Blood–brain barrier opened by stimulation of the parasympathetic sphenopalatine ganglion: a new method for macromolecule delivery to the brain

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Object. Drug delivery across the blood–brain barrier remains a significant challenge. Based on earlier findings, the authors hypothesized that parasympathetic innervation of the brain vasculature could be used to augment drug delivery to the brain.

Methods. Using a criocotomy–cerebrospinal fluid superfuse paradigm in rats with an intravenous injection of tracer the authors demonstrated that stimulation of the postganglionic parasympathetic fibers of the sphenopalatine ganglion (SPG) increased the concentration of fluorescein isothiocyanate–dextran (4–250 kD) in the superfuse by two- to sixfold. A histological examination indicated the presence of dextran in the parenchyma. In another experiment the amount of Evans blue dye in the brain following SPG activation was similarly significantly elevated. The chemotherapeutic agents anti-HER2 monoclonal antibody and etoposide were also delivered to the brain and reached therapeutic concentrations. Brain homeostasis was not disturbed by this procedure; a measurement of nicotinamide adenine dinucleotide reduction did not show a decrease in the tissue metabolic state and brain water content did not increase significantly.

Conclusions. Sphenopalatine ganglion activation demonstrates a promising potential for clinical use in the delivery of small and large molecules to the brain.

Key Words • blood–brain barrier • parasympathetic nervous system • sphenopalatine ganglion • electrical stimulation • rat

T he BBB is one of the major defense mechanisms of the brain; it regulates blood–brain molecular traffic and maintains the brain’s delicate ionic and metabolic environment. Unless recognized by a specific transport system, hydrophilic molecules and lipophilic molecules larger than approximately 500 D display extremely limited penetration across the BBB. Hence the BBB has long been a major obstacle in the delivery of drugs to the brain, allowing its penetration only to small, weakly plasma-bound lipophilic molecules, and restricted entry to larger and more polar compounds and those strongly bound to plasma proteins.

Many attempts had been made to enable specialized medications to penetrate the BBB by changing either the barrier or the drug by circumventing the barrier by direct administration into brain tissue. An example of the first method is the intracarotid injection of a hyperosmolar solution, causing shrinkage of brain endothelial cells and allowing a transient increase in the permeability of the BBB.5,15 An example of the second method is the conjugation of amino acid or other moieties to the desired molecule so that it can use a BBB transport system to cross the barrier.4 An example of direct administration is a chemotherapy-laden gel directly placed inside a tumor resection site.1

In this paper we report the results of a new approach for BBB opening based on electrical stimulation of the postganglionic parasympathetic fibers of the SPG. This ganglion, classically known as the source of parasympathetic fibers to nasal and eye mucosa and the lacrimal gland, also supplies parasympathetic innervation to the brain vascular system, covering all the anterior cerebral circulation and some of the posterior circulation.7,10 Electrical stimulation of parasympathetic fibers of the SPG has been shown to induce vasodilation in cerebral vessels in rats,17 cats,3 dogs,10 and monkeys.20 This effect may involve the secretion of NO and/or vasoactive intestinal peptide.20 It has been shown,
Materials and Methods

All procedures were performed after approval of our protocol had been obtained from the local ethics boards.

Stimulation of the SPG

A uniform method of stimulation was used in all experiments reported here. In brief, a combined mucoperiosteal incision of the superior and inferior eyelid was performed, a flap was raised, and the medial canthus of the orbit was detached. Evolution of the orbit together with its contents was gently retracted posteriorly and laterally, and the anterior ethmoidal nerve was exposed. The peristeme of the ethmoidal foramen that surrounds the ethmoidal nerve was carefully separated from the bone, while keeping the nerve within its periosteal envelope. Custom-made bipolar nickel-coated hook electrodes, the poles of which were 1 mm apart and the 2-mm tips exposed, were hooked onto the postganglionic parasympathetic fibers immediately lateral to the orbital opening of the ethmoidal foramen. The cathode was placed on the nasal (medial) side. During stimulation, care was taken to maintain steady positioning of the electrode to prevent tearing of the nerve and maintain electrode–nerve contact. Control animals underwent sham operations without hooking the electrodes.

