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Bystander Attenuation of Neuronal and Astrocyte Intercellular Communication by Murine Cytomegalovirus Infection of Glia▽

Winson S. C. Ho and Anthony N. van den Pol*

Department of Neurosurgery, Yale University School of Medicine, New Haven, Connecticut 06520

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Astrocytes are the first cells infected by murine cytomegalovirus (MCMV) in primary cultures of brain. These cells play key roles in intercellular signaling and neuronal development, and they modulate synaptic activity within the nervous system. Using ratiometric fura-2 digital calcium imaging of >8,000 neurons and glia, we found that MCMV-infected astrocytes showed an increase in intracellular basal calcium levels and an enhanced response to neuroactive substances, including glutamate and ATP, and to high potassium levels. Cultured neurons with no sign of MCMV infection showed attenuated synaptic signaling after infection of the underlying astrocyte substrate, and intercellular communication between astrocytes with no sign of infection was reduced by the presence of infected glia. These bystander effects would tend to cause further deterioration of cellular communication in the brain in addition to the problems caused by the loss of directly infected cells.

Cytomegalovirus (CMV) is one of the most prominent causes of congenital defects in the developing nervous system and a common opportunistic agent of infection in the brains of immunocompromised individuals (those infected with human immunodeficiency virus or organ transplant recipients) (19, 30). CMV infection leads to a host of brain problems, including microcephaly, deafness, retardation, microgyria, and epilepsy (1, 8, 19).

Murine CMV (MCMV) preferentially targets astrocytes in early stages of infection (33). In the past few years, the traditional view that astrocytes in the brain merely provide structural support for neurons has given way to the realization that astrocytes play critical and active roles in processing information, communicating with other glia, and modulating synaptic communication (6, 7, 28). Astrocytes respond to and release transmitters such as glutamate and extracellular ATP through increases in intracellular Ca^{2+} (4, 20, 26, 31). Astrocytes modulate synaptic activity and respond to the release of transmitters from neurons (4, 9, 24, 26). Astrocytes communicate with one another; intercellular Ca^{2+} elevations initiated in a single astrocyte can propagate to neighboring cells in a wavelike manner mediated by ATP release and enhanced by gap junctions (2, 11, 12, 13, 16, 17). Long-distance intercellular calcium waves occur spontaneously or are triggered by neurotransmitters such as ATP (2, 31) and glutamate (10, 12, 21). In addition, astrocytes actively modulate synaptic transmission between neurons (3, 5, 9, 14, 24, 25).

As astrocyte signaling plays a role in microglia activation and neurogenesis, the preferential MCMV affinity for astrocytes may play a crucial role in the pathogenesis of MCMV, and, therefore, understanding the impact of MCMV on astrocyte signaling may contribute to a greater understanding of the disease process. Although we are unaware of studies investigating ion shifts due to MCMV infection of brain cells, CMV infections of fibroblasts may increase cytoplasmic calcium, thereby possibly enhancing CMV replication (22). In the present study, we examined the changes in astrocyte Ca^{2+} responses to glutamate, ATP, and depolarization by high K^+ in the course of MCMV infection. We also tested the hypothesis that infection of the underlying astrocyte substrate alters synaptic communication among cocultured neurons. Finally, we examined whether intercellular Ca^{2+} waves between communicating glia are impeded by MCMV infection.

Primary cultures were prepared from Swiss albino mouse brains harvested on postnatal day 5 and on embryonic day 17 for astrocyte and neuron preparations, respectively. Care was used to ensure that all cultures used in a particular comparison were of the same age and cell density. Astrocytes were maintained in vitro with minimal essential medium for up to 2 to 3 weeks before use, as described elsewhere (34). Neuronal cultures were maintained in serum-free neurobasal medium (Gibco). One micromolar 5-chlorocytosine arabinoside was used to prevent astrocyte overgrowth when high neuron density was desired. Fura-2 ratiometric calcium imaging was employed to monitor intracellular calcium levels. Cells were incubated with 5 μM fura-2 acetoxymethyl ester for 30 min at 37°C in standard HEPES buffer solution (10 HEPES, 137 mM NaCl, 25 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, pH 7.4). Cultures growing on glass coverslips were then loaded into a laminar-flow perfusion chamber for image acquisition. Ratiometric images were taken every 3 s by alternating between 340-nm and 380-nm excitation wavelengths using a Sutter filter wheel controlled by a Sutter Lambda 10-2 microprocessor. Ratiometric values were then calibrated to Ca^{2+} with a standard curve established using a fura-2 calcium calibration kit (Invitrogen). Images were acquired using a QImaging Retiga EX digital camera and processed with Openlab and IGOR Pro software running on an Apple G5 computer. In some cases, image contrast was corrected by using Adobe Pho-

* Corresponding author. Mailing address: Department of Neurosurgery–Room LSOG 212, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520. Phone: (203) 785-5823. Fax: (203) 737-2159. E-mail: anthony.vandenpol@yale.edu.

