of the intended bilateral VP sites was misplaced such that it partially occupied the VP, but also occupied or bordered the LPO/MPO, robust locomotor activation was likely to be observed and accompanied by abnormal movements (Table 2, section B).

Bicuculline infusions into the lateral ventricle

When tested with a one-way ANOVA following infusions into the lateral ventrical (n=3), total horizontal and vertical ambulatory counts for bicuculline (1509±235 and 49±10, respectively), although not significantly different from saline (1930±394 and 127±37), trended downward, which is opposite of the result observed following infusions of bicuculline into the LPO/MPO.

Discussion

Anatomy

At the time when basal forebrain-elicited locomotor activation was first observed (see references in the Introduction), the concept of a 'ventral striatopallidum' was relatively new (Heimer et al., 1972; Heimer and Wilson, 1975; Switzer et al., 1982), as was a companion conceptualization, the extended amygdala (de Olmos and Ingram, 1972; Alheid and Heimer, 1988). These novel 'functional-anatomical' entities, founded on the basis of sound cytoarchitectural, histochemical, immunohistochemical and connectional criteria in rodents and primates (Heimer and Alheid, 1991; Heimer et al., 1991; 1997; 1999; Alheid et al., 1995; 1998; 1999; Sakamoto et al., 1999), essentially usurped territory classically assigned to the preoptic area and 'subpallidal' region. The immunohistochemical observations described herein were not intended to revisit basal forebrain organization comprehensively, but rather were to be used to generate a map to localize our infusion sites. In this regard, the markers we selected produced conspicuous labeling differences distinguishing VP, LPO/MPO and extended amygdala, consistent with the extensive literature cited above. It is important to point out, however, that hard lines separating brain structures in maps are poor reflections of the actual situation in the basal forebrain, wherein structures more often than not blend into each other along imperceptible boundaries referred to as transition zones (Alheid et al., 1994; Zahm et al., 2011; 2013). This is particularly true of the caudomedial part of the VP which merges with the LPO along precisely such a transitional boundary, where an exceedingly dense GABAergic striatopallidal innervation terminating on typical pallidal-type neurons gives way to a less dense innervation of neurons characteristic of the LPO (Zahm, 1989). Also in this region, typical VP outputs to, e.g., the subthalamic nucleus and substantia nigra reticulata, diminish in favor of efferents aligned more exclusively with the trajectory of the medial forebrain bundle (Zahm, 1989). Consistent with the caudalward decline in numbers of GABAergic inputs from VP toward LPO, Mogenson and Yang (1991) reported that the spontaneous firing rate of neurons is greater moving from VP to the subpallidal region, which in part includes the LPO. In view of these differences between VP and LPO, we anticipated differences in how infusions of bicuculline into each would influence locomotor activation.

We were surprised to encounter locomotor activations following bicuculline infusions into the MPO, which, as a modulator of neuroendocrine function with important roles in sexual and parental behavior and various forms of aggression (Newman, 1999), is distinct from the LPO. We speculate that MPO-elicted locomotor activation may reflect a transitional character of the boundary between LPO and MPO.

Locomotion

The present results indicate that unilateral infusions of bicuculline into the LPO/MPO increase horizontal locomotion and rearing with far greater probability and elicit locomotor activations of substantially greater magnitude than unilateral infusions of bicuculline into any other basal forebrain structure, including the VP. Fold difference values for horizontal locomotion following infusions into the LPO/MPO reliably surpassed 3.5 and, frequently, were more than 5 (Table 1). In contrast, unilateral and bilateral infusions of bicuculline into the VP typically produced fold differences in locomotion relative to saline of less than 2 (Tables 1 and 2) and these were unassociated with increased rearing. In addition, bilateral infusions of bicuculline into the VP produced abormal movements. Direct comparison of LPO/MPO-elicited and VP-elicited total locomotion/session was consistent with the fold difference evaluations in that LPO/MPO infusions produced significantly greater amounts of horizontal locomotion and rearing during the 20 minute test period than did VP infusions (Fig. 3). The present results agree with a previous study done in our laboratory (Reynolds et

al., 2006) and a series of earlier papers in which locomotor activation elicited by $GABA_A$ antagonists and glutamate agonists was said to be localized to the "lateral preoptic area/ substantia innominata", but in illustrations was mapped to the preoptic area exclusively (Shreve and Uretsky, 1988; 1989; 1991; Willins et al., 1992). Finally, the present finding of minimal to moderate locomotor activation following unilateral and bilateral infusions of bicuculline into the VP are reflected by recent reports involving infusions of $GABA_A$ antagonists into the VP that left locomotor activation unmentioned (Kemppainen et al., 2012) or tested for and failed to detect it (June et al., 2003).