Craniotomy–CSF Superfusate Experiments

Surgical Procedure. Twenty-two Wistar–Furth rats each weighing approximately 300 g were used. The rats were given an anesthetic agent (inactin; thiobutabarbital 100 mg/kg administered intraperitoneally), a tracheotomy, and mechanical ventilation with room air and supplemental oxygen. A catheter was placed in the left femoral artery for the measurement of systemic blood pressure and for blood sampling. Another catheter was placed in the left femoral vein for tracer administration. The left ethmoidal foramen was exposed and a craniotomy was performed as previously described.10,11 In brief, the craniotomy was performed, the dura mater incised, and the cerebral microcirculation exposed. Inlet and outlet ports were placed in the craniotomy well to allow a constant flow of superfusate over the cerebral pial microcirculation and cortical surface. The superfusion fluid (artificial CSF) was heated to 37°C and bubbled continuously with 95% nitrogen and 5% carbon dioxide to maintain gases within normal limits.

Experimental Procedure. The electrodes were hooked onto the ethmoidal nerve fibers; thereafter, FITC–anti-HER2 mAb was infused. The procedure was similar to the one used for infusing the FITC–dextran. The animals' brains were removed and sectioned for fluorescence microscopy imaging.

Opening of the BBB by Sphenopalatine Ganglion Stimulation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>4-kD dextran (ng/ml) (3 stimulated animals, 5 control animals)</th>
<th>70-kD dextran (ng/ml) (3 stimulated animals, 4 control animals)</th>
<th>250-kD dextran (ng/ml) (4 stimulated animals, 4 control animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation</td>
<td>‡ Probability value for the comparison between stimulation and control ratios.</td>
<td>† Probability value for the comparison between peak superfusate concentrations in the stimulation and control groups.</td>
<td>† Probability value for the comparison between peak superfusate concentrations in the stimulation and control groups.</td>
</tr>
<tr>
<td>* Values are expressed as means ± standard error.</td>
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independently, that an increase in NO concentration in cerebral vessels due to administration of NO donors10 or histamine11 increases the permeability of the BBB. It was, therefore, our hypothesis that stimulation of the SPG would cause an increase in BBB permeability, in addition to vasodilation, and thus enable delivery of molecules to the brain.
Opening of BBB by sphenopalatine ganglion stimulation

**TABLE 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stimulation Group</th>
<th>Control Group</th>
<th>Overall Stimulation/Control Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superfusate</td>
<td>Blood</td>
<td>Superfusate/Blood Ratio</td>
</tr>
<tr>
<td>4-AD dextran (ng/ml) (3 stimulated animals, 3 control)</td>
<td>126.5 ± 49.7</td>
<td>1.60 ± 0.3</td>
<td>87.20 ± 35.2</td>
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<tr>
<td></td>
<td>(p = 0.044)†</td>
<td>(p = 0.02)†</td>
<td></td>
</tr>
<tr>
<td>7-AD dextran (ng/ml) (5 stimulated animals, 4 control)</td>
<td>93.9 ± 64.7</td>
<td>14.75 ± 1.1</td>
<td>6.56 ± 5.5</td>
</tr>
<tr>
<td>250-AD dextran (ng/ml) (3 stimulated animals, 4 control)</td>
<td>29.8 ± 7.3</td>
<td>7.36 ± 0.8</td>
<td>2.37 ± 1.3</td>
</tr>
<tr>
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<td>(p = 0.001)†</td>
<td>(p = 0.051)†</td>
<td></td>
</tr>
</tbody>
</table>

* Values are expressed as means ± SD unless otherwise indicated.
† Probability value for the comparison between peak superfusate concentrations in the stimulation and control groups.
‡ Probability value for the comparison between stimulation and control ratios.

Closed Craniotomy Experiments

**Evans Blue Dye.** Six Wistar rats each weighing approximately 300 g received stimulation and six served as sham-operated controls. The rats were anesthetized using pentobarbital (60 mg/kg) and the right carotid artery and jugular vein were catheterized. The cerebral vessels were carefully separated from the meninges, cerebral cortex, and dura mater. Then, a craniotomy was performed, and a right lateral ventricle was perforated. A 2-mm dural incision was made in the pericranium over the left hemisphere, and the dura was opened. A 2-mm dural incision was then made in the pericranium over the left hemisphere, and the dura was opened. A 2-mm dural incision was made in the pericranium over the left hemisphere, and the dura was opened.

