Enhancement of Fear Extinction with Deep Brain Stimulation: Evidence for Medial Orbitofrontal Involvement

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Deep brain stimulation (DBS) of the ventral capsule/ventral striatum (VC/VS) reduces anxiety, fear, and compulsive symptoms in patients suffering from refractory obsessive-compulsive disorder. In a rodent model, DBS-like high-frequency stimulation of VS can either enhance or impair extinction of conditioned fear, depending on the location of electrodes within VS (dorsal vs ventral). As striatal DBS activates fibers descending from the cortex, we reasoned that the differing effects on extinction may reflect differences in cortical sources of fibers passing through dorsal–VS and ventral–VS. In agreement with prior anatomical studies, we found that infralimbic (IL) and anterior insular (AI) cortices project densely through ventral–VS, the site where DBS impaired extinction. Contrary to IL and AI, we found that medial orbitofrontal cortex (mOFC) projects densely through dorsal–VS, the site where DBS enhanced extinction. Furthermore, pharmacological inactivation of mOFC reduced conditioned fear and DBS of dorsal-VS-induced plasticity (pERK) in mOFC neurons. Our results support the idea that VS DBS modulates fear extinction by stimulating specific fibers descending from mOFC and prefrontal cortices.

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INTRODUCTION

Deep brain stimulation (DBS) of the ventral capsule/ventral striatum (VC/VS) reduces fear and anxiety symptoms in patients suffering from refractory obsessive-compulsive disorder (OCD; Denys et al, 2010; Greenberg et al, 2010), but little is known about the mechanisms. Previous studies have proposed that the primary effects of DBS are on cortical fiber bundles passing through the striatum (see Greenberg et al, 2010b for review). Furthermore, OCD patients show deficient extinction of conditioned fear, which depends on specific areas of the prefrontal cortex (Milad et al, 2013). Using a rat model, we previously found that high-frequency DBS-like electrical stimulation of the dorsal–VS (just above the anterior commissure) enhanced fear extinction, whereas stimulation of the ventral–VS impaired fear extinction (Rodriguez-Romaguera et al, 2012). Interestingly, these striatal sub-regions did not follow core/shell compartmentalization. Furthermore, DBS effects were not observed with pharmacological inactivation of dorsal–VS or ventral–VS (Rodriguez-Romaguera et al, 2012), suggesting that DBS modulates different sets of cortical fibers passing through these VS areas. We therefore sought to determine the cortical sources of fibers passing through the dorsal–VS and ventral–VS sub-regions.

The infralimbic(IL) cortex and prelimbic (PL) cortex project through VS (Berendse et al, 1992; Mailly et al, 2013; McGeorge and Faull, 1989; Sesack et al, 1989; St Onge et al, 2012; Vertes, 2004) and could potentially mediate DBS effects on extinction. It is well established that inhibition of IL impairs fear extinction (Burgos-Robles et al, 2007; Laurent and Westbrook, 2009; Quirk et al, 2000; Sierra-Mercado et al, 2011), whereas stimulation of IL enhances extinction (Kim et al, 2010; Maroun et al, 2012; Milad and Quirk, 2002; Milad et al, 2004). In contrast, inhibition of PL reduces fear expression (Corcoran and Quirk, 2007; Laurent and Westbrook, 2009; Quirk et al, 2000; Sierra-Mercado et al, 2011), whereas PL stimulation increases fear expression and impairs extinction (Vidal-Gonzalez et al, 2006). Other areas projecting through the VS include the orbitofrontal cortex (OFC) and the anterior insular (AI) cortex (Gabbott et al, 2005; Hoover and Vertes, 2007, 2011; Mailly et al, 2013; McGeorge and Faull, 1989; Schilman et al, 2008), but their role in fear extinction has not been studied. Furthermore, the projection patterns of these areas through dorsal–VS and ventral–VS are not clearly defined. We therefore injected the retrograde tracer wheat germ agglutinin (WGA) into the dorsal–VS or ventral–VS, to assess retrograde labeling in the cortex. Cortical areas...
projecting predominantly through the dorsal–VS site (in which DBS enhanced fear extinction) were further studied using pharmacological inactivation and immunocytochemistry.