Methodological considerations

Bicuculline and its methyl derivatives competitively block GABA at the $GABA_A$ receptor, in contrast to picrotoxin, which utilizes a mixed/non-competitive mechanism involving the associated chloride channel (Zukin et al., 1974; Simmonds, 1982; Krishek et al., 1996). Accordingly, bicuculline methochloride was determined to be a more potent and selective antagonist of GABA than picrotoxin (Dray, 1975). In a direct comparison of efficacies following forebrain infusions, picrotoxin produced more robust locomotor activations, but a less coherent dose response curve, than bicuculline methbromide (Austin and Kalivas, 1990). In view of these considerations, we selected to use bicuculline methbromide in this and our earlier study (Reynolds et al., 2006), despite knowing that mainly picrotoxin had been used in earlier, similar studies (Mogenson and Nielsen, 1983; Mogenson et al., 1985; Shreve and Uretsky, 1988; 1991; Austin and Kalivas, 1990; 1991)

The present results, obtained using microinfusions, reflect methodological problems typical of the technique. Thus, although we have no measure of the volumes of tissue effectively disinhibited by our infusions, the modest volume of vehicle (0.25 μl) and amount of bicuculline (67 ng) we used were slightly less than used in other studies where the compounds were shown in illustrations to have occupied approximately spherical volumes between 0.5 and 1.0 mm in diameter (e.g., Johnson et al., 1996; Chapman and Zahm, 1996). While this encourages confidence that our compound activated a volume of tissue somewhat less than shown in those studies, it seems possible that the geometry of our infusions may have been shaped by preferential diffusion along longitudinally-running fascicles of myelinated and unmyelinated axons that pervade basal forebrain. Thus, uncertainty attends the size and shape of the volume of tissue disinhibited by our infusions. This and the omnipresent potential for clogging of injection cannlae by brain tissue may in part underlie the substantial variability of our behavioral results. Furthermore, we cannot be certain if indeterminate subpopulations of neurons were preferentially activated by bicuculline. In this regard, e.g., we presume that the functional effects of a bicuculline infusion are due more to its capacity to release neurons with high intrinsic firing rates from GABA-mediated tonic suppression than by actions on tonically quiescent neurons.

We attempted to avoid disrupting the lateral ventricles by setting our guide cannulae at an angle of 20° from vertical in the mediolateral plane and, for most infusions, succeeded. However, the most medial of our cannula placements, which, by happenstance, were among those targeting LPO/MPO, frequently did disrupt the lateral ventricle, such that locomotor activation observed following such infusions could have reflected leakage of bicuculline into the ventricular system. To control for this, we infused bicuculline directly into the lateral ventricle in three rats and observed no significant effect on locomotion as compared to saline.

Our results complement those of Mogenson and Nielson (1983), who infused the $GABA_A$ antagonist, picrotoxin, in a sequence of increasing doses at various sites throughout the 'subpallidal' region, which in their description encompasses the sublenticular substantia innominata, lateral preoptic area, bed nucleus of stria terminalis and ventral globus pallidus.

They ultimately took no position on where in basal forebrain is most sensitive to such infusions, but their Figure 1 indicates that infusions in and near the lateral preoptic area required the least amount of picrotoxin to elicit robust activations of locomotion. We infused bicuculline at a single concentration found in our previous study to maximize locomotion upon infusion into the LPO/MPO (Reynolds et al., 2006) and then monitored the magnitude of locomotor responses generated at various immunohistochemically identified sites throughout the subpallidal region. Because we observed that locomotor activations were not limited exclusively to infusions of bicuculline into LPO/MPO, but occasionally were seen after infusions at some distance from it, it is necessary to entertain the possibility that the substrate for bicuculline-elicited locomotor activation extends into parts of the basal forebrain at some distance from the LPO/MPO, possibly as a distributed neuronal network comprising elements that are less sensitive or consistently activateable at increasing distance from the LPO/MPO. Occasional strong locomotor activation in response to bicuculline infusion at sites distant from the LPO/MPO could be due to stimulation of sensitive nodes in such a presumptive network. It is perhaps more likely, however, that the decreasing probability of activating locomotion following infusions at distant sites simply reflects greater distances that bicuculline would need to diffuse in order to activate the LPO/MPO. Robust activations following infusions in very distal sites could reflect fortuitously facilitated diffusion, e.g., along glial interfaces or fiber tracts. It should also be apparent that these alternative mechanisms are not mutually exclusive and that either would benefit from deposition of more voluminous or concentrated $GABA_A$ antagonist infusions at sites increasingly distant from the LPO/MPO, consistent with our results and Mogenson's and Nielsen's (1983) map.

