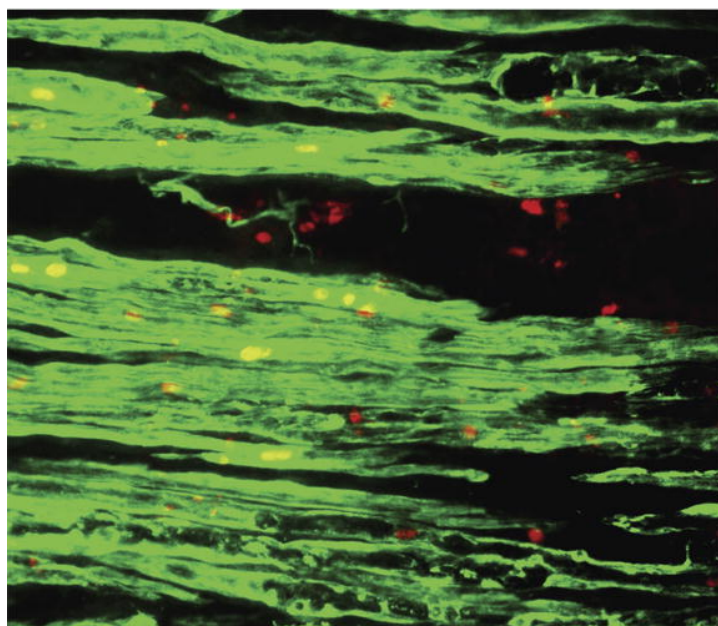


Brain Research



JANUARY 10, 2008 | VOLUME 1188
ISSN 0006-8993

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RESEARCH

Research Report

Histological evidence for drug diffusion across the cerebral meninges into the underlying neocortex in rats

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ARTICLE INFO

Article history:

Accepted 17 October 2007

Available online 26 November 2007

Keywords:

Epidural cup

NMDA

Neocortex

Focal epilepsy

Transmeningeal drug delivery

ABSTRACT

Transmeningeal pharmacotherapy has been proposed to treat neurological disorders with localized pathology, such as intractable focal epilepsy. As a step toward understanding the diffusion and intracortical spread of transmeningeally delivered drugs, the present study used histological methods to determine the extent to which a marker compound, *N*-methyl-D-aspartate (NMDA), can diffuse into the neocortex through the meninges. Rats were implanted with bilateral parietal cortical epidural cups filled with 50 mM NMDA on the right side and artificial cerebrospinal fluid (ACSF) in the contralateral side. After 24 h, the histological effects of these treatments were evaluated using cresyl violet (Nissl) staining. The epidural NMDA exposure caused neuronal loss that in most animals extended from the pial surface through layer V. The area indicated by this neuronal loss was localized to the neocortical region underlying the epidural cup. These results suggest that NMDA-like, water soluble, small molecules can diffuse through the subdural/subarachnoid space into the underlying neocortex and spread in a limited fashion, close to the meningeal penetration site.

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1. Introduction

It has recently been proposed that transmeningeal pharmacotherapy, utilizing microprocessor-controlled subdural drug deliveries with continuous feed-back from the treated neural tissue, might be used for the therapy of brain disorders that involve localized cerebral cortical pathology, such as focal epilepsy and some types of stroke and tumors (Ludvig et al., 2006; John et al., 2007). A prerequisite of developing this novel therapy is to understand the diffusion, intracortical spread and clearance of transmeningeally administered drugs. Pre-

vious *in vitro* studies on human postmortem material showed that the cranial dura is readily permeable to a range of water soluble small molecules (Moore et al., 1982); however, the intracortical spread of drugs was not investigated. Cornblath and Ferguson (1976) used biochemical methods to estimate the penetration of topically applied [³H] acetylcholine (Ach) into the neocortical layers in cats. While the size of the drug-exposed area was not determined, the authors did detect the presence of radioactivity in all cortical layers after 3–5-min exposures of the pia mater to the radiolabeled compound. In their epidural drug delivery experiments, Eder and his

