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## **Golgi staining-like retrograde labeling of brain circuits using rabies virus: Focus onto the striatonigral neurons**

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### **Highlights:**

Viral tract-tracing is an added value for multidimensional study of the connectome

Chains of connected neurons can be visualized in a Golgi staining-like fashion

SAD and CVS rabies virus strains allow detailed visualization of dendritic morphology

A new fluorescent transneuronal recombinant of CVS-N2c provides Golgi-like labeling

### **Abstract**

#### **Background**

The introduction of viral transneuronal tracers in the toolbox of neural tract-tracing methods has been an important addition in the field of connectomics for deciphering circuit-level architecture of the nervous system. One of the added values of viral compared to conventional retrograde tracers, in particular of rabies virus, is to provide a Golgi staining-like view of the infected neurons, revealing the thin dendritic arborizations and the spines that are major post-synaptic seats of neuronal connections.

#### **New method**

Here, we comparatively illustrate the characteristics of the labeling obtained in the same model system, the basal ganglia circuitry, by different retrograde viral tracing approaches, using the Bartha strain of pseudorabies virus, the SAD and CVS strains of rabies virus and by the conventional retrograde tracer cholera toxin B. To best contrast the differences in the capacity of these tracers to reveal the dendritic morphology in details, we focused on one population of first-order infected neurons in the striatum, which exhibit high spine density, after tracer injection in the substantia nigra.

#### **Results and conclusion**

None of the viruses tested allowed to detect as many neurons as with cholera toxin B, but the SAD and CVS strains of rabies virus had the advantage of enabling detailed Golgi-like visualisation of the dendritic trees, the best numerical detection being offered by the transneuronal rCVS-N2c-P-mCherry while poor labeling was provided by rCVS-N2c-M-GFP. Results also suggest that, besides different viral properties, technical issues about constructs and detection methods contribute to apparently different efficiencies among the viral approaches.

#### **Key Words**

Retrograde tract-tracing; Rabies virus; transneuronal; Golgi-like; Basal Ganglia; Striatal medium-sized spiny neuron

## 1           1. Introduction

2  
3 Tract-tracing approaches, together with electrophysiology and electron microscopy, are powerful  
4 experimental tools to unveil the connectivity of neurons whose coordinated activity underlies brain  
5 circuit function. Advances in this field have greatly benefited from the expanding exploitation of  
6 viruses (Lanciego and Wouterlood, 2020). In particular, the development of viral transneuronal tracing  
7 methods, which use the capacity of some neurotropic viruses to propagate sequentially across  
8 connected neurons and to function as self-amplifier in the infected neurons, has made it possible to  
9 delineate chains of neurons within specific networks. The visualization of the neuronal dendrites, in  
10 particular of their spines, is crucial to understand the organization and plasticity of neuronal circuits.  
11 Dendritic spines, first described by Santiago Ramón y Cajal in 1888 (Cajal, 1888), are the postsynaptic  
12 sites of most excitatory synapses in the central nervous system and are dynamic structures that can  
13 exhibit changes in shape and number in physiological and pathological conditions (Berry and Nedivi,  
14 2017). The gold standard to detect the fine details of neuronal dendritic arborization is still the silver  
15 staining technique that has been used by Cajal, called Golgi metallic impregnation method. This  
16 method or its updates (Rapid Golgi staining, Golgi-Cox method) allows staining a limited number of  
17 neurons in their entirety, in a still unexplained random manner. The transneuronal retrograde tracers,  
18 particularly rabies virus (RABV), have the advantage of enabling both the detection of neurons based  
19 on their connectivity and the morphological characterization of their dendritic processes, which is an  
20 added value for multi-dimensional studies in combination with other neuroanatomical techniques.