Functional considerations

The data indicate that unilateral activation by disinhibition of some subset of basal forebrain neurons centered mainly in the LPO/MPO provides a potent facilitatory modulation of locomotion and rearing, presumably exerted via descending projections from LPO/MPO to a neuronal network located more caudally in the brainstem that controls the actions of brainstem and spinal cord motor effectors (Holstege, 1991, 1992). This relatively distal impact on locomotor circuitry would account for how a profound disruption of normal patterns of neuronal activity in a particular structure, LPO/MPO, powerfully affects the quantity but not the quality or physical integrity of locomotion (Holstege et al., 2004). Stated differently, the data suggest that LPO/MPO has no role in synthesizing motor patterns, but rather regulates level of activation in a different network that does synthesize locomotion. Identical considerations may apply to the modest increase in locomotion observed following unilateral bicuculline infusions into the VP, although, as noted above, this could instead reflect diffusion of bicuculline from the VP to the LPO/MPO. Alternatively in this regard, bicuculline-elicited locomotor activations were less prevalent following infusions in the rostral part of the VP as compared to its caudal part (Fig. 2), where numerous corticopetal cholinergic neurons are present (Zaborszky et al., 1991). The action of bicuculline on these cholinergic neurons might lead to the locomotor activations elicited by infusions at caudal VP sites.

Similar to unilateral infusions, bilateral infusions of bicuculline into the VP in our hands produced small increases of locomotion and, in contrast to the unilateral infusions, profoundly altered the quality of movements, such that abundant compulsive pivoting and gnawing, occasionally accompanied by forepaw grasping and, on one occasion, a gait abnormality we refer to as 'galloping', were observed. Mechanisms underlying such abnormalities are at present unknown, but their relatively minimal association with generalized locomotor activation and the extent to which they resemble distorted normal movements suggests that descending projections from the VP exert less control over

brainstem networks that modulate the abundance of locomotion and more, presumably direct, control over those responsible for synthesizing motor patterns. In view of this, one may be inclined to speculate that descending projections from the VP are concerned with intended movement, or, stated differently, intentional actions (see also Root et al., 2013). Insofar as most of these involve movements to one side or the other, it is intuitively agreeable that descending projections from the VP are mainly unilateral (Groenewegen et al., 1993), but problematic as to why it takes bilateral infusions of bicuculline to elicit a VP effect on motor function. In contrast, LPO/MPO has bilateral distribution of downstream projections (Geisler and Zahm, 2006b), consistent with its role in the modulation of bilaterally potent general locomotion.

Mental correlates of LPO/MPO-elicited locomotion?