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Abbreviations: NMDA, *N*-methyl-D-aspartate; ACSF, artificial cerebrospinal fluid; GABA, γ -aminobutyric acid; Ach, acetylcholine; PBS, phosphate-buffered saline

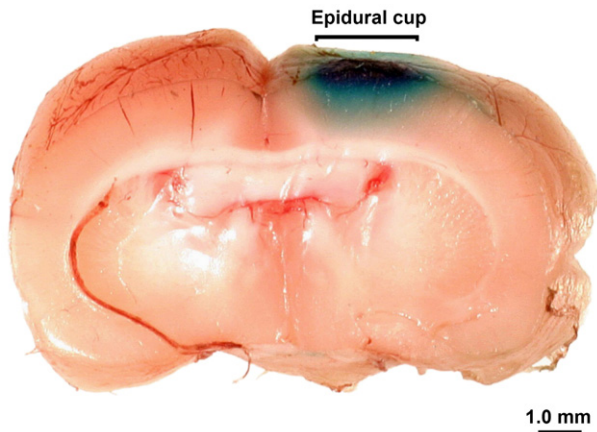


Fig. 1 – Diffusion of methylene blue from an epidural cup into the underlying neocortex, following a 15-min incubation period. Note the localized penetration of the dye into the cortical tissue and that the geometry of the stained area is similar to that of the area of neurodegeneration induced by epidural NMDA incubation (Figs. 2A and C).

colleagues (1997) monitored the diffusion of a dye, methylene blue, into the cerebral cortex and found that it penetrated into the ventricular system. It remained unclear whether this extensive penetration was due to rupturing the meninges and the underlying cortical tissue, as the dye, along with the examined drug solutions, was delivered as a bolus via an injection cannula, a method prone to produce such ruptures.

We have previously shown that Ach delivered via an epidural cup causes focal seizures in freely moving rats and that this effect can be (a) prevented by administering pentobarbital into the cup prior to Ach delivery and (b) terminated by administering either pentobarbital or GABA into the cup during an ongoing seizure (Ludvig et al., 2006; John et al., 2007). The epidural cup technique (Tanganelli et al., 1992) was used because it offers transmeningeal drug deliveries in freely moving animals without causing significant damage to the meninges and the underlying neural tissue. To date, in these epidural cup studies, no neuroimaging, autoradiography or histological methods have been used to visualize transmeningeal drug penetration through the intact dura, arachnoid and pia maters and to reveal the extent of such drug penetrations into the brain.

