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Short Communication

Anterograde transsynaptic tracing in the murine somatosensory system using Pseudorabies virus (PrV): A "live-cell"-tracing tool for analysis of identified neurons in vitro

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The Pseudorabies virus (PrV) strain Bartha is widely used as a tool for retrograde transneuronal tracing in mammals. Traced neurons can be identified in cell culture allowing the analysis of their physiological features ("live-cell"-tracing). Compared to PrV-Bartha, PrV-Kaplan is known for higher virulence and transsynaptic spread in both retrograde and anterograde direction. Herein we assess the authors assess PrV-Kaplan for transsynaptic anterograde "live-cell"-tracing. Following intranasal application in mice, labelled trigeminal and brainstem neurons could be identified in vitro. Detailed electrophysiological analysis indicated that viral infection did not affect neuronal properties, making PrV-Kaplan eligible for functional analysis of identified neurons within somatosensory systems. *Journal of NeuroVirology* (2007) **13,** 579–585.

Keywords: mice; nasal mucosa; patch-clamp; Pseudorabies virus; trigeminal system

Findings

Pseudorabies virus (PrV), a swine herpesvirus of the *Alphaherpesvirinae* subfamily, is the causative agent of Aujeszky's disease. PrV has a broad host range which includes nearly all mammals (except higher primates and humans), as well as other vertebrate species (reviewed in Mettenleiter, [1994]). However, pigs are considered to be the natural host of PrV and are the only animals that are able to survive a productive infection. Natural infection with PrV occurs via the oronasopharyngeal route. After primary infection of epithelial cells, the viral particles invade peripheral neurons via nerve endings innervating the mucosae and ascend toward the central nervous system (CNS), resulting in a non-suppurative meningoencephalitis (Pensaert and Kluge, 1989; Enquist, 1994).

PrV's neurotropic nature and its property to spread within synaptically connected neurons have been exploited in numerous tract tracing studies in mammals, in order to define functional neuronal circuits. PrV-Bartha, an attenuated live-vaccine strain, proved to be particularly appropriate for this purpose because of its reduced virulence and specific transneuronal spread in the retrograde direction of information processing within the nervous system (Enquist, 2002). Native PrV-Bartha as well as marker protein-expressing variants, have been effectually

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used to define the organization of CNS circuits after intracerebral (Jasmin *et al*, 1997; DeFalco *et al*, 2001; Krout *et al*, 2003) or peripheral (Jansen *et al*, 1995; Jansen *et al*, 1997; Smith *et al*, 2000; Billig *et al*, 2000; Cano *et al*, 2001; Irnaten *et al*, 2001; Horvath *et al*, 2003) injection. However, identification of CNS circuits involved in processing of sensory information requires anterograde transneuronal tracing from peripheral sensory organs towards the CNS. Both retrograde and anterograde spread can be accomplished by wild-type α-herpesviruses (Sabin, 1938), like PrV-Kaplan, providing a potential tool for anterograde tracing in sensory neuronal systems.

Primary sensory neurons of the trigeminal nerve are cranial analogues of DRG neurons in the peripheral nervous system (Darian-Smith *et al*, 1973) and provide most of the somatosensory information of the head. They mediate mechanical, thermal, and chemosensory information from many tissues, including the meninges, the cornea and conjunctiva of the eyes, the facial skin, and the mucous membranes of the oral and nasal cavities. Thereby single trigeminal neurons (TGNs) mediate sensations from selective areas of the head (receptive fields) and are specialized for different qualities of somatosensory information (modalities) (Patapoutian *et al*, 2003). Our current knowledge about differentiated processing of this "where" and "what" information on the cellular level is limited due to the problem of identification of and accessibility to single neurons of a defined destination. Recently, we have shown that PrV-Bartha strains could be utilized to identify TGNs that innervate the mucous membranes of the murine nasal cavity or the epithelial cells of the facial skin (Damann *et al*, 2006a). Electrophysiological analysis of these neurons, which display features that depend on their peripheral innervation pattern, in cell culture could solve the puzzle of their chemosensory capabilities, displaying neuronal features which depend on their peripheral innervation pattern. However, postsynaptic neurons in the brainstem, the first relay for trigeminal information processing, could not be identified due to impeded anterograde transsynaptic spread of Bartha strains. Identification of synaptically connected higher-order neurons in vitro would allow easy access to these cells and would facilitate a detailed physiological characterization in order to further our understanding of mechanisms underlying differentiated trigeminal somatosensation.

