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Experimental Models of Neurodegenerative Diseases: A Simple Method to Check Both Viability of Neural Tissue and Cannulae Location After Stereotaxic Surgeries

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The reagent MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) is reduced to formazan in living cells, giving a purple colored product. The product absorbance is susceptible to be measured at a certain wavelength by a spectrophotometer. We report in this paper the use of MTT as a suitable method for checking both the viability of neural tissues as well as the correct location of stereotaxically placed cerebral cannulae. Evidence is given that this method can be useful not only because it is a new way of utilizing a vital staining to evaluate cannulae location, but also because MTT is a simple, effective and cheaper way of assessing the arousal level of neural tissues under study. In doing so, we can effectively test the stimulatory and/or inhibitory actions of several reagents in what we finally named as MTT-micro assay on living tissues: the MTT-malt.

Keywords: Neurodegenerative Diseases, Animal Models, Viability, MTT, Stereotaxic Surgery.

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1. INTRODUCTION

Stereotaxic surgery is one of the most employed methods to directly deliver several reagents to discrete brain areas.[1,5,16] Therefore, it becomes critical to asses the correct cannulae location in the brain, so we can correlate the presence of the reagent and its eventual effect. To verify the correct placement of the cannulae most researchers use conventional dyes like India ink, methylene blue, trypan blue, among others.[6,10,14] However, these methods are generally used for histological studies and are not compatible with subsequent analysis of the utilized samples. The use of the dye MTT that fulfill both conditions—it allows both stereotaxic cannulae localization and subsequent biochemical studies—would prove to be useful in getting new insights related to neurobiology research.

Testing the right localization of a brain cannulae and, at the same time, trying not to disturb biological processes on the same tissues, is possible because of a 50 year old biological reaction that uses a tetrazolium salt called MTT [3-(4,5-dimethylthiazol-2-yl)]. The reaction is based on the yellow color of MTT and its reduction to purple crystals of formazan in a mitochondrial and cytoplasmic redox reaction in living cells. The colored product has an absorbance of 560 nm and can be read by any spectrophotometer (Fig. 1). Lecture of this colorimetric assay allows to estimate the metabolic activity of the tissue.[4,8,9]

Different applications of MTT arise directly from its chemical characteristics.[2] It is an insoluble salt which cross the cellular membrane, enters the cell, and undergo a redox reaction. In doing so, the original yellow reagent changes its color to that of the purple reaction product formazan. Interestingly, the change of color is proportional to the magnitude of the redox reaction. Thus, the objectives of the present work were: (a) to use MTT to assess the right localization of cannulae in the brain; and (b) to use the aforementioned reaction, to be able to measure brain metabolic activity. In dealing with the second objective we performed two kind of metabolic assays in brain areas related to our regular work: (1) *in vitro* metabolic activity in some dissected brain areas such as striatum, substance nigra and lateral septum; and (2) *in vivo* assays using intact animals that underwent microinjection with MTT on

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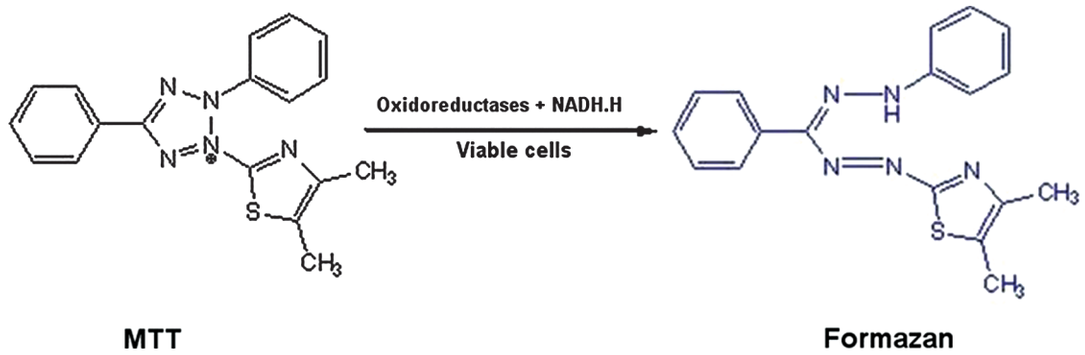

Fig. 1. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide structure and schematic reactions of its reductions to formazan by oxidoreductase system.

lateral septum. Its metabolic activity was measured after removing the nucleus from the brain.