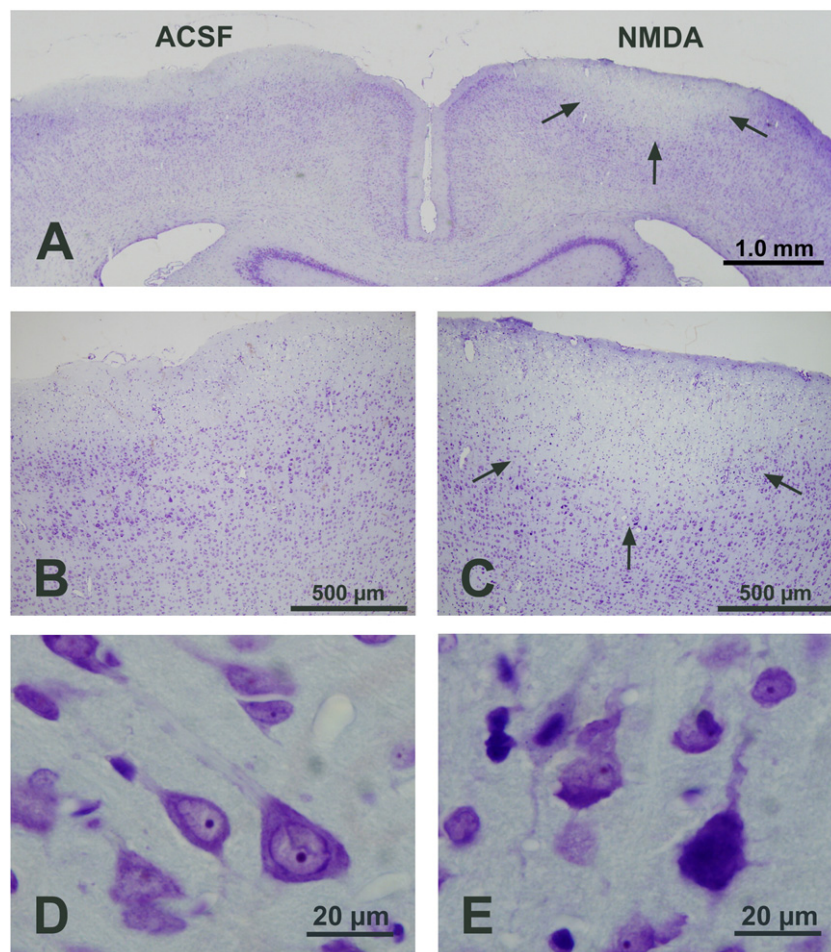


Fig. 2 – Rat brain section stained with cresyl violet for Nissl substance. (A) Image of the superior portion of the brain. Note the arc-shaped region of cell loss (outlined by arrows) in the area under the epidural cup. (B) View of the ACSF-treated left cortex highlighting relative sparing of the layered organization of the cortex. (C) The NMDA-induced neuronal loss, as manifested through cortical layer IV. (D) Intact layer V cortical pyramidal cells with normal soma and apical dendrite morphology. (E) NMDA-induced neurodegeneration in layer V: note the dark pyknotic nuclei and the altered (“corkscrew”-like) dendritic morphology.

The goal of the present study was to provide histological evidence of transmeningeal drug diffusion in the rat brain and to clarify the extent to which drugs delivered by this method spread within the underlying cortical tissue. We examined the diffusion and spread of *N*-methyl-*D*-aspartate (NMDA) and, for comparison, methylene blue, delivered using the epidural cup method. NMDA was chosen because it is comparable to Ach and GABA in terms of water-solubility and molecule size, and thus its transmeningeal diffusion characteristics are likely similar to those of Ach and GABA used in our prior studies. Furthermore, NMDA can induce neurodegeneration that is readily detectable with histological methods. Indeed, the neurodegenerative effects of NMDA in the rat neocortex have been previously examined, albeit with different methods: by applying powdered NMDA on the surface of the dura mater (Sofroniew and Pearson, 1985; Storey et al., 1992) and by briefly administering this compound in a 150 mM solution onto the dura mater under anesthesia (Huang et al., 1998). In our histological analysis, we divided the parietal cortical layers into superficial and deep layers, with the superficial layers comprising the supragranular layers (I–III; Somogyi et al., 1982; Huang et al., 1998) and the internal granular layer IV itself, whereas the deep layers comprising layers V and VI. This division was merely based on our preliminary studies (Experimental procedures), which already indicated a more apparent drug penetration into layers I–IV than into layers V and VI.

2. Results

Epidural delivery of a methylene blue solution resulted in an intracortical diffusion pattern that was restricted to the area underneath the epidural cup (Fig. 1). Within the 2.0 mm deep neocortex, the average penetration depth of the dye was 1.3 ± 0.2 mm (mean \pm S.E.M.). The variability in penetration depth appeared to be related to the extent of spillover of the dye, however limited it was, onto the surface of the dura surrounding the cup: larger spillover led to smaller penetration depth.