Locomotor activation was viewed by some earlier investigators as the behavioral expression of a sense of impending consummation from which intended motor acts may be launched (Roberts and Carey, 1965; Mary Christopher and Butter, 1968; Hotsenpiller et al., 2001). In view of such ruminations, it is tempting to speculate further that the specificity and sensitivity with which locomotor activation is elicited from the LPO/MPO, combined with the convergence there of outputs from reward- and threat-sensitive functional-anatomical macrosystems (Zahm et al., 2013), makes the LPO/MPO a likely substrate to blend competing and cooperating influences descending via the macrosystems from the cerebral cortex, consistent with a proximal contribution of LPO/MPO to motivational processes. However, it is also known that activation of LPO/MPO stimulates VTA dopamine (DA) neurons (Reynolds et al., 2006), resulting in ‘DA release in the accumbens’ (Blaha et al., 1990; Laitinen et al., 1990; Rivest et al., 1991; Sotty et al., 1998; 2000), which, according to Berridge and Robinson (1998) embodies the psychic state of ‘wanting’ and is invariably accompanied, e.g., by increased locomotor activity (Kelly et al., 1975; Kalivas et al., 1983; Kalivas and Duffy, 1990; Steinberg et al., 1994), energized responses to second-order conditioned stimuli (Cador et al., 1991; Taylor and Robbins, 1984; 1986), establishment of conditioned place preference (Glimcher et al., 1984; Heidbreder et al., 1992), potentiation of brain stimulation reward (Rompre et al., 1992; Rompre and Gratton, 1992; Rompre, 1995), self-administration (Glimcher et al., 1997) and both behavioral and neurochemical sensitization (Kalivas et al., 1982; Kalivas & Taylor, 1985; Elliott & Nemeroff, 1986; Kalivas & Duffy, 1990). But then, infusion of $GABA_A$ receptor agonists or excitatory amino acid receptor antagonists into the LPO/MPO and VP, to both of which the accumbens densely projects (Zahm et al., 2013), abolishes these kinds of effects (Mogenson and Nielson, 1983; Swerdlow and Koob, 1984; Shreve and Uretsky, 1988; Austin and Kalivas, 1989; Willens et al., 1992). Furthermore, the sense that dopamine release is essential to the mediation of all these functions is called to question by the observation that LPO/MPO-elicited locomotor activation persists in complete blockade of DA receptors (Zahm et al., 2012). Moreover, intracranial electrical self-stimulation sites are abundant in LPO (Bushnik et al., 2000) and electrical self-stimulation of the LPO (and other sites) persists, albeit with attenuated vigor, in the presence of DA depletion and reward-blocking doses of antipsychotic drugs (e.g., Fibiger et al., 1976; 1987; Gallistel et al., 1985, but see Breese et al., 1971). Together, these observations lead one to wonder if the mental state underlying motivation is not attributable, at least in part, to events occuring in the LPO/MPO. Indeed, they beg a question as to what extent accumbens outputs associated with rewarding aspects of basal forebrain function (e.g., Johnston et al., 1996; Tindell et al., 2004; 2005; 2006; Lodge and Grace, 2006, see also Root et al., 2012) are linked to LPO/MPO, VP, both, or, possibly, a transition area intervening between the two (Zahm, 1989). In any event, whether or not the initial attribution by the brain of ‘reward’ status is an exclusively DAergic event, signals subserving or permitting the perception of reward (or its absence), however triggered, are promptly transmitted, via complex connectivity to DAergic neurons in the

ventral mesencephalon (Phillipson, 1979; Zahm et al., 2001; Geisler and Zahm, 2005; 2006a; b; Geisler et al., 2007; Jhou et al., 2009; Zahm et al., 2011; Watabe-Uchida et al., 2012; reviewed in Sesack and Grace, 2010; Lavezzi and Zahm, 2012), which, in turn, disseminate the information in other parts of the brain. LPO/MPO may have a quite proximal role in these processes.

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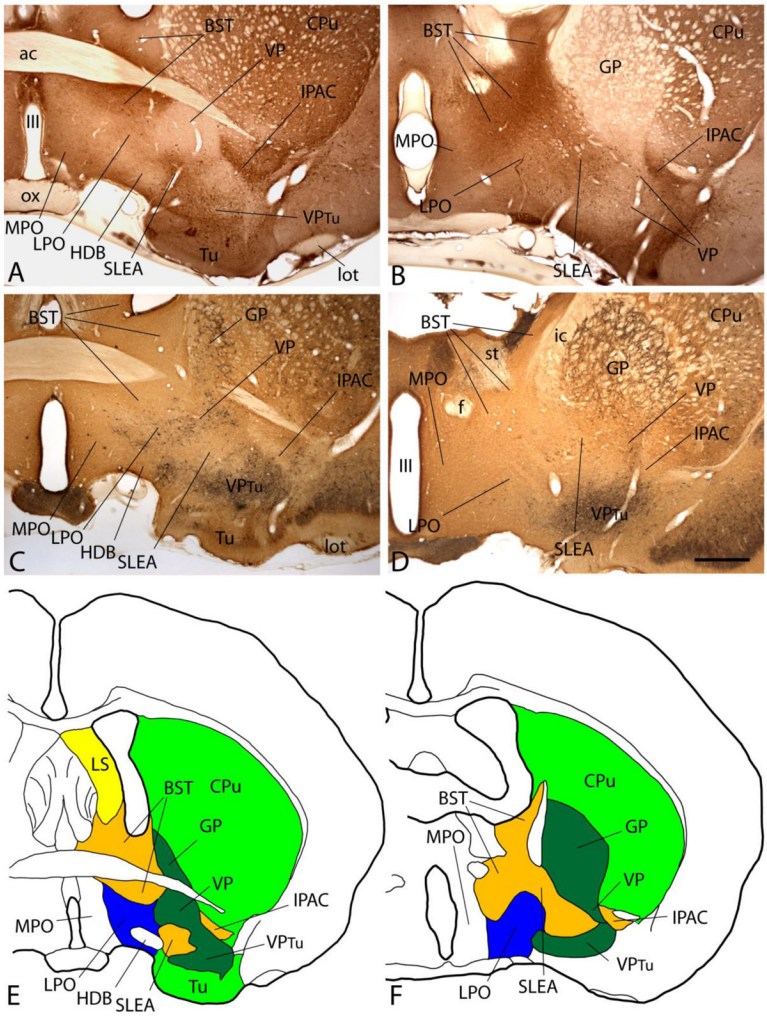