2. MATERIALS AND METHODS

2.1. Animals

Adult male Sprague-Dawley rats (60–70 day old; 280–320 g) were housed in groups of four per cage until surgery. After surgery rats were housed alone. Room temperature was maintained at 22 ± 1 °C with lights on from 7.00 a.m. to 7.00 p.m. Food and water were freely available throughout the experiments. Animals for these experiments were kept and handled according to the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, USA.[7] All efforts were made to minimize animal suffering.

2.2. Reagents

The reagents utilized were pregnenolone (Preg) and pregnenolone sulfate (Preg-S), lidocaine (Lid), 7-amino-phosphono-heptanoic acid (AP7), bicuculline (Bic), sodium azide (SIGMA, St. Louis, MO, USA), penicillin G (benzathine, Richet, Argentina), chloral hydrate (Anedra, Argentina), (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) MTT (Sigma-Aldrich Co M5655). KREBS buffer was saturated with 95% O_2 and 5% CO_2.

Stocks of Preg and Preg-S were initially dissolved in propylene glycol to a concentration of 0.4 mM. The doses used in the experiments were obtained by dilution in sterile saline in order to make negligible the final amount of propylene glycol. Control animals were injected with sterile saline containing 1% propylene glycol in equivalent concentrations to that used in experimental groups.

2.3. Lateral Septum Surgery

Rats were anesthetized with chloral hydrate (5 ml/kg, ip) and fixed in a stereotaxic frame (David Kopf, USA). Guide cannulae were made from stainless steel (0.80×38 mm) and were unilaterally implanted in right lateral septal nucleus (LS). The cannulae implanted in LS were introduced to a point 1.5 mm above the LS itself in order to minimize mechanical damage of the nucleus. The following coordinates from bregma were used in accordance with Pellegrino's atlas:[13] AP +1.8 mm, L −0.5 mm, DV −3.7 mm. Cannulae were fixed to the skull with dental cement and a stainless steel screw. At the end of the surgery, cannulae were sealed with a stainless steel wire to protect them from obstruction. To prevent infections, all animals received an intramuscular (im) injection of 0.2 ml penicillin G benzathine.

2.4. MTT Assay *in Vitro*

Animals were sacrificed by decapitation, afterwards brain were removed and placed on a cold plate. Both cerebral hemispheres were removed to access different brain structures such as substantia nigra (SN), striatum (ST) and lateral septum (LS) in order to evaluate only their basal metabolic activity—SN, ST and LS—or under treatment metabolic activity—only LS. This structure, was injected with saline ($n = 13$), Preg 12 μM ($n = 7$), Preg-S 12 μM ($n = 9$), Lidc 2 μg/μl ($n = 7$), AP7 1 μg/μl ($n = 7$), Bic 9,8 μg/μl ($n = 7$). The group of sodium azide was incubated with a volume of 100 μl of azide 65 mM ($n = 4$) instead of being injected. The time elapsed between drug administration and metabolic activity assays was 1 minute.

2.5. MTT Assay *in Vivo*

Animals were implanted with the guide cannulae in the right lateral septum. After 7 days of recuperation they were injected using a microsyringe (Hamilton, Reno, NV, USA) 1 μl of a solution of MTT (1 mg/ml dissolved in PBS). After 10 minutes animals were sacrificed, and their brains placed in a cold plate washed continuously with cold KREBS solution to maintain tissue and cell viability. In order to access LS, both brain hemispheres were removed. Correct cannula placement in right LS was verified by

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identifying a purple halo corresponding to MTT injected previously. Subjects with purple halo outside boundaries of the lateral septum were discarded. Both LS were removed and metabolic activity was measured separately.

