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In vivo differential susceptibility of sensory neurons to rabies virus infection

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Abstract There is controversy with regard to the entry pathway of the rabies virus (RABV) into the central nervous system (CNS). Some authors have suggested that the virus inoculated at the periphery is captured and transported to CNS only by motor neurons; however, it has been reported that dorsal root ganglia (DRG) sensory neurons capture and transport the virus to the spinal cord (SC) and then to the brain. It is probable that preferences for one pathway or another depend on the site of inoculation and the post-infection time. Therefore, in the present study, we evaluated different vertebral segments and post-infection times, along with the location, number, and subpopulation of sensory neurons susceptible to infection after inoculating RABV in the footpads of adult mice. It was noted that the virus inoculated in the footpad preferentially entered the CNS through the large-sized DRG sensory neurons, while infection of the motor neurons occurred later. Further, it was found that the virus was dispersed in spinal cord trans-synaptically through the interneurons, arriving at both sensory neurons and contralateral motor neurons. In conclusion, we observed that RABV inoculated in the plantar footpad is captured preferentially by large sensory neurons and is transported to the DRG, where it replicates and is spread to the SC using transynaptic jumps, infecting sensory and motor neurons at the same level before ascending to the brain.

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Introduction

The rabies virus (RABV), which belongs to the genus *Lyssavirus* and the family Rhabdhoviridae, possesses a negative-sense RNA that codes for the structural proteins N (nucleoprotein), P (phosphoprotein), and L (viral polymerase) and for the proteins M (matrix) and G (glycoprotein), which are located at the periphery of the virion (Lyles and Rupprecht 2007). The ectodomain of the glycoprotein G promotes virus and cell membrane fusion and confer intracellular transport properties to the internalized virions and stimulates the immune response of the host (Lyles and Rupprecht 2007; Wunner et al. 1988). Additionally, the cytoplasmic domain of the G protein increases the pathogenic potential of the virus promoting the survival of the infected neuron like a subversive strategy (Lafon 2004; Préhaud et al. 2010)

RABV alters the physiology of the nervous system and causes the deaths of more than 55,000 people around the world annually (Rupprecht et al. 2002; Faber et al. 2004), and the infection with RABV begins when glycoprotein G binds with molecules of the cellular surface, such as nicotinic acetylcholine receptor (nAChR; Lenzt et al. 1982) or neuronal cell adhesion molecule (Thoulouze et al. 1998), which promote the union and later endocytosis of the virus in susceptible cells (Lafon 2005). Next, the virus travels via retrograde axonal transport to the neural somas, which are located in different areas of the nervous tissue (Ugolini 1995). Some authors in vitro and in vivo have reported that the motor neurons, located in cortex (Kelly and Stick 2003) or in the ventral horn of the spinal cord (SC), are the cells with greatest susceptibility to infection and are responsible

for virus spreading within the tissue until it reaches the brain (Jackson 2003; Shankar et al. 1991; Tang et al. 1999; Mazarakis et al. 2001; Guigoni and Coulon 2002). In other cases, it has been suggested that the sensory neurons of the dorsal root ganglia (DRG) participate both in vivo and in vitro in the capture and transport of the virus from the periphery to the SC and the brain (Tsiang et al. 1989, 1991; Castellanos et al. 1997; Velandia et al. 2007).

In 1963, Dean et al. developed a model of infection with RABV in mice to evaluate whether the sensory and motor neurons show differences in susceptibility to the virus. In this model, the authors previously sectioned the ventral or dorsal roots of the lumbar SC and inoculated RABV in the plantar footpad. Later, using immunohistochemistry, they evaluated the presence of viral antigens in slices of SC and DRG, finding that both tissues were susceptible to infection, independent of the type of lesion (Dean et al. 1963). This finding suggests that both types of neurons can capture and transport the virus to the brain and that the preference of RABV for one of the pathways depends on the site of inoculation and the post-infection time.

After rabies exposition, the inoculation of the virus takes place through bites or scratches in the infected animals, principally in the arms and legs, thus the inoculated virus must transport itself to the central nervous system (CNS) using axonal transport to the SC. However, until now, it is unknown if there is differential susceptibility for RABV through sensory and motor pathways. Therefore, the aim of the present study was to evaluate the location, number, and populations of sensory and motor neurons infected by RABV at different post-infection times after the virus were inoculated in the hind footpad. The obtained results suggest that the virus inoculated in the hind footpad entered early and preferentially in the CNS through the larger-diameter sensory neurons. It was also found that the virus spread both by retrograde and anterograde axonal transport and by the infection of neighboring neurons following the synaptic connections of each cellular group. This finding provided evidence of the presence of the infection in the interneurons in the SC, which carried the virus to the contralateral motor neurons and even to the contralateral sensory neurons, while the virus simultaneously ascended to the brain until encephalitis occurred. These findings led to the conclusion that the preference of RABV for one of the nerve pathways depends on the site of inoculation and that RABV uses different spreading mechanisms to augment its pathogenic potential within nerve tissue.