Figure 1.

A–D: Photomicrographs illustrating sections through the basal forebrain processed to exhibit nitric oxide synthase (NOS, A and B) or parvalbumin and calbindin-D 28kD (PV and CB, C and D) immunoreactivity (-ir). B and D are cut at a more caudal level than A and B. Note that NOS-ir is strong in extended amygdala stuctures such as the bed nucleus of stria terminalis (BST) and interstitial nucleus of the posterior limb of the anterior commissure (IPAC) and weak in the ventral pallidum (VP). Lateral preoptic area (LPO) exhibits an intermediate density of NOS-ir. PV-ir neurons (black dots) in C and D occupy the VP and, to lesser extent, LPO. E and F: The immunohistochemical patterns in A and B and C and D were used as a basis to draw the maps reflecting the respective levels of the forebrain in E and F. Scale bar: 1 mm for all photomicrographs. Additional abbreviations: ac - anterior commissure; CPu - caudate-putamen; f - fornix; GP - globus pallidus; HDB - horizontal limb of the diagonal band; ic - internal capsule; lot - lateral olfactory tract; LS - lateral septum; MPO - medial preoptic area; ox - optic chiasm; SLEA - sublenticular extended amygdala; st - stria terminalis; Tu - olfactory tubercle; VPtu - ventral pallidum in the deep layer of the olfactory tubercle; III - third ventricle.

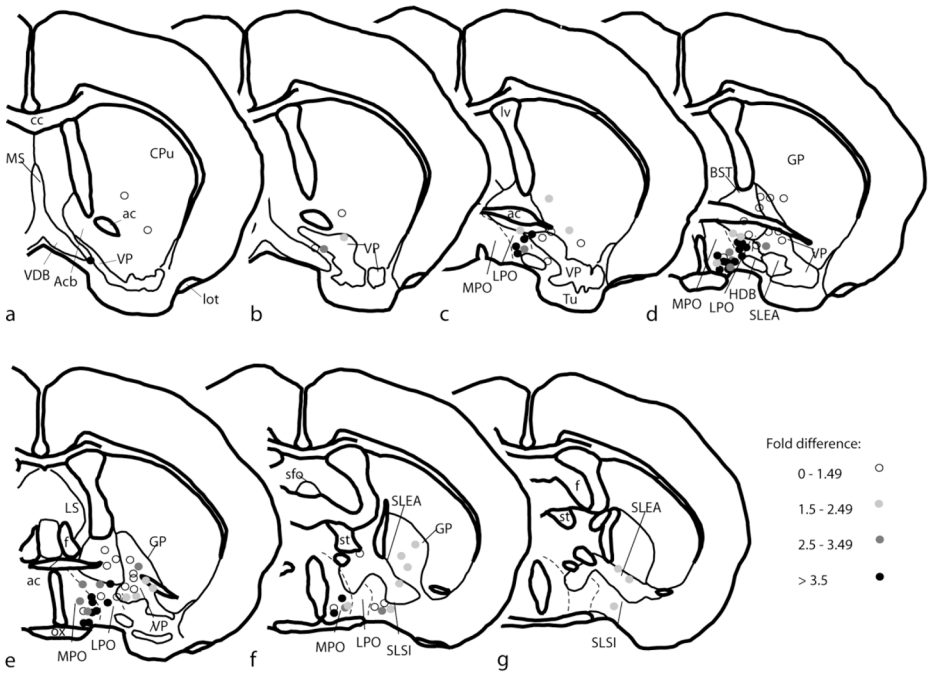

Figure 2.