2.6. Metabolic Activity

Assay was modified from the work of Sylvester PW.[15] Immediately after areas of interest were removed, they were placed in a 100 μl solution of MTT (1 mg/ml) and 100 μl of KREBS buffer saturated with 95% of O_2 and 5% of CO_2 during 60 minutes at 37 °C. Crystals generated by the MTT reagent were dissolved by adding isopropyl alcohol until completing a total volume of 1 ml, and letting solution to be at rest during all night. Next morning, each sample was shacked during 2 minutes to dissolve completely the crystals. Supernatants were used for spectrophotometric lecture at 560 nm (JENWAY 7315, ST15 OSA-UK), while the tissue of each sample was processed to determine protein concentration by the Lowry method.[11] Standard values were corrected by using bovine serum albumin as our basal values.

2.7. Statistical Analysis

For the statistical analysis the software GraphPad Prism 5 was used. Both *in vivo* and *in vitro* MTT assays were analyzed using the test of Shapiro–Wilk in order to evaluate whether or not data were normally distributed. Since our results indicated that our data did not follow a parametric distribution, we compare two groups by using a Mann-Whitney test. When more than two groups were to be compared a test of Kruskal-Wallis followed by a post-hoc test of Dunn was performed. In all cases data are expressed as the mean $\pm$ SEM. A value of $p < 0.05$ was considered for assigning statistical significance.

3. RESULTS

3.1. Physiological Metabolic Activity

The first goal in this study was to evaluate physiologic metabolic activity in the different brain areas, particularly the lateral septum, striatum and substantia nigra. It was important to note that no drug treatment was performed in these experiments. Results show that the MTT assay was able to detect efficiently different levels of cellular metabolic activity for the aforementioned cerebral regions. Since different values between cores were observed possibly can be due by tissue specific-particularities within each brain structure (Fig. 2).

3.2. Lateral Septum and Metabolic Activity Modulations

The group of animals treated with *in vitro* microinjections of different reagents into the lateral septum showed

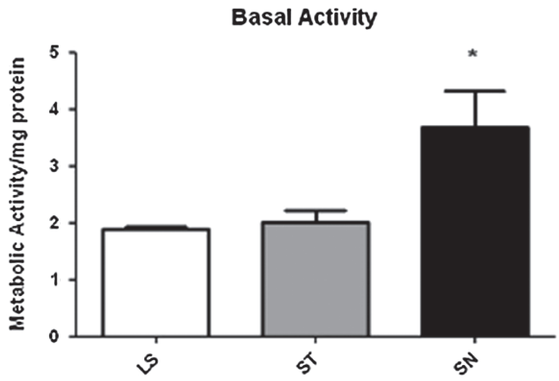

Fig. 2. Differential metabolic activity of lateral septal nuclei (LS), striatum (ST) and substantia nigra (SN) demonstrated by MTT assay. Cellular activity was expressed as metabolic activity/mg protein. *($p > 0.05$).

different responses. The administration of pregnenolone 12 μM—a well known neurosteroid that modulates $GABA_A$ receptors—and bicuculline 9.8 μg/μl—a potent antagonist of $GABA_A$ receptors- did not show any significant changes in metabolic activity. However, the sulfated form of the neurosteroid, pregnenolone sulfate 12 μM, well known for being a positive modulator of glutamatergic receptors and negative modulator of $GABA_A$ receptors, significantly diminished metabolic activity regarding control groups. Similar changes were observed when lidocaine 2 μg/μl—a local anesthetics-, and AP7 1 μg/μl—a potent NMDA receptor antagonist were used. As an internal control of mitochondrial activity sodium azide, a respiratory chain blocker, as expected, showed a significant decreased metabolic activity in the lateral septum (Fig. 3).