Our study was designed to assess the suitability of PrV-Kaplan for transsynaptic tracing within somatosensory circuits in the trigeminal system. The data are compared to results from a tracing approach using PrV strain Bartha, which already proved to be an appropriate "live-cell"-tracing tool (Damann *et al*, 2006a). We analyzed the basic electrophysiological properties of identified neurons in primary cell culture and evaluated PrV-Kaplan as a "live-cell"-tracer in somatotopically defined and synaptically connected neurons.

For this study, the PrV-Kaplan derived recombinant PrV-KaΔgGgfp was generated according to PrVΔGCam used in an earlier study (Damann *et al*, 2006a). Expression of the reporter gene (green fluorescent protein; [GFP]) was controlled by the human cytomegalovirus immediate-early 1 promoter/enhancer (PhCMV), inducing transcription of DNA in the early stages of infection. Swiss CD-1 mice, 0 to 5 days old, were unilaterally intranasally inoculated with 2 μl of high-titered PrV-KaΔgGgfp (resulting in $\sim$106 plaque-forming units [pfu]) grown on PK15 cells. Cryosections through the head of infected animals sacrificed at different time points after infection (6, 8, 10, 12, 16, 20, 24, 36, 42, 47, 51, and 55 hours post infection [hpi]) were analyzed for GFP fluorescence in order to determine the onset of marker protein expression at different levels of the trigeminal system. We detected GFP fluorescence in the ipsilateral trigeminal ganglion at 12 hpi and in the brainstem at 36 hpi (Figure 1A, D). The time indicated represents the earliest time point of incidence of fluorescent cells. These findings indicate transsynaptic spread of PrV-Kaplan to neurons of higher order and underline the capability of PrV wild-type strains to ascend in the anterograde direction within the nervous system of mice. At later stages, marker protein fluorescence indicated presence of virus also at the contralateral side of infection (after 24 and 47 hpi in the trigeminal ganglion and the brainstem, respectively; data not shown). Viral spread to the contralateral side might reflect dispersion of infectious nasal secretions to the contralateral nasal cavity or other areas of the murine head and, therefore, was considered to be unspecific for neurons that innervate the ipsilateral nasal cavity.

Next, we determined the appropriate time windows for preparation of trigeminal and brainstem neurons, that allow identification of virally labelled neurons in vitro. Primary cell cultures of trigeminal and brainstem neurons were prepared at 12 hpi and 36 hpi, respectively (Figure 1B, E). Immediately after plating, fluorescence was observed in a few trigeminal neurons allowing identification of nasal TGNs, similar to our recent findings using PrV-Bartha strains (Damann *et al*, 2006a). Preparing mice at 36 hpi, cultures of brainstem neurons contained a few cells showing GFP fluorescence, identifying higher-order neurons that are involved in trigeminal sensory processing. The number of fluorescent neurons within trigeminal and brainstem cell cultures further increased within the next few hours of culturing, a fact that is most likely due to emerging GFP expression in neurons that were reached by PrV very shortly before tissue preparation. In order to prevent secondary viral infection in the culture dish, the cell culture medium for trigeminal and brainstem cell cultures were supplemented with the direct non-competitive viral DNA-polymerase inhibitor