Materials and methods

The procedures described below were previously approved by the Ethics' Committee of Universidad El Bosque, taking

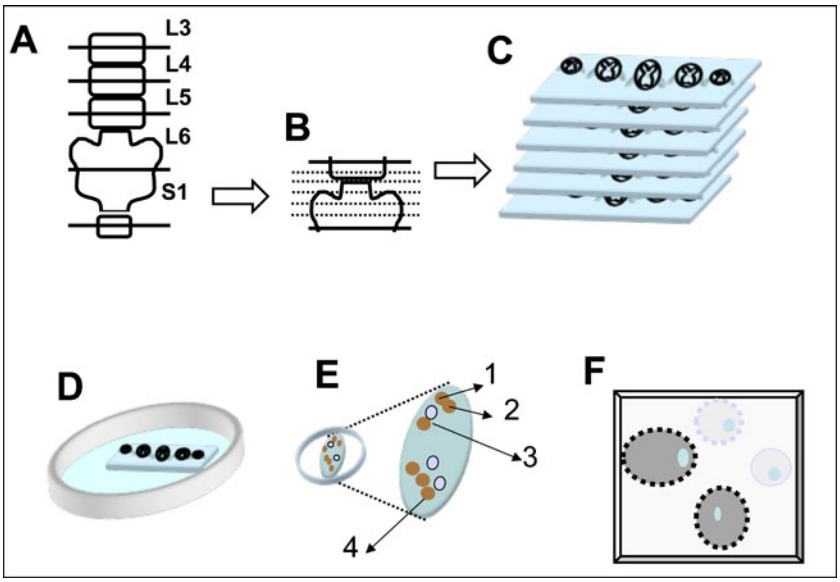

Fig. 1 Scheme of the research approach and methodology used in this study. **a** The whole vertebral column of infected and mock-infected mice was dissected and decalcified. **b** Each vertebral level of sacrolumbar region were separated and serial slides obtained in a cryostat. **c** Tissue slices were recovered on 16 glass slides, putting on the slices 1, 17, 34, 51, and 72 in the first one and the following slices (2, 18, 35, 52, and 73) in the second one. **d** Eighth different slides (40 tissue slices) from each vertebral level and p.i. period were processed by immnunohistochemistry. **e** Rabies virus positive and negative neurons were counted. **f** Digital images were captured using a video camera coupled to a microscope. The profile of each neuron in a slide were then drawn and analyzed in Scion-Image software