A–G. Diagrams of a series of frontal sections ordered from rostral to caudal that were drawn on the basis of immunohistochemical observations as illustrated in Fig. 1. Round symbols indicate the positions of the tips of ejection cannulae from which bicuculline and saline were infused into the brain to generate data illustrated in Fig. 3 and Tables 2 and 3. The symbols are coded such that increasing density of gray tone indicates increasingly robust locomotor responses to infusion of bicuculline. Fold difference refers to the amount of distance traversed in an activity chamber according to the following relationship: [beam breaks/20 minutes following bicuculline infusion]/[beam breaks/20 minutes following saline infusion]. Structures and abbreviations are as provided in Fig. 1 legend.

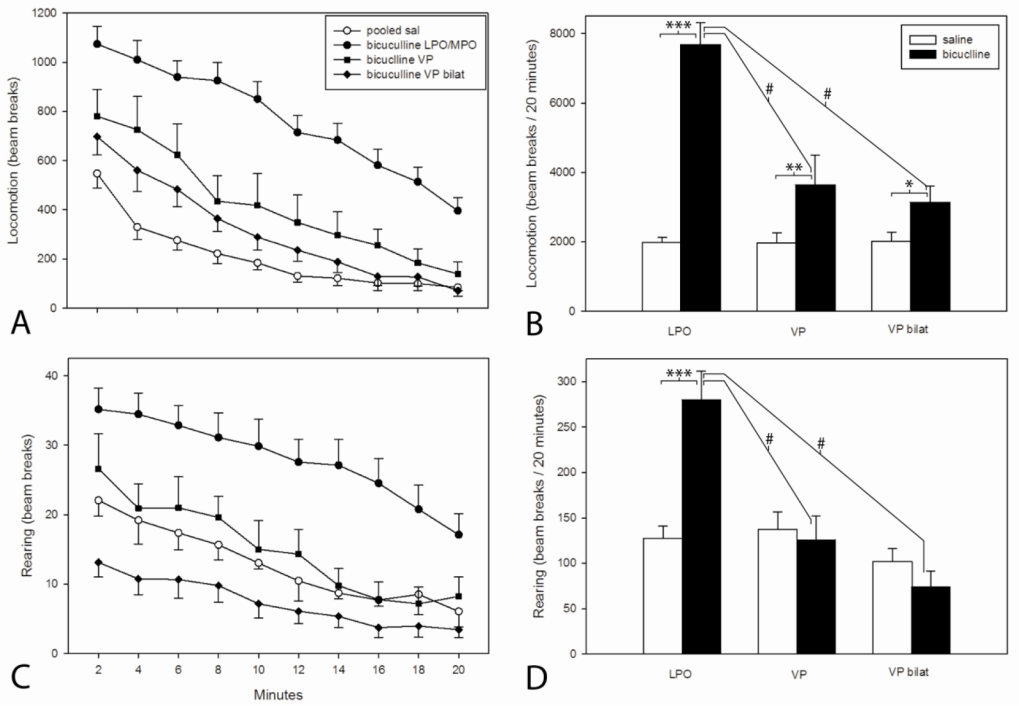

Figure 3.

A and C: Plots illustrating ambulatory locomotor activation following infusion of bicuculline hydrobromide (67 ng/0.25 μl) into the lateral preoptic area (LPO), ventral pallidum unilaterally (VP) and bilaterally (VP bilat) and an equivalent volume of saline into the same structures. In the absence of differences between groups, the data for saline infusions into LPO, VP and VP bilat were pooled. After the infusions, the cannulae were left in place for one minute and then the rats were placed immediately into the activity monitors. Bins are 2 min in duration. B and D: Bar graphs reflecting total locomotion reflected in the respective adjacent line/scatter graphs (A and C). Pairs of bars were tested with paired t-tests and the bicuculline infusions were tested with a Kruskal-Wallis one-way ANOVA on ranks with post hoc comparisons by Dunn's test. Paired t-test: *** P=<0.001, ** P=0.025, * P=0.017. Kruskal Wallis test with Dunn's post hoc comparison: # P=<0.001.

