Double-Blind Pilot Trial of Oral Tolerization with Myelin Antigens in Multiple Sclerosis

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Multiple sclerosis (MS) is thought to be an autoimmune disease mediated by T lymphocytes that recognize myelin components of the central nervous system. In a 1-year double-blind study, 30 individuals with relapsing-remitting MS received daily capsules of bovine myelin or a control protein to determine the effect of oral tolerization to myelin antigens on the disease. Six of 15 individuals in the myelin-treated group had at least one major exacerbation; 12 of 15 had an attack in the control group. T cells reactive with myelin basic protein were reduced in the myelin-treated group. No toxicity or side effects were noted. Although conclusions about efficacy cannot be drawn from these data, they open an area of investigation for MS and other autoimmune diseases.

Multiple sclerosis is an inflammatory disease of the central nervous system (CNS) of presumed autoimmune etiology (*1*). Because of its inflammatory nature and evidence for immune activation both within the CNS and in the peripheral immune compartment, many clinical trials have attempted to ameliorate the disease process by immunosuppression (*2*). The majority of immunosuppressive drugs used are nonspecific and have toxic side effects that preclude their prolonged use or their use early in the disease process, a time at which they are more effective (*3*). These problems are common to any organ-specific inflammatory disease of presumptive cell-mediated autoimmune etiology, such as type I diabetes, rheumatoid arthritis, and uveitis. The goal of immunotherapy is to develop an antigen-specific, nontoxic method to suppress the immune response that is postulated to be central to these diseases.

In MS, myelin basic protein (MBP) and proteolipid protein (PLP) are probably the major targets of a putative cell-mediated autoimmune response. When animals are injected with adjuvant plus MBP or PLP, experimental autoimmune encephalomyelitis (EAE) is induced, which can be similar, pathologically, to MS. EAE is the primary model to examine approaches for the treatment of MS (*4*), although not all approaches that ameliorate EAE are applicable to or efficacious in the treatment of MS.

A classic method of inducing tolerance is through the oral administration of antigens (*5*, *6*). Oral tolerance presumably evolved to prevent systemic immune reactions to ingested proteins necessary for nutrition and thus survival. Oral administration of MBP suppresses acute EAE (*7*, *8*), and chronic, relapsing EAE can be suppressed after the onset of disease by oral administration of MBP or myelin (*9*). This approach suppresses other experimental autoimmune diseases, including models of arthritis (*10–12*), uveitis (*13*), and diabetes (*14*) without apparent toxicities. On the basis of these findings, a double-blind pilot trial of oral tolerization to 300 mg of bovine myelin (*15*) given daily for a year was initiated in 30 individuals with early relapsing-remitting MS (*16*). Bovine myelin was chosen because of its availability and because it contained both MBP and PLP. The primary clinical outcome measures were number of major exacerbations and change in disability as measured on the extended disability status scale (EDSS) (*16*, *17*). Double blinding was accomplished (*18*).

The individuals of the two groups were randomized for age, disease duration, EDSS, and number of exacerbations in the previous 2 years (Table 1). Included individuals had at least two clearly defined attacks (*17*) in the 24 months before entry, similar to other recent trials (*19*, *20*). The placebo group contained more females and more subjects that carried the class II major histocompatibility complex (MHC) antigen HLA-DR2. Fewer myelin-treated patients had major attacks (6 of 15) as compared to placebo-treated patients (12 of 15) ($P = 0.06$) (*21*) (Tables 2 and 3). Although the overall change in EDSS was not greater with myelin than with placebo ($P = 0.27$), subgroup analyses showed that two characteristics, gender and MHC phenotype, may have been related to treatment outcome. None of the eight males in the myelin treated group had an attack, whereas six of seven females had an attack (Table 3). All measures of disease activity were different between the males and females in the myelin-treated group, including use of steroids, disability status scale changes, and physician impressions. Male patients treated with myelin had an average improvement of 1.00 point on the disability scale. The three males in the placebo group all did significantly worse when compared to myelin-treated males as measured by number of attacks, treatment with steroids, worsening on the disability scale, and physician impression.