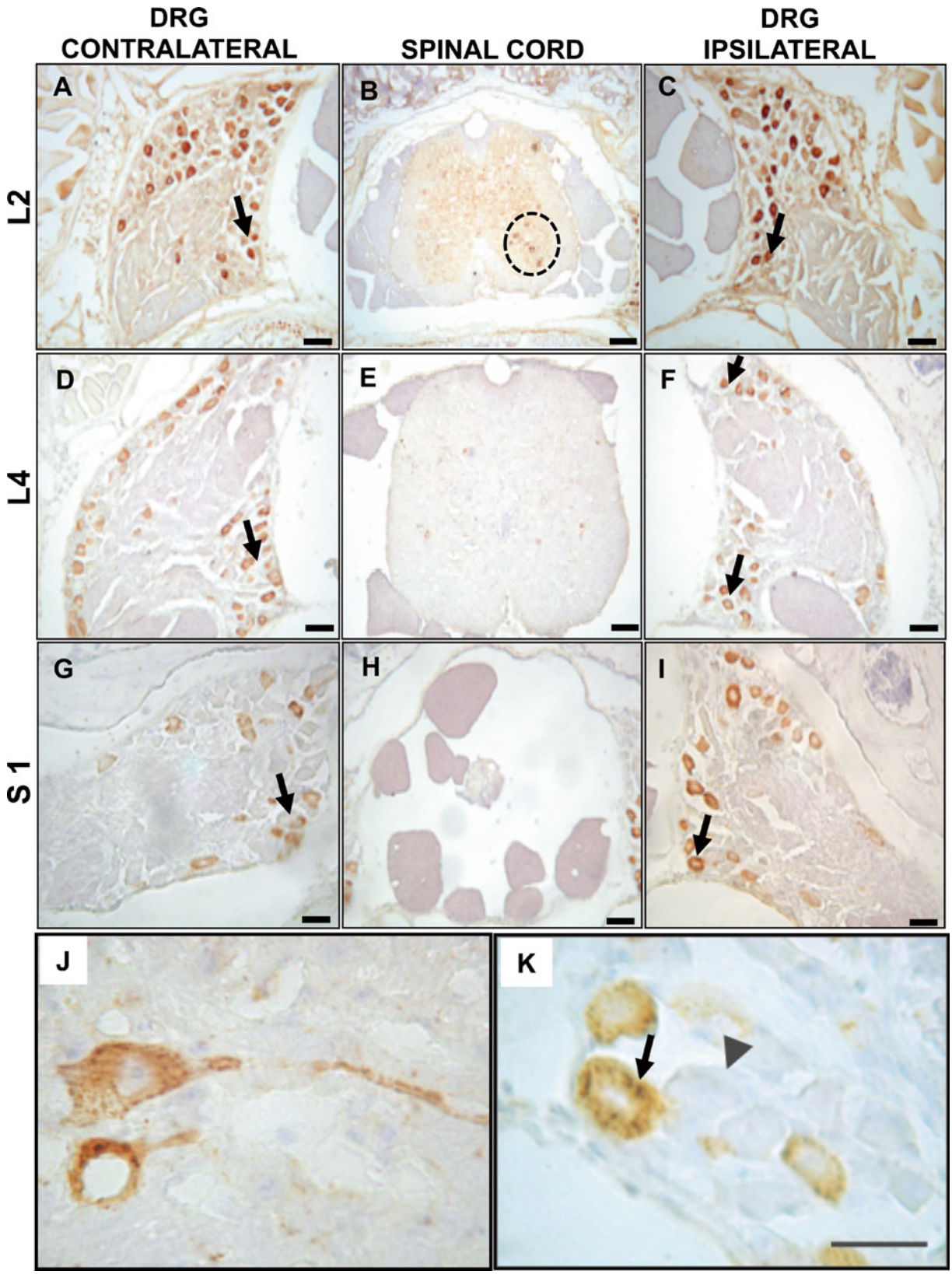

Fig. 2 Immunoreactivty for RABV in vertebral column S1, L4, and L2 segments of mice after 120 h p.i. Note the immunopositive cells in both the ventral (*dotted circle*) and dorsal horn of spinal cord. *Arrows* point out to many infected sensory neurons in all shown ipsi- and contralateral dorsal root ganglion. Immunopositive sensory neurons are grouped in ventrolateral, ventromedial, and dorsolateral zones of ganglia. *Bottom images* show a detail of infected motor neuron (*left*) and sensory neurons infected (*arrow*) and non-infected (*arrowhead*) from the S1 vertebral level. *Bar* corresponds to 100 μm

into account the Resolution 8430 of 1993 of the Colombian Ministry of Health.

Infection with RABV

A pair of mice (strain ICR) were injected in the posterior right footpad, the first with 30 μl of the viral inoculum (CVS strain, $10^{6.7}$ DL_{50}) obtained in mouse brain diluted in DMEM supplemented with 2 % fetal calf serum (FCS; Castellanos et al. 1996), and the second was injected with a 10 % brain homogenate in DMEM supplemented with 2 % FCS (mock), which was used for the controls. The procedure was repeated three times. The animals were maintained for 24, 48, 72, 96, and 120 h post-infection (p.i.) with food and water ad libitum. At the end of each period, the animals were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (15 mg/kg) and were perfused intracardially with 4 % paraformaldehyde (PFA). After that, the complete vertebral columns (bone, muscular, and nervous tissue) were dissected and post-fixed for 48 h. They then underwent a 15-day process of decalcification with a solution of 10 % formic acid and 4 % PFA and were stored in 20 % sucrose at 4 °C until use (Velandia et al. 2002).

Immunohistochemistry

To determine the exact localization of the infected neurons, serial slices of 12 μm were obtained from the sacral vertebra 1 (S1) to the lumbar vertebra 1 (L1). The slices obtained were then mounted on slides that were pretreated with poly-L-lysine (100 μg/ml) according to the distribution shown in Fig. 1. Next, the slices obtained were hydrated with PBS and permeabilized with Triton X-100 (0.1 %). Endogenous peroxidases were inactivated with a solution of 50 % methanol and 0.5 % H_2O_2, and nonspecific sites were blocked with 5 % horse serum in PBS. The slices were then incubated overnight at 4 °C with a polyclonal antinucleocapsid antibody (BioRad 72114), and they were then washed and incubated with a secondary biotinylated anti-rabbit antibody (Vector BA-1000). Next, immunoreactivity was detected with the ABC kit from Vector (PK-4000) using 3,3-diaminobenzidine as a chromogen. Finally, the slices were given contrast with Mayer's Hemalum and preserved in Kaiser gelatin. For each infected and non-infected (mock) animal, we processed by immunohistochemistry eight different slides with five slices each for each vertebral segment for each time p.i.

Determination of the percentage and morphometric profile of sensory neurons

Under the microscope, using 40 independent sections of each vertebral segment for each time p.i., the infected and non-infected sensory neurons with nuclei and defined nucleoli were counted. Morphometric profiles were determined in the following way. For each vertebral segment and time p.i., ten independent sections belonging to the same ganglion were digitized using a Philips LTC 0435 camera and the software program Studio DC10plus (Pinnacle Systems®). Next, for each one of the images, the outline of each neuron was delimited (perimeter, micrometer), and using the ImagePC software program (www.scioncorp.com), the diameter were obtained using the formula D=perimeter/π. Finally, the data were stored in Excel® and analyzed with the statistical program Simstat ®. Also, the slices of the vertebral segments of the sacrolumbar region of the non-infected animals were processed in a similar way.

