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Effects of G207, a conditionally replication-competent oncolytic herpes simplex virus, on the developing mammalian brain

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Viral oncolytic therapy for malignant brain tumors involves local intratumoral delivery of a genetically engineered virus with tumor cell–specific lytic activity. Promising preliminary results have been achieved in preclinical models with G207, a replication-competent herpes simplex virus type 1 constructed with multiple directed mutations. Although the safety of G207 has been demonstrated in adults, application of viral oncolytic therapy to children with brain tumors has been delayed because of previous lack of data concerning the impact of a replication-competent oncolytic virus on the developing mammalian brain. In this study there was no significant difference in long-term physical development, cognitive performance, or exploratory behaviors between mice that received intracerebral inoculation of G207 or control saline at 4 days of age. However, histological examination and magnetic resonance imaging revealed frequent unilateral ventriculomegaly ipsilateral to the site of injection in only the G207 group. These results suggest that although it is unlikely that G207 will have significant adverse effects on neurodevelopmental outcomes of pediatric patients with brain tumors, an initial study of G207 in children should exclude those patients with tumors in or near the ventricular system as well as patients less than 2 years of age. Furthermore, patients in such a study will need to be closely monitored for the development of hydrocephalus. *Journal of NeuroVirology* (2007) **13,** 118–129.

Keywords: animal model; brain tumors; growth; human herpesvirus 1; investigational therapies; learning

Introduction

Tumors of the central nervous system (CNS) are the most common type of tumors in children, occurring at an annual rate of 1.7 to 4.3 per 100,000 children (Kaatsch *et al*, 2001; Packer, 1999; Mehta *et al*, 2002). CNS tumors are also the leading cause of cancer-related mortality in children, with 3-year and 10-year survival rates of 69% and 59%, respectively (Kaatsch *et al*, 2001; Packer, 1999). Because brain tumors can involve functionally significant cortex and the tumor/brain boundary may be poorly defined, even tumors within the cerebral hemispheres are often incompletely resected when postoperative imaging studies are reviewed. Patients without total or near total resection typically carry a worse prognosis (Pollack *et al*, 1995). Even gross total resection may not be curative for many tumors due to their microscopic invasion of surrounding brain parenchyma. Most malignant tumors will recur without adjuvant therapy, and some subsets (for example, pontine gliomas) exhibit poor response even with

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aggressive adjuvant combinations of radiation therapy and chemotherapy (Jennings *et al*, 1996; Dunkel *et al*, 1996; Wolff *et al*, 2002; Mandell *et al*, 1999). Furthermore, adjuvant therapy often has untoward long-term effects (Gurney *et al*, 2003; Packer *et al*, 2003). For these reasons there is great impetus for the exploration of novel approaches to pediatric brain tumor therapy that will provide improved response and decreased toxicity.

Viral oncolytic therapy is one such novel approach, and involves local intratumoral delivery of a genetically engineered virus with tumor cell–specific lytic activity. Promising preliminary results have been achieved with G207, a replication-competent herpes simplex virus type 1 (HSV-1) constructed with multiple directed mutations (Mineta *et al*, 1995). G207 exhibits loss of neurovirulence secondary to deletion of both $\gamma_1$34.5 loci and an inability to replicate in nondividing cells secondary to disruption of the U_L39 gene with a *lac*Z insertion (Mineta *et al*, 1995; Chou *et al*, 1990). An additional benefit arising from loss of U_L39 function is marked hypersensitivity to the antiviral agent ganciclovir, allowing rescue if herpes encephalitis were to develop as a result of intracerebral inoculation with G207 (Mineta *et al*, 1995). Such properties allow for the possibility of viral replication and resulting cell lysis that is limited to primary neoplasms arising from normal neural tissue. Accordingly, both increased survival and inhibition of tumor growth have been demonstrated in mice with intracerebral gliomas following intracerebral injection of G207 (Mineta *et al,* 1995; G Yancey Gillespie, unpublished data). Equally significant, the safety of intracerebral injection of G207 has been documented in both mice and owl monkeys (Mineta *et al*, 1995; Sundaresan *et al*, 2000; Hunter *et al*, 1999). In a subsequent phase I trial that enrolled adults with recurrent malignant glioma, none of the 21 subjects demonstrated any evidence of toxicity directly attributable to G207 (Markert *et al*, 2000). In this trial, only patients with recurrent hemispheric tumors at least 1 cm away for the ventricle were eligible. Likewise, a second phase I study using a similar replication-competent HSV-1 carrying only the $\gamma_1$34.5 mutation did not reveal any toxicity in any of the nine adults who were treated (Rampling *et al*, 2000). Although G207 holds great therapeutic potential for children with malignant brain tumors, the impact of a replication-competent oncolytic virus on the developing mammalian brain has not yet been investigated. This study was designed to examine the effect of G207 on motor and cognitive development in a murine model.

Results

Tolerance of G207 inoculation

Four different dose levels of G207 (from 5.3×10^4 plaque-forming units [pfu] of G207 to 5.3×10^6 pfu/2 μl) were used in the intracerebral inoculation of 41 mice between 2 to 4 days of age in order to calculate the dose expected to produce 50% mortality within 21 days (LD_{50}). Survival at 21 days is described in Table 1. These data produced an LD_{50} estimate of 3.02×10^6 pfu/dose.

Table 1 Intracerebral inoculation in mice 2 to 4 days old: LD_{50} dose-finding experiment

Plaque-forming units of G207/dose	Mice surviving at 21 days/total mice injected
5.3×10^4	7/7
5.3×10^5	9/10
1.59×10^6	12/14
5.3×10^6	3/10

Calculated $LD_{50} = 3.02 \times 10^6$ pfu/dose.

Mice to be tested for physical, cognitive, and behavioral development were injected at 4 days of age. Seven pups (three females, four males) were inoculated with G207 at a dose that was 25% of the LD_{50}: 7.6×10^5 pfu. This group is hereafter referred to as the G207 developmental study group. Eight pups (four females, four males) were inoculated with 2 μl of phosphate-buffered saline (PBS) that did not contain G207, and served as the control group. Both the G207 and the control groups exhibited 100% survival throughout the duration of the experiment. There was an additional group of 10 pups that were inoculated at 4 days of age with G207 at a dose that was 75% of the LD_{50}: 2.2×10^6 pfu. These mice exhibited only 40% survival at 20 days of life and 30% survival overall. Because of the small number of surviving mice in this latter group, developmental data from this group are not included in this report. However, histological analysis of the brains of the long-term surviving mice was performed after 14 months and is discussed below.