Similarly, a difference in the clinical response to myelin was observed in relationship to expression of the DR2 phenotype. The HLA-DR2 phenotype is more common in individuals with MS as compared to control groups; 66% of subjects carried HLA-DR2. In the myelin-treated group, none of the six subjects that lacked HLA-DR2 had an attack, but six of the nine HLA-DR2$^+$ subjects did. Individuals that were HLA-DR2$^-$ registered significant improvement when treated with myelin as compared to the placebo treatment, when attacks, EDSS changes, and physician impressions were analyzed.

However, the factors contributing to the improvements of the myelin-treated group could not be sorted out because the individuals were not initially randomized for gender and HLA phenotype. Six of eight male myelin-treated patients were HLA-DR2$^-$, whereas all the females were HLA-DR2$^+$. Two of the three males in the placebo group were DR2$^-$. Thus, the males in the myelin-treated group were primarily DR2$^-$, which confounds the analysis.

To determine whether oral tolerance could affect the T cell response to myelin autoantigens, primary proliferation (7-day) assays in response to MBP and PLP and frequency analysis of MBP-reactive T cells to human MBP and MBP peptides were

Table 1. Baseline patient characteristics.

	Placebo	Myelin-treated	*P* (*21*)
Age (years)	32.1 ± 4.9	33.5 ± 4.9	0.31
Sex (% females)	80	47	0.13
Disease duration (years)	6.8 ± 3.7	6.1 ± 3.1	0.68
EDSS	2.7 ± 0.96	2.4 ± 0.95	0.37
Exacerbations	2.7 ± 0.78	3.4 ± 1.5	0.14
HLA-DR2$^+$ (%)	73	60	0.70

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measured. Seven-day assays show minimal reactivity in MS patients (*22*) and there was no increase of proliferation to MBP and PLP in treated patients. Thus, to determine if a decrease in reactivity occurred, we used a frequency analysis assay that assesses reactivity to an autoantigen in those cases in which primary proliferation assays are insensitive. MBP and PLP T cell lines and clones generated with this methodology have MHC-restricted MBP reactivity (*23*).

A total of 11,520 short-term MBP T cell lines were generated just before treatment commenced and 5944 lines were generated, from the first 19 subjects enrolled, after completion of the protocol at 1 year (Table 4). Response to therapy and changes in MBP frequency were not clearly linked. In placebo-treated individuals MBP frequencies increased, decreased, or remained the same. In myelin-treated subjects, frequencies either decreased or remained the same; no major increases were observed. The overall frequency of MBP-reactive T cells in the oral myelin-treated group decreased ($P = 0.027$), but not in the placebo-treated group (Table 4). Reactivity of the T cell lines to the MBP peptide from residues 84 to 102 [MBP(84-102)] was also analyzed. The mean frequency of T cells recognizing MBP(84-102) was higher in the placebo group (2.010 ± 1.19) than the oral myelin-treated group (0.256 ± 0.080) before initiating treatment, probably because of the higher number of HLA-DR2$^+$ individuals in the placebo group (*23*). When changes in reactivity to MBP(84-102) were analyzed, there was a mean decrease in frequency of 0.17 ± 0.078 in the oral myelin- treated group that approached significance ($P = 0.06$), whereas in the placebo group there was a mean increase of 2.66 ± 2.25 that was not significant ($P = 0.268$). Reactivity to other peptides did not significantly change. Subgroup analysis regarding changes in frequency of MBP-reactive T cells with treatment outcome was not possible due to the small number of subjects in each group.

There were no consistent changes in T cell expression of CD3, CD4, CD8, CD26, or CD45RA determinants. Humoral responses to myelin were also measured; serum antibodies to MBP, PLP, or myelin were not detected by an enzyme-linked immunosorbent assay. There were no abnormalities of liver function, kidney function, or hematologic indices in the myelin-treated individuals.