21  
22 Available viral retrograde transneuronal tracers include the herpes simplex virus type 1, the  
23 pseudorabies virus (PRV) and RABV, this latter and its recombinants being currently the most widely  
24 utilized. Worldwide, the use of RABV as a marker of neural networks is mainly based on two ‘fixed’  
25 strains (Figure 1A) coming from ‘street’ rabies virus isolated from i) a rabid cow in 1882 (Paris): The  
26 Challenge Virus Standard (CVS) and ii) a rabid dog in 1935 (USA): Street-Alabama-Dufferin (SAD)  
27 virus. Several CVS subtypes are available, including CVS-11 and variants of CVS-24 (as CVS-N2c).  
28 These fixed strains have been adapted from the street ones by repeated passages first in mice brains  
29 and then in cell cultures, leading to the selection of strains with stable properties. The first use of CVS-  
30 11 as neuronal tracer was reported by Astic and collaborators in the olfactory network (Astic et al.,  
31 1993), followed by a study from Ugolini (Ugolini, 1995) with injection in the hypoglossal nerve to  
32 reveal, by immunodetection of the viral nucleocapsids, the spread of the virus over time in the  
33 interconnected neurons. This latter study was a follow-up of series of experiments using the  
34 transsynaptic property of the herpes simplex virus type 1 or PRV to decipher neural networks (Aston-  
35 Jones & Card, 2000; Card et al., 1990; Card & Enquist, 2014; Ugolini et al., 1989). Due to some  
36 limitations of PRV, such as its uptake by fibers of passage, neurotoxicity and glia infection at  
37 advanced stages, data from experiments using RABV incrementally increased at the expense of those  
38 using PRV. After molecular cloning of RABV SAD B19 (Conzelmann et al., 1990; Schnell et al.,  
39 1994) the second key advance for the use of RABV has been the development of different  
40 recombinant variants of SAD B19 (Ceccaldi et al., 1998; Etessami et al., 2000) and the selection of  
41 CVS-N2c variant (Morimoto et al., 1998), highlighting the crucial role of the RABV envelope  
42 glycoprotein (G-protein) in the viral infection in neurons. Design and generation of recombinant  
43 RABV vectors with deletion of the G-protein-coding sequence from the SAD B19 genome and  
44 inclusion of fluorescent reporter genes (Wickersham et al., 2007a, b) have given new tools to study the  
45 neuronal networks. Indeed, G-deleted ( $\Delta$ G) RABV coated with its G-protein can infect neurons from  
46 their presynaptic terminals but loses its transsynaptic property (Etessami et al., 2000). It became a  
47 useful first-order retrograde tracer (Wickersham et al., 2007a), and a potent monosynaptic retrograde  
48 marker following *in vivo trans*-complementation with G within the infected neurons either from a  
49 plasmid, a helper virus, or a mouse transgene. The next step has been to direct the primary infection to  
50 a genetically-targeted neuronal population and to restrict the viral spread to the presynaptic neurons  
51 connecting this population, which overcame key limitations in the use of transsynaptic tracers by  
52 enabling unambiguous tracing of monosynaptic inputs to specific cell types. Recombinant  $\Delta$ G-SAD  
53 B19 pseudotypes, which carry the envelope protein of an avian retrovirus (EnvA), have been created  
54 to make the RABV unable to infect mammalian cells unless they have been engineered to express  
55 EnvA's receptor, ‘TVA’, and the ability of the virus to spread to the monosynaptically connected

1 neurons has been conferred by *Trans*-complementation with G within the primary infected neurons  
2 (Wickersham et al., 2007b). This RABV-based monosynaptic tracing approach, which has also the  
3 advantage of safety for the users, has been extensively applied in mapping circuits in various parts of  
4 the nervous system (for review see Callaway & Luo, 2015). In parallel, recombinant CVS-N2c called  
5 rN2c, containing the synthetic codon-optimized or -deoptimized versions of the N2c G gene have been  
6 developed on mouse neuroblastoma cells (Morimoto et al., 2000, 1999, 1998), showing that G-protein  
7 expression level is not critical in viral pathogenicity (Wirblich and Schnell, 2011). More recently,  
8 recombinant  $\Delta$ G-CVS-N2c has been shown to exhibit enhanced retrograde transfer compared with  
9  $\Delta$ G-SAD B19 (Reardon et al., 2016). On the other hand, new chimeric glycoprotein variants have been  
10 generated to improve monosynaptic tracing with  $\Delta$ G-SAD B19 (Kim et al., 2016). These studies  
11 provided additional evidence that the CVS strain of RABV is far more infectious than SAD B19.