Statistical analysis

The proportion of infected sensory neurons from the sacrolumbar region (ipsi- and contralateral) at each time p.i. (72, 96, 120 h p.i.) were compared using an ANOVA test and post-hoc LSD and Student's t test with a value of $p<0.05$. To compare the distribution of the diameters of the infected neurons in comparison to the total for each ipsilateral and contralateral ganglion from the sacrolumbar region, the nonparametric Kolmogorov–Smirnov test was used. Finally, the diameters of the infected neurons were compared by applying Student's t test with a value of $p<0.05$.

Results

Immunoreactivity for RABV in DRG and SC of the sacrolumbar region

Dorsal root ganglia

To determine the kinetic of infection with RABV in the sensory neurons of the DRG, 40 serial slices of each vertebral level and time p.i. were processed using immunohistochemistry. Between 24 and 48 h p.i., no viral antigen was detected in any of the vertebral levels evaluated. Meanwhile, at 72 h p.i., viral antigen was detected only in the sensory neurons of the ipsilateral DRG of the L5 and L4 vertebral levels. At 96 h p.i., two phenomena took place: first, numerous infected sensory neurons were observed in all the ipsilateral DRG of the sacrolumbar region and second, numerous infected sensory neurons were observed in the contralateral DRG of the seven analyzed vertebral levels. On the other hand, at 120 h p.i., a significant increase in the number of sensory neurons infected in the ipsi- and contralateral DRG was observed. In all cases, the infected sensory neurons were observed in the ventromedial, ventrolateral, and dorsolateral zones of the ipsilateral and contralateral ganglia, which suggest a specific topography of the neurons that innervate the plantar footpad and a specific connectivity between the neurons of the DRG of the same vertebral level; this phenomenon was named mirror infection (Fig. 2).

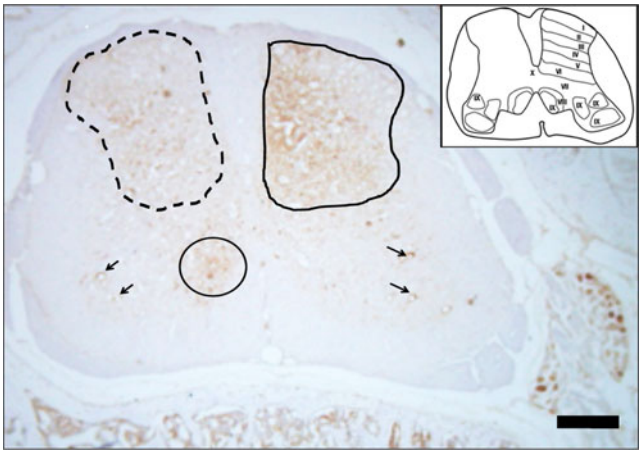

Fig. 3 Section of L2 region at 96 h p.i. Rabies virus antigen was detected in ipsilateral dorsal horn (*solid line*) and contralateral side (*dotted line*). Also, it is pointed out specific immunoreactivity in ventral horn lamina VIII (*circle*) and motor neurons infected in both ipsi- and contralateral ventral horn (*arrow*). *Box*. Rexed laminae of the spinal cord: dorsal horn (I to VI), intermediomedial nucleus (VII-X); ventral horn, interneurons VIII and motor neurons IX. *Bar* corresponds to 100 μm

Spinal cord

In the SC, we detected viral antigens only at 96 and 120 h p.i., between the L4 to L1 lumbar segments, with some differences. For example in the L4 and L3 segments, some infected neurons were detected in the ventral and dorsal ipsilateral horns, while in segments L2 and L1, a greater number of infected neurons were observed in both sides of the SC. In addition, infected neurons were detected in lamina VIII of the contralateral ventral horn, which suggests that the virus is disseminated by the tissue using different mechanisms and involving synaptic connections with commissural interneurons (Fig. 3).