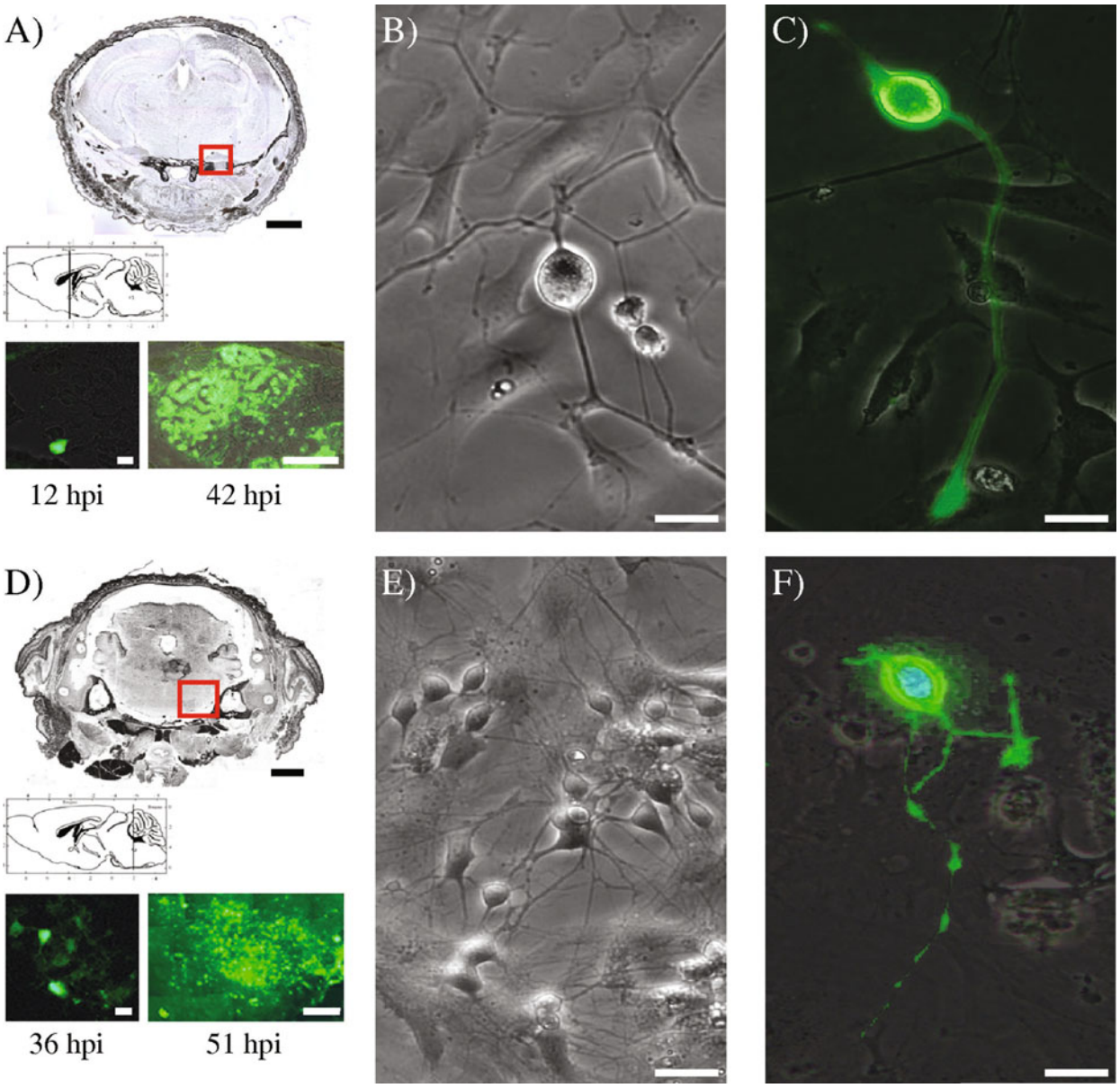

Figure 1 Tracing of trigeminal neurons and synaptically connected higher order neurons in the brainstem. A) Representative cryosection through the head of an intranasally infected mouse (P3; Bregma ~ 0 mm). Red square indicates area of marker protein expression in the ipsilateral trigeminal ganglion. Enlarged view: earliest incidence of infected trigeminal neurons in cryosections through the head of an animal sacrificed 12 hpi. At 42 hpi a massive infection of the trigeminal ganglion could be detected. B) Phase contrast light microscopy of trigeminal neuron in primary cell culture 2 days after plating. C) Fluorescence image showing traced trigeminal neuron 3 days after plating. General appearance of traced cultured neuronal cells in phase contrast light microscopy did not differ from non-infected neurons; traced cells could be identified in fluorescence microscopy only. D) Representative cryosection through the head of an infected mouse (P4; Bregma −5.8 mm). Red square indicates area of marker protein expression in the ipsilateral trigeminal brainstem complex. Enlarged view: earliest incidence of infected brainstem neurons in cryosections through the head of an animal sacrificed 36 hours post infection. At 51 hours post infection a massive infection in the trigeminal brainstem complex could be detected. E) Phase contrast light microscopy of brainstem neurons in primary cell culture 4 days after plating. F) Fluorescence image showing a traced brainstem neuron 5 days after plating. General appearance of traced cultured neuronal cells in phase contrast light microscopy did not differ from non-infected neurons; traced cells could be identified in fluorescence microscopy only. Bars in A: 1000 μm (overview); 20 μm, 250 μm (enlarged view; left and right, respectively); B, C and E: 20 μm; D (overview): 600 μm; 10 μm, 100 μm (enlarged view; left and right, respectively); F: 10 μm