Some animal EAE models of oral tolerance to MBP operate through antigen-driven bystander suppression (*24*). Oral administration of MBP generates a CD8$^+$ T cell that can adoptively transfer protection to naïve animals (*25*) and when stimulated with MBP in vitro releases the cytokine transforming growth factor–β (TGF-β) (*26*). Antibodies to TGF-β abrogate oral tolerance in vivo (*26*). Furthermore, TGF-β and interleukin-4 (IL-4) are increased in the brains of animals fed MBP (*27*) and oral tolerance to alloantigen in transplantation models is associated with IL-4 expression in the graft (*28*). Thus, theoretically, one would not need to know the autoantigen against which the autoimmune response is directed for oral tolerance to be effective as long as it is an antigen capable of inducing a regulatory T cell that secretes a suppressive cytokine when it encounters the oral tolerogen in the microenvironment of the target organ. In support of this mechanism, oral administration of MBP suppresses EAE induced by a PLP peptide (*29*). Thus, even if another CNS protein is the target of the autoimmune attack, orally administered MBP could still be effective. Also, in arthritis models, oral administration of type II collagen suppresses adjuvant as well as collagen-induced arthritis (*12*), and in the NOD mouse oral administration of insulin suppresses diabetes (*14*), even though there is no evidence that autoimmunity to insulin participates in that model. If more specific mechanisms, such as clonal anergy, are required for oral tolerance to be effective (*30, 31*), the specific autoantigen involved in the pathogenesis of the disease would have to be administered.

In animal studies, dosage and species of antigen are important variables in generating oral tolerance. In the EAE model, there is a hierarchy of suppression by MBP from different species, with syngeneic MBP generally more efficacious than xenogeneic MBP (*32*). Dosages used in the present trial were derived empirically by extrapolation from dosages used in animal studies. Oral tolerance is dose-specific and loss of tolerance may occur with increased dosages (*10–12, 14*). Oral tolerance also can be enhanced by feeding immune adjuvants such as lipopolysaccharide, which appear to stimulate additional populations of cells to down-regulate immune responses (*27, 33*).

Further investigation is needed to determine if specific subgroups respond different-

Table 2. Individual characteristics and clinical parameters of placebo- and myelin-treated subjects.

Subject	Age	Sex	HLA-DR	DR2	Attacks*		Disability status scale†			Steroids	Physician impression‡
					Pre	Post	Pre	Post	Change		
				Placebo-treated							
1	34	F	DR2, 7; DRw53	+	1,1	0	2.0	1.5	Stable	0	B
2	36	F	DR7, w6; DRw52	−	1,1	0	2.0	1.5	Stable	0	B
3	28	F	DR2	+	2,2	0	4.0	4.0	Stable	0	B
4	34	F	DR2	+	1,1	1	2.0	1.0	Improved	1	C
5	34	F	DR2, w11; DRw52	+	2,1	1	2.0	1.5	Stable	0	B
6	34	F	DRw6; DRw52	−	0,2	1	2.0	1.5	Stable	0	C
7	25	F	DR2, 4; DRw53	+	2,2	1	2.0	1.5	Stable	2	C
8	36	F	DR2, 7	+	1,1	1	2.0	2.5	Stable	1	D
9	28	F	DR2, 7; DRw53	+	1,1	2	2.0	1.0	Improved	0	C
10	43	F	DR 2, 3; DRw52	+	2,1	2	3.5	3.0	Stable	0	C
11	26	M	DR2, w6; DRw52	+	0,3	2	4.0	4.0	Stable	2	D
12	32	M	DR1, 3; DRw52	−	2,1	3	4.0	5.0	Worse	2	D
13	35	F	DR2, 7; DRw53	+	2,2	3	2.5	3.5	Worse	2	E
14	31	M	DR3, 4; DRw52	−	0,3	3	2.5	4.0	Worse	4	E
15	26	F	DR2, 5; DRw52	+	1,1	3	4.5	6.0	Worse	1	E
				Myelin-treated							
1	37	M	DR1	−	0,2	0	3.0	0.0	Improved	0	A
2	30	M	DR1, 7; DRw53	−	0,2	0	4.5	3.0	Improved	0	B
3	28	M	DR2, w12; DRw52	+	3,3	0	3.0	2.0	Improved	0	A
4	30	M	DR7; DRw52	−	2,3	0	1.0	0.0	Improved	0	B
5	28	M	DR1, 12; DRw52	−	2,1	0	3.0	2.0	Improved	0	B
6	37	M	DR3, 4; DRw52	−	2,1	0	1.5	1.0	Stable	0	B
7	32	M	DRw6, w11; DRw52	−	0,2	0	1.5	1.0	Stable	0	B
8	39	M	DR2, 4; DRw53	+	1,2	0	1.5	2.0	Stable	0	C
9§	28	F	DR2, 7; DRw53	+	2,4	0	3.5	1.5	Improved	2	C
10	35	F	DR2, 3; DRw52	+	1,1	1	2.0	1.5	Stable	3	C
11	42	F	DR2, 11; DRw52	+	2,2	1	2.5	3.0	Stable	0	D
12	35	F	DR1, 2	+	1,1	2	2.0	3.0	Worse	0	D
13	38	F	DR2; DRw52	+	1,1	3	3.0	5.5	Worse	3	E
14	26	F	DR2	+	2,2	4	1.5	1.0	Stable	3	D
15	37	F	DR1, 2	+	2,3	4	2.5	6.0	Worse	4	E