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toshop; all images in a set were treated simultaneously and in the same fashion to avoid data bias.

CMV alters astrocyte responses to neuroactive substances. In the first experiment, cultures enriched in astrocytes were infected with MCMV (2.5×10^4 PFU/ml) (Smith strain; ATCC). We monitored the basal Ca^{2+} level at 2 and 3 days postinfection (dpi) and found an increase from a Ca^{2+} level of 105 ± 1 nM (mean ± standard error) in noninfected controls to 146 ± 3 nM in MCMV-infected cells at 3 dpi ($n = 4{,}050$, $P < 0.05$, analysis of variance [ANOVA] with the Bonferroni procedure) (Fig. 1A). In a separate experiment, we examined the changes in the basal Ca^{2+} levels by using different concentrations of the virus. Basal Ca^{2+} levels at 1 dpi showed an increase from 125 ± 1.9 nM in control cells ($n = 658$) to 132 ± 2.1 nM at a multiplicity of infection (MOI) of 1 ($n = 605$, $P < 0.05$) and to 221 ± 2.7 nM at an MOI of 5 ($n = 811$, $P < 0.01$) (Fig. 1D). To avoid minor differences in Ca^{2+} responses from one region of the coverslip to another, each group is based on more than six imaging regions from two to four coverslips.

Calcium responses to stimulation by glutamate (100 μM), high K^+ (55 mM), and ATP (10 μM) were examined (Fig. 1B, C, E, and F). Infected cells (MOI of 0.5) showed a greater rise in intracellular calcium concentration than did noninfected control cells when stimulated with ATP, glutamate, and high K^+, suggesting that CMV enhances the calcium response to a number of neuroactive substances ($n = 1{,}088$, $P < 0.05$, ANOVA with Bonferroni procedure) (Fig. 1G to I); only responding cells were included in the data means. This finding is interesting as it suggests that the astrocytes become hypersensitive to neurotransmitter stimulation in the course of infection. This sensitization appears to be general to all three agents tested, suggesting an altered sensitivity of the glutamate and extracellular purinergic ATP receptor responses as well as the voltage-gated calcium channels stimulated by high K^+. At 3 dpi, cells no longer showed an exaggerated calcium response to ATP, glutamate, or high K^+. Given the central roles that glutamate and ATP play in intercellular glia-glia and neuron-glia communications (14, 15, 23, 29), the altered astrocyte responses to these neurotransmitters may impede normal functioning of the nervous system.

Figure 1J shows the percentage of cells responding to the different stimulations during the course of CMV infection. The response to ATP was robust, with almost all cells still responding at 3 dpi, whereas the percentage of cells showing a detectable rise in Ca^{2+} levels due to high K^+ and glutamate fell to 66% and 20%, respectively, at 3 dpi. This suggests that there is a sequence (glutamate → high K^+ → ATP) in which astrocytes become unresponsive to the three stimulations as infection proceeds. Another notable observation is that in response to ATP, a markedly higher percentage of infected astrocytes show repetitive oscillations in Ca^{2+} levels (Fig. 1C), from 3% ± 0.8% in noninfected control cells to 29% ± 5.1% at 2 dpi ($n = 1{,}006$, $P < 0.05$, two-tailed t test) (Fig. 1K). These oscillations occur in single cells and are characterized by a rise and fall in calcium levels over a period of about 15 s. This suggests that infected cells did not sustain a consistently elevated Ca^{2+} response but, rather, oscillations in the Ca^{2+} response in the form of multiple peaks. At 3 dpi, the oscillations were not sustained and the response to ATP came mostly in a single peak. Using a recombinant MCMV strain (K181 MC.55 [*ie2*$^-$ GFP$^+$]) that expresses the reporter green fluorescent protein (MCMV-GFP) (33), the detection of MCMV infection was correlated with the expression of the reporter gene. In a separate experiment in which astrocytes were infected with the MCMV-GFP strain, cells were divided into three groups based on the measured fluorescence intensity of the GFP signal, determined by using a 12-bit digital camera (low, 0 to 500; medium, 500 to 1,500; and high, 1,500 to 4,095). The level of Ca^{2+} oscillations, based on the standard deviation in Ca^{2+} concentration under ATP perfusion, showed a statistically significant increase in the high-GFP group compared to the low- or medium-GFP groups ($n = 474$, $P < 0.05$, ANOVA with the Bonferroni procedure) (Fig. 1L), suggesting that cells with a higher level of infection showed more oscillations in calcium level. Glial Ca^{2+} oscillations may serve to orchestrate the release of, or response to, transmitters (18, 24).