MATERIALS AND METHODS

Subjects

Fifty five male Sprague–Dawley rats (~325 g; Harlan Laboratories) were housed and handled as previously described (Quirk et al., 2000). Rats used in the fear conditioning experiment were fed standard rat chow in a restricted manner (18 g/day), to facilitate pressing a bar for food on a variable interval schedule of reinforcement (VI-60) and to maintain a constant behavioral baseline against which freezing could be reliably measured. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico—School of Medicine in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Surgery and Drug Infusion

Rats were initially anesthetized with isoflurane inhalant gas (5%) in an induction chamber and positioned in a stereotaxic frame. Isoflurane (2%–3%) was delivered through a face mask for anesthesia maintenance. For our neuroanatomical study, unilateral infusions of WGA were aimed at the dorsal–VS (−6.5 mm DV, ±2.0 mm ML, and +1.2 mm AP) or ventral–VS (−8.0 mm DV, ±2.0 mm ML, and +1.2 mm AP; Paxinos and Watson, 1998). A small volume of 20 nl was used to restrict the tracer from spreading outside of mOFC. For our neuroanatomical study, sections (50 μm thickness, 200 μm apart) were initially treated with 10% methanol and 3% H2O2 in 0.1 M PB, to inhibit endogenous peroxidase, and rinsed extensively in PBS with Triton X-100 (PB-TX; Sigma, St Louis, MO). Preincubation occurred in PB-TX with 10% normal goat serum (NGS) for 30 min and incubation in primary anti-WGA (1:50 000; Sigma-Aldrich) in PBS-TX with 10% NGS for four nights at 4°C. The tissue was then treated with biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) and incubated with avidin–biotin complex solution (Vectorstain ABC kit; Vector Laboratories). Standard DAB procedures were used to react the tissue and visualization was optimized by using cobalt chloride and nickel ammonium sulfate. Sections were mounted onto gel-coated slides, dehydrated, defatted in xylene, and coverslipped with mounting medium (Permount, Fisher Scientific, Fair Lawn, NJ). To visualize the cytoarchitectonic subregions of cortical area, adjacent sections were reacted with neuronal marker NeuN. Sections were incubated with anti-NeuN antibody (1:1000; Chemicon, Rosemont, IL) overnight at room temperature and treated with the same procedures described above.

For our pERK immunocytochemistry experiment, alternate sections were initially blocked in a solution of 2% NGS (Vector Laboratories, USA) plus 0.3% triton (Triton X-100, Sigma-Aldrich) in 0.12 M potassium buffer SAL for 1 h, as previously described (Rodriguez-Romaguera et al., 2012). The sections were incubated overnight at room temperature with anti-pERK serum raised in rabbit (1:100; p-44/42MAPK, 4370L; Invitrogen, Carlsbad, CA) and treated with the same procedures described above.

For our neuroanatomical study, the tissue from a total of 24 WGA-injected animals was examined. Striatal injections with either contamination or weak labeling were eliminated from the analysis. Contamination refers to injections in which the tracer was not limited to the dorsal–VS or ventral–VS regions, but had leaked into the adjacent striatal regions. Weak labeling refers to poor uptake of the tracer as indicated by poor labeling in the midbrain. A total of four animals (two in dorsal–VS and two in ventral–VS) had injection sites that were confined to the dorsal or ventral region with adequate uptake. The cortex was divided into specific regions based on the rat atlas (Paxinos and Watson, 1998) and labeled cells were plotted with stereological analysis (see below). Cortical areas were defined according to cytoarchitectonic boundaries in adjacent sections, as indicated by NeuN labeling (Paxinos and Watson, 1998). We analyzed the number of labeled cells in the medial (mPFC), OFC, and AI prefrontal cortices. The mPFC was subdivided into cingulate (Cg1), PL, and IL cortices, the
OFC into MO and VLO OFCs, and the AI into ventral (AIv) and dorsal (Ald) AI cortices.

