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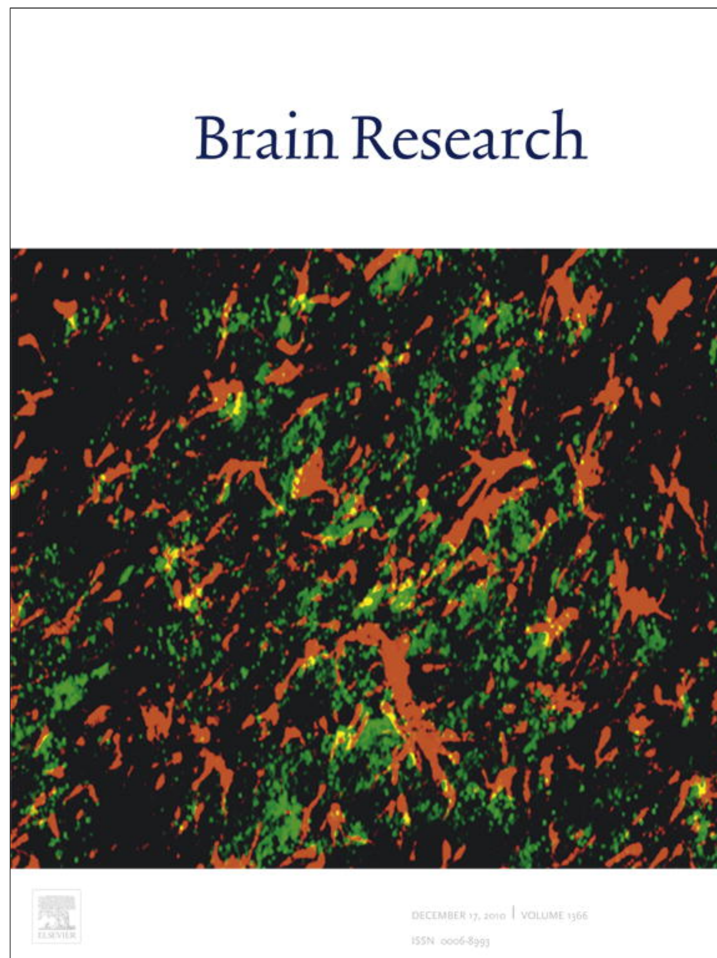

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BRAIN RESEARCH

Research Report

Brain metastases of mouse mammary adenocarcinoma is increased by acute stress

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ABSTRACT

Brain metastases from mammary adenocarcinoma constitute the chief cause of morbidity and mortality. Some evidence suggests that stress may contribute to disease progression and metastases. Here we show that acute restraint stress (30 min) induces statistically significant increase in brain metastases of systemically administered luciferase-tagged 4T1-BR-3P mouse mammary adenocarcinoma cells as evidenced by the total brain-associated photons from 5.6×10^7 photons in unstressed controls to 1.7×10^8 photons in C57BL/6 ($p=0.0018$) and from 7.6×10^7 to 2.1×10^7 photons in BALB/c ($p=0.004$) mice. Acute stress may increase metastases by disrupting the blood–brain-barrier (BBB), through release of corticotropin-releasing-hormone (CRH) activating perivascular brain mast cells.

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1. Introduction

Metastases, especially in the brain, contribute to the high morbidity and mortality of most frequent forms of cancer (Gupta et al., 2005; Lassman and DeAngelis, 2003). In fact, more than 30% of breast cancer patients develop metastases, about 5% in the brain, with poor associated prognosis (Schouten et al., 2002; Nishizuka et al., 2002). However, how cancer cells disseminate to the brain remains unknown (Weber and Ashkar, 2000). In one case, the occurrence of breast cancer in humans was preceded by stress associated with the loss of a loved one in 68% of cases (Naesse et al., 2003). The possible effect of stress on brain metastases was reviewed recently (Theoharides et al., 2008), but there has not been any direct proof.