In order to preclude the possibility that the observed changes in calcium levels were due to factors other than the process of MCMV infection, several control experiments were performed. Using MCMV-GFP, the basal Ca^{2+} levels of uninfected cells in infected mouse astrocyte cultures (2 dpi), selected by the absence of a GFP signal, were compared to those of noninfected control cultures. At 2 dpi, the mean basal Ca^{2+} level did not increase in the uninfected cells of infected cultures compared to that of the control cultures; mean Ca^{2+} concentrations were 169 ± 7 nM ($n = 253$) and 141 ± 19 nM ($n = 151$), respectively ($P = 0.28$). In a second control experiment, we used human astrocytes infected with MCMV; MCMV has been shown previously to infect human astrocytes and cause the expression of some viral genes but not to replicate (33). Human astrocytes did not show any increase in Ca^{2+} after MCMV infection (MOI, 5; 1 dpi; $n = 302$) compared with noninfected human astrocytes ($n = 298$). The same concentration of MCMV in mouse cells caused a substantial rise in Ca^{2+} levels under similar conditions (Fig. 1D). These data are consistent with the view that intracellular Ca^{2+} is particularly sensitive to replicating MCMV. In a third control experiment, MCMV-GFP, inactivated with UV light for 5 h, was used to compare the Ca^{2+} responses of astrocytes to high K^+ levels and ATP. At 2 dpi, responses in cultures inoculated with the inactivated virus showed no increase compared to the control: mean Ca^{2+} levels in response to high levels of K^+ were 313 ± 12 nM ($n = 127$) in inoculated cells and 298 ± 13 nM in control cells ($n = 113$); the mean Ca^{2+} levels in response to ATP were 504 ± 22 nM ($n = 134$) in inoculated cells and 497 ± 20 nM in control cells ($n = 113$, $P = 0.78$). Inactivation was confirmed by the failure of MCMV-GFP to express GFP in any cell, while the same amount of virus before UV treatment did express GFP at the same dpi. These experiments suggest that changes in Ca^{2+} levels are not attributable to contaminants in the inoculum but rather that active replicating virus is responsible. In addition, it is also shown that the MCMV-mediated increase in Ca^{2+} increase occurs selectively in infected astrocytes and not in bystander astrocytes in the same dish.

Astrocyte infection alters neuronal communication. Astrocytes play an important role in modulating communication between neurons (4, 9, 24, 26). To test the hypothesis that MCMV infection (MOI, 0.5) of astrocytes may change neuronal communication, mixed astrocyte-neuron cultures were in-