**Etoposide.** Six experimental and five control Wistar–Furth rats were used in this experiment. The FITC–anti-HER2 mAb was infused. The procedure was similar to the one used to administer the FITC–dextran. The animals’ brains were removed to prepare histological fluorescence slides and for homogenization and fluorescence measurements.

**Brain Water.** Brain water content was measured in several setups: 1) immediately after a 1-hour stimulation; 2) 1 hour after the end of the 1-hour stimulation; 3) 24 hours after the 1-hour stimulation; and 4) immediately after a 3-hour stimulation. After the animals had been killed, their brains were dissected into four regions: right and left hemispheres, cerebellum, and brainstem. Tissue samples were then dried in a desiccating oven at 105°C for 24 hours.

**Statistical Analysis**

We used t-tests for most comparisons. Data provided by the Evans blue dye experiment were analyzed using the Tukey–Kramer test. All data are presented as means ± SDs.

**Results**

**Permeability of the BBB Measured in the CSF Superfusate**

The craniotomy–CSF superfusate paradigm is a well-known technology for the determination of BBB permeability. Activation of the SPG significantly increased the concentration of the dextran for all molecular weights in the superfusate, indicating an increase in BBB permeability (Fig. 1 and Table 1). The increase in permeability appeared
Brain Physiology During SPG Stimulation

Mitochondrial Function. In stimulated animals NADH fluorescence decreased by 10.16 ± 19.42% after 20 minutes and by 29.04 ± 49.48% after 40 minutes of stimulation. Fifteen minutes after the cessation of stimulation the decrease was 43 ± 65.94% compared with baseline; these changes were not significant. For the control animals, the measurement revealed minute changes with decreases of 0.58 ± 3.59% at 20 minutes and 2.67 ± 4.96% at 40 minutes; the poststimulation measurement showed an increase of 0.93 ± 9.75% (not significant). Thus, the NADH redox state did not show a significant change subsequent to stimulation. If anything, there was a tendency toward improved oxygenation of brain tissue, as indicated by a decrease in NADH level typical of vasodilation, as evidenced by the increase in LDF values.

Brain Water Content. No significant change in brain water content was measured following SPG stimulation in any of the experimental paradigms (data not shown). A slight nonsignificant increase in brain water content was found after 3 hours of stimulation, from 78.54 to 79.63%.

Discussion

Stimulation of the SPG increased the permeability of the BBB, markedly enhancing entry of macromolecules into the brain, as demonstrated for FITC–dextran, Evans blue dye–albumin, and the chemotherapy agents anti-HER2 mAb and etoposide. Furthermore, our findings indicate that this effect is reversible, as demonstrated by the rapid decline of FITC–dextran concentrations in the superfusate at the end of the stimulation. We interpret our findings as a major advance in overcoming the obstacle of the BBB in the delivery of therapeutic agents to the brain tissue, with a substantial promise for rapid application in many currently lethal and disabling central nervous system disorders.

The SPG belongs to the parasympathetic subdivision of the autonomic nervous system. Its main ramifications are traditionally known to be the mucosal membranes of the nose, the lacrimal gland, and facial skin vessels. Its effects are dilatory and secretory, causing nasal secretions, lacrimation, and flushing of the intracranial vessels. Cerebrovascular effects, as mimicked by an infusion of carbachol, are also shared by parasympathetic fibers emanating from the SFG.

In a study of the dura mater in rats, Delepine and Aubin showed an increased BBB permeability in response to electrical stimulation of the SPG with capsaicin, which diminished, but did not block the effects of an infusion of carbachol. This indicates that cholinergic transmission is central to the enhancement of BBB permeability. The lack of fluorescence in tissue from control animals is in clear contrast with the prominent fluorescence in tissue from animals that received stimulation. Original magnifications correspond to these scales.

FIG. 3. a: Photographs of the same area of tissue corresponding to animals that received stimulation. Laminar staining is observed to be dependent on molecular weight, with the larger 250-kD dextran penetrating less than the smaller molecules.

The dextran concentration in the superfusate quickly decreased on cessation of stimulation (Fig. 2), an indication of the reversibility of the SPG stimulation effect on BBB permeability. The continuous increase, compared with the basal (nonstimulated intervals) superfusate dextran concentration, indicated a cumulative effect of repeated SPG stimulations. Presence of FITC–dextran in brain tissue following stimulation is demonstrated in Fig. 3a.