Table 1

Fold changes in horizontal locomotion associated with bicuculline as compared to saline infusions

LPO-MPO			
10111 - **3.3**	11257 - **6.19**, 0.34	12014 - 0.82, **3.17**, 0.85	12058 - 1.53, **3.35**, 0.42
10112 - **7.7**	11258 - **5.13**, **4.87**	12017 - 1.32, 2.02, 1.24	12059 - **3.16**, 1.55, 0.05
10117 - 1.41	11259 - 0.91	12018 - 1.03, 1.86, 1.91	12060 - 0.61, **4.35**, 0.4
10120 - **3.31**	11272 - 0.87, **6.6**	12021 - **4.01**, 6.3, 2.46, **3.49**	12061 - **4.41**, **3.65**, 0.33
10121 - 0.95, 0.82, 0.92	11273 - 2.79, 1.73, 0.44	12022 - 2.8, 1.08, **3.93**, **3.87**	12062 - **4.95**, **4.4**, 1.09
10124 - 1.11, 0.81, 1.59	12001 - 0.48, 1.11	12023 - **4.73**, **3.25**, 2.45, 1.3	12063 - **9.11**, **6.14**, **3.48**
10130 - 2.42	12002 - 0.63, **6.33**	12024 - **10.56**, **8.21**, **5.75**, **4.01**	12064 - **7.72**, **2.11**, **6.58**
10135 - **5.13**	12009 - 1.19, **3.34**	12028 - **3.04**, **6.18**	12065 - **4.26**, 1.99, **3.95**
11250 - **9.6**, 2.4	12010 - 1.53, **3.78**	12031 - **3.87**, **9.7**, **3.39**, **6.4**	12066 - **4.54**, **3.73**, **3.25**
11251 - **4.4**, 0.99	12011 - **7.04**, **7.4**	12032 - 1.86, **3.25**, 1.23, **7.22**	12067 - **7.97**, 2.65, 0.74
11256 - 1.91, 3.1	12012 - **5.05**, **3.37**	12057 - 1.16, **3.52**, 0.31	12098 - **8.94**

VP		**BST**	**SLEA**
10110 - 0.74	13036 - 1.71, 1.48, 1.07	10113 - 0.9, 0.95	10114 - 0.51
10116 - 1.53	13037 - 2.08, 2.29, 1.8	10131 - 0.33, 1.01	10115 - 0.67
10132 - 2.82	13038 - 0.049, 0.087	12006 - 1.14	10133 - 0.48
12096 - 0.59	13042 - 1.2, 1.43	12099 - 0.46	13047 - 1.6, 1.37
12097 - 1.44	13043 - 0.43, 0.98	12101 - 1.09	13049 - 1.48, 1.34
12100 - 1.07	13048 - 1.98, 1.6	13041 - 0.76, 1.02	13050 - 1.38, 2.62
13029 - 1.04, 2.56, 2.47			13052 - 1.65, 2.55

CPu	**Other**	
12005 - 0.95, 0.85, 1.09	11289-VDB - 2.3, 0.79	12037-SLSI - 0.67, 1.19
13021 - 0.72, 0.64	11290-VDB - 0.95, 0.54	13023-GP - 1.14, 1.33
13024 - 0.72, 0.64	12027-VDB - **5.27**, **4.08**	13030-GP - 1.88, 2.9, **3.24**
13025 - 0.63, 1.31	12033-SLSI - 1.07, 1.41, 1.48, 0.67	13035-GP - 1.74, 1.79, 1.94
13039 - 0.62, 1.65	12034-SLSI - 0.3, **3**, 0.15, 0.82	13040-GP - 0.95, 1.37
	12035-SLSI - 2.36, 2.36	13051-GP - 1.33, 2.1
	12036-SLSI - 2.5, 0.89	

Case numbers are given to the left in each column. Each case number is followed by one or several fold values, each reflecting a separate comparison of saline-and bicuculline-elicited of locomotion. Fold difference is [beam breaks/20 minutes following bicuculline infusion]/[beam breaks/20 minutes following saline infusion] (see Methods). Increases of 3.0 or greater are bolded and underscored for emphasis. Structures in which infusions were made can be referenced in Figures 1E and F and 2.