Stress has been associated with increased metastatic spread of tumors (Ben-Eliyahu et al., 1991; Wu et al., 2001), possibly through reduced host resistance (Palesh et al., 2007),

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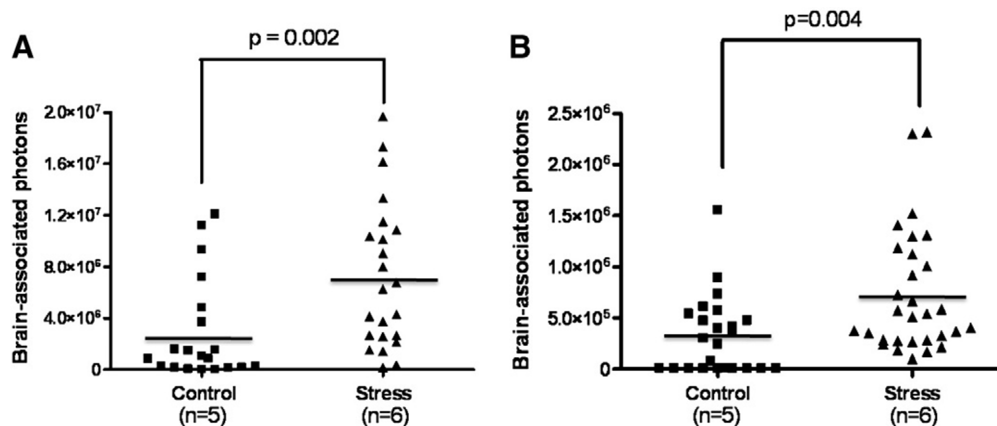

Fig. 1 – Scattergrams of control ($n=5$) and stressed ($n=6$) (A) C57BL/6 mice, and (B) BALB/c mice.

or greater vascularization due to increased vascular endothelial growth factor (VEGF) levels (Thaker et al., 2007). Stress can promote cancer growth (Saul et al., 2005; Antoni et al., 2006; Kruk and Aboul-Enein, 2004; Nielsen and Gronbaek, 2006), and induces breast cancer resistant to chemotherapy (Su et al., 2005). Stress also increased susceptibility to UV-induced squamous cell carcinoma in a mouse model and in humans (Saul et al., 2005).

The blood–brain-barrier (BBB) is defective in metastatic tumors (Long, 1979), permitting brain metastases (Menter et al., 1995), such as in mammary carcinoma (Boogerd, 1996). Stress was known to increase permeability of the BBB (Skultetyova et al., 1998). It was originally proposed that brain mast cells may regulate the BBB permeability (Theoharides, 1990). It was subsequently shown that stress induces BBB disruption through corticotropin-releasing hormone (CRH) secreted under stress, activating brain mast cells (Esposito et al., 2001a, 2002), and that leukocyte adherence contributes to BBB disruption (Mayhan, 2000).

Here we show that acute restraint stress significantly increases brain metastases of systemically administered luciferase-tagged mouse mammary adenocarcinoma cells in mice.

2. Results

Intracardiac administration of luciferase-tagged mouse 4T1-BR-3P breast carcinoma cells in C57BL/6 mice resulted in statistically significant increase in brain-associated photons (metastases) in mice exposed to 30 min restraint stress applied right after cell injection, as compared to controls (Fig. 1A). The total number of brain-associated photons in control C57BL/6 mice was 5.6×10^7 with mean±SEM of $2.8\times10^6\pm8.8\times10^5$ photons ($n_1=5$, $n_2=20$) and increased to a total of 1.7×10^8 photons with mean±SEM of $7.2\times10^6\pm1.3\times10^6$ ($n_1=6$, $n_2=24$, $p=0.0018$). A representative set of images from C57BL/6 mice show photons associated with the brain sections (Fig. 2A, B).