Closed Cranium Tissue Homogenate Experiments

In line with the FITC–dextran superfusate experiments, the amount of Evans blue–stained brain tissue also increased after SPG stimulation to a similar extent (Fig. 4). Interestingly, a clear difference was seen between the lateral distribution of postganglionic parasympathetic fibers innervating the lacrimal gland, and facial skin vessels. Its effects are additionally known to be the mucosal membranes of the nose, with capsaicin, which mimics the effects of sympathetic stimulation.

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and flushing of the face. More recently, its relevance for intracranial vessels has been recognized. Suzuki and colleagues and later Hara, et al., mapped the projections of parasympathetic fibers from the ganglion to most of the cerebrovascular bed. Later, a vasodilatory effect of SPG stimulation on these vessels was shown. Based on these lines of evidence, we hypothesized that electrical stimulation of the SPG would induce both vasodilation and increased BBB permeability of cerebral vessels innervated by its fibers.

In a study of the dura mater in rats, Delepine and Aubineau showed an increase of 200% in plasma protein extravasation in response to electrical stimulation of the SPG. This effect was abolished by an infusion of atropine and mimicked by an infusion of carbachol. This indicates that cholinergic transmission is central to the effect, most likely at the ganglionic level. Furthermore, to rule out a possible antidromic firing from nociceptors as a mediator neurogenic inflammation, some of the rats were pretreated with capsaicin, which diminished, but did not block the effect. Our histological investigations and measurements of brain homogenate concentration clearly demonstrated that the effect we describe is not limited to changes in meningeal vessels.

Opening of BBB by sphenopalatine ganglion stimulation

FIG. 3. Fluorescence microscopy images and photomicrographs of brain tissue. a: Frontal cortex as it appears with light microscopy (I) and with fluorescence of the same region due to FITC–dextran (10 kD) with (II), and without (III) stimulation. b: Cortical areas in animals treated with anti-HER2 mAb. Tissue shown with light microscopy (I and III) and corresponding area viewed with fluorescence microscopy (II and IV) for stimulated and nonstimulated animals, respectively. The lack of fluorescence in tissue from control animals is in clear contrast with the prominent fluorescence in tissue from animals that received stimulation. Original magnifications × 100. H & E used for light microscopy (al, bl and III).
A major concern for any disruption of the BBB is the possibility of the protective function of this barrier, its presumed raison d’être. This could lead to penetration of unwanted molecules; changes in the ionic, metabolic, or osmotic balance; or invasion of immunogenic or infectious elements. These possible effects have not yet been systematically studied. Our understanding of the potential clinical uses of this method is that repeated short activations can be made at the height of blood concentration of the relevant medication. This could be designed as cycles lasting several minutes up to a few hours, occurring once every several days or weeks. For such a working paradigm, one can consider migraine attacks, which represent neurogenic inflammation of cerebral vessels and probably cause a similar transient effect on the BBB; despite possible opening of the BBB, migraineurs do not show any sign of long-term injurious effects on brain function between attacks. Similarly, most patients who contract meningitis or encephalitis sustain some transient damage to their BBB, usually lasting several days or longer, and recover completely. An extrapolation from these clinical examples to our model indicates no long-term damage to the brain.

As seen in Fig. 2, the present experiments demonstrate the short duration of BBB disruption; the clear decrease in dextran concentration in the superfusate immediately on cessation of stimulation indicates the reversibility of the effect. This is a major safety feature in favor of this proposed method for BBB opening. Nevertheless, we conducted two additional experiments to explore the potential injurious effects of SPG stimulation on brain physiology. We found no injurious effect on the metabolic state of the brain, as shown by the NAD/NADH balance and no significant brain edema caused by SPG stimulation. Therefore, SPG stimulation appears to be a safe procedure for future administration to humans, and a regimen of repeated short activations should not cause brain damage. The surgical procedure to expose the ethmoidal nerve in the rat was designed as an acute procedure, the size and accessibility of the human equivalent mean that implantation of an electrode could be done as a minor procedure. The greater palatine canal, which extends from the roof of the oral cavity up into the sphenopalatine fossa, can serve as a convenient route for introduction of a stimulating electrode to the ganglion.