Abbreviations: BST - bed nucleus of stria terminalis; CPu - caudate-putamen; GP - globus pallidus; LPO-MPO - lateral and medial preoptic area; SLEA -sublenticular extended amygdala; SLSI - sublenticular substantia innominata; VDB - vertical limb of the diagonal band; VP ventral pallidum

Table 2

Bilateral infusions of bicuculline: locomotion and abnormal movements

Case	Infusion sites		Abnormal movements				Fold differences in sequential trials					
	Right side	**Left side**	**pivoting**	**gnawing**	**grasping**	**galloping**	**1**	**2**	**3**	**4**	**5**	**6**
A.												
11017	lVP	lVP, VPtu					0.11					
11018	vmCPu	vmCPu					0.69					
11038	vBST, VP	vmCPu, dVP	X	X			0.39	0.36				
11039	VP	vmCPu, dVP					1.53	1.30				
11053	VP	VP	X	X			**5.95**	2.90				
11063	Acb, VP, CPu	VP, VPtu	X	X			1.42	0.51	0.38			
11068	VPtu	mTu (Str&VP)					2.39	1.78	1.37			
11070	lVP,VPtu	VP, VPtu					0.94	1.85	2.05			
11231	CPu, lVP	VP					**4.37**	1.57				
12044	VP	VP, VPtu	X	X			0.93	1.60	1.30			
12045	VP	VP	X	X			1.02	0.87	0.67			
12047	VP, CPu	VP	X	X			1.43	1.51	1.07			
12048	VP, VPtu	vCPu, VP	X	X			0.95	2.58	2.21			
12049	vmCPu, VP	vmCPu, VP	X	X			2.41	2.95	**3.96**			
13094	vmCPu, VP	vmCPu, VP	X	X		X	0.83	2.35	1.80	**3.48**	1.39	0.10
13096	vGP, VP	vGP, VP		X	X		1.04	0.68	2.55	2.46	0.06	
13097	VP	VP	X	X			0.92	0.88	1.58	0.40	0.17	0.14
13098	VP	VP		X	X		0.62	0.83	2.09	2.04	0.06	0.23
13106	VP	VP		X			1.53	0.88				
13107	VP	VP	X	X			1.77	1.76				
13109	VP	VP	X	X			0.68	0.77				
13110	VP	VP	X	X			0.45	0.66				
B.												

Case	Infusion sites		Abnormal movements		Fold differences in sequential trials					
	Right side	**Left side**	**pivoting**		**1**	**2**	**3**	**4**	**5**	**6**
			gnawing							
			grasping							
			galloping							
11052	VP, LPO, vBST	VP, LPO	X	X	2.12	2.99				
11062	VP, LPO	clVP			**4.82**	**10.7**	2.82	**4.59**		
11066	clVP, HDB	cmVP, LPO	X	X	0.98	**4.29**	**4.50**			
11067	vCPu, cAcb	VP, cmCPu, BST	X	X	0.63	0.28	1.77	1.34		
11069	lVP, VPtu	mTu (Str), LPO	X	X	**3.47**	**6.73**	**4.34**			
11230	VP, LPO	CPu	X	X	1.17	**6.18**				
11232	VP, vCPu, BST	VP, vCPu, BST			**3.47**	**3.77**				
12046	VP, LPO	VP	X	X	1.04	2.84	**4.94**			

A. Cases (not included in Table 1) in which infusion sites involved the ventral pallidum (VP) and other indicated structures, but not the lateral preoptic area (LPO), on both sides of the midline. Abnormal movements are as described in the the text. Fold difference is beam breaks/20 minutes following bicuculline infusion]/[beam breaks/20 minutes following saline infusion] (see Methods). Fold difference values more than 3.0 are bolded and undescored for emphasis.

B. Cases in which the infusion site on at least one side of the midline involved the LPO or transition area between the VP and LPO.

Note the greater density of bolded entries in section B.

Additional abbreviations: Acb - accumbens; BST - bed nucleus of stria terminalis; c - caudal; CPu - caudate-putamen; GP - globus pallidus; HDB - horzontal limb of the diagonal band; m - medial; l - lateral; Str - striatal, as in the striatal part of the olfactory tubercle; VPtu - ventral pallidum occupying the deep layer of the olfactory tubercle