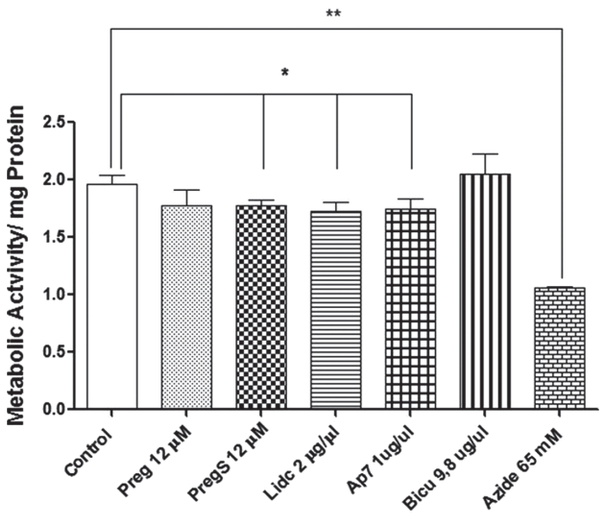

Fig. 3. Sodium azide—a potent inhibitor of mitochondrial respiratory chain—reduces significantly the metabolic activity in lateral septum when compared to controls ($p < 0.05$). Similar results were obtained with pregnenolone sulfate ($p < 0.05$), AP7 ($p < 0.05$) and lidocaine ($p < 0.05$). No differences were found when pregnenolone and bicuculline were used in lateral septum.

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3.3. Microinjection with MTT on Lateral Septum and Metabolic Activity Evaluation

By micro injecting 1 μl of MTT in the right lateral septum it was possible to find the correct location of the cannula (Fig. 4). In all cases a small dark halo resulting from the direct reaction of MTT with the living tissue, which did not extend beyond the limits of the core was observed. Accordingly, and after certifying the right cannulae location, the study of metabolic activity by using again the MTT assay was continued. The subsequent analysis in both left and right septal nuclei did not show any difference between them (Fig. 5).

4. DISCUSSION

Since many years ago, it has been a common practice to use steel cannulae implanted into the brain of experimental animals in order to administer drugs or chemical reagents. To report liable results, it was—and still is—indispensable to make sure that the reagent was administered to the right place. Several methods, all of them based on staining substances were developed, like india ink, methylene blue, trypan blue, just to mention a few of them.[6, 10, 14] Notwithstanding the objective was accomplished, a not always perceived problem remained: By estimating the right cannula location, the tissue involved was almost irremediably lost for any further purpose. Here we showed an old method used in a new way, designing it as MTT-micro assay on living tissues (MTT-malt).

The reagent 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow colored drug, has been used for years because of its—oxide-reduction properties in appropriate conditions—producing a purple colored product called formazan (Fig. 1).[4, 8, 9] The reaction product can be quantified by reading in colorimetric assays. Most of this process takes place—in living cells—in the oxidoreductase systems of the cell. Our recent work[3, 12] has been related to two main areas:

(1) pharmacological properties of sulfated neurosteroids applied on the lateral septum and memory[12] and

(2) possible neuroprotective role of neuroactive steroids regarding damage of rats' striatum by injecting 6-OHDA (animal models of Parkinson's disease).[3]

In performing our experiments we dealt with the aforementioned problem: after identifying the right place of our micro injections, the tissue was lost for any other experimental purpose. On the other hand, and in parallel, we were utilizing the traditional MTT assay in order to measure tissue viability. At that time, it became apparent the possibility of using a vital assay in order to localize the injection place, and additionally to use tissues to assess their metabolic activity. As shown in Figure 4 (top left panel), technique was successful. Even more interesting, after removing the corresponding brain structures, the biological activity of both intact and injected nuclei was not different at all (Fig. 5) it was obvious that MTT was a useful assay to test the metabolic activity of different brain structures (Fig. 2). Furthermore, LS and ST showed