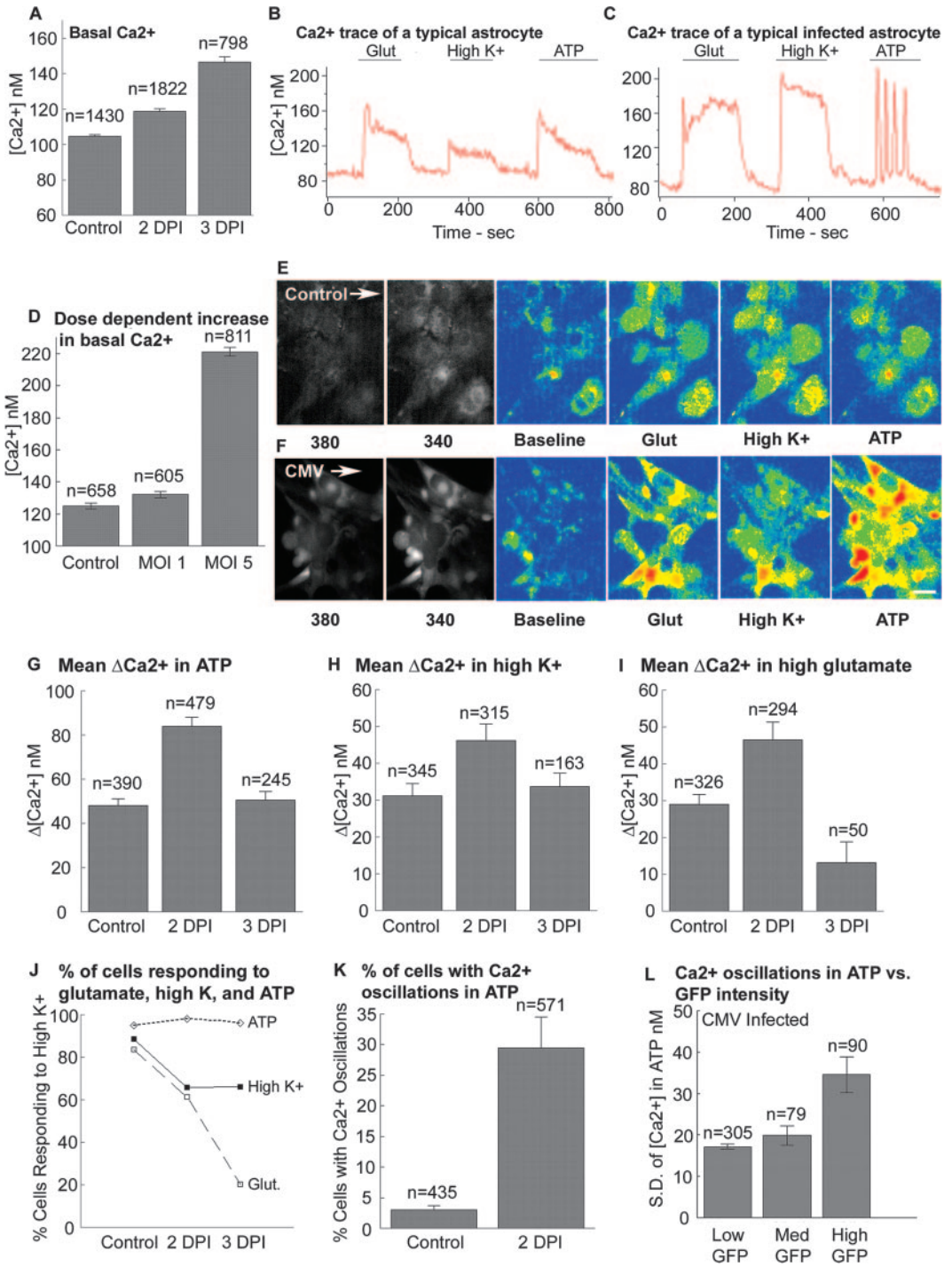

FIG. 1. MCMV alters astrocyte Ca^{2+} levels and responses. (A) The mean basal intracellular Ca^{2+} level in astrocytes is increased by MCMV at 2 and 3 dpi. (B and C) Representative Ca^{2+} traces of astrocyte responses to glutamate (Glut), a high level of K^+, and ATP in uninfected (B) and infected (C) cells. (D) The mean basal intracellular Ca^{2+} increase in astrocytes is dependent on viral concentration. An MOI of 5 results in a greater increase in basal Ca^{2+} than an MOI of 1 compared to noninfected control cells. (E and F) Typical raw 340-nm and 380-nm excitation images and ratiometric images of 340/380 calibrated to Ca^{2+} in pseudocolor show a color spectrum from blue (representing low Ca^{2+}) to red (representing high Ca^{2+}) of uninfected and infected cells, respectively (scale bar, 50 μm). (G to I) At 2 dpi, infected cells show increased responses to ATP, a high level of K^+, and glutamate. This exaggerated response was lost by 3 dpi. (J) The proportion of cells responding to glutamate (Glut) and a high level of K^+ decreased at 2 and 3 dpi. However, almost all cells retained responsiveness to ATP stimulation. (K) The percentage of cells displaying a high level of oscillations in Ca^{2+} concentration during ATP stimulation increased after infection. (L) Using a GFP-expressing MCMV, the degree of Ca^{2+} concentration oscillations in response to ATP as determined by the standard deviation of Ca^{2+} concentration is correlated with GFP intensity, suggesting that increased levels of MCMV infection are correlated with a greater degree of Ca^{2+} oscillations.