foscarnet (400 μg/ml) as shown previously (Damann *et al*, 2006a). Under these conditions, plating of dissociated trigeminal and brainstem cells allowed reliable in vitro identification of in vivo traced fluorescent cells.

The general appearance of traced cultured neuronal cell bodies in phase-contrast light microscopy did not differ from non-infected neurons; traced cells could be identified in fluorescence microscopy only (Figure 1C, F). To investigate a possible influence

of viral infection on cellular physiology, whole-cell patch-clamp recordings of labelled TGNs and brainstem neurons were performed. Experiments were designed to compare traced cells with non-infected control neurons. For tracing primary TGNs from the nose, no transsynaptic passage of viral particles was needed and, therefore, data obtained with PrV-KaΔgGgfp infection could additionally be compared with data obtained following tracing neurons with the retrograde tracer PrV-Bartha (PrV-ΔGCam).

We recorded from cells 1 to 4 days in vitro (div). Within one experimental group, data obtained from different time points revealed no significant differences and therefore could be combined for statistical analysis. Electrophysiological data obtained from TGNs of different experimental groups (traced with PrV-Bartha, traced with PrV-KaΔgGgfp, non-infected control) were analyzed and tested for significance using an unpaired Student's t-test. Data from brainstem neurons traced with PrV-KaΔgGgfp were compared with recordings from uninfected control neurons of the same brain region. Significance was defined for $p < 0.05$. Exemplary recordings from traced and uninfected control cells are illustrated in Figure 2A and B for trigeminal and brainstem neurons, respectively. We found that the resting membrane potential of infected and non-infected trigeminal and brainstem neurons did not significantly differ. The same was true for the threshold for activation of voltage-dependent sodium channels (Na_v) measured in voltage-clamp experiments. There was also no significant difference in the amplitude of the