*Patients are arranged according to the number of major attacks they had post treatment. For patients with an identical number of attacks, they are arranged according to changes on the EDSS. Pretreatment attacks (Pre) represent the number of attacks the patient had in each of the previous 2 years. †An increase or decrease on the EDSS of 1.0 from pretreatment value was considered improved or worse; a change of 0.5 was considered stable. ‡Physician's impression of patient course before breaking the code: A, much improved; B, slightly improved; C, unchanged; D, slightly worse; and E, much worse. §Because of pregnancy at 6 months, myelin was discontinued at that time. Values, however, represent a year of observation.

Table 3. Clinical outcomes in patient subgroups (*21*).

Group	Attacks*	ΔEDSS†	Steroids‡	Physician Impression§				
				A	B	C	D	E
			Myelin-treated					
Total	6/15	−0.23 ± 0.43	5/15	2	5	3	3	2
Males	0/8	−1.00 ± 0.35	0/8	2	5	1	0	0
Females	6/7	+0.64 ± 0.71	5/7	0	0	2	3	2
DR2$^-$	0/6	−1.25 ± 0.38	0/6	1	5	0	0	0
DR2$^+$	6/9	+0.44 ± 0.57	5/9	1	0	3	3	2
			Placebo-treated					
Total	12/15	+0.03 ± 0.22	8/15	0	4	5	3	3
Males	3/3	+0.83 ± 0.44	3/3	0	0	0	2	1
Females	9/12	−0.17 ± 0.22	5/12	0	4	5	1	2
DR2$^-$	3/4	+0.38 ± 0.52	2/4	0	1	1	1	1
DR2$^+$	9/11	−0.09 ± 0.24	6/11	0	3	4	2	2

*Number of patients having major attacks. Male myelin-treated patients compared to male placebo-treated patients, $P = 0.006$; DR2$^-$ myelin patients compared to DR2$^-$ placebo patients, $P = 0.033$. †Average ΔEDSS ± SE. Male myelin patients, before treatment compared to after, $P = 0.03$; male myelin patients compared to male placebo patients, $P = 0.03$; DR2$^-$ patients before treatment compared to after, $P = 0.03$; DR2$^-$ myelin patients compared to DR2$^-$ placebo patients, $P = 0.036$. ‡Number of patients requiring steroids. Male myelin patients compared to male placebo patients, $P = 0.006$. §Physician impression was as described in Table 2. Male myelin patients compared to male placebo patients, $P = 0.005$; DR2$^-$ myelin-treated patients compared to DR2$^-$ placebo-treated patients, $P = 0.028$.

Table 4. Change in frequency of myelin basic protein–reactive T cells with oral tolerization to myelin. Whole mononuclear cells (200,000 per well) were cultured in 96-well round-bottom plates. Individual wells were tested for reactivity to human MBP on day 12 by splitting each well and incubating with either media or media with human MBP for 72 hours. Duplicate wells were pulsed with [^{3}H]thymidine for the last 18 hours of culture, harvested, and counted with an LKB scintillation counter. Frequencies were calculated by dividing the percent positive wells by 200,000 as described (*23*). Change in frequencies (Δ frequency) was calculated by subtracting the frequency of MBP-reactive T cells before treatment from the frequency of MBP reactive T cells at 1 year after initiating therapy. The Δ frequency for the placebo group was +1.0 ± 3.2 (not significant) and for the myelin-treated group was −0.64 ± 0.23 ($P = 0.027$).