12  
13 This paper aims at characterizing the propagation and Golgi-like staining properties of CVS-11 in  
14 comparison with other retrograde viral tracers, either first-order or transsynaptic, including two  
15 fluorescent rN2c, rCVS-N2c-P-mCherry (Nikolic et al., 2017, 2016) and rCVS-N2c-M-GFP  
16 (unpublished) (Figure 1B), recently engineered by Blondel and collaborators to avoid the step of  
17 immunodetection of CVS. We have chosen as a common study model the basal ganglia, a set of brain  
18 structures engaged in loop circuits with the cerebral cortex, which fulfill essential role in adaptive  
19 control of motor, cognitive, and motivational behavior and are involved in the pathophysiology of  
20 movement disorders and different neuropsychiatric disorders (Jellinger, 2019a,b; Macpherson &  
21 Hikida, 2019). The striatum represents the main input station of the basal ganglia. The great majority  
22 (> 90%) of the striatal neurons are GABAergic projection neurons, called medium-sized spiny neurons  
23 (MSNs), given the great numbers of spines studding their dendrites. They are segregated into two  
24 numerically equivalent populations with different neuronal targets and neurochemical features: i) the  
25 striatonigral neurons, which express dopamine D1 receptor and the neuropeptides substance P and  
26 dynorphin and ii) the striatopallidal neurons that express dopamine D2 receptors and enkephalin  
27 (Gerfen and Scott Young, 1988). These two populations are respectively at the origin of the so called  
28 ‘direct’ and ‘indirect’ pathways by which the striatum regulates the activity of the substantia nigra  
29 (SN) pars reticulata, the main basal ganglia output structure in rodents. In the classical model proposed  
30 in the 90’s (Albin et al., 1989; Alexander and Crutcher, 1990), the direct and indirect pathways have  
31 an opposite balancing role in the control of motor behaviour. Although being increasingly challenged,  
32 this model provided striking predictions about basal ganglia dysfunction in movement disorders  
33 (Calabresi et al., 2014; Quartarone et al., 2019). In addition to their extrastriatal projection sites, axons  
34 of MSNs also profusely branch within the striatum, with subtype specific distribution of their  
35 collaterals within or outside the area occupied by the dendritic field of the cell of origin (Bishop et al.,  
36 1982; Van Dongen et al., 2008). The connections they form with other MSNs have been reported to  
37 show subclass-dependent asymmetries in probability and strength (Taverna et al., 2008) and the  
38 functional relevance of the lateral inhibition emerging from the collateral transmission between these  
39 neurons, long considered as weak, has been recently emphasised (for review see Burke et al., 2017).  
40 The striatal projection neurons receive several extrinsic inputs, notably from pyramidal cortical  
41 neurons, as well as intrinsic inputs from striatal interneurons, allowing to evaluate the transsynaptic  
42 propagation efficiency of the different viruses tested. Besides their well-known connectivity, these  
43 neurons have highly spiny dendritic arborization, which are advantages in our comparative analysis of  
44 the ability of the different tracers to provide Golgi-like staining of primary infected neurons. To study  
45 the viral propagation while avoiding any problem of contamination from the injection site, we focused  
46 on the connectivity of striatonigral neurons, which are long distance projecting neurons compared to  
47 the striatopallidal ones. Figure 2 schematizes the virus propagation in the network (2B) and gives  
48 illustrative examples of the staining observed in the injected substantia nigra (2A), the striatum (2D)  
49 and the cerebral cortex (2C). All the original results presented concern the dorsal striatum.

## 50 51 52 **2. Materials and methods**

53  
54 Experiments were carried out in adult male Wistar rats (6 weeks old), adult male C57Bl/6 mice (10  
55 weeks old) and *Drd1a*-tdTomato line 6 BAC transgenic mice in which the striatonigral neurons are

1 identified by D1 dopamine receptor promotor-specific fluorescent reporter (Ade et al., 2011). Surgical  
2 procedures adhered to the European guidelines for the care and use of laboratory animals (EU  
3 Directive 2010/63) and were approved by the ethics committee in Neurosciences at the Institut de  
4 Neurosciences de la Timone, INT-Marseille (n° 02167-01). Vaccinated personnel conducted all rabies  
5 viruses handling, surgery, injection and animal care procedures at the appropriate biosafety  
6 containment level (level 2) at the INT.