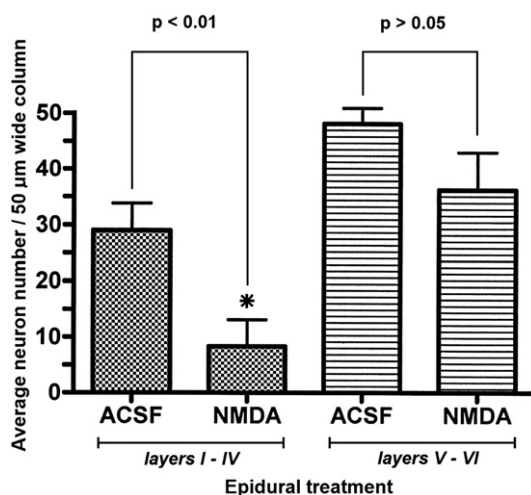


Fig. 3 – Statistical analysis of the neuron counts in the ACSF-treated and the NMDA-treated cortices. Neuron counts in the superficial and deep layers are separated. Bars indicate mean \pm S.E.M.

(We note that while spillover from the interior of the epidural cup to the surface of the skull did not occur during surgery, as this could be prevented by careful sealing with dental acrylic, unrecognized slow spillover in between the bone of the skull and the dura mater could occur, since this was difficult to control with our method of gently placing the cup on the dura mater).

The overall diffusion pattern obtained with NMDA treatments were similar to those obtained with methylene blue. Thus, incubation with the epidural cup with NMDA induced neurodegeneration in an arc-shaped region within the drug-exposed cortex (Figs. 2A and C). In 7 of 8 animals, the area of neurodegeneration, marked with neuronal loss, extended from the pial surface through layer IV and into layer V. Additional neuronal damage was evident based on the presence of sparse pyknotic neurons (Fig. 2E). In some cases, the pyknotic neurons were found in a rim around the lesion; in others, they were more randomly distributed. The total number of neurons counted in the NMDA-incubated region (44.54 ± 11.23 per column) was significantly lower ($p < 0.05$) than that in the ACSF-incubated area (77.17 ± 6.57 per column). The extent to which NMDA diffused from the brain surface was reflected in the gradual neuronal loss across the cortical layers. In the superficial cortical layers, significantly fewer neurons were identified on the NMDA-incubated side (8.3 ± 4.8 ; Fig. 3) than on the ACSF-incubated side (29.0 ± 4.9 ; $p < 0.01$; Fig. 3). In the deep cortical layers, the number of neurons was reduced in the NMDA-treated cortex (36.2 ± 6.9), but this value was not statistically different from that in the contralateral, ACSF-treated area (48.1 ± 2.9 ; $p > 0.05$; Fig. 3). It is worth noting that in 5 of the 8 animals some neuronal loss was observed in layer II on the ACSF-treated side; however, cortical layer organization and morphology of the neurons appeared normal (Figs. 2A, B and D).

3. Discussion

The present data are consistent with the neurodegenerative effects of NMDA delivered into the cortex with other methods (Sofroniew and Pearson, 1985; Storey et al., 1992; Huang et al., 1998) and provide further evidence for the diffusion of small, water-soluble molecules, like NMDA, across the cerebral meninges into the underlying neocortical tissue. This supports the central idea behind transmeningeal pharmacotherapy, i.e., that the physico-chemical and anatomical properties of the cerebral meninges permit the spatially and temporally controlled delivery of therapeutic compounds through the subdural/subarachnoid space for the treatment of neurological disorders with focal pathology (Ludvig et al., 2006). Consequently, in our previous studies (Ludvig et al., 2006; John et al., 2007), the abilities of epidurally delivered pentobarbital and GABA to control focal neocortical seizures were primarily due to the penetration of these compounds into the seizure focus.

The characteristic shape and size of the area of NMDA-induced neurodegeneration and methylene blue diffusion suggest that drug delivery via the epidural cup leads to localized intracortical drug penetration. Thus, the delivered drug molecules do not diffuse through the corpus callosum into deep brain structures, but remain in the cortical zone underlying the drug-exposed dura mater. This distribution

pattern, if confirmed with other compounds in future studies, has important practical consequences. Namely, it indicates that the intracerebral diffusion of transmeningeally delivered agents can be controlled by the location, shape and size of the subdural/subarachnoid drug delivery apparatus, permitting site-specific (e.g., seizure-focus-specific) intracranial drug treatments. Nevertheless, due to the complexity of the human pia mater/neocortex interface (Ramsey, 1965; Lopes and Mair, 1974; Weller, 2005), elaborating the degree of drug diffusion in the intact human brain requires further investigation.