Subject	MBP frequency (per 10^7 cells)	
	Pretreatment	12 months
	Placebo-treated	
2	3.13	0.79
5	27.10	11.50
6	0.87	1.04
7	5.73	17.20
8	12.70	34.70
9	3.75	0.17
10	1.04	0.10
12	5.21	4.90
13	2.72	0.52
14	1.39	3.04
	Myelin-treated	
3	1.56	1.67
5	4.17	2.08
6	1.74	1.04
7	1.65	0.73
10	0.69	0.52
11	1.21	0.87
12	2.08	0.94
14	1.39	0.74
15	2.08	2.26

ly. In animals, males and females have no apparent difference in oral tolerization to myelin antigens. These issues will only be answered in further clinical trials in which patients are prospectively randomized on the basis of gender and HLA-DR type. The subgroup responses we observed may not be seen at different dosages of bovine myelin or when human recombinant MBP is tested.

There were no toxicities associated with treatment. Systemic toxicities were not expected after the ingestion of a protein, although it was theoretically possible that administration of myelin antigens could exacerbate the disease process. Similarly, no toxicities or exacerbation of disease have been observed in open label pilot trials of oral tolerization to type II collagen in rheumatoid arthritis (*34*) or S-Ag in uveitis (*35*). At the end of the study there was no evidence of sensitization to myelin antigens in the patients either in terms of cellular immune responses or the generation of antibodies to myelin, as has occurred in other studies when MBP was repeatedly injected subcutaneously (*36*). Upon completion of the study, the male patients in the myelin-treated group have remained on myelin in an open-label fashion for a total of 2.5 years with no apparent toxicity and continued stabilization of their disease. The three males in the placebo group subsequently treated with myelin experienced disease stabilization in open-label treatment.

Whether decreasing the frequency of MBP-reactive T cells is associated with a response to the treatment should be addressed by further investigation in larger clinical experimental trials. The PLP frequency and generation of T cells that secrete suppressive cytokines may be linked to the response and should also be measured in future studies.

It must be strongly emphasized that this study does not demonstrate efficacy of oral myelin in the treatment of MS. Nonetheless, our data open an area of clinical investigation for the treatment of MS and other cell-mediated organ-specific autoimmune diseases.

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15. Myelin was prepared from bovine sources by Biopure, Inc., Boston, MA. Bovine brains, obtained from healthy cows and local certified herds, had the white matter dissected at 4°C. Myelin was prepared from white matter by ultracentrifugation on a sucrose gradient. The purified myelin was pasteurized by heating to 60°C for 8 hours and then lyophilized. The lyophilized product was examined by SDS gel electrophoresis and amino acid analysis. Biologic activity of the myelin was assessed by its ability to suppress acute EAE in the Lewis rat by oral administration (*7, 9*). Purified myelin was screened for a panel of bacterial and viral contaminants. Each opaque gelatin capsule (size 0) contained 300 mg of myelin only, and dissolved in the stomach. The placebo consisted of a powdered bovine milk product (Star Market dryfat milk). The capsules were opaque red. Patients were given the capsules in a −20°C pack and were instructed to keep the capsules in their home freezers, and take one capsule each morning before breakfast.
16. Thirty patients with relapsing-remitting MS were selected from the multiple sclerosis clinical unit at the Brigham and Women's Hospital from approximately 1500 patient visits per year. The clinical trial was approved by the human studies institutional review board of the Brigham and Women's Hospital. Subjects were randomized in pairs matched according to number of attacks in the previous 2 years, level of disability, and age. A complete neurologic exam with recording of the EDSS was performed before entry and at 3, 6, 9, and 12 months. The EDSS is a measure of neurologic disability. Scores of 1 to 5 represent mild to moderate disability; individuals with scores of 6 or greater require canes, crutches, or wheelchairs. Both subjects and physicians were blinded as to the treatment. Two physicians (G.A.M. and D.M.D.) followed all subjects and were the primary care neurologists for the year of the study. Subjects informed physicians of changes in their neurologic condition, were evaluated, and then some were treated with oral prednisone (a 3-week tapering schedule beginning with 60 mg) or intra-

venous methylprednisolone (1 g daily for 5 days followed by a 1-week prednisone tapering schedule). Patients continued to take the myelin or placebo pills while receiving steroids.
17. The primary clinical outcome measures were the number of major exacerbations and change in the EDSS. Only well-defined attacks affecting pyramidal, cerebellar, brainstem, or visual functional systems were counted as major exacerbations. Sensory and bladder symptoms without objective findings on neurologic exam were not included. Patients were considered improved or worse if they changed two steps (one full point) on the EDSS. At the conclusion of the study and before unblinding, a global assessment rating was assigned for the course of each patient during the study.
18. Neither patient nor physician knew the treatment group. Compliance was measured by counting pills and by questionnaire. Patients rarely did not take a pill, missing at most 5 days during the year of the trial. Patients who did well predicted they were taking medication, irrespective of whether they were in the myelin or placebo group.
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Association Between Brain Temperature and Dentate Field Potentials in Exploring and Swimming Rats