In order to investigate if the observed effect was strain-specific, especially since the 4T1-BR-3P cells used were derived from BALB/c mouse mammary gland, we repeated these experiments using BALB/c mice. Again, acute stress led to a statistically significant increase in brain-associated photons (Fig. 1B). The total number of brain-associated photons in control BALB/c mice was 7.6×10^6 ($n_1=5$, $n_2=24$) with a mean± SEM of $3.2\times10^5\pm7.7\times10^4$ photons compared to a total of 2.1×10^5 ($n_1=6$, $n_2=30$) with a mean±SEM of $7.3\times10^5\pm1.1\times10^5$ photons ($p=0.004$) in stressed mice (Fig. 1B). A representative set of images from BALB/c mice show photons associated with the brain sections of control (Fig. 2C, D).

BALB/c mice were also imaged for metastases in inner organs such as the lungs, heart, liver, kidney and spleen, but there were no apparent differences between stressed and control mouse (data not shown).

3. Discussion

Here we show for the first time that a brief period of restraint stress significantly increases brain metastases of luciferase-tagged 4T1-BR-3P mouse mammary adenocarcinoma cells in both C57BL/6 and BALB/c mice. The 4T1-BR-3P mouse mammary adenocarcinoma mimics the characteristics of human breast cancer cells, and we recently showed that luciferase tagged 4T1-BR-3P cells can be followed at sites of metastases by imaging (Tao et al., 2008).

We did not intend to compare the two strains of mice in terms of susceptibility to mouse 4T1-BR-3P breast carcinoma cells in both BALB/c and C57BL/6 mice. Stress increased the brain metastases about 3-fold with more total brain metastases in C57BL/6 than BALB/c mice. This finding may be related to the fact that the cancer cells used were derived from BALB/c mice. C57BL/6 mice are also considered to have the largest autonomic responses to stress (van Bogaert et al., 2006).

We do not have direct evidence that the acute stress paradigm we used increased BBB permeability in these experiments. However, use of exactly the same protocol in C57BL/6 mice had previously been shown to increase BBB permeability that was absent in mast cell deficient mice (Kandere-Grzybowska et al., 2003).

The BBB appears to be disrupted in neuroinflammatory diseases (De Vreis et al., 1997; Kermode et al., 1990; Moor et al., 1994; Kwon and Prineas, 1994), especially multiple sclerosis and cancer (Long, 1979; Menter et al., 1995). The BBB is formed by a complex array of tight junctions among endothelial cells,