7  
8 **2.1. Neural tracers.** The rabies virus mainly used in the experiments was the French isolate of fixed  
9 strain CVS-11 (figure 2; Ugolini 2010). The unmodified CVS-11, amplified in BSR cells, was used as  
10 a cell culture supernatant in minimal essential medium, titrated at  $4 \times 10^7$  plaque forming units  
11 (pfu)/ml.

12 The recombinant strain of CVS-24 N2c expressing a Phospho-mCherry fusion protein (rCVS-N2c-P-  
13 mcherry) and the one with the Matrix-GFP fusion protein (rCVS-N2c-M-GFP) were used at  $5 \times 10^7$   
14 pfu/ml and  $2 \times 10^7$  pfu/ml respectively. The full-length recombinant N2c (prN2c) infectious clone was  
15 described previously (Wirblich and Schnell, 2011). The authentic phosphoprotein (P) or matrix-protein  
16 (M) coding sequence was replaced with the P-mCherry or M-GFP fusion encoding sequence,  
17 respectively (Figure 1B). The rCVS-N2c-P-mCherry has been described previously (Nikolic et al.  
18 2016). For rCVS-N2c-M-GFP, the M-GFP fusion was generated by insertion of the e-GFP  
19 downstream the residue 26 of the M protein, which is located in the disordered N terminus of M  
20 (Graham et al., 2008) as described for VSV to obtain a viable M-eGFP fusion protein (Soh and  
21 Whelan, 2015). In brief, the GFP gene was first inserted downstream the nucleotide 78 of the M gene  
22 in the pCDNA3.1 vector (Invitro gene). Then two overlapping fragments were amplified by PCR: the  
23 first one going from the Spe1 site of the full-length genomic plasmid prN2c to the end of the M-GFP  
24 and the second one from the end of M to the PST1 site located at the end of the G gene coding  
25 sequence. The genomic plasmid which has been digested with Spe1 and Pst1 restriction enzymes and  
26 the PCR products were assembled using Gibson Assembly kit (New England Biolabs).

27 Recombinant viruses were recovered as described previously (Fouquet et al., 2015; Wirblich et al.,  
28 2008). Briefly, N2A cells ( $10^6$  cells) were transfected using lipofectamine 2000 (Invitrogen) with 0.85  
29  $\mu\text{g}$  of full-length rCVS P-mCherry or rCVS-M-GFP, in addition to 0.4  $\mu\text{g}$  pTIT-N, 0.2  $\mu\text{g}$  pTIT-P, 0.2  
30  $\mu\text{g}$  pTIT-L and 0.15  $\mu\text{g}$  pTIT-G, which encode respectively the N, P, L and G proteins of CVS-N2C  
31 RABV strain. These plasmids were cotransfected with 0.25  $\mu\text{g}$  of a plasmid encoding the T7 RNA  
32 polymerase. Six days post-transfection, the supernatant was passaged on fresh N2A cells, and  
33 infectious recombinant viruses were detected three days later by the fluorescence of the P-mCherry  
34 protein or M-GFP.

35 The fluorescent recombinant  $\Delta\text{G}$  SAD B19 coated with its G-protein (SAD $\Delta\text{G}$ .eGFP) [generous gift  
36 from K.K. Conzelmann; (Hagendorf and Conzelmann, 2015)] was injected at  $3 \times 10^8$  pfu/ml.

37 The Bartha strain of GFP-pseudorabies virus (Bartha GFP-PRV), a gift from T.C. Mettenleiter (Jöns  
38 and Mettenleiter, 1997) was injected at  $5 \times 10^6$  pfu/ml.

39 Cholera toxin subunit B (CTB) conjugated to Alexa Fluor 594 (Life technologies, Cat# C34777) was  
40 injected at the concentration of 1  $\mu\text{g}/\mu\text{l}$  in phosphate buffered saline.