Physical development

The average body weight at birth and at three later time points is shown for each group in Figure 1. Although the average weight in the G207 developmental study group was consistently greater than that in the control group, this difference was not significant ($P = .39$ by repeated-measures analysis of variance). Brain weight was measured at the time of sacrifice; although G207 mice averaged a lower brain weight to body weight ratio, this difference also was not statistically significant ($P = .26$ by Student's t test) (Figure 2). The hang-time test was performed as an estimate of overall limb strength. The G207 developmental study group averaged a longer best time than the control group (226 versus 163 s), but this difference was not significant ($P = .32$ by Student's t test) (Figure 3). Of note, the G207 males performed significantly better on the hang-time test than did the control males (170 versus 38 s, $P = .03$ by Student's t test). Although this difference cannot be attributed to a difference in body weight ($P = .95$ for comparison

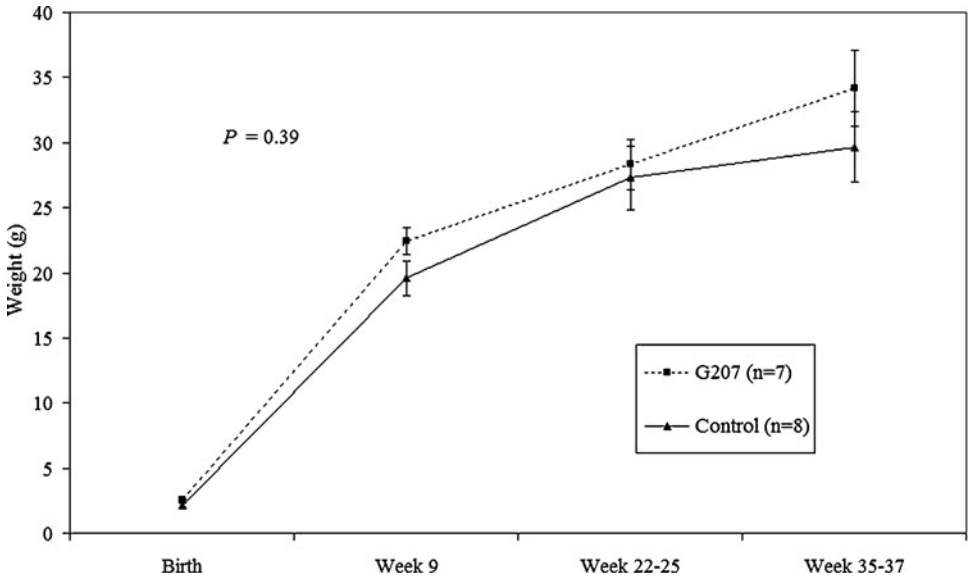

Figure 1 Body weight over time. Each point represents the average body weight (in grams) for each group, and the trend over four time points is shown. There was no significant difference between the two groups ($P = .39$, repeated-measures analysis). Error bars represent standard error.

of average group weights by Student's *t* test), the small number of male mice in each group requires a cautious interpretation.

Cognitive development

Cognitive development was assessed by performance in the eight-arm radial maze. As a group, G207 mice performed better on average than did the control mice in every parameter that was measured: fewer working memory errors (53 versus 95—Figure 4A), fewer recall memory errors (30 versus 37—Figure 4B), and fewer total errors (83 versus 132—Figure 4C) (all error counts cumulative until learning criteria met). Additionally, fewer days were required to meet the learning criterion (21 versus 27—Figure 4D). However, none of these differences were significant ($P = .24$ for working memory errors, $P = .39$ for recall memory errors, $P = .26$ for total errors, and $P = .35$ for days required to meet the criterion [all comparisons based on Student's *t* test]). Figure 5 shows error frequency trends over the first 25 days of testing in both groups, and reflects the presence of a

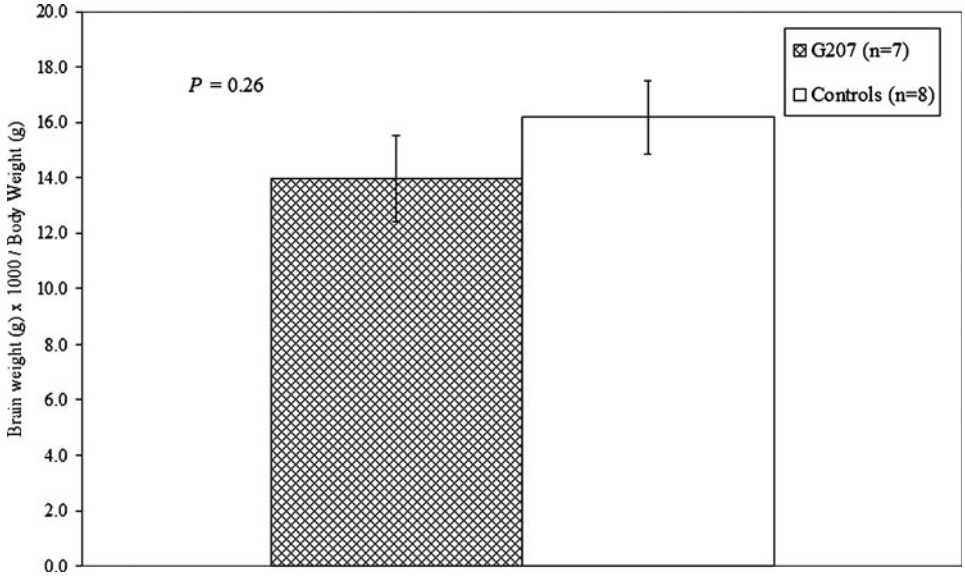

Figure 2 Brain weight at time of sacrifice. All mice were sacrificed at 35 to 37 weeks of age, and the average brain weight (corrected for body weight) of each group is shown. There was no significant difference between groups ($P = .26$, Student's *t* test). Error bars represent standard error.