Examination of the penetration depth of the methylene blue and NMDA solutions showed that water-soluble drugs delivered through the meninges can reach the internal pyramidal cell layer, and this penetration, according to the dye experiment, can take place within 15 min. This indicates that transmeningeally administered drugs do have the potential to intervene, within a reasonably short diffusion time, into the functions of neurons integrated in layers II, III, IV and V, all of which seem to contribute to the genesis and propagation of epileptiform electrical discharges (Eccles, 1984; Connors, 1997; Ribak et al., 1979; Telfeian and Connors, 1998; Rakhade et al., 2005). Consequently, drugs delivered through the subdural/subarachnoid space might especially be useful for the treatment of intractable neocortical seizures. Regarding the variation in both methylene blue and NMDA penetration depths across experiments, despite our fairly constant experimental conditions, the observation that spillover of the dye between the dura mater and the skull led to decreased penetration may offer a clue to at least one source of this variation. Namely, as such a spillover decreases the height of the fluid column inside the epidural cup, the hydrostatic pressure exerted by this fluid column on the meninges decreases proportionally. This can cause limited transmeningeal diffusion.

We observed some neuron loss in the superficial cortical layers underlying the ACSF-incubated epidural cup. While this effect was clearly minimal compared to the marked neurodegeneration in the NMDA-treated cortex, it should not be overlooked. It seems that exposing the dura mater to an epidural device and/or an incubating solution can induce unwanted cellular damage, possibly via various mechanisms, such as mechanical compression (Kundrotiene et al., 2002) or the drainage of extracellular molecules: the very mechanism that is exploited for neurotransmitter collection with epidural cups (Tanganelli et al., 1992). Understanding and counteracting these unwanted cellular effects are necessary to make transmeningeal pharmacotherapy a safe method.

4. Experimental procedures

4.1. Animals

Adult, male Long–Evans rats ($n=18$), weighing 300–400 g, were used according to an experimental protocol approved by the Institutional Animal Care and Use Committees at NYU School of Medicine and SUNY Downstate Medical Center. Six animals were used to determine the optimal experimental design for NMDA administration. This optimal design was employed in 8 rats: the described results were obtained in these animals. Four additional rats were used to examine the penetration of a

methylene blue solution from the epidural cup into the underlying cortex.

4.2. Surgical and post-surgical protocols

Each rat for the MMDA study was anesthetized with 50 mg/kg pentobarbital, i.p. Additional injections of 0.1 mg/kg atropine, s.c. and 30,000 units/100 g Penicillin G Benzathine/Penicillin G, i.m., served to reduce secretion in the respiratory system and prevent infection, respectively. The rat was placed in a stereotaxic apparatus, the skull was exposed and a 5 mm diameter craniotomy was drilled in the right parietal bone with its center 2.5 mm posterior to the bregma and 2.5 mm lateral to the midline, according to the rat brain atlas of Paxinos and Watson (1998). This was followed by drilling a similar craniotomy into the left parietal bone. Every effort was made to perform these craniotomies without causing damage to the dura mater. Next, a silicone cylinder, 8 mm high, 5 mm outer diameter and 3 mm inner diameter (“epidural cup”), was positioned into each craniotomy, gently placed on the exposed dura mater, and secured with dental acrylic to the skull and two nearby anchoring screws. The surgical techniques ensured that no bleeding occurred inside the cup and no dental acrylic spilled into its inner area. Normal artificial cerebrospinal fluid (ACSF; pH=7.4; total osmolarity=311.2 mOsm; 150 mM Na, 155 mM Cl, 1.4 mM Ca, 3.0 mM K, 0.8 mM Mg, 1.0 mM P) was delivered into each cup to test whether any leakage occurred from its interior to the surface of the skull. If there was no such leakage, due to proper dental acrylic sealing, the cups were emptied, and we proceeded by delivering a 50 mM NMDA solution (in a volume of 50 μ l, dissolved in ACSF) into the right side epidural cup. To maintain the osmolarity of the NMDA solution at 311.2 mOsm, the NaCl concentration in the solvent ACSF was appropriately reduced by 50 mM. This was followed by delivering the same volume of ACSF (control solution) into the cup on the left side. We selected 50 μ l for delivery volume, partly to be consistent with our previous pharmacological study where also 50 μ l volume was used for epidural GABA and Ach deliveries, and partly because the design of the epidural cup, the size of the craniotomy, the number of consecutively delivered drugs and the technique of their delivery made this delivery volume optimal.