The number of cells labeled with WGA was quantified by using an unbiased stereology method (Stereoinvestigator Software, Micro Bright Field, Williston, VT), with a modified Nikon microscope (Nikon E800) under dark field illumination. Contours were drawn for each cortical area leaving a small transitional space (~200 µm) between areas. A fractionator probe was used to estimate the number of labeled cells with systematic random sampling. Quantification of cells was performed at ×20 magnification in 100 µm² counting frames, which constituted 64% of the reference space. We used the PL cortex to confirm that stereology estimates were accurate by comparing with manual counts of the entire structure. For each cortical region, the fraction of cells (% of total cells counted) was used to analyze the distribution of cortical cell labeling. The fraction of cells between each injection site was compared for dorsal–VS and ventral–VS regions.

For our pERK immunocytochemistry experiment, images were generated for mOFC and the number of pERK immunoreactive neurons in each brain hemisphere was quantified as previously described (Rodriguez-Romaguera et al., 2012). The density of pERK-positive neurons was calculated by dividing the number of positive neurons by the total area counted.

Behavior

Rats underwent bar-press training, auditory fear conditioning, and extinction in standard operant chambers (Coulbourn Instruments, Whitehall, PA) inside sound-attenuating boxes (Med Associates, St Albans, VT). Further details regarding the apparatus have been previously described (Quirk et al., 2000). On day 1, rats were presented with five non-reinforced tones (4 kHz, 75 dB, 30 s; habituation) followed by seven tones that co-terminated with footshocks (0.5 s, 0.56 mA; conditioning). On day 2, rats were infused with either SAL or MUS 30 min before being presented with 15 tones in the absence of footshocks (extinction). On day 3, rats were presented with eight tones measuring 0.5 mm in length and separated by 20 ms counting frames, which constituted 64% of the reference space. The amount of time rats spent freezing to the tone was expressed as a percentage of the tone presentation. Statistical significance was determined with Student’s two-tailed t tests, one-way ANOVA, or repeated-measures ANOVA, followed by Tukey post-hoc analysis, when appropriate (STATISTICA; Statsoft, Tulsa, OK).

RESULTS

Figure 1a shows findings from our previous study (Rodriguez-Romaguera et al., 2012), in which DBS of ventral–VS impaired fear extinction, and DBS of dorsal–VS enhanced fear extinction. To identify the cortical areas projecting through these different VS subregions, we injected the retrograde tracer WGA into either dorsal–VS or ventral–VS, and used stereotechnical techniques to count the number of retrogradely labeled cells in different cortical areas (Figure 1b and c).

Ventral–VS Receives Projections from PL, IL, and AI

Figure 2a and b show drawings of retrogradely labeled neurons resulting from WGA injections into dorsal–VS or ventral–VS. Group data revealed that there were no significant differences between the two striatal sites in the number of labeled cells in PL (dorsal–VS: 46%, ventral–VS: 45%, t(3) = 0.59, p = 0.62; Figure 2c), Gc1 (dorsal–VS: 4%, ventral–VS: 3%, t(3) = 0.96, p = 0.44), or caudal VLO (cVLO; dorsal–VS: 15%, ventral–VS: 16%, t(3) = -0.02, p = 0.98). Regarding PL, the ventral–VS projections originated largely from middle and deep layers, but not superficial layers. In contrast to PL, Gc1, and cVLO, there were significant differences between striatal sites in the number of labeled cells in the IL and AI (Ald + Alv) cortices. Specifically, ventral–VS injections yielded significantly more IL and AI labeling than dorsal–VS injections (IL = dorsal–VS: 3%, ventral–VS: 24%, t(3) = -4.90, p = 0.04; AI = dorsal–VS: 4%, ventral–VS: 17%, t(3) = -5.66, p = 0.03; Figure 2b and c). This suggests that modulation of IL and AI may contribute to the impairment of extinction by DBS of ventral–VS, perhaps through DBS-induced inhibition of IL, as previously proposed for lateral OFC (McCracken and Grace, 2007).