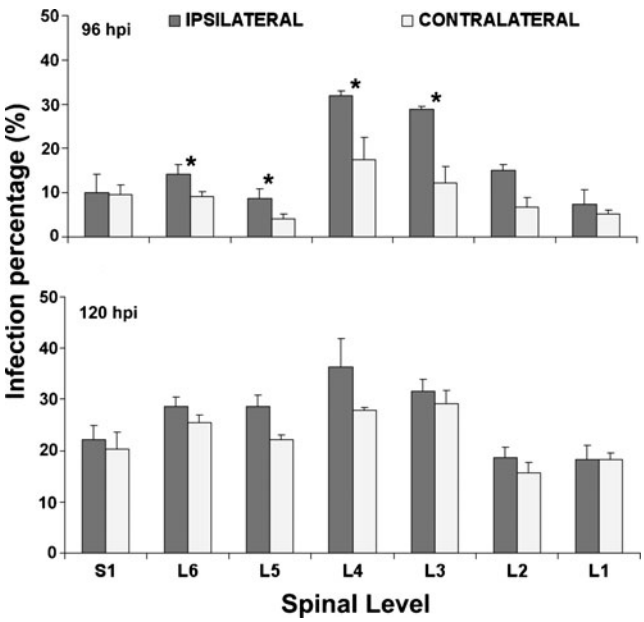

Fig. 4 Percentages of infected sensory neurons at different times post-infection. Infected and non-infected neurons were counted at 96 h p.i. (**a**) and 120 h p.i. (**b**), from sacrolumbar region, ipsilateral (*black bars*) and contralateral DRGs (*white bars*) sides. *Significant differences in the percentages between ipsi- and contralateral sides of the same vertebral level were analyzed using ANOVA test and post hoc LSD and Student's *t* test with a value of $p<0.05$

Table 1 Percentage of sensorial neurons infected at 72, 96, and 120 h post-infection in ipsi- and contralateral DRG of the sacrolumbar region

Ipsilateral	72 h p.i.	96 h p.i.	120 h p.i.	Contralateral	72 h p.i.	96 h p.i.	120 h p.i.
S1	0	10±4	22±3	S1	0	10±3	20±3
L6	0	14,1±1	29±2	L6	0	9±1	25±1
L5	0	9±1	29±2	L5	0	4±1	22±1
L4	0	32±1	36±6	L4	0	17±5	28±1
L3	0	29±3	31±2	L3	0	12±4	29±2
L2	0	15±4	19±2	L2	0	7±2	16±2
L1	0	7±1	18±3	L1	0	5±1	18±1

Forty independent sections were analyzed by every time and spinal segment of two animal infected. The percentage of infected sensory neurons from the sacro-lumbar region at 96 and 120 h p.i. were compared using an ANOVA test and post hoc LSD and Student's *t* test with a value of $p<0.05$

Percentages of infection and morphometric profile of the infected sensory neurons

Percentages of infection

The percentages of infection of the DRG sensory neurons of the sacrolumbar region were the following: at 72 h p.i., infection was lower than 1 % in the ipsilateral DRG of lumbar segments L5 and L4 and we did not find immunoreactive neurons at the other vertebral levels. On the other hand, at

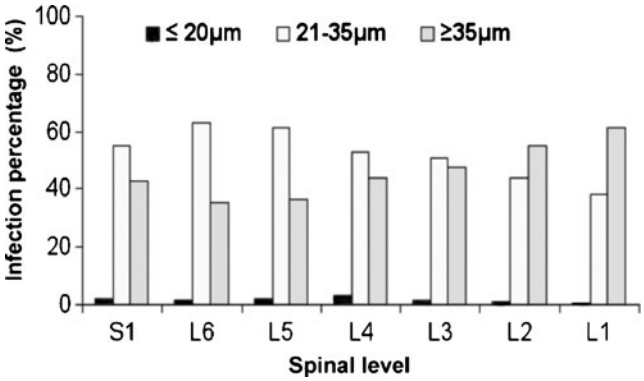

Fig. 5 Frequency distribution of sensory neuron diameters in DRG from sacrolumbar region. Note that the neuronal subpopulation is different in each of the DRG. For example, the most caudal ganglia (S1, L6, and L5) contains mainly intermediate neurons (21–35 mm). At the same time, L4 and L3 ganglia have similar percentages of intermediate and large neurons, while there are mainly large neurons (>35 μm) in L2 and L1 DRG

96 and 120 h p.i., a significant increase was observed in the infection percentages in all the ipsilateral DRG, and finally, at 120 h p.i., it was noted that the percentages of the infection of the contralateral DRG were similar to those obtained in the corresponding ipsilateral ganglia (Fig. 4, Table 1).

Morphometric profile

For this analysis, the frequencies distribution of the DRG sensory neurons of the sacrolumbar region of non-infected adult mice was initially defined. Thus, it was observed that 46 % of the total population of neurons corresponded to large neurons (≥35 μm), 52 % corresponded to intermediate neurons (between 25 and 35 μm), and the remaining 2 % corresponded to small neurons ≤25 μm (Fig. 5). Further, upon evaluating the frequencies distribution of the sensory neurons positive for RABV in the ipsilateral and contralateral DRG of each vertebral segment at 96 and 120 h p.i., it was observed that between 80 and 100 % of the infected neurons corresponded uniquely to large neurons (≥35 μm; Fig. 6). In comparing the mean diameters of the infected sensory neurons of the ipsilateral DRG against their respective contralaterals, it was observed that infected neurons from ipsilateral lumbar vertebral levels L6, L5, L4, and L2 were significantly larger than contralateral infected neurons (Kolmogorov–Smirnov, $p<0.05$; Table 2).