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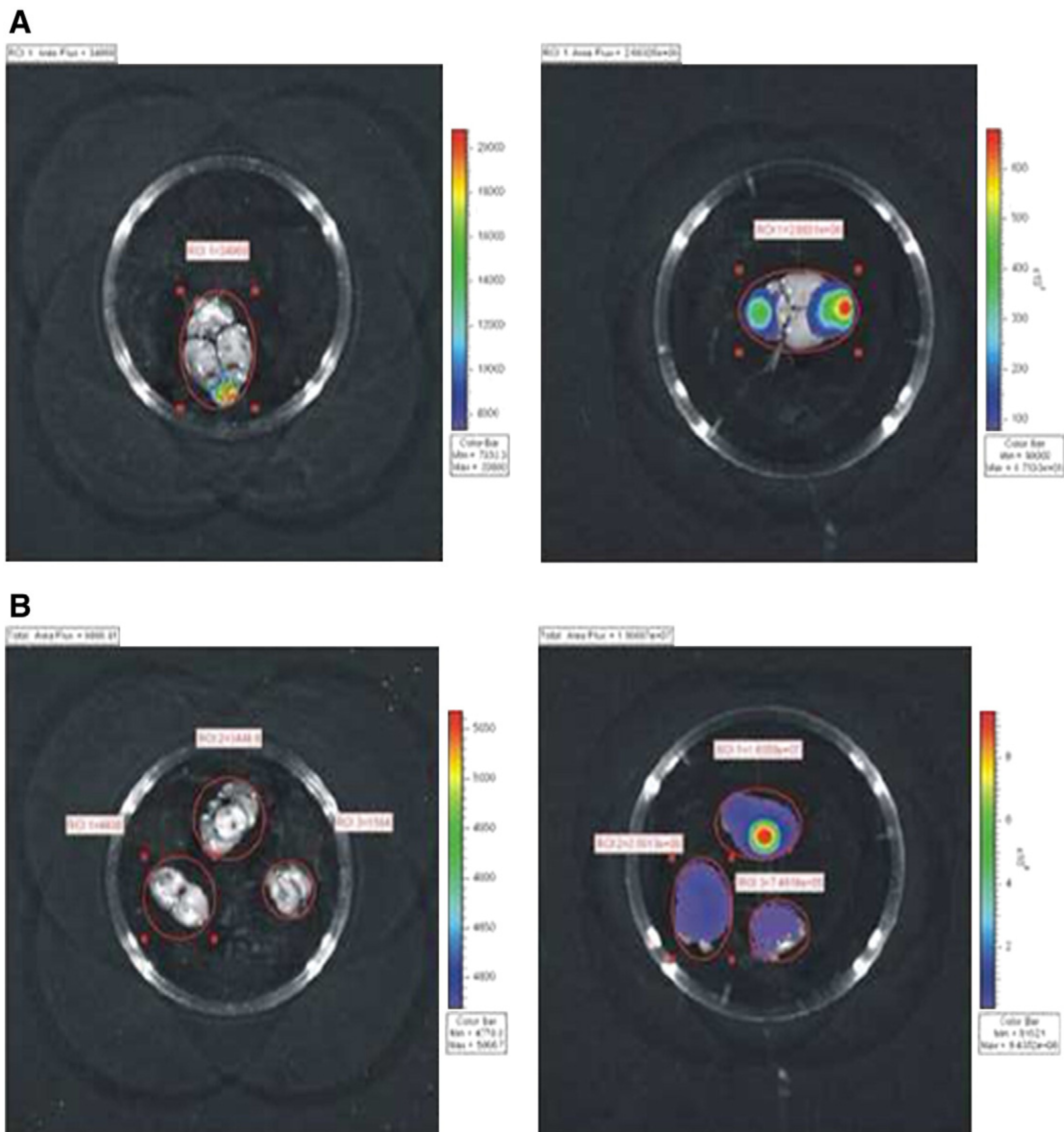
astroglia, pericytes and perivascular mast cells (de Boer and Breimer, 1998a; Petty and Lo, 2002; Wahl et al., 1988). The BBB also actively regulates cytokine production and flow (Quan, 2006). Stress had been reported to increase BBB permeability (Skultetyova et al., 1998), possibly through leukocyte adherence to endothelial cells of the BBB (Mayhan, 2000). Furthermore, acute stress disrupted the BBB through mast cell activation by corticotropin-releasing-hormone (CRH) (Theoharides and Konstantinidou, 2007), secreted under stress. For instance, acute stress activated rat intracranial mast cells, and led to increased BBB permeability (Theoharides et al., 1995); this effect was inhibited by pretreatment with CRH antiserum or the CRH receptor-1 (CRH-R1) antagonist, Antalarmin (Esposito et al., 2001a). Site injection of CRH in the rat hypothalamus induced BBB permeability that was blocked by site pretreatment with the mast cell stabilizer disodium cromoglylate (cromolyn), and was absent in W/W^v mast cell deficient mice (Esposito et al., 2002). Functional CRH-R1 is expressed on human mast cells (Theoharides et al., 2004), and its activation leads to selective release of vascular endothelial growth factor (VEGF) (Cao et al., 2005), which is also vasodilatory. A number of cytokines could disrupt the BBB (de Boer and Breimer, 1998b), and many of these (e.g. IL-6, IL-8) are released by mast cells (Theoharides and Kalogeromitros, 2006). However, we have no evidence at the present of which mediators or other molecular events are involved in the phenomenon we describe, or whether chronic stress has a similar effect. However, non-invasive investigation of BBB permeability using Technitium gluceptate and a gamma camera in live rats indicated that BBB was disrupted within 15 of restraint stress, and returned to normal in a few hours (Esposito et al., 2001b).