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Attempts to correlate behavioral learning with cellular changes, such as increased synaptic efficacy, have often relied on increased extracellular potentials as an index of enhanced synaptic strength. A recent example is the enlarged excitatory field potentials in the dentate gyrus of rats that are learning spatial relations by exploration. The altered hippocampal field potentials do not reflect learning-specific cellular changes but result from a concomitant rise in brain temperature that is caused by the associated muscular effort. Enhanced dentate field excitatory potentials followed both passive and active heating and were linearly related to the brain temperature. These temperature-related effects may mask any learning-induced changes in field potential.

The hippocampal formation is a phylogenetically old part of the cerebral cortex. Although there is strong evidence for its involvement in learning the spatial relation between objects (spatial learning) (*1*), neurophysiological correlates to learning such as synaptic weight changes have been difficult to find in freely moving animals (*2*). However, it was reported that exploration of an unfamiliar environment was associated with an increased dentate field excitatory postsynaptic potential (f-EPSP) after perforant path stimulation but with decreased population spike amplitude and latency. The effect was interpreted to reflect a changed synaptic weight due to the learning experience (*3*). We now examine an alternative possibility: These changes may be due to a temperature effect (*4*). This possibility would also explain why the enlarged f-EPSP was associated with a decreased population spike (*5*). Both exercise and feeding elevate the brain temperature in rats, whereas inactivity and sleep result in lower temperatures (*6*). The major factor that controls brain temperature is muscular heat production, which warms the cerebral arterial blood (*7*). In rats that were swimming in a Morris maze, we observed field potential changes that are exactly opposite to those reported above, namely, f-EPSP reduction with spike increase. Similar field potential changes are seen during brain cooling (*4*).

For these reasons we have recorded the hippocampal temperature in freely moving rats and correlated it with dentate field potentials during exploration and swimming (*8*). In both situations there was a strong, linear correlation between the behaviorally induced potential changes and the brain temperature.

When rats explored items on a platform the slope of the f-EPSP was increased (Fig. 1A), which confirms earlier reports (*3*). However, this enhancement was paralleled by an increase in brain temperature. In addition, the f-EPSP latency, the population spike amplitude, and latency all decreased. The brain temperature rose during 10 to 20 min of exploration from 37.0° ± 0.1°C (mean ± SEM) with a mean rate of 0.11° ± 0.01°C per minute (n = 20); the largest increase was 3.2°C. After the exploration the brain temperature and the f-EPSPs declined along the same exponential time course, with both the f-EPSP and spike reaching base-line levels after 20 to 80 min. The temperature and f-EPSP curves were always parallel (43 sessions in nine of nine rats). The correlation factor (r) between the brain temperature and the f-EPSP slope during exploration was never <0.5 and in most runs was >0.75. For the exploring rat in Fig. 1, the r values between the brain temperature and the following signal elements were: f-EPSP slope, 0.76 ($P < 0.001$); population spike latency, −0.78 ($P < 0.001$); and population spike amplitude, −0.34 ($P < 0.01$). In rats with two thermistors, implanted at the same depth in the same or opposite hemispheres, the bilateral activity-induced temperature changes were nearly identical (difference <0.15°C). Therefore, the temperature at the contralateral homotopic point could be used as a reference value. Warming the brain by radiant heating (Fig. 1B) gave a similar parallel increase in brain temperature and f-EPSP slope (n = 19). Increasing the temperature of the animal by letting it run on a treadmill yielded comparable effects (n = 15), with larger changes seen after the faster running speed (Fig. 1C). During treadmill runs of animals that were not fully habituated, there was an initial reduction of the f-EPSP slope, similar to the results of Green, McNaughton, and Barnes (*3*). Similar parallel changes of brain temperature and f-EPSP were seen in the responses of the olfactory bulb to lateral olfactory tract stimulation in exploring rats (n = 5; Fig. 1D).

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