41 Experiments were conducted on at least 20 animals for CVS-11, groups of 8-12 animals for  
42 SAD $\Delta\text{G}$ .eGFP, rCVS-M-GFP, rCVS P-mCherry, and 3-5 animals for Bartha GFP-PRV and for CTB.  
43 The kinetic study for CVS-11 was performed on subgroups of 4 rats at each time point post-injection  
44 and the study of the co-expression with D1 receptor was done on 6 transgenic mice.

45  
46 **2.2. Surgical procedure.** Surgery was performed under ketamine+xylozine anesthesia (intraperitoneal  
47 injections, 100 and 10 mg/kg, respectively). The animals, placed in a stereotaxic frame (David Kopf  
48 Instruments), received a unilateral injection of the tracer in the SN. For the rat, the stereotaxic  
49 coordinates of the injection site were (from the interaural) anteroposterior (AP) +2.2 mm, lateral (L)  
50 2.0 mm and dorsoventral (DV) +3.3 mm, with the incisor bar at +5.0 mm above the interaural plane,  
51 according to the atlas by De Groot (1959), and for the mice (from bregma coordinates) AP: +2.8 mm,  
52 L: 1.35 mm and DV: +4.35 mm, with the incisor bar at -1.0 mm above the interaural plane, according  
53 to the atlas by Paxinos and Franklin (2001). Each tracer was pressure-injected in a final volume of 300  
54 nl for the rat and 200 nl for the mouse at a flow rate of 100 nl/min using a programmable injector.

1 **2.3. Histology.** Animals were processed for histology at the following time points after injection of the  
2 tracers: 10 days for CTB, 24, 42 and 72 h for CVS-11 and 40–42h for the other viruses. They were  
3 anesthetized with an overdose of ketamine and xylazine and then intracardially perfused with a saline  
4 solution (50 ml for rat, 5 ml for mouse) followed by a fixative solution containing 4%  
5 paraformaldehyde (PFA) in 0.125 M phosphate buffer, pH 7.4 (300 ml for rat, 30 ml for mouse). After  
6 perfusion, the skull was opened and the dissected brain was post-fixed in PFA (4%) overnight at 4°C  
7 and then placed in a 30% sucrose solution for 48 h. The brains were frozen in powder dry ice and  
8 stored at -80°C. Coronal (40 µm thick) tissue sections were cut at -20°C with a cryostat (CM3050S,  
9 Leica), collected in cryoprotective solution and stored at -20°C. The immunodetection of the  
10 unmodified CVS-11 rabies virus was done with an antibody (31G10) directed against viral P-protein  
11 (Raux et al., 1997) that spreads throughout the cytoplasm of RABV-infected neurons (Salin et al.,  
12 2008). The brain sections were incubated for 12 h in a mouse anti-RABV P-protein monoclonal  
13 antibody diluted 1:10,000 in PBS. After rinsing, the sections were incubated for 2h with the secondary  
14 antibody donkey anti-mouse IgG coupled to Alexa Fluor 594 or Alexa Fluor 488 diluted 1:200  
15 (Jackson ImmunoResearch Europe, Inc.). For rCVS-M-GFP, immunodetection of GFP was performed  
16 in an experiment for the signal amplification. In brief, sections were incubated overnight with a  
17 chicken anti-GFP antibody diluted 1:500 (Aves Labs Inc., USA). After rinsing, they were incubated  
18 with a secondary fluorescent antibody, donkey anti-chicken IgG coupled to Alexa Fluor 594 diluted  
19 1:200 (Jackson ImmunoResearch Europe, Inc.). In another experiment, the P protein of rCVS-M-GFP  
20 was immunodetected as described above, using the donkey anti-mouse secondary antibody coupled to  
21 Alexa Fluor 594.

22 The sections were then mounted on SuperFrost Plus glass slides (ThermoFisher Scientific). The  
23 staining examination focused mainly on the striatum and the overlying motor cortical areas.

24  
25 Golgi staining was performed on 4-mm thick coronal blocks of brain tissue including the striatum,  
26 using FD Rapid GolgiStain Kit (NeuroTechnologies Inc.) based on Golgi-Cox impregnation. Blocks  
27 were then frozen and processed for cryostat sectioning (40µm thick sections) as detailed by the  
28 manufacturer. Light microscope observations were performed on sections from 3 animals.