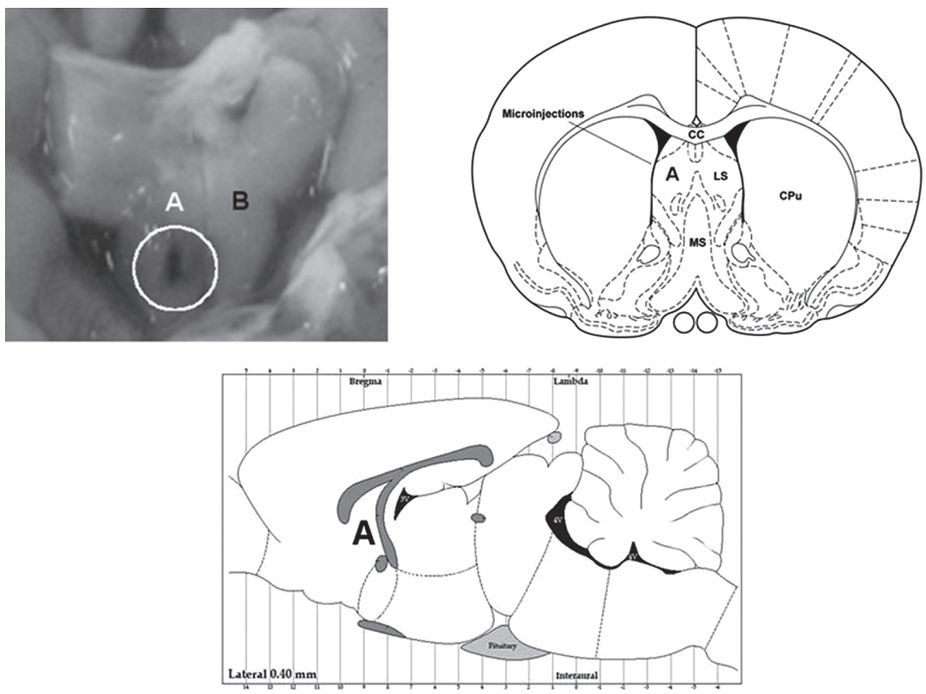

Fig. 4. Dissected brains allow us to expose lateral septum. We observed a positive dark (purple) spot located in right lateral septum (A) which coincides with injection side. Diffusion ratio for MTT was less than 1.5 mm^2. Intact lateral septum does not show any color at all (B). Top right (frontal) and bottom (lateral) panels shows injected areas (A) regarding the lateral septum (LS).

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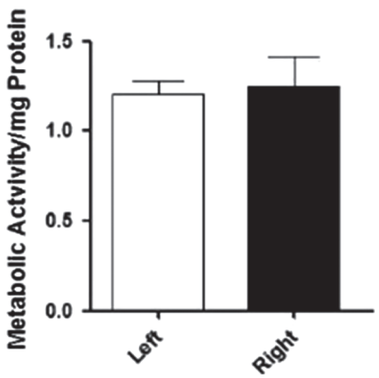

Fig. 5. After removing lateral septum the MTT activity assay did not find any difference between left—intact- and right—saline injected animals—LS nuclei ($p > 0.05$).

comparable metabolic activities, but SN, displayed significant increases in its metabolic activity (Fig. 2; $p < 0.05$).

Several tests were performed in order to clarify whether or not LS tissues (previously identified by MTT injections) remained viable. Results showed that sodium azide significantly decreased metabolism—as expected—possibly by diminishing mitochondrial respiratory activity (Fig. 3) in a similar fashion to pregnenolone-sulfate, lidocaine and AP7 injections (Fig. 3). Interestingly, there was no difference when pregnenolone was injected, which is in line with our previous results regarding memory.[12] Possibly the sulfation state of the reagent could be ascribed to a sort of molecular switch, chemical mechanism present in modulatory endogenous molecules of several brain receptors.

In brief, we showed here for the first time that MTT assay is suitable of being used to identify in a dependable way the site of injection in discrete brain structures and, sequentially, allow the researcher to continue working with the tissues, since they remain completely viable. By continuing the assay with the MTT protocol, after identifying the structure, metabolic activity can be studied in an accurate way. It is expected that the MTT-micro assay method on living tissues (MTT-malt), would be useful to the research community involved in neuroscience all around the world.

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