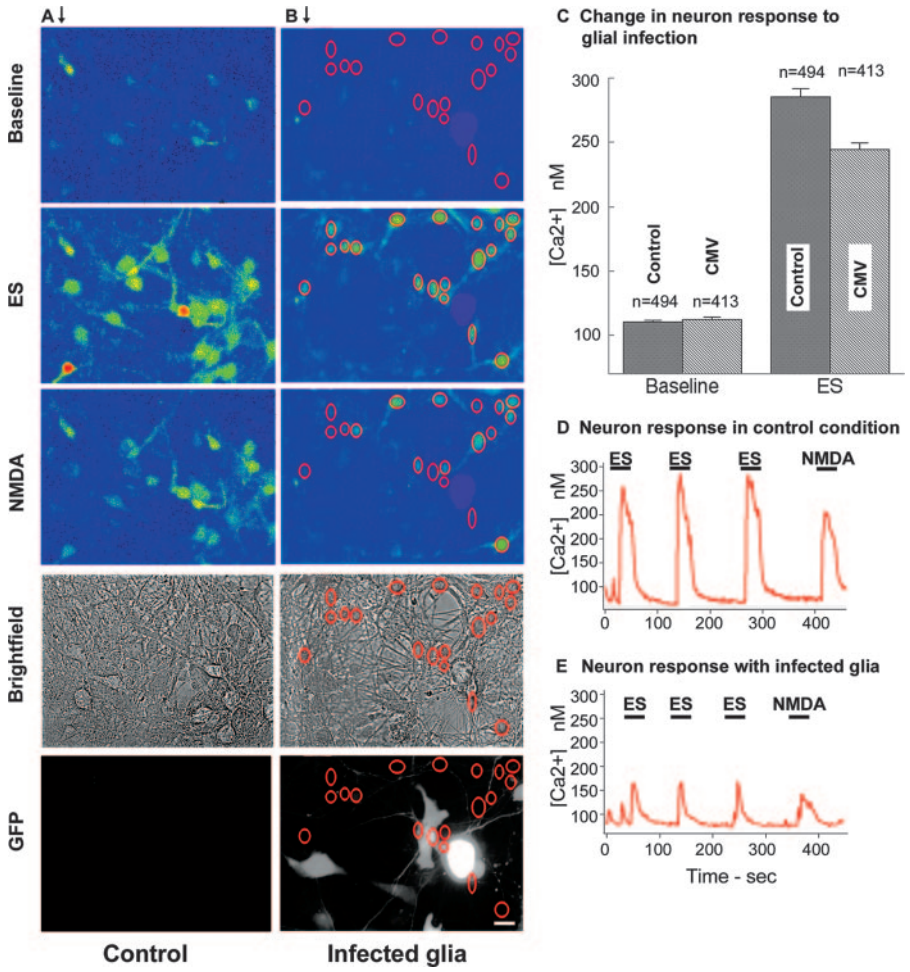

FIG. 2. Astrocyte infection reduces neuron synaptic responses. (A and B) Ratiometric images of 340/380 nm calibrated to Ca^{2+} show neuronal responses to ES and *N*-methyl-D-aspartic acid (NMDA). Synaptically linked neurons are identified by their responsiveness to both ES and NMDA, with confirmation based on morphology in brightfield images. The GFP intensity indicates the level of MCMV infection. Cells in red circles in panels B are neurons responding to ES and NMDA that are uninfected by MCMV as shown by the absence of GFP signal. Underlying astrocytes are, however, heavily infected, and show a bright GFP signal. Scale bar, 20 μm. (C) The change in the ES response of neurons on infected astrocytes was lower than that on control neurons growing with uninfected astrocytes. The change in Ca^{2+} concentration decreased from 175 ± 6 nM in control cells to 132 ± 6 nM in neurons with infected astrocytes. (D and E) Representative Ca^{2+} traces of neurons responding to ES and NMDA with normal astrocytes (D) and with MCMV-infected astrocytes (E).

fected with low levels of MCMV-GFP. Experiments were performed at a time when there was clear infection of the underlying astrocytes but little or no infection of the overlaying neurons (Fig. 2A and B). The GFP image in Fig. 2B clearly shows that the GFP signal comes exclusively from the astrocytes, not from neurons. The cells were electrically stimulated using electrodes on opposite edges of the recording chamber with a Grass SD9 stimulator. A rise in Ca^{2+} levels was triggered by passing a current of 0.06 V/mm^2 through the chamber for 3 ms at 7 Hz. Figure 3A and B show that the rise in Ca^{2+} levels with electrical stimulation (ES) is attributable to synaptic activation. In the presence of tetrodotoxin (TTX) to block spike-mediated transmitter releases, the electrically stimulated Ca^{2+} rise was completely eliminated. Similarly, the ionotropic glutamate receptor antagonists (AP5 [DL-2-amino-5-phosphonopentanoic acid] at 100 μM and CNQX [6-cyano-7-nitroquinoxaline-2,3-dione] at 10 μM) blocked the stimulated response (not shown). Figure 2C shows that the synaptic activity of neurons with infected underlying astrocytes becomes attenuated in comparison to that of the control cells. MCMV infection of astrocytes reduced the ES-mediated Ca^{2+} rise in neurons from 176 ± 6 nM in control cells to 131 ± 6 nM (n = 907, $P < 0.05$, two-tailed t test), suggesting that MCMV altered neuronal communication by the infection of astrocytes, even before the neurons showed any sign of infection. Since TTX blocks spike-mediated neurotransmitter release and glutamate receptor antagonists block the response to synaptic glutamate release, together these data support the view that the electrically evoked calcium rise is due to the synaptic release of glutamate and not to a direct effect of electrical stimulation on voltage-gated calcium channels.