Potential applications of this stimulation method in human disease are wide, including improvement in the delivery of the following agents: 1) drugs used in chemotherapy for primary and secondary brain tumors; 2) immune molecules tailored to attack specific targets such as the amyloid protein in cases of Alzheimer disease; 3) growth factors to stop degeneration and enhance regeneration in cases of neurodegenerative diseases such as Parkinson, Alzheimer, Huntington, amyotrophic lateral sclerosis, and others; 4) genes for modification of genetically based diseases; and 5) future molecules to be developed once it is realized that the BBB can be overcome. Furthermore, molecules that are in excess in the brain, such as the amyloid protein in cases of Alzheimer disease; 3) growth factors to bypass the P-glycoprotein to some extent and endothelium, opening of tight junctions could allow such molecules to bypass the P-glycoprotein to some extent and reach higher concentrations in the brain. A very useful clinical application of this method can therefore be foreseen for both hydrophilic and lipophilic agents.

The method of measurement we used for the craniotomy–superfusate experiments was based on the model developed by Mayhan and Heistad, in which the brain surface is constantly superfused with artificial CSF. Previous studies in which this model has been used have shown that dextran extravasation during perturbation of the brain (acute hypertension, infusion of hyperosmolar solutions, and application of inflammatory mediators) reflects a disruption of the BBB, primarily in cerebral venules and veins. Care was taken, therefore, to maintain a constant blood pressure during our experiments. Although the craniotomy–superfusate model has the advantage of reflecting the time course of the stimulation effect, it could be criticized because it involves a surgical procedure to expose the brain immediately before the experiment. This could cause some local tissue damage and artificially raise extravasation, which would explain the gradual increase in the baseline concentration of the dextran in the superfusate, and tissue staining, however, are free of this potential difficulty and clearly show the BBB disruption effect. Fluorescence microscopy imaging (Fig. 3) demonstrated florescence not only in the capillaries and pia mater, but also in the brain parenchyma itself. This is evidence of the penetration of FITC–dextran across the parenchymal BBB.

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Chemotherapeutic Agents
- anti-HER2 mAb (6 stimulated animals, 5 control; 5 etoposide (6 stimulated animals, 1 control)
- * Values are expressed as means ± standard deviation
- † Probability values are not detectable
- ‡ Probability values are not detectable
- † The lowest detectable value

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Fig. 4. Closed cranium experiments: Evans blue dye in the brain after stimulation of the right SPG. Upper: Bar graph of tissue homogenate concentrations showing significant differences between stimulated and control right hemispheres (a, p < 0.01) and stimulated and control left hemispheres (b, p < 0.05). Lower: Macroscopic view of the brain showing a clear blue stain in the stimulated hemisphere and the cerebellum and no dye in the other hemisphere.
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| TABLE 2 |
|-----------------|-----------------|-----------------|-----------------|
| **Brain homogenous concentrations of anti-HER2 mAb and etoposide** | **Stimulation Group** | **Control Group** | **Stimulation/Control Ratio** |
| **Chemotherapeutic Agent** | Cortex | Brainstem | Cerebellum | Cortex | Brainstem | Cerebellum | Cortex | Brainstem | Cerebellum |
| anti-HER2 mAb (6 stimulated animals, 5 control) | 26.0 ± 19.5 | 43.7 ± 27.3 | 8.7 ± 5.7 | 30.9 ± 133.2 | ND | ND | 5.2 ± 2 (p = 0.002) | 2.4 ± 1.2 (p < 0.001) | 2.6 ± 0.7 (p = 0.02) |
| etoposide (6 stimulated animals, 1 control) | | | | | | | 5.0 | 17.9 | 3.4 |

* Values are expressed as means ± SD (pg/ml for anti-HER2 and ng/ml for etoposide) unless otherwise indicated. Abbreviations: NA = not applicable; ND = not detectable.
† Probability values are for the comparison between the stimulation and control groups.
‡ The lowest detectable concentration was 45 ng/ml.

BBB can be overcome. Furthermore, molecules that are in excess in the brain, such as the amyloid protein in cases of Alzheimer disease, can be expected to clear out into the plasma if the BBB obstacle is removed.

Conclusions

Using several lines of evidence, the present study shows that the BBB can be temporarily opened by electrical stimulation of the SPG in the rat. Limited safety studies attest to the lack of substantial side effects. Potential application of this technique in humans is wide, including brain tumors, and neurodegenerative and immune-based brain diseases.

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References


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