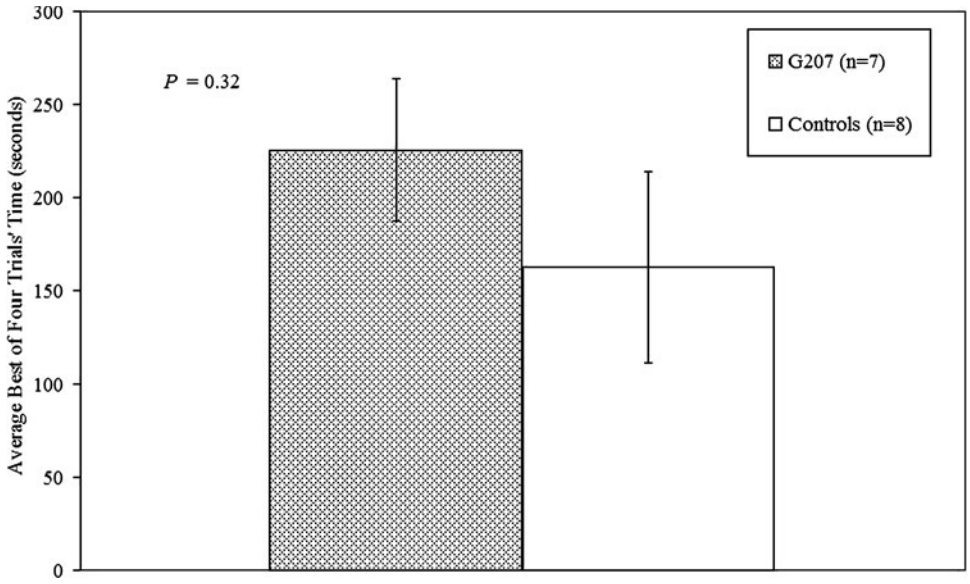

Figure 3 Hang time test—average best time of four trials. There was no significant difference between groups ($P = .32$, Student's t test). Error bars represent standard error.

bimodal distribution within the control group that may explain that group's relatively poorer overall performance.

Behavioral development
Exploratory behavior in the open-field maze test task demonstrated only small and nonsignificant differences between G207 and control mice. The G207 mice spent more time in the periphery, traveled a greater distance in the periphery, traveled a greater distance overall, and spent less time moving slowly. There were no significant differences between the groups in any of the parameters measured (data not shown).

Histological evaluation
Histological examination was performed on the brains of all mice in the control and G207 groups from the developmental studies. Of the eight control mice from the developmental studies, seven of the brains were normal histologically (Figure 6A); one mouse had a discernible unilateral ventriculomegaly (not shown). In the G207 developmental study group, five of seven mice had ventricular abnormalities evident on histological examination. Three mice had right unilateral ventriculomegaly, one mouse had enlargement only of the fourth ventricle, and one mouse had enlargement of both the right lateral ventricle and the fourth ventricle. The latter mouse also had evidence of intraventricular hemorrhage, potentially related to the freehand injection. This also could have been an effect of G207, although no evidence of encephalitis was seen. One of the mice with unilateral ventriculomegaly showed extensive ependymal cell necrosis with associated thickening of the ependymal cell layer and gliosis (Figure 6B). Additionally, of the 10 mice that had received 75% of the LD_{50}, 3 survived for greater than 1 year and their brains were also available for histological examination: 2 mice showed extensive unilateral ventriculomegaly, 1 associated with intraventricular hemorrhage.

Magnetic resonance imaging (MRI) evaluation
The histological ventricular changes in the G207 group were unexpected given the results of the physical, cognitive and behavioral testing. Post hoc MRI evaluation was then undertaken on the brains of the surviving mice which had received graduated doses of G207 in the LD_{50}-finding experiments. These mice were approximately 12 to 13 months of age at the time the MRI was performed. In confirmation of the gross and histologic changes seen in the mice involved in the developmental-behavioral studies, ventriculomegaly was observed (Figure 7).

Morphometric analyses of the changes in ventricle size relative to the brain for each of the MRI slices in which the ventricle could be observed were conducted as described in Materials and Methods. Figure 8 shows the ventricle/brain ratios for the surviving adult mice (~1 year) that were initially used in the LD_{50} dose-finding study in neonatal mice. Compared with the control mice (PBS-treated, $n = 11$), there was a trend of increasing ventriculomegaly as the dose of G207 was increased. The means of the ratios of the 2% LD_{50} ($P = .16$, $n = 3$) or the 175% LD_{50} ($P = .48$, $n = 2$) treatment groups were not significantly different from control (PBS-injected) mice, although the power of the analyses was low (0.16 to 0.05) compared to the desired power of 0.80. The difference between control and 20% LD_{50} ($n = 4$) groups, however, was significantly different ($P = .04$).

Several mice were sacrificed after MRI to establish a correlation between radiographic and histologic appearance. Of note, ventricular changes similar to

those observed in the experimental G207 group were evident on histological examination of mice receiving only one-tenth the dose of G207 (data not shown).

Discussion

Brain tumors are the leading cause of cancer-related mortality in children, and malignant subsets often recur in spite of aggressive adjuvant therapy that not infrequently causes unacceptable toxicity. Viral oncolytic therapy is a novel therapeutic approach that involves local intratumoral delivery of a genetically engineered virus with tumor cell–specific lytic activity. The safety of one such agent, G207, has been demonstrated in adults with recurrent high-grade gliomas. Although it could therefore be hypothesized that G207 can be safely given to pediatric patients as