CRH receptors are also expressed by a number of human cancers (Reubi et al., 2003). Human mast cells can synthesize and secrete CRH (Kempuraj et al., 2004) which is a growth factor (Slominski et al., 2006) and enhanced migration of murine melanoma cells (Yang et al., 2006). It was also recently reported that CRH promotes the invasion of mammary adenocarcinoma cells (Androulidaki et al., 2009).

Mast cells are important for allergic reactions (Blank and Rivera, 2004), but also in innate immunity (Galli et al., 2005; Mekori and Metcalfe, 2000; Redegeld and Nijkamp, 2003), inflammation (Theoharides and Cochrane, 2004) and autoimmunity (Benoist and Mathis, 2002). Mast cells could also be conducive to tumor growth (Conti et al., 2007; Theoharides and



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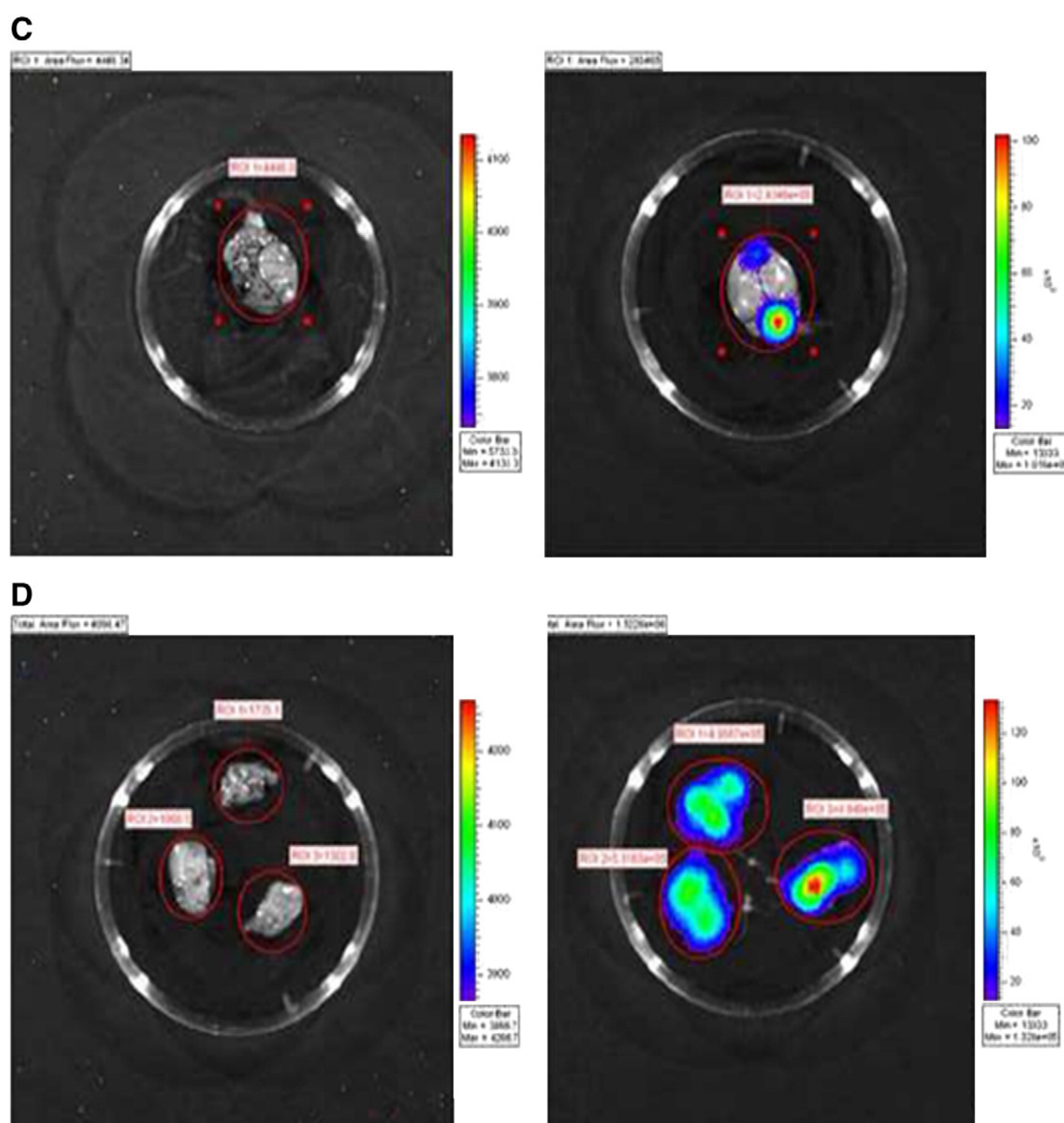