Figure 3C and D show an additional control in which neuron-enriched cultures, in the absence of an underlying astrocyte substrate, were inoculated with the MCMV at the same MOI as that described above. When used for calcium imaging, neurons did not show GFP expression or a cytopathic effect.

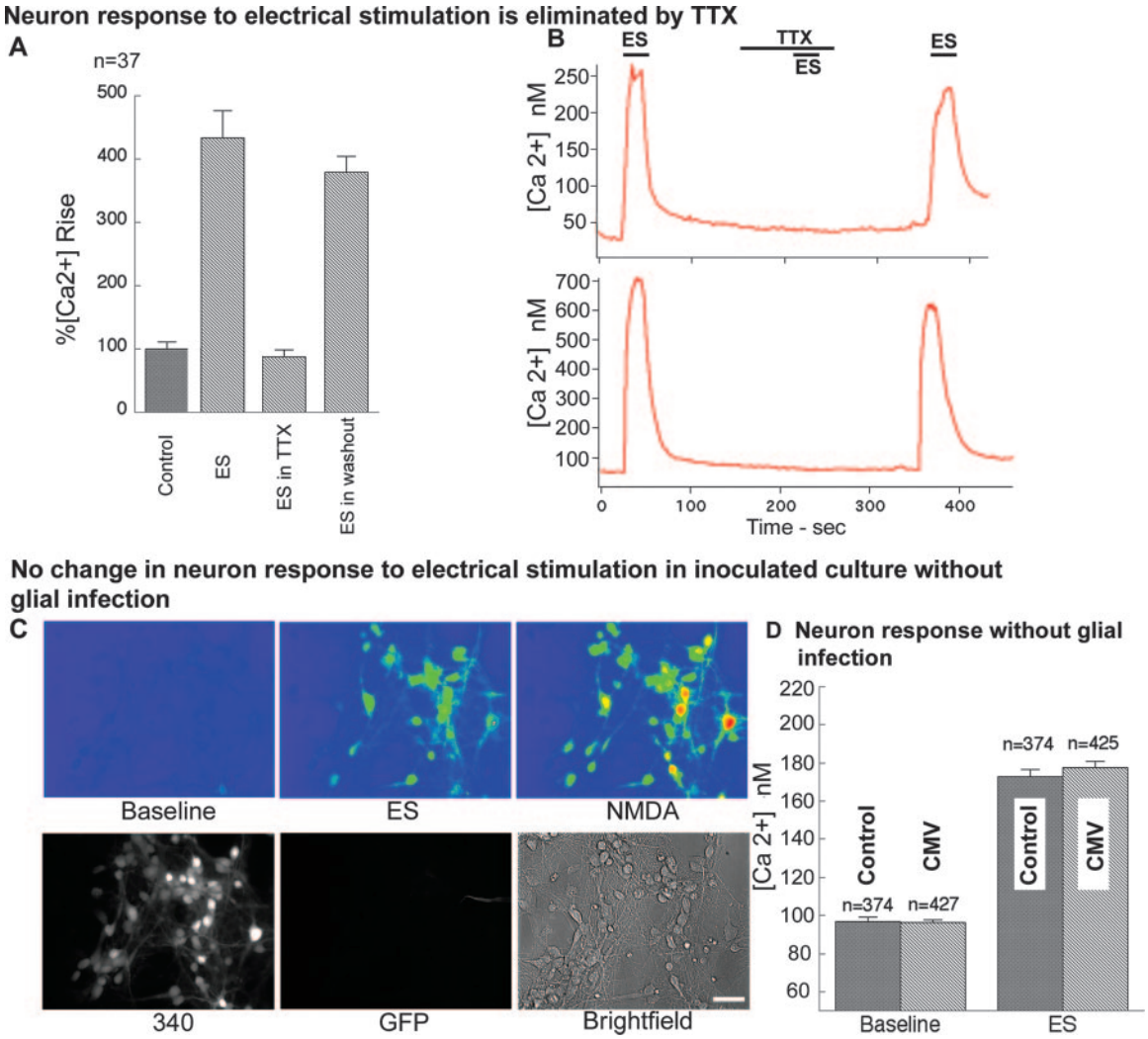

FIG. 3. Calcium rise due to electrically stimulated neurotransmitter release. Control experiments demonstrated that the ES response is attributed to increased synaptic activity (A and B) and that the ES response of neurons is unaffected by MCMV inoculation in the absence of infected underlying astrocytes (C and D). (A) In the presence of TTX, the neuron response to ES is completely abolished. This indicates that the Ca^{2+} increase in response to ES is attributed to an increase in synaptic activity and not to the direct activation of voltage-gated Ca^{2+} channels, supporting the use of the ES response as a measure of synaptic activation. The ES response recovered after the washout of TTX. (B) Two representative Ca^{2+} traces showing a complete block of the ES response in the presence of TTX as well as the recovery after the TTX washout. (C) Ratiometric images of 340/380 nm calibrated to Ca^{2+} showing the neuronal responses to ES and NMDA (*N*-methyl-D-aspartic acid). The 340-nm and brightfield images show the absence of underlying astrocytes; the GFP image shows that the experiment is performed at a time when no MCMV reporter gene expression or cytopathic effect can be detected. Scale bar, 30 μm. (D) The mean ES response of neurons is unchanged in the absence of astrocyte infection.