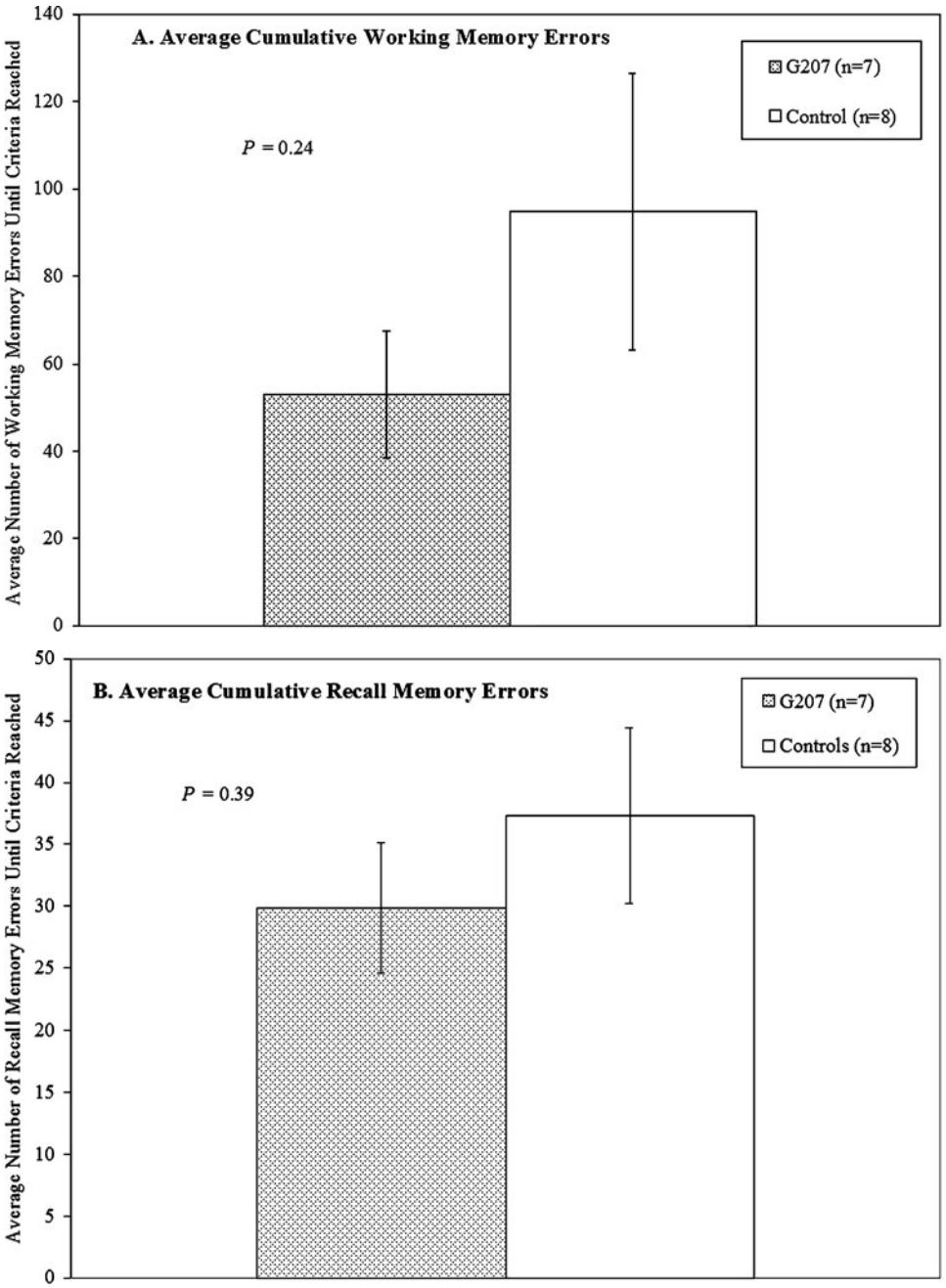

Figure 4 (**A–D**) Performance on the eight-arm radial maze. Working memory errors, recall memory errors, and the learning criterion are defined in Materials and Methods; total errors are working memory errors and recall memory errors combined. All group averages were compared by Student's *t* test, and none of the measures were significantly different between groups (cumulative working memory errors, $P = .24$; cumulative recall memory errors, $P = .39$; cumulative total errors, $P = .26$; days until criterion met, $P = .35$). All error bars represent standard error (*Continued*).

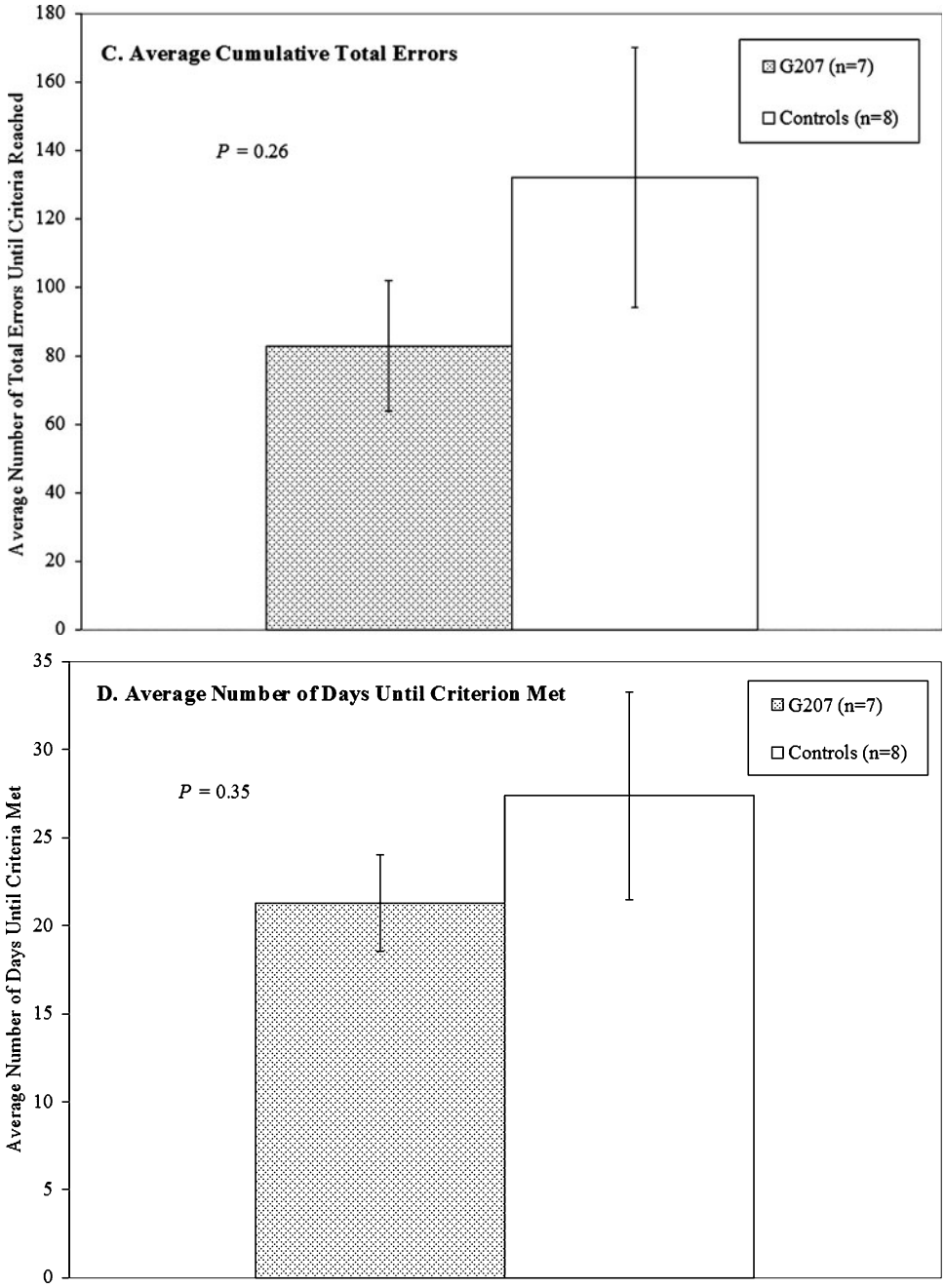

Figure 4 (Continued).