Fig. 2 – Representative images of brain sections of C57BL/6 mice (A, B), as well as brain sections and inner organs of BALB/c mice (C, D). Left hand side panels are images from control mice, while right hand side panels are images from stressed mice. C57BL/6 mice. (A) 2nd pair—the whole brain (dorsal plane) of control and stressed mice, (B) 3rd pair—3 brain sections of control and stressed mice. BALB/c mice. (C) 2nd pair—the whole brain (dorsal plane) of control and stressed mice, (D) 4th pair—3 brain sections of control and stressed mice.

Conti, 2004), by promoting angiogenesis (Theoharides and Conti, 2004) as well as inducing immune suppression (Wasiuk et al., 2009). Mast cell deficient mice were reported to have reduced metastases (Starkey et al., 1988). Moreover, a mast cell stabilizer inhibited growth and metastases of rat mammary adenocarcinoma (Dabbous et al., 1991) and pancreatic islet tumors (della Rovere et al., 2007).

Metastases, especially in the brain, continue to be a major source of morbidity and mortality for mammary adenocarcinoma (Gupta et al., 2005; Lassman and DeAngelis, 2003; Schouten et al., 2002; Nishizuka et al., 2002). The present results provide direct support for the premise presented recently that stress contributes to brain cancer metastases through disruption of the BBB (Theoharides et al., 2008).

It would be important to investigate the effect of acute as compared to chronic stress, as well as use mast cell deficient and CRH–/– or CRHR-1–/– mice on brain metastases. Moreover, brain expression of pro-inflammatory cytokines and/or vascular adhesion molecules may help explain how BBB disruption occurs. Such experiments would not only provide evidence of specific mechanisms, but may identify potential new therapeutic targets.

4. Experimental procedures

Female C57BL/6 or BALB/c mice (6 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Maine) and were

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housed in 14:10 dark:light cycle with food and water *ad libitum* in virus-free sections of a modern animal facility. The 4T1 mouse adenocarcinoma cells used in this study (designated 4T1-BR-3P) were derived from the luciferase-expressing 4T1-12B cell line (ATCC #CRL-2539 derived from BALB/c mouse mammary gland) that spontaneously produces highly metastatic tumors (Hoffman, 2002) by *in vivo* selection and isolation for brain metastases (Tao et al., 2008). The protocol described below was approved by the Tufts Animal Use Committee.