29  
30 In brain sections of mice injected with rCVS-N2c-P-mCherry, the two neurochemically distinct  
31 compartments of the striatum, namely the striosomes -also termed patches- and the matrix, were  
32 revealed by immunodetection of the mu opioid receptor (highly expressed in striosomes). Sections  
33 were incubated with a monoclonal rabbit anti-mu opioid receptor antibody (1/1000; Abcam  
34 ab134054), followed by goat anti-rabbit IgG coupled to Alexa Fluor 488 (Jackson ImmunoResearch  
35 Europe, Inc.).

36  
37 Except for Golgi staining, brain sections were examined under a confocal microscope (LSM 780, Carl  
38 Zeiss) and the acquired images were processed using Adobe Photoshop.

### 39 40 41 **3. Results and Discussion**

#### 42 43 **3.1. Lack of staining at the site of RABV injection**

44  
45 Upon injection in the brain, RABV is quickly sequestered within permissive neuronal terminals. The  
46 lack of accumulation of the virus prevents precise determination of the RABV spread at the injection  
47 site (Ugolini, 2010). Accordingly, at all the time points that we have considered (24 h, 42 h and 72 h),  
48 no specific viral labeling is detected at the site of injection of CVS-11 (Figure 2 A). The absence of  
49 staining at the injection site complies with most *in vivo* studies reporting that glial cell infection by  
50 RABV is not prominent in the CNS. It is also consistent with the fact that interneurons have not been  
51 identified in the SN and that axon collaterals of projection neurons, although providing feedback  
52 inhibition (Brown et al., 2014), are very sparse (Mailly et al., 2003). Surprisingly, by co-injecting  
53 methylene blue aqueous solution with CVS-11 to visualize the injection area, we observed RABV  
54 accumulation at the injection site in an area restricted to the SN. This accumulation, together with the

1 lack of RABV-positive neurons in the striatum (not shown), suggests that the presence of methylene  
2 blue prevents infection by the virus.

### 3.2. Time course of the RABV staining using CVS-11

6 It has been previously reported that labeling of first-order neurons usually requires up to 2 days after  
7 RABV injection at peripheral sites and that subsequent successive transneuronal transfer to higher-  
8 order neurons occurs at regular intervals of at least 12h (Coulon et al., 2011; Tang et al., 1999;  
9 Ugolini, 2011, 2010). Here, immunodetection of unmodified CVS-11 was carried out at 24, 42 and 72  
10 h post-injection.

12 At 24 h, a significant number of striatal neurons and very few scattered neurons in cortical layer 5 are  
13 labeled, although faintly. The staining pattern is similar to the one that can be obtained with CTB and  
14 with the recombinant SADΔG.eGFP, indicating that, at this time point, only the primary infected  
15 neurons projecting to the SN are detected. The sparse neuronal labeling in the cortex is consistent with  
16 previous data reporting weak corticonigral projections (Naito and Kita, 1994; Watabe-Uchida et al.,  
17 2012). In both the striatum (Figure 3A1, A2) and the cerebral cortex (Figure 3B1), the intracellular  
18 labeling is largely restricted to the cell body and proximal dendrites, detection of dendritic spines  
19 being only casual. The perikarya of infected neurons show non-uniform labeling with intensely-  
20 stained round inclusion bodies that are called Negri bodies. These structures, typical of RABV  
21 infection, are sites of viral transcription and replication containing all the replication machinery  
22 proteins (L, N and the P protein) and viral RNA (Lahaye et al., 2009). In addition, Negri bodies have  
23 been recently described as liquid droplets formed by phase separation (Nikolic et al., 2017).