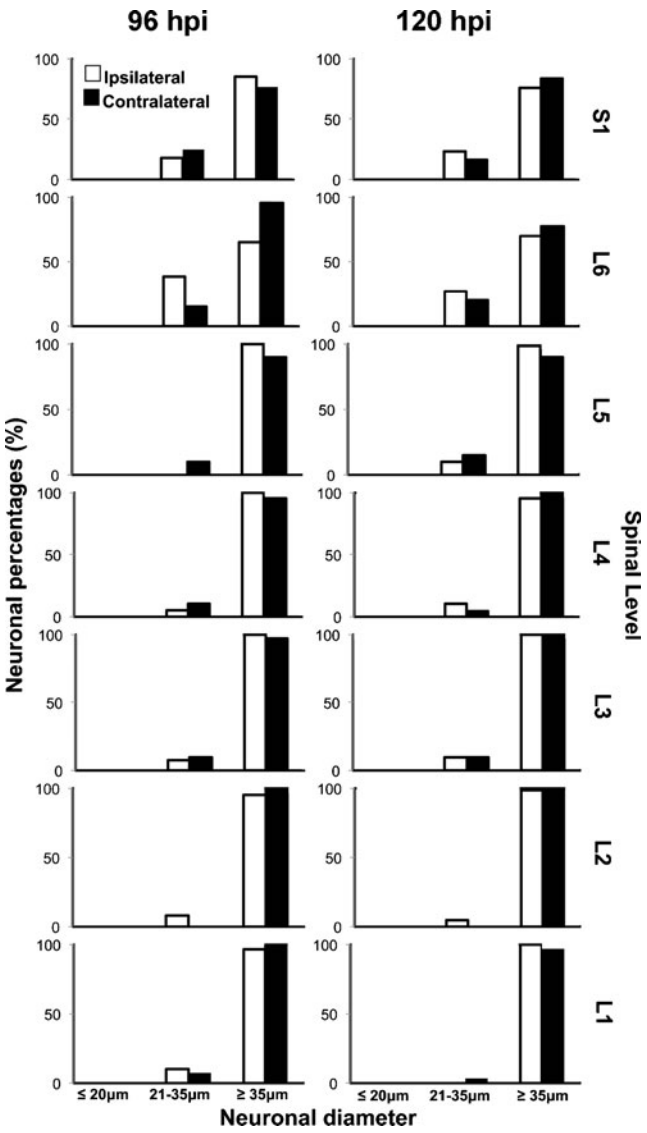

Fig. 6 Frequency distribution histogram of infected sensory neuron diameters at 96 and 120 h p.i. In all the vertebral levels and both post-infection periods, the majority of infected neurons have large diameters (>35 μm)

Discussion

Location, number, and populations of infected motor and sensory neurons

In the present study, we established the location, number, and populations of sensory and motor neurons susceptible to infection by RABV in different vertebral segments and at various post-infection times by inoculating the virus in the right hind footpad of adult mice. In our model, we only observed viral antigen in some neurons of the SC and the DRG from the 3 days p.i., perhaps owing to the period of eclipse of the infection or possibly to the early replication of the virus in muscle and skin, which limited the detection of the viral antigen (with the technique employed) present in the sensory and motor neurons, as had been reported previously (Tsiang 1988; Charlton et al. 1997). On the contrary, at 96 h p.i., viral antigen was detected in the SC and the ipsilateral and contralateral DRG of the sacrolumbar region, with a significant increase in the percentages of infection of the sensory neurons in the different vertebral sections at 120 h p.i.

Table 2 Average of the diameters at 96 and 120 h p.i., of ipsi- and contralateral sensory neurons of each spinal level of the sacrolumbar region

	S1	L6	L5	L4	L3	L2	L1
96 h p.i.							
Ipsilateral	35	35	36	41	36	38	36
Contralateral	31	31*	32*	36*	35	36*	35
120 h p.i.							
Ipsilateral	34	32	37	37	35	38	40
Contralateral	34	32	37	36*	35	38	40

Morphometric profiles were determined in the following way. For each vertebral segment and time p.i., 10 independent sections belonging to the same ganglion were digitized then we compare the distribution of the diameters of the infected neurons in comparison to the total for each ipsilateral and contralateral ganglion from the sacrolumbar region, the nonparametric Kolmogorov–Smirnov test was used. Finally, the diameters of the infected neurons were compared by Student's *t* test with a value of $p<0.05$. *Asterisks* indicates significant differences between ipsi- and contralateral infected neurons ($p<0.05$)

Our results at 96 h p.i., suggest that the L4 and L3 DRG sensory neurons preferentially innervate the hind footpad, which coincides with what has been previously reported in mice and confirms the ability of RABV as a neurotracer (Puigdellívol-Sánchez et al. 1998; Ugolini 2010). However, at 120 h p.i., we observed an increase in the number of infected sensory neurons in these DRG and in the other ganglia of the sacrolumbar region. These findings suggest that is possible that during the course of the infection, the virus replicates itself and propagates in the muscle and skin of the footpad, which increases the number of nerve endings involved and the number of infected sensory neurons located in other DRG of the sacrolumbar region; or it is probable that the virus present in the neurons of the dorsal horn of the SC was dispersed in a retrograde manner to the DRG in the other levels of the sacrolumbar region.