Mice were anesthetized with a single intraperitoneal (ip) injection of 0.3 ml each ketamine/xylazine (10 mg/kg and 80 mg/kg). Mouse mammary carcinoma 4T1-12B-3P cells were cultured in RPMI-1640 (Gibco Invitrogen, Grand Island, NY) medium containing DMEM (high glucose) supplemented with 10% fetal calf serum, as well as penicillin/streptomycin. Cancer cells (5×10^5 in 0.1 ml DMEM) were administered by intracardiac injection and mice were returned to their cages. The approximate rate of clearance of the injected cells is about 1–2 days. Within several min after intracardiac administration of cancer cells, mice were injected ip with luciferin 1.5 mg (Caliper Life Sciences, Hopkinton, MA) in order to produce bioluminescence when acted upon by the luciferase expressing cancer cells. These steps were performed in order to check whether the cancer cells had been properly injected into the left ventricle of the heart and were in the systemic circulation. Imaging of luciferase-dependent bioluminescence (which lasts 30–45 min) was performed using a Xenogen Ivis 200 Biophotonic Imager (Caliper Life Sciences). Animals were imaged under 2% isofluorane gas anesthesia 5–10 min after ip injection of luciferin.

4.1. *Imaging*

Right after the intracardiac injections, mice were divided into two groups: (A) controls ($n=5$) kept in cages in a different room, and (B) restraint stress ($n=6$), in which every animal was separately placed in a small Plexiglass immobilizer (Harvard Apparatus, Cambridge, Ma) for 30 min.

The mice were injected with luciferin just before they were killed on the final day of the experiment as follows: On day = 11 (in the case of C57BL/6 mice) or on day = 6 (in the case of BALB/c mice) after cancer cell administration. The interval between injecting cancer cells with/without subsequent acute restraint stress and the day when mice were sacrificed varied between C57BL/6 and BALB/c mice, based on the clinical stage of the animals when they as a group first presented symptoms of disease as follows: (a) change in the appearance of their fur coat, generalized weakness, or (c) limb paralysis as focal neurological signs. The mice were first imaged (following anesthesia) alive (image #1) and then sacrificed one by one, not in each other's presence, by CO_2 asphyxiation and decapitation.

In order to make sure that all metastatic cell-associated photons were evaluated, each brain was removed from the skull and imaged from the back in C57Bl/6 (Fig. 2A), and BALB/c (Fig. 2C). The brain was then sectioned as described below and each part was imaged separately:

(A) The brain was divided into 3 parts: the anterior (coronary/vertical incision on the level of the optic chiasm), the middle, and the posterior—including the cerebellum and part of the brain stem, and medulla oblongata (coronary/vertical incision on the level of the border between the cerebral cortex and the cerebellum).
(B) The inner anterior part, the inner posterior part, and the frontal face of the middle part were first exposed for imaging and scanned (Fig. 2B in C57BL/6, Fig. 2D in BALB/c).
(C) The middle part of the brain was then reverted showing the rear of it, and the anterior and posterior parts of the brain were sectioned showing both new surfaces of each of them, and scanned.
(D) Finally, the middle part of the brain was sectioned exposing the newly formed surfaces, and all pieces were imaged without changing the position of the sections of the anterior and posterior parts of the brain.

The method of sectioning the brain described above virtually examined the whole brain since it exposed all brain aspects. The thickness of brain slices was the smallest possible while keeping the shape and the orientation of the pieces. The middle sections were examined also from both sides to permit maximal count of the signal from cancer cells deep inside the brain. Inner organs such as the lungs, heart, liver, kidney and spleen were excised and imaged only in BALB/c mice.

4.2. *Statistics*

The original data from bioluminescence scanning are expressed as "counts". The computer program used to analyze the data was Living Image® 2.50.1-IGOR Pro 4.09a (WaveMetrics, Laek Oswaego, OR, USA). It transforms the "counts" into "photons" that are comparable between different scanning sessions and animals. The numbers in the boxes of the scanned images (see figures) indicate the number of photons emitted in the corresponding brain sections, while the box at the upper left hand side corner lists the "Total Area Flux" or the calculated total brain-associated number of photons. The results are presented as scattergrams with the median shown by a horizontal line. The calculated total number of photons in the experimental mice was compared to that in the control using the non-parametric Mann–Whitney *U* test. Statistical significance is denoted with $p<0.05$ (n_1 = number of mice used; n_2 = number of brain images evaluated).

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