25 At 42 h, the numbers of detected neurons increased in the striatum (Figure 3A3) and even more in the  
26 cerebral cortex (Figure 3B2) compared to 24 h. In both territories, first-order labeling is increased,  
27 enhancing the detection of primary-infected neurons. In addition, second-order less intense labeling is  
28 detected. In the cortex, the great majority of RABV-positive neurons seats in layer 5. Their staining is  
29 attributable mostly to transsynaptic infection by RABV of the cortical projection neurons connecting  
30 the primary infected striatonigral neurons, as indicated by comparison with the few cortical neurons  
31 detected after injection of the retrograde tracers SADΔG.eGFP and CTB. In the striatum, the labeled  
32 neurons are in majority spiny neurons that are likely the primary-infected striatonigral neurons. Their  
33 cytoplasm is now homogeneously labeled, without individualized Negri bodies, presumably due to  
34 recognition of P protein within or outside Negri bodies by the antibody used. They exhibit intense  
35 Golgi-like staining, allowing detailed visualization of their dendritic tree and specialisations such as  
36 spines. By contrast, only the proximal segment of their axon can be detected, consistent with previous  
37 work reporting that viral proteins do not spread in the axon extent (Ugolini, 1995). The fewer second-  
38 order infected neurons do not show spines and exhibit Negri bodies, and were previously reported to  
39 mainly include cholinergic and parvalbumin interneurons (Salin et al., 2009).

40 To further characterize the infected neurons at this time point, intranigral injection of CVS-11 was  
41 performed in *Drd1a*-tdTomato line 6 mice. In these mice, td-Tomato fluorescence has been reported to  
42 be highly specific to striatonigral neurons, since the most abundant interneuron types do not exhibit  
43 detectable level of fluorescence and only around 2% of the neurons co-express D1 and D2 in *Drd1a*-  
44 *tdTomato*<sup>+/+</sup>/*Drd2*-EGFP<sup>+/+</sup> mice (Ade et al., 2011). The great majority of the RABV-labeled neurons  
45 at 42 h post-viral inoculation are D1 receptor-expressing MSNs (Figure 4A), confirming that they  
46 belong to the striatonigral population. Quantitative analysis shows that  $87.6 \pm 2.23\%$  over 789 RABV-  
47 labeled neurons are expressing D1 receptor (3 mice, 4 sections examined per animal). The 12% of  
48 RABV-positive striatal neurons that do not express D1 receptor are weakly labeled neurons, in which  
49 the visualization of the dendritic arborization is limited, presumably corresponding to second-order  
50 neurons. Populations of striatal interneurons are likely to constitute most of this pool of second-order  
51 neurons, as we previously reported that for each 100 striatonigral RABV-positive neurons counted,  
52 around 3 are cholinergic and 9 are parvalbumine GABAergic interneurons (Salin et al., 2009). The  
53 complementary experiment in which CVS-11 is injected in the globus pallidus of *Drd1a*-tdTomato line  
54 mice (n=3 animals) to label striatopallidal neurons from their terminals shows that, with very few  
55 exceptions, RABV-infected striatal neurons are not D1-expressing neurons (Figure 4B). Cross-

1 connections between the striatonigral and striatopallidal neurons, via axon collaterals are thus unlikely  
2 to contribute significantly to the pool of second-order infected neurons in the striatum at 42 h post-  
3 injection of the virus in either striatal target.

4  
5 At 72 h, the density of positive neurons and the tangles of neurites in the striatum are so important that  
6 it is now difficult to individualize the neurons and their dendritic extension (Figure 3A4). In the  
7 cerebral cortex (Figure 3B3), numerous neurons are labeled in layers 2-6. At this time point,  
8 considering its replication and transfer kinetics, the RABV has passed 4-5 synapses, which make it  
9 impossible to interpret the results given the rich connectivity of the basal ganglia. Such delay post-  
10 infection appears unsuitable to decipher the chain of connections within a given neuronal network in  
11 the rodent central nervous system.

12  
13 Therefore, a time window around 2 days post-injection appears as the most appropriate to properly  
14 delineate the multisynaptic circuits in our model system, allowing both Golgi-like visualization of the  
15 first-order neurons and good detection of at least second-order neurons. To note that no major  
16 differences in the viral staining between mouse and rat was observed in our experiments.