This allows for the possibility that there may be a low level of infection of neurons that was not detected by measuring GFP expression and that could potentially alter neuronal signaling. Electrically evoked and synaptically mediated calcium level increases did not differ between control and MCMV-inoculated cultures; the change in Ca^{2+} level was $+76 \pm 4$ nM in control cultures and $+81 \pm 4$ nM in MCMV-inoculated cultures ($n = 801$, $P > 0.3$, two-tailed t test). This supports the view that an infected-astrocyte substrate is most likely responsible for the attenuation of neuronal synaptic communication.

Intercellular calcium signaling attenuated by CMV infection. Intercellular calcium waves mediated by ATP release underlie long-distance glial signaling. The impact of CMV on intercellular signaling between astrocytes was examined. Calcium waves in astrocyte-enriched cultures were triggered by light mechanical stimulation with a glass pipette (Fig. 4A to C) that led to an intercellular Ca^{2+} wave. MCMV infection (MOI, 1) strongly attenuated calcium wave propagation (Fig. 4A to C). In MCMV-infected astrocytes, the mean distance traveled in a single triggered wave decreased from 434 ± 36 μm to 119 ± 23 μm, the mean number of cells in a wave decreased from 53 ± 5 cells to 19 ± 2 cells, and the maximum propagation velocity dropped from 15 ± 3 μm/s to 8 ± 1 μm/s ($n = 54$, $P < 0.05$, two-tailed t test). Given that glutamate and ATP are critical messengers in the propagation of calcium waves, it is possible that the change in the responses to ATP and glutamate de-