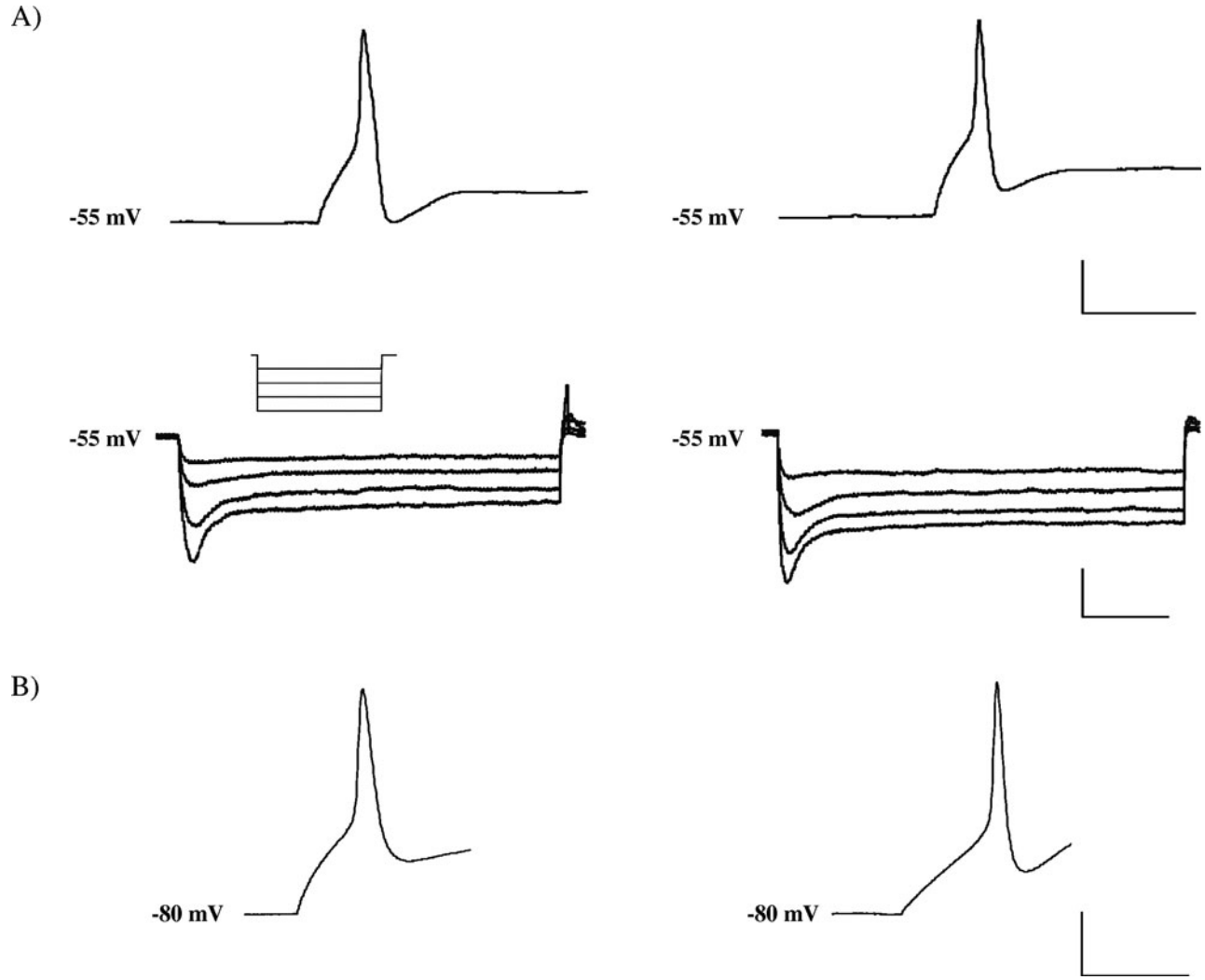

Figure 2 Electrophysiological recordings of infected and un-infected trigeminal and brainstem neurons. A) Representative traces from patch-clamp recordings of trigeminal neurons. Traced TGNs (PrV-Kaplan, left) could not be distinguished from uninfected control neurons (right). Upper picture: Action potentials induced by current injection (50 pA). Lower picture: I_h channel activation in current-clamp mode. Lower picture insert: injection of currents ranging from −20 pA to −80 pA; duration 2s; Bars: upper picture: 50 mV/20 ms; lower picture: 40 mV/650 ms. B) Representative traces from patch-clamp recordings of brainstem neurons. Traced brainstem neurons (PrV-Kaplan, left) could not be distinguished from uninfected control neurons (right). Action potentials induced by current injection (50 pA). Bars: 25 mV/10 ms.