Additionally, in our model, we observed the presence of infected neurons of the SC at 120 h p.i., located in the dorsal and ventral horns of the L4 to L1 lumbar segments, which suggests that the motor neurons which innervate the hind footpad are located in these medullar regions and are infected later or might be infected by virus transported by the sensory neurons from the DRG. Therefore, our results suggest that the dispersion of RABV inoculated in the hind footpad depends mainly on DRG sensory neurons of the sacrolumbar region. Similar data were reported previously by

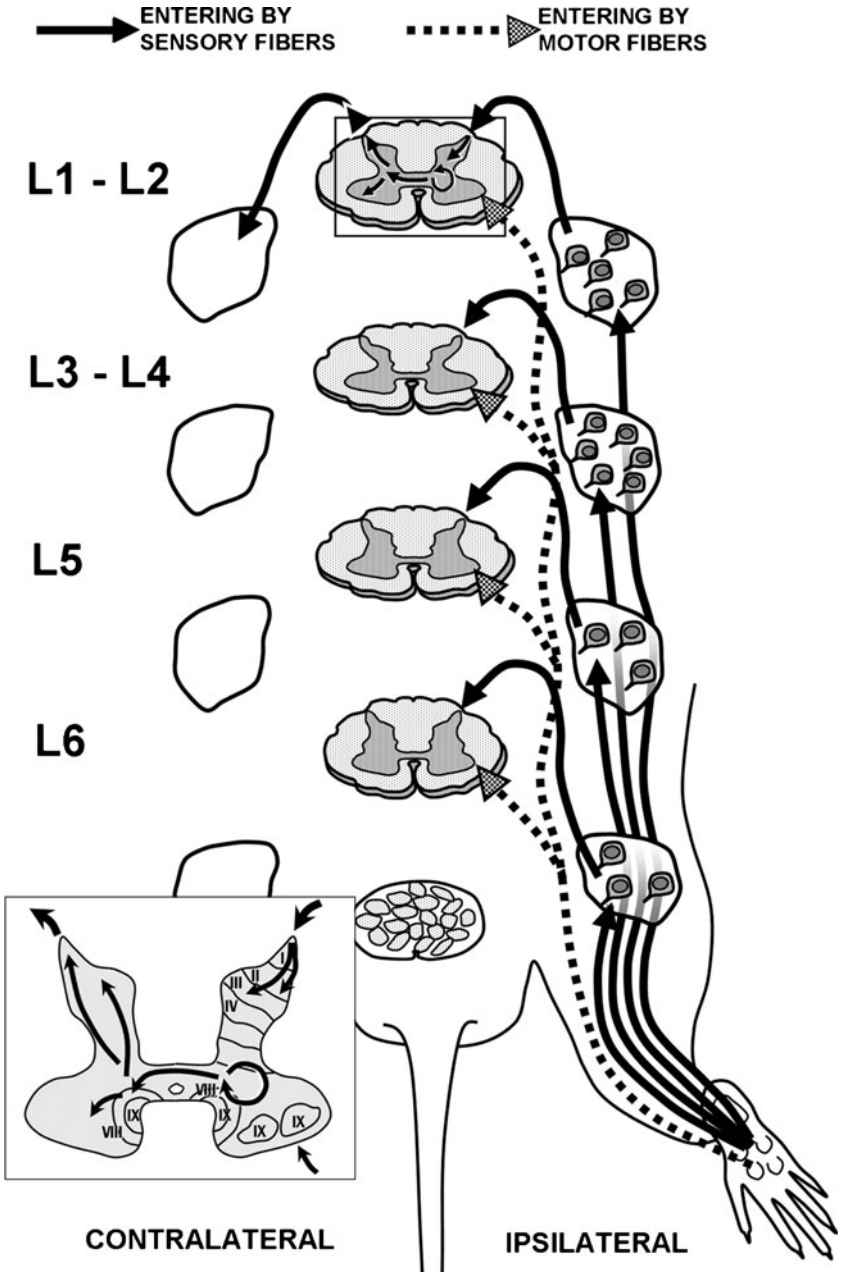

Fig. 7 Model for transport and dispersion for RABV inoculated in the hind footpad of adult mice. The virus inoculated in the hind footpad can replicate and propagate in the muscle or be captured directly by sensory nerve endings present at the site of inoculation. Once in the axoplasma, the virus is taken by retrograde transport to the sensory neurons located in the ipsilateral DRG of the sacrolumbar region. Then, the virus in these neurons can replicate immediately or can travel to the neurons of the corresponding dorsal horn (laminas I to III), from where it is transported to the interneurons of lamina VIII of the SC. These neurons would favor the spreading of the virus to other neurons of the medulla and the contralateral DRG