Dorsal–VS Receives Projections from PL and mOFC

In contrast to ventral–VS, projections from PL to dorsal–VS originated largely from middle and superficial layers, but not deep layers. Furthermore, dorsal–VS tracer yielded significantly more retrograde labeling in the mOFC (MO + rVLO), compared with ventral–VS (mOFC; dorsal–VS: 43%, ventral–VS: 11%, t(3) = 10.99, p = 0.008; Figure 2a and c). Thus, DBS enhancement of extinction observed with the
dorsal–VS site could be due to DBS-induced inhibition of mOFC (McCracken and Grace, 2007), if mOFC normally opposes extinction. Prior studies, however, have not examined the role of mOFC in fear expression or fear extinction.

**Pharmacological Inactivation of mOFC Reduces Fear Expression**

To determine the role of mOFC in fear expression and extinction, we infused the fluorescently labeled GABA-A agonist MUS into mOFC just before extinction training (on Day 2). We included only rats showing spread of MUS to MO and rVLO subregions of mOFC (Figure 3a). Inactivation of mOFC significantly reduced freezing from the start of the extinction session (Figure 3b). Repeated-measures ANOVA revealed a main effect of group ($F(1, 12) = 12.46, p = 0.004$). Post-hoc comparisons confirmed that MUS-infused rats showed significantly less freezing than controls in trial blocks 1, 2, 3, 5 and 7 (all $p$'s < 0.05), suggesting that mOFC activity acts to augment fear expression. The following day, both SAL and MUS rats showed significantly less freezing than the previous day ($t(17) = 2.47, p = 0.04$), demonstrating extinction retrieval (Figure 3b). There was no difference, however, between MUS and SAL groups in extinction retrieval ($F(1, 12) = 0.04, p = 0.84$), indicating that mOFC is not a site of extinction memory.

**DBS of Dorsal–VS Induces Plasticity in mOFC**

Our mOFC MUS finding is consistent with the idea that DBS-induced inhibition of mOFC could account for reduced freezing observed when DBS is on (see Figure 1a), but what accounts for reduced freezing the following day when DBS is off? One possibility is that DBS (but not extinction itself) induces plasticity in inhibitory circuits within mOFC (Gradinaru et al., 2009; McCracken and Grace, 2007), which could account for the long-lasting reduction in freezing. To investigate whether DBS alone induces plasticity in mOFC, we measured the expression of the neuronal plasticity marker pERK (Sweatt, 2004). Naive rats were given 3 h of DBS or sham stimulation in either dorsal–VS or ventral–VS (same as Figure 1a), and were killed immediately after. We observed that DBS of dorsal–VS (but not ventral–VS) triggered pERK labeling in mOFC neurons. One-way ANOVA revealed a significant effect of treatment in the...

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**Figure 1** Stereological analysis of cortical neurons projecting through dorsal– and ventral–ventral striatum (VS). (a) Left: deep brain stimulation (DBS) electrode placements in VS. Circle diameter indicates the estimated spread of current from electrode tip. DBS of dorsal–VS was applied dorsal to anterior commissure (orange circles) and DBS of ventral–VS was applied ventral to anterior commissure (green circles). Right: freezing plots for Sham ($n = 16$), dorsal–VS DBS ($n = 6$), and ventral–VS DBS ($n = 8$) groups. Dorsal–VS DBS reduced freezing on day 2 and facilitated extinction recall on day 3. Ventral–VS DBS had the opposite effects. Data are shown in blocks of two trials as mean ± SEM. *$p$ < 0.05. Adapted from Figure 1b and c of Rodríguez-Romaguera et al., 2012. (b) Left: representative micrographs showing the spread of wheat germ agglutinin (WGA) into dorsal–VS (top) or ventral–VS (bottom). Right: Injection sites and spread of all cases used in Figure 2c. (c) Illustration of the cortical maps generated using non-biased stereology techniques. Sample obtained from a rat with a WGA injection into ventral–VS.
number of pERK-labeled neurons within mOFC ($F_{(2,11)} = 15.90$, $p < 0.001$; Figure 4). Post-hoc tests confirmed that DBS of dorsal–VS significantly increased the number of pERK-labeled neurons compared with sham-operated controls ($p < 0.001$). Further studies are needed to discriminate between plasticity in excitatory vs inhibitory networks within mOFC.