After these procedures, the rats were allowed to wake up and behave freely in their cage for 24 h. During this period, convulsions were not observed. This is consistent with prior experiments, which showed that intracortical NMDA delivery does not cause convulsions if the delivery method assures no spillover into subcortical structures (Ludvig et al., 1992), perhaps because neocortical NMDA receptors decrease Ach release and may also be involved in facilitating the release of GABA (Materi and Semba, 2001).

In the 4 rats for the methylene blue penetration study, the above surgical and post-surgical methods were modified as follows: (a) only a right-side cup was implanted, (b) the 50 μ l of dye solution was kept for only 15 min in the cup, and (c) after this 15-min epidural methylene blue exposure the cup was emptied, the rat was immediately euthanized and the brain was removed followed by a frontal cut across the center of the implanted/stained area for examination under a dissection microscope (Fig. 1).

4.3. Histology and statistical analysis

At the end of the 24-h epidural NMDA and ACSF incubations, the rats were euthanized with 120 mg/kg pentobarbital, i.p., transcardially perfused with phosphate-buffered saline (PBS) followed by 10% formalin. The epidural cup assembly was carefully detached and the fixed brain was removed. A 4 mm thick block that included the right and left cortical areas under the epidural cups was cut from each brain and embedded in paraffin. Sections 8–10 μm thick were cut, mounted on glass slides and every tenth section was stained with cresyl violet for Nissl substance. This stain is used to differentiate between neurons and glial cells based on the amount and location of Nissl substance as well as nuclear size and appearance. For each animal, three sections were used to evaluate a sampling of neuronal loss. The analysis of relative neuronal loss was undertaken in order to document the average cortical depth that NMDA penetrated following epidural administration.

Morphologically identifiable neurons in two 50 μm -wide columns were counted in all cortical layers from the pial surface to the underlying white matter, under both the NMDA-filled and the ACSF-filled epidural cups. Normal and pyknotic neurons were counted within a 50 μm -wide box extending the length of the display monitor projecting the live image from a 40 \times objective. The slide was aligned with the pial surface horizontal on the monitor, and moved vertically in order to quantify neurons in all layers in the column. The location of the columns was chosen based on the arc shape of the cortical lesion caused by administration of NMDA. On the NMDA-treated side, the first column was located at the center of the lesion and the second column midway between the lesion center and lateral edge. Corresponding columns were examined on the contralateral, ACSF-treated side. Data collected from both columns of each hemisphere were used for analysis. The average number of neurons across all six cortical layers was obtained for both the NMDA-treated and ACSF-treated cortices. In addition, the average numbers of neurons within the superficial (I–IV) layers and that within the deep (V–VI) layers were also determined. For statistical comparison of the neuron counts in the NMDA-treated and ACSF-treated cortices, two-tailed paired t-test was employed, using the following dependent variables: (1) mean total number of neurons per column, (2) mean number of neurons per column in the superficial layers and (3) mean number of neurons per column in the deep layers.

Acknowledgments

We thank Lorraine Braithwaite-Harte and Hayley B. Miskiewicz for their assistance. This study was supported by NYU/FACES.

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