well, no data exist on the impact of a replication-competent oncolytic virus such as G207 on the developing mammalian brain. This study was designed to examine the effect of G207 on physical, cognitive, and behavioral development in a neonatal murine model.

There were two experimental groups in the developmental study: seven mice injected with G207 at 25% of the LD_{50} and eight control mice injected with an equal volume of vehicle without G207. No significant differences were found between the groups in physical development (body weight, brain weight, limb strength), cognitive performance (eight arm radial maze), or exploratory behavior (open-field maze). In fact, many of the nonsignificant trends showed better performance by the G207 developmental study group. These data suggest that G207 may not adversely affect the physical development, cognitive performance, or behavioral development of pediatric patients.

Given how well the G207-treated mice performed, it was surprising to find ventricular changes on

histologic examination of brains from mice predominantly in the G207 group, findings that were corroborated by MRI investigation of surviving mice that had received G207 in the LD_{50}-finding experiment. Several explanations may be proposed for this incongruity. It is possible that the physical, cognitive, and behavioral testing was either underpowered or inadequately sensitive to detect subtle differences between the groups. Secondly, imaging and histologic determination of ventricle size was not done concordantly with developmental testing, and thus it is possible that the changes in ventricular size occurred after developmental testing had been completed. More than likely, these changes may have occurred early, but subsequent neurologic development allowed for compensation of the injury induced by the virus injection.

Two variables in the current model should also be recognized for their likely bias towards overly precautionary conclusions regarding the potential clinical use of G207 in pediatric populations. First, the current study involved the free-hand injection of

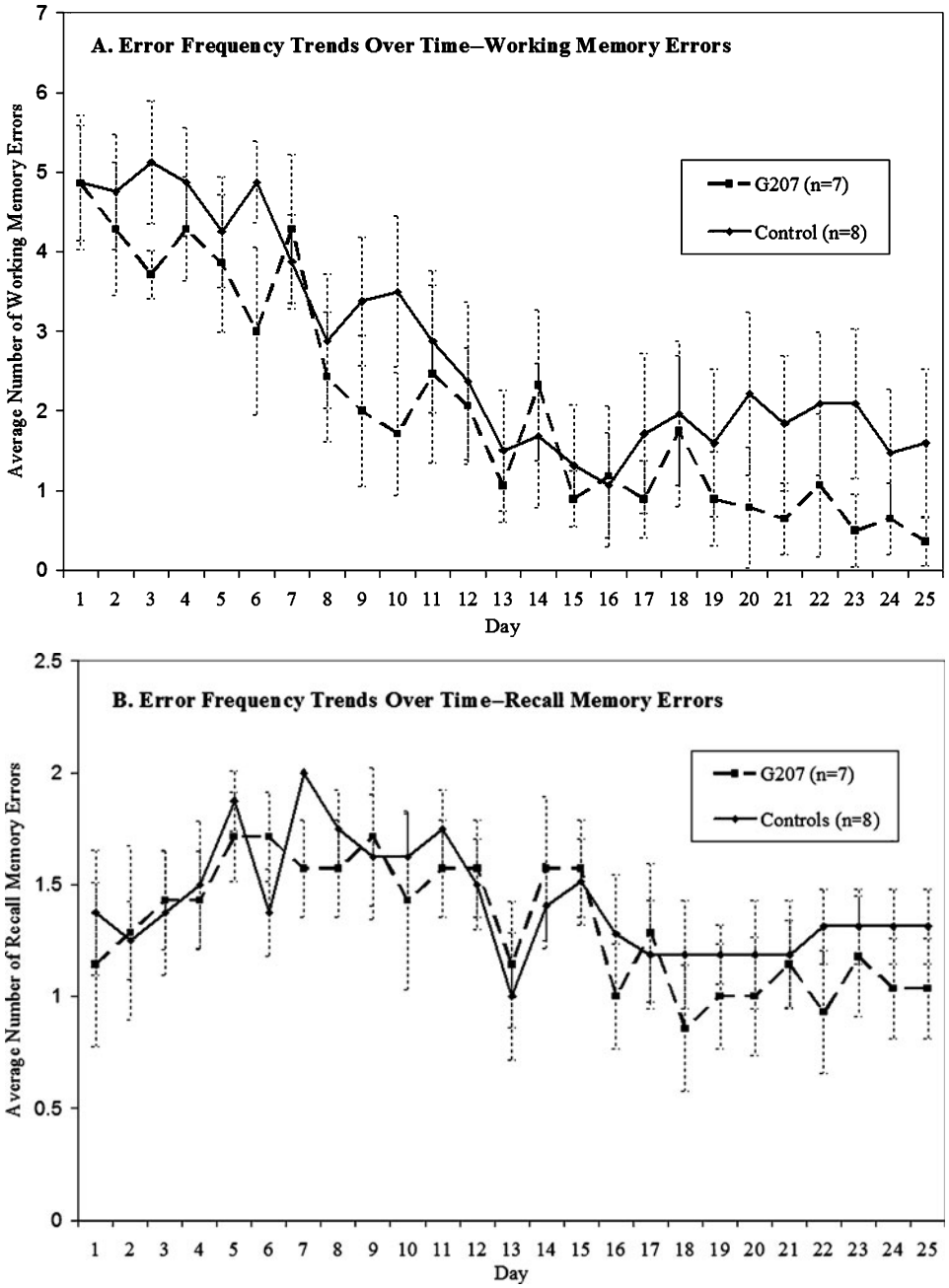

Figure 5 (**A–C**) Error frequency trends over the first 25 days of the eight-arm radial maze testing. Of note is the presence of a bimodal distribution of increased error frequency within the control group. Error bars represent standard error (*Continued*).