**DISCUSSION**

We investigated cortical projections through the dorsal and ventral subregions of ventral striatum, to better understand the effects of DBS on extinction of conditioned fear (Rodriguez-Romaguera et al., 2012). We found that dorsal and ventral subregions of VS receive different sets of inputs from mPFC and OFC. The dorsal–VS receives strong projections from mOFC, whereas the ventral–VS receives strong projections from IL and AI. Thus, enhancement of fear extinction with DBS of dorsal–VS could be due to modulation of mOFC fibers, whereas impairment of fear extinction with DBS of ventral–VS could be due to modulation of IL/AI fibers.

Pharmacological inactivation of mOFC reduced freezing, demonstrating that mOFC is part of fear expression circuitry. mOFC could drive fear through its projections to the basolateral nucleus of the amygdala or PL (Hoover and Vertes, 2011). Therefore, one could speculate that DBS of dorsal–VS inhibits the mOFC. McCracken and Grace (2007) have shown that DBS-like stimulation of dorsal–VS reduces OFC firing rate, and suggest that this occurs via activation of recurrent collaterals projecting onto inhibitory interneurons in OFC. Thus, reduced freezing in the presence of DBS could be due to inhibition of mOFC. Projections from deep layers of PL through dorsal–VS may also be
involved. Deep layers of PL project to the lateral hypothalamus (Gabbott et al., 2005), which mediate conditioning-induced increases in autonomic responses (LeDoux et al., 1988). Another target of PL deep layers is the dorsal midline thalamus (Gabbott et al., 2005), an area shown to be important for fear expression (Padilla-Coreano et al., 2012; Do-Monte et al., 2015). Thus, the beneficial effects of DBS in dorsal–VS (rather than ventral–VS) may rely on mOFC and PL convergence at this site, which has been observed in rats, monkeys, and humans (Draganski et al., 2006; Haber et al., 2006; Mailly et al., 2013).

In addition to reducing freezing, DBS of dorsal–VS has also been shown to strengthen extinction memory (Rodriguez-Romaguera et al., 2012). Potentiation of inhibitory circuitry within OFC or PL by DBS may account for strengthening of extinction. Consistent with this, we observed expression of pERK in both mOFC (this study) and PL (Rodriguez-Romaguera et al., 2012) following DBS. As pharmacological inactivation of mOFC (this study) or PL (Sierra-Mercado et al., 2011) does not alter extinction memory, DBS-induced inhibition of these areas likely does not replace extinction. In support of this, administering DBS alone does not induce extinction (Rodriguez-Romaguera et al., 2012). Alternatively, DBS may strengthen extinction memory via indirect effects on IL, as suggested by DBS-induced increases in Fos, pERK, and BDNF levels in IL (Do-Monte et al., 2013; Rodriguez-Romaguera et al., 2012).

There is considerable debate over the homology of orbitofrontal areas in rodents and primates. Cross-species comparisons of connectivity suggest that rodent mOFC is homologous to the medial region of the OFC in humans (Brodmann area 14) (Price, 2007; Wallis, 2012; Wise, 2008). Hyperactivity within the human mOFC is thought to drive the excessive compulsive behavior that is the hallmark of OCD (for reviews, see Baxter et al., 1996; Menzies et al., 2008; Milad and Rauch, 2012). DBS of the VC/VS increases BOLD signaling in mOFC of OCD patients (Rauch et al., 2006); however, whether this represents activation of excitatory or inhibitory networks is not known. Furthermore, a recent study showed that optogenetic activation of mOFC neurons induces OCD-like behaviors in mice (Ahmari et al., 2013). Therefore, DBS-induced inhibition of mOFC may explain the reduction in symptoms observed in refractory OCD patients receiving DBS (Denys et al., 2010; Greenberg et al., 2010a; Haber and Behrens, 2014). In support of this, it was recently shown that DBS of VC/VS reduces functional connectivity in cortico-striatal circuits in OCD patients (Figee et al., 2013). Thus, augmentation of exposure-based therapies with DBS (Denys et al., 2010) may be due to enhancement of fear extinction via mOFC inhibition.

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The authors declare no conflict of interest.

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