Table 1 Electrophysiological analysis of traced and un-infected trigeminal neurons. Electrophysiological characterization of traced trigeminal neurons using either PrV-Bartha (Ba) or PrV-Kaplan (Ka), and uninfected (control, c) TGNs revealed no significant differences. APs: Action potentials; SD: Standard deviation; SEM: Standard error of the mean

	c	traced (Ba)	traced (Ka)		c	traced (Ba)	traced (Ka)
	Membrane potential/mV				Width of AP at 75% of amplitude/ms		
n	136	20	45	n	87	14	30
Mean	−56.5	−55.0	−53.8	Mean	2.45	1.96	1.58
SD	4.6	9.7	4.4	SD	0.84	0.86	0.89
SEM	0.4	2.2	0.7	SEM	0.09	0.23	0.16
Range	29	33	19	Range	3.78	3.00	3.16
	Threshold for activation of VGSCs/mV				Maximal amplitude (overshoot) of APs due to current injection/mV		
n	78	12	38	n	91	14	30
Mean	−28.2	−33.0	−34.6	Mean	39.2	37.9	38.1
SD	9.9	11.3	10.2	SD	9.3	15.3	11.2
SEM	1.1	3.3	1.7	SEM	1.0	4.1	2.0
Range	40	40	40	Range	44	48	41
	Maximal sodium current amplitude/nA				Amplitude of "sag" due to current injection resulting in potential of −100 mV/mV		
n	76	16	37	n	66	5	26
Mean	−3.60	−3.78	−5.84	Mean	11.7	14.1	15.8
SD	2.09	2.69	2.30	SD	5.3	4.3	5.1
SEM	0.24	0.67	0.38	SEM	0.6	1.9	1.0
Range	12.36	9.24	8.47	Range	22	11	20.0
	Membrane potential due to current injection eliciting APs/mV				Threshold for Ih−activation due to current injection/mV		
n	91	14	30	n	68	8	26
Mean	−27.0	−34.6	−27.8	Mean	−79.4	−81.9	−75.7
SD	8.1	7.9	8.0	SD	7.1	10.3	6.5
SEM	0.9	2.1	1.5	SEM	0.9	3.7	1.3
Range	45	29	28	RANGE	29	31	28

resulting inward current. In addition, recordings in current-clamp mode indicated no detectable influences of PrV on electrophysiological properties. The threshold potential to elicit action potentials was not changed between control and traced neurons. Action potential (AP) overshoots had almost equal maximal amplitudes. The width of APs at 75% of maximum amplitude was also within the same range. The threshold for activation of Ih-channels in current-clamp recordings and the amplitude of the sag (Ih-induced change in membrane potential) after hyperpolarization to −100 mV was not significantly altered in trigeminal neurons. The data are summarized in Table 1 (trigeminal neurons) and Table 2 (brainstem neurons). In summary, none of the electrophysiological properties tested differed significantly between groups, indicating that under our cell culture conditions (using foscarnet), PrV-KaΔgGgfp–traced neurons show no detectable alterations in physiological properties when compared to uninfected or PrV-Bartha–infected neurons.