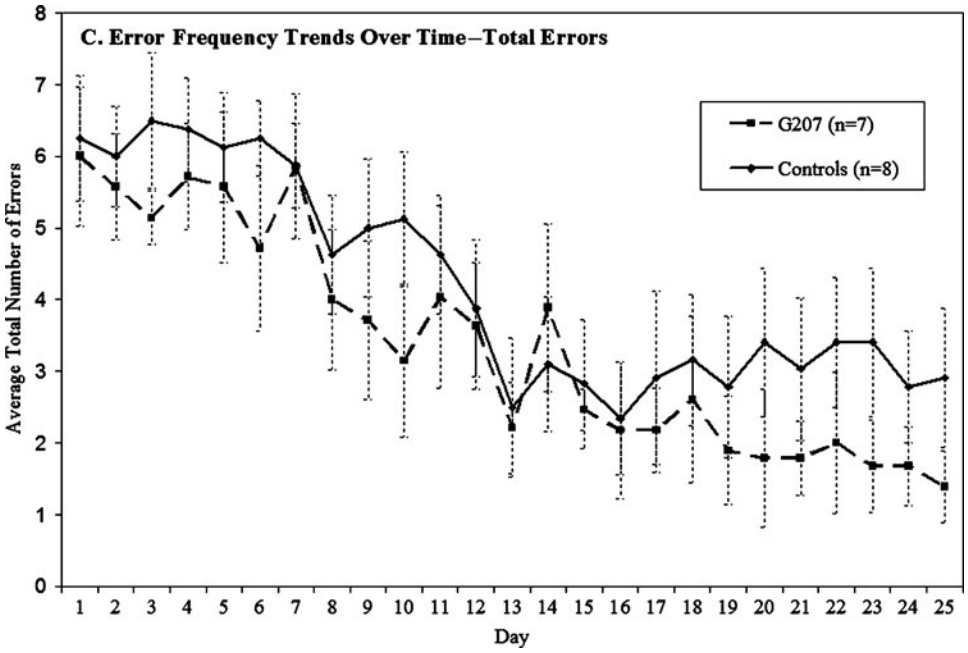

Figure 5 (Continued).

G207 into normal brain parenchyma and ventricle. No attempt was made to stereotactically deliver the virus to a particular site, and given the size of the four day-old murine brain, it is likely that (1) varying amounts of virus were delivered to parenchyma and ventricle among the mice in this cohort, and (2) local trauma was produced by the injection needle (30G) and the volume inoculated (2 μl). Even so, changes in ventricular histology may be an inappropriate surrogate marker that does not correlate closely with neurocognitive outcomes. Second, it is possible that the neonatal murine brain is simply a poor model for human therapeutic studies in this age group. Our murine model assumed developmental equivalence between mice pups at 2 to 4 days of age and young children. However, infant mice of that age have not yet even undergone eye opening, and CNS development might be more comparable to a third trimester human fetus than the typical pediatric patient presenting with a CNS tumor.

An improved understanding of the neurophysiologic response to G207 in the developing mammalian brain will help establish the significance of the observed ventricular changes. The exact mechanism and temporal window of susceptibility remain incompletely understood. Avoidance of ventricular inoculation and the resulting intraventricular inflammatory response might greatly limit the histologic effects; although such precision is not technically feasible in this preclinical mouse model, it could easily be achieved in select pediatric patients. Toxicity may also in part depend on the evolving immune status and CNS plasticity in developing mammals. Current ongoing work is attempting to elucidate the identity

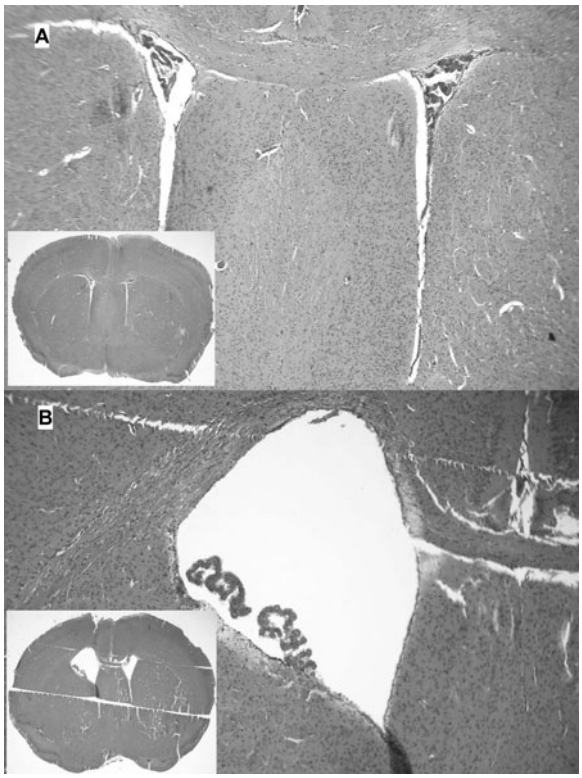

Figure 6 Brain sections of mice injected at days 2 to 4 of age with PBS (control) or G207 HSV. Mice were killed at 180 days of age and their brains processed as described in Materials and Methods. Inset photos show 1× full-section photograph of magnified images. **A,** Coronal brain section of (control) mouse injected with PBS. **B,** Coronal section through striatum of mouse injected with 7.6×10^5 pfu G207, showing ventriculomegaly, extensive ependymal cell necrosis with thickening of the ependymal cell layer and gliosis.