Rossiter et al. (2009), who, after inoculating RABV in the hind footpad, detected viral antigens at the fourth and fifth day p.i. in the DRG sensory neurons of the sacrolumbar region, while the motor neurons were detected at the sixth day p.i. (Rossiter et al. 2009). However, these authors did not manage to clearly establish the participation of the motor or sensory neurons in the dispersion of RABV within the CNS because they evaluated just a few slices of the sacrolumbar region at different times p.i. On the contrary, our model allows us to suggest, with a high degree of certainty, that above all, the sensory neurons promote the infection and spreading of RABV within nervous tissue when it is inoculated in the hind footpad.

Additionally, our data confirm the ability of RABV to trace specific neural networks, thanks to the ability of the virus to disperse itself rapidly within the tissue using transynaptic connections (Ugolini 2010; Rossiter et al. 2009; Loewy 1998; Kelly and Strick 2000). Surprisingly, our results suggest other forms of dispersion and possibly new neurological connections between the ipsilateral and contralateral DRG because at 96 and 120 h p.i., we detected the presence of both motor neurons and sensory neurons infected at the same vertebral levels but also on the contralateral side.

It is possible that contralateral infection in these areas is attributable to the infection of the interneurons located in lamina VIII of the vertebral segments L2 and L1 that connect both sides of the medulla. The commissural interneurons, located in lamina VIII of the SC, participate through excitatory and inhibitory signals in the coordination of movements that involve both sides of the body and control the activity of the ipsilateral and contralateral motor neurons (Eide et al. 1999; Eide and Glover 1996). Infection of interneurons from lamina VIII of the SC was previously reported by Coulon et al. (2011); however, our results are currently the first to show that beyond the connection between motor neurons and ipsilateral and contralateral interneurons, these interneurons also connect the sensory neurons of the contralateral DRG. It is possible that the participation of the interneurons increases the ability of dispersion of the virus within the nervous tissue, in this way adding to the neuroinvasive and neuropathogenic character of the virus, even prior to colonizing the brain, because in altering the cellular functions of the neurons present in each one of the infected vertebral levels, it affects at an early stage the functions of the organs and tissues that are innervated by them. However, new studies are required, which would allow for the identification of the transsynaptic connections between the neurons of the medulla and the ipsilateral and contralateral DRG and the identification of the mechanisms used by the virus to disperse and transport itself into and between these cells.

Morphometric profile of the neurons of infected ganglia of the dorsal root

Morphometric analysis of the infected neuronal populations in vivo in the DRG of the sacrolumbar region showed that the sensory neurons of ≥35 μm were preferentially infected by RABV. These neurons, according to our analysis of frequencies distribution, were considered to be large neurons, which were susceptible to infection independent of p.i., time, vertebral segment, and laterality of the DRG (ipsilateral or contralateral). These results coincide with those reported previously by Martínez-Gutiérrez and Castellanos (2007) and Tuffereau et al. (2007), who showed that the population of large neurons present in primary cultures of DRG neurons from adult mice are more susceptible to infection by RABV (Martínez-Gutiérrez and Castellanos 2007; Tuffereau et al. 2007). Large sensory neurons are associated with the innervation of neuromuscular spindles and joints, transmitting mechanoreceptive and proprioceptive information. It is possible that these neurons possess some specific molecular characteristics that favor the union and endocytosis of the virus and possibly favor the replication and transport of the virus within nervous tissue. In this regard, it has been demonstrated that this neuronal subpopulation is $p75^{NTR}$ positive and that the p75 receptor is one of the molecules that is postulated to be a receptor for RABV, however, not all the positive $p75^{NTR}$ neurons are susceptible to the virus; other cells, such as Schwann cells, which are rich in $p75^{NTR}$, are partially refractory to the infection (Tuffereau et al. 2007). The molecular reasons for the marked tropism of the virus for large neurons of the DRG remain to be defined.

Finally, our results allow us to suggest a possible model for transport and dispersion for RABV inoculated in the hind footpad of adult mice, summarized in Fig. 7. The virus inoculated in the hind footpad can replicate and propagate in the muscle or be captured directly by the proprioceptive or mechanoreceptive sensory nerve endings present at the site of inoculation (fibers that belong to larger neurons). Once in the axoplasma, the virus is taken by retrograde transport machinery to the sensory neurons soma located in the ipsilateral DRG of the sacrolumbar region. The virus in these neurons can replicate immediately or can travel to the neurons of the corresponding dorsal horn (laminas I to III), from where it is transported to the interneurons of lamina VIII of the SC. These neurons would favor the spreading of the virus to other neurons of the medulla and the contralateral DRG, and once within the tissue, the virus disperses itself to the various organs and to the brain, causing dysfunction of organs, paralysis, and death of the infected individual.

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