Research on PrV has accelerated rapidly in the past 20 years. More recently, PrV-Bartha has been extensively used in laboratory animal models to study viral pathogenesis. Its remarkable propensity to infect synaptically connected neurons has led to the use of PrV as a tracer of neuronal circuits. Moreover recombinant PrV strains expressing fluorescent proteins can be used as "live-cell"-tracing tools for in vitro investigations of selectively labelled neurons (Damann *et al*, 2006a; van den Top *et al*, 2003; Irnaten *et al*, 2001; Smith *et al*, 2000). However, its application is limited due to its selective transport in the retrograde direction. For labelling sensory pathways from the periphery up to the brain, a transsynaptic tracer must have the capacity to invade the nervous system in the anterograde direction.

This work demonstrates the usability of the bidirectional tracer PrV-Kaplan for tracing defined sensory neuronal populations within the trigeminal ganglion and synaptically connected higher-order neurons in the brainstem. Patch-clamp analysis revealed that under our cell culture conditions, infection of neurons by PrV-Kaplan had no influence on the biophysical properties of traced cells when compared with virus-free cell cultures, making PrV-Kaplan-traced neurons ready for functional in vitro investigations.

In conclusion, PrV-Kaplan enables rapid labelling and in vitro identification of neurons of the trigeminal system of mice. In contrast to infected TGNs grown with conventional culture medium, the functionality of infected cells was retained for several days under foscarnet treatment. Under these conditions, labelled neurons displayed physiological properties highly similar to uninfected control neurons. In respect to earlier publications reporting herpes simplex virus (HSV)–mediated reduction

Table 2 Electrophysiological analysis of traced and un-infected brainstem neurons using PrV-Kaplan. Electrophysiological characterization of traced brainstem neurons using PrV-Kaplan (Ka), and uninfected (control, c) brainstem neurons revealed no significant differences. APs: Action potentials; SD: Standard deviation; SEM: Standard error of the mean

	c	traced (Ka)
	Membrane potential/mV	
n	21	6
mean	−49.6	−46.5
SD	11.5	12.5
SEM	2.5	5.1
range	40	32
	Threshold for activation of VGSCs/mV	
n	20	7
mean	−36.0	−25.7
SD	11.4	15.1
SEM	2.6	5.7
range	40	40
	Maximal sodium current amplitude/nA	
n	20	7
mean	−1.92	−1.35
SD	1.49	1.46
SEM	0.33	0.55
range	4.57	3.48
	Membrane potential due to current injection eliciting APs/ mV	
n	10	4
mean	−41.5	−40.7
SD	11.1	16.5
SEM	3.5	8.2
range	43	39
	Width of AP at 75% of amplitude/ms	
n	10	4
mean	2.85	2.73
SD	1.43	1.11
SEM	0.45	0.56
range	4.80	2.50
	Maximal amplitude (overshoot) of APs due to current injection/ mV	
n	10	4
mean	−3.2	−1.9
SD	9.3	5.8
SEM	3.0	2.9
range	35	14

of neuronal excitability of cultured DRG neurons (Mayer *et al*, 1986; Storey *et al*, 2002), our detailed electrophysiological characterization of PrV-infected and foscarnet-treated neurons has demonstrated unchanged excitability (foscarnet treatment itself has also no effect on the physiology of neurons as shown before [Damann *et al*, 2006]). Our methodological approach now allows experiments to be carried out, with a focus directed on intrinsic properties of brainstem neurons that receive information from the nasal cavity. A former study already demonstrated unique features of barrelette cells in the brainstem that might have a function in determining the temporal resolution of tactile related responses along the trigeminothalamic pathway (Lo *et al*, 1999). In future, recombinant PrV-Kaplan variants expressing different fluorescent marker proteins may be used for double-tracing studies, similar to studies using variants of PrV-Bartha (reviewed in Song *et al*, 2005; Damann *et al*, 2006a). Inoculation of PrV-Kaplan at two distinct areas of trigeminal innervation and subsequent physiological analysis of two neuronal populations should bring us a step closer to the understanding of differentiated trigeminal somatosensation. This technique may also be transferred to other mammalian primary somatosensory afferent neurons to combine fast tract tracing with physiological in vitro analysis.

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