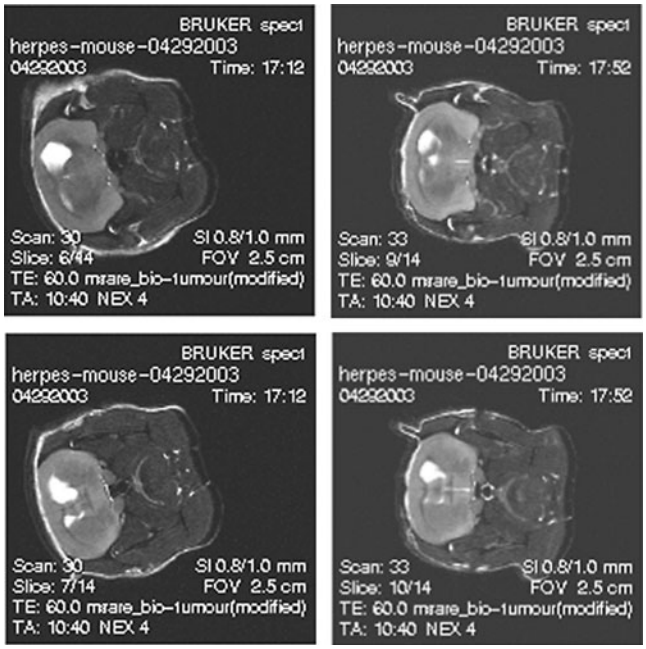

Figure 7 Serial 0.5 mm thick MRI coronal images of adult mice injected at 2 days of age with G207. Two of the mice injected intracerebrally with G207 at 2 to 3 days of age were subjected to non–contrast-enhanced NMR imaging using a T2-weighted sequence as described in Materials and Methods. These images of the striatal areas show unilateral ventriculomegaly on the right side (ipsilateral to injection). These images and the contiguous ones in which the ventricular space could be visualized were used to construct a three-dimensional volumetric measurement to estimate increased ventricular size.

of the cells supporting G207 replication after intracranial inoculation in mice pups, and the mechanism by which the observed histologic changes are effected at varying ages. Attempts are also being made to distinguish between acute and chronic pathologic changes

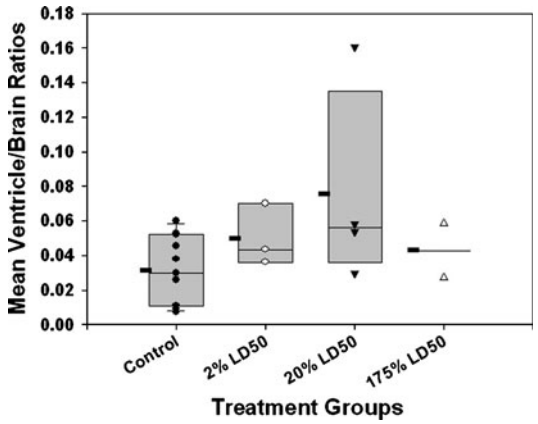

Figure 8 Computed ventricular volumes in 1-year-old mice injected with saline or varying doses of G207 HSV at 2 to 4 days of age. The average ratios of ventricle to total brain volume (within the imaging slab) for surviving mice in the dose-finding treatment groups are shown as box plots for each group with individual mouse values shown as symbols. Each box represents the median *(horizontal line)* and standard deviation of each of the four groups. The mean value for each group is represented by a short horizontal line to the left of each box.

that follow G207 inoculation. Further animal studies in *Aotus*, simian primates that are exquisitely sensitive to herpes virus, are ongoing to assess the safety of inoculating G207 directly into or around the ventricular systems.

This preclinical study suggests that in select pediatric patients with recurrent malignant brain tumors who have adequate brain volume and tumor location sufficiently removed from the periventricular region, G207 may be a novel therapeutic approach with acceptable potential toxicity. A similar study planned in juvenile *Aotus* will give further insight into the effects of G207 on the developing brain, and perhaps complete the foundation for a pediatric phase I protocol that could closely mirror the trial that has already been performed in adult patients (Markert *et al,* 2000). Clearly, the current study confirms that patients in such a trial would need to be closely monitored for development of hydrocephalus—already a common problem for pediatric brain tumor patients—or encephalitis, in which case acyclovir therapy would be available as a "rescue" drug.

Materials and methods

Construction of recombinant virus

G207 is derived from the parent herpes simplex virus type 1 strain F, and carries a deletion of both $\gamma_1$34.5 loci and a disabling *Lac*Z insertion in U_L39. The construction of this recombinant virus using the $\gamma_1$34.5-deletion HSV (R3616) has been previously described in detail (Mineta *et al*, 1995; Chou *et al,* 1990). The G207 used in this study was manufactured by MediGene, AG, with similar processes used to produce clinical grade virus.

Intracerebral inoculation of G207 and vehicle

Nontransgenic C57BL/6 female mice were crossed with nontransgenic DBA/2 male mice to produce the C57BL/6 × DBA/2 F_1 (B6D2F1) mice that were utilized in this study. For intracerebral inoculation, a 30-gauge needle was positioned just anterior to the coronal suture and ~1 mm to the right of the sagittal suture and advanced through the uncalcified skull to a depth of 1.5 mm in a freehand fashion. A volume of 2 μl of vehicle or G207 diluted in vehicle was then delivered. These coordinates approximate the subependymal plate of the frontal horn of the right lateral ventricle and more than likely results in a pass of the needle through the right ventricular space. Mice used for determination of the LD_{50} were injected between 2 and 4 days of age, whereas study mice were injected only at 4 days of age. The neurological development of mice at this age was felt to approximate the level of development in very young children, but was chosen somewhat arbitrarily given the lack of comparative data. Pups were returned to their mothers until weaning, at which time siblings were housed by gender.

Physical development
In addition to the daily weights recorded during testing in the radial arm maze (see below), mice were weighed at birth, 9 weeks, 22 to 25 weeks, and 35 to 37 weeks. All developmental-behavioral experimental mice were sacrificed at 35 to 37 weeks, and brain weight was recorded.

Hang-time test
Mice were tested in the hang-time test between 22 and 26 weeks of age. The testing apparatus consisted of a 14.5 cm × 14.5 cm wire screen (mesh squares 0.8 cm × 0.8 cm) bounded by a 3.5 cm wide wooden frame. Aluminum foil was attached to the proximal and distal borders of the frame and extended outwards proximally and distally such that it was impossible for the mouse to climb onto the opposite surface of the screen. The wire screen and wooden frame were attached to a stationary base by hinged arms of 40 cm length; the hinge allowed rotation of the arms, frame and screen as a single unit through a 135° arc. The height of the center of the screen above the bench top at the end of the 135° arc was 34.5 cm, and bedding of 2 cm depth was present under the screen at this position. In each trial, the mouse was placed on top of the screen and facing away from the hinge; the screen lay flat (arms at 0°). The screen was then rotated through 135° over 3 s such that the mouse was then on the downwards-facing surface of the screen, which formed a 45° angle with the bench top below. The time until the mouse released from the screen was measured in seconds; the trial was terminated if the mouse remained on the screen after 300 s. Two trials were conducted on the first day of testing, followed by two further trials 7 days later, and each mouse's single greatest hang time was included in the final analysis.