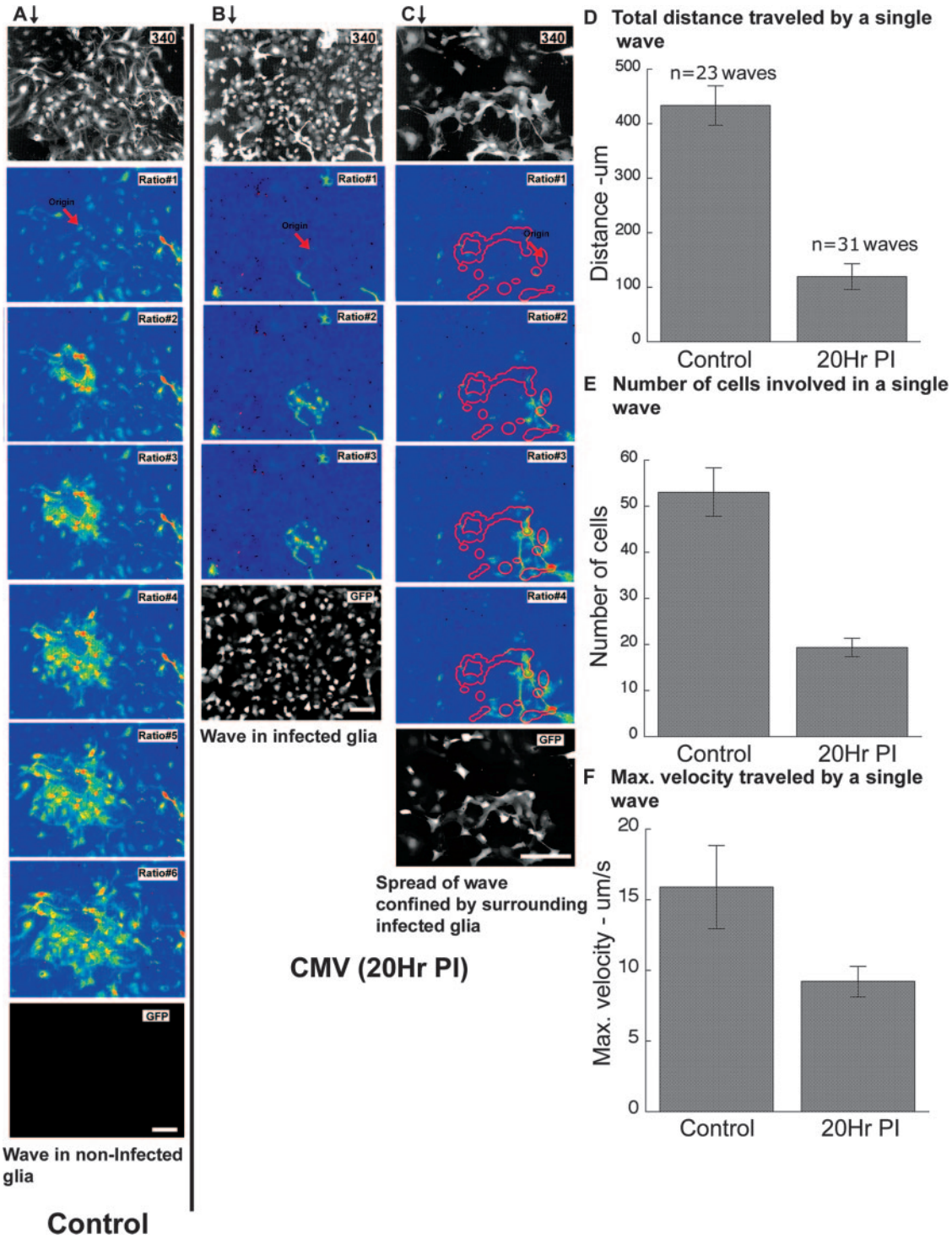

FIG. 4. Intercellular Ca^{2+} waves are attenuated by MCMV. (A to C) Sequences of ratiometric images of 340/380 nm excitation in pseudocolor calibrated to Ca^{2+} show the progression of intercellular waves triggered by light mechanical stimulation in uninfected control astrocytes (A) and infected astrocytes (20 h postinfection) (B and C). Red arrows indicate points of stimulation. GFP images show the distribution of infected astrocytes. Intercellular Ca^{2+} waves are significantly inhibited in infected cells. Infected cells spatially confine the spread of a wave in other cells (C). Scale bar, 150 μm. Note that the magnification is higher in panel C than in panels A and B. (D) The mean distance traveled by a wave is decreased in infected astrocytes. (E) The mean number of cells in a wave decreases in infected astrocytes. (F) The maximum velocity traveled by a wave decreases in MCMV-infected astrocytes.

scribed earlier contributes to the disruption of wave propagation. Figure 4C also shows an interesting consequence of MCMV infection on the spatial distribution of waves in infected cells. Wave propagation is spatially limited by infected cells, demonstrating that those cells can form a barrier between astrocytes with no sign of infection, over which wave propagation is attenuated. To test further the effect of infection on intercellular glial signaling, astrocytes were grown in thin strips, and MCMV was focally applied by a micropipette to a single region of each strip. Three days later, noninfected astrocytes were stimulated, and the distance the wave traveled was measured. By focusing only on cells not expressing the MCMV-GFP reporter, calcium waves were found to be strongly attenuated in proximity to infected cells, reducing the distance the wave traveled from 554 ± 19 μm ($n = 6$) when the wave did not encounter infected cells to 264 ± 16 μm ($n = 6$) when the wave met infected glia. Thus, MCMV reduces the velocity of and distance traveled by calcium waves in astrocytes. Importantly, the intercellular calcium wave is attenuated in astrocytes showing no sign of infection.

In summary, we show that MCMV infection of astrocytes reduces intercellular communication both between astrocytes and between neurons. The attenuation of neuronal communication appears to be a bystander effect, as little detectable infection was found in neurons secondary to astrocyte infection. This would be consistent with the view that astrocytes modulate the synaptic activity of neurons and that infection of astrocytes by MCMV causes deterioration in neuronal communication. As glial signaling has been shown to play an important role in synaptogenesis, microglial activation, and neurogenesis (20, 27, 35), the attenuation of glial signaling reported here may constitute an additional underlying substrate for central nervous system dysfunction associated with CMV infection of the brain. CMV infections of the developing brain can cause microencephaly and microgyria; these may result in part from retardation of intercellular glial signaling, which has been shown to play a substantive role in neurogenesis (35).

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