Eight-arm radial maze
Testing in the eight-arm radial maze began when the mice reached 12 weeks of age. The apparatus used for these experiments has been previously described (Wyss *et al,* 1992). Briefly, a central octagonal platform 36 cm wide anchored eight arms of 54 cm length and 12 cm width. The floor of each arm was rimmed by walls 2.5 cm high. A 2 cm high ridge positioned 2 cm before the end of each arm created a food cup baited with a sunflower seed out of sight of the mouse. Entrance to each arm was controlled by an individually adjustable transparent Plexiglas guillotine door of 20.5 cm height; the eight guillotine doors together formed the walls of the central octagonal platform. Numerous extramaze sensory cues were present. Each mouse was deprived of food (water available *ad libitum*) to decrease body weight by 15% prior to initiation of testing; daily weights and limited feeding allowed maintenance of this weight throughout the duration of testing in the eight arm radial maze. The first 2 days of testing involved the "shaping phase"; during this time all doors remained open for a 15-min interval, allowing the mouse to become familiar with the apparatus. Six of the eight arms were consistently baited with food for each mouse: one sunflower seed was placed at the entrance and another seed midway out each baited arm on day 1 of the shaping phase, whereas on day 2 one seed was midway out each baited arm with another seed in the food cup at the end of each baited arm. Day 1 of testing commenced on the day following the shaping phase, and one sunflower seed was placed in the food cup of each baited arm.

At the start of each test session, a mouse was placed in the center of the platform with all doors closed. After 10 s, all doors were open until the mouse chose one arm (defined as placement of all four paws into the arm), at which point all other doors were closed. When the mouse returned to the central platform, the last door was also closed; after a 10-s interval, all doors where then reopened and the mouse made a new decision. A working memory error was committed when the mouse chose an arm that had already been visited in that testing session; a recall memory error was defined as a visit to an unbaited arm (this applied only to the first visit to an unbaited arm). Because there was no reinforcement, the maximum number of seeds that could be obtained in each testing session was 6. A testing session was terminated as soon as one of the following three conditions was met: (1) All baited arms were visited; (2) 12 individual choices were made; or (3) 10 min passed. Successful completion of an individual testing session was defined as visits to baited arms in five of the first six choices. The criterion for learning the maze was defined as successful completion of the test for four consecutive days. Testing was performed daily until the mouse successfully met the maze-learning criterion or until testing proceeded for 50 days, whichever came first.

Open-field maze
Open-field maze testing was performed when mice were between 31 and 34 weeks of age. The square open field was 50 cm × 50 cm with 38 cm high walls; both floor and walls were constructed of white acrylic. Each mouse underwent a single trial per day for 3 consecutive days. Each trial involved placing a mouse in the center of the open field with subsequent recording of unmotivated behavior over 5 min. Location (central versus peripheral) and movement parameters (velocity, distance) were continuously captured by an overhead video camera and analyzed by tracking software (SD Instruments, San Diego, CA, USA). Tracking software was set to arbitrarily define the central zone as a square-shaped region beginning at a distance of 12.5 cm from each wall, and time spent in peripheral and central zones of the open field was recorded in each trial. Velocity was measured continuously in centimeters per second, and is described as time spent resting (<2 cm/s), moving slowly (2 to 5 cm/s), or moving quickly (>5 cm/s).

Magnetic resonance imaging and image analysis
Magnetic resonance imaging of mice that survived the initial HSV LD_{50}-dose–finding study was performed on an 8.5-T vertical bore MR scanner for small animals (Bruker Biospin, Billerica, MA, USA), equipped with the AVANCE spectrometer and running Paravision 3.0 software. Mice were anesthetized with a ketamine (100 mg/kg)/xylazine (5 mg/kg) mixture via intraperitoneal injection and placed in a 20-mm head coil using a specially designed head holder with adjustable ear pieces for head immobilization. A thermostat-monitored water bed maintained animal temperature and animal stability was monitored with a respiratory monitor. The rapid acquisition with relaxation enhancement (RARE) T_2-weighted imaging sequence was used to acquire coronal images of mice (Hennig *et al*, 1986). Twenty slices were acquired with a field of view of 25 mm and TR/TE of 4500/60 ms, matrix 256×256, slice width 0.5 mm, interslice gap 0.05 mm, in-plane resolution 98 μm, rare factor 8, 4 averages, acquisition time 9.6 min. This sequence provides excellent visualization of cerebrospinal fluid.

The MedX software (version 3.4.2; Medical Numerics, Sterling, VA), running on a Sun Blade 1000 (Sun Microsystems, Palo Alto, CA), was used for ventricular volume computation and analyses. Region of interest (ROI) techniques were used for ventricular quantification by manual outlining. Two specific regions of interest were outlined—ventricles and whole brain. To compute statistics for each LD_{50} dosage group, these ROIs were then summed for all slices and ratios of ventricular volume to the volume of brain within the imaging slab were calculated.

Histological evaluation
Mice in the developmental-behavioral study were killed at 35 to 37 weeks of age by carbon dioxide inhalation. Mice that were long-term survivors of the LD_{50}-finding study were killed at 11 to 12 months of age. Immediately after the mice were killed, brains were removed, weighed, and fixed in formalin. Brains were sectioned coronally in 2 mm thick slices and processed for paraffin embedding. Slides containing 8 to 10 μm thick sections, with four contiguous slices per slide, were prepared, deparaffinized, and processed for hematoxylin and eosin staining. Images were captured with a digital camera attached to a Olympus Opti-Phot microscope.

Statistical methods
The dose of G207 at which 50% mortality could be expected was calculated by the Spearman-Karber formula (Dougherty, 1964). When testing provided independent continuous variables for each mouse (brain weights, best hang-time interval, errors/days until criteria in the radial arm maze), a normally distributed population variance was assumed for both groups and comparison was made by use of the Student's *t* test with a two-tailed distribution. When repeated testing of the same variable occurred over time (body weight, open-field maze parameters), univariate and multivariate repeated measures analyses were performed with SYSTAT version 8.0 statistical program to examine the effect of both group and time.

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