### 17 18 19 **3.3. Differences in staining among retrograde viral tracers at 42 h post-injection**

20  
21 Differences in neurotropism among retrograde viral tracers have long been demonstrated. More recent  
22 work, comparing tracing characteristics of such viruses in the same model systems, showed region  
23 preferences in their neurotropic properties (Sun et al., 2019). Here, the tracing characteristics of Bartha  
24 GFP-PRV, SAD  $\Delta$ G.eGFP, rCVS-N2c-P-mCherry and rCVS-N2c-M-GFP were compared to that of  
25 unmodified CVS-11, at 42 h post-injection in the SN.

26  
27 The Bartha GFP-PRV was a commonly used tracer to delineate multi-synaptic central circuits from  
28 peripheral targets (Card & Enquist, 2014). It has been rarely applied to trace basal ganglia  
29 connectivity. Its injection in the SN leads to a significant labeling of striatonigral neurons at 42 h post-  
30 injection, but no cortical labeling. Compared to RABV injection at the same time point, the PRV is  
31 less efficient and does not provide nice Golgi-like labeling: the number of stained striatal neurons is  
32 lower and only few dendritic branches and sparse spines are visualized (Figure 5A). The marked  
33 differences between the Bartha GFP-PRV and the RABV staining could be due to differences in the  
34 titers of the viruses, the slowness of the PRV replication or to differences in the intraneuronal  
35 localisation of the fluorophore.

36  
37 Using SAD $\Delta$ G.eGFP as first-order retrograde viral tracer, numerous stained striatal neurons and very  
38 few cortical neurons are visualised. Compared to CVS-11 or rCVS-N2c-P-mCherry, SAD $\Delta$ G.eGFP  
39 provides similar intraneuronal staining intensity, with a nice Golgi-like visualisation of dendritic  
40 arborizations and spines (Figure 5B), but fewer striatal positive neurons are detectable (Figure 6A  
41 compared to 6B-C). Although the lack of transsynaptic labelling of striatal neurons connected to the  
42 primary-infected striatonigral neurons with SAD $\Delta$ G.eGFP could contribute in part to the differences  
43 with the transsynaptic CVS used, the present observations are in agreement with previous reports  
44 showing that the SAD B19 is less efficient to infect neurons compared to CVS (Kim et al., 2016;  
45 Reardon et al., 2016; Zhu et al., 2019). However, once in the neuron, it presumably shows viral  
46 replication potency similar to CVS, as suggested by the comparable Golgi-like staining.

47  
48 Injection of rCVS-N2c-P-mCherry induces after 42 h a strong Golgi-like staining of a large number of  
49 striatal neurons (Figures 5C, 6C) and intense labeling of numerous cortical neurons, mainly in layer 5.  
50 The numbers of infected neurons in both the striatum and the cortex are approaching those seen 72 h  
51 rather than 42 h after CVS-11 injection. However, the layer pattern of cortical labeling is similar to the  
52 one observed at 42 h post-injection of CVS-11 (Figure 2D), suggesting that labeled neurons are mostly  
53 second-order infected corticostriatal neurons. Previous studies have suggested a higher transneuronal  
54 transfer rate of CVS 24 N2c compared to CVS-11 (Callaway, 2008). Although we cannot rule it out,  
55 our data rather support higher infection capacity or replication rate of rCVS-N2c-P-mCherry than of



1 CVS-11. Studying in details the kinetics of transfer of rCVS-N2c-P-mCherry for identifying the order  
2 of connections in comparison with CVS-11 is required to further address this issue.

3 Injection of rCVS-N2c-M-GFP leads to suboptimal staining compared to CVS-11 or even more to  
4 rCVS-N2c-P-mCherry, both numerically and qualitatively, suggesting at first sight that this  
5 recombinant is much less infectious (Figure 5D). This is consistent with the results obtained for the  
6 recombinant VSV M-GFP which has been shown to be attenuated with regard to viral replication and  
7 viral growth (Soh and Whelan, 2015). It can be assumed that the insertion of the fluorophore inside the  
8 M protein is not inert and results in reduced infectivity or/and speed of replication/propagation of this  
9 RABV recombinant. In contrast, SADΔG.eGFP, in which GFP replaces the viral glycoprotein gene, is  
10 known to be stable. However, immunodetection of the GFP is shown to amplify the labeling obtained  
11 with rCVS-N2c-M-GFP, indicating that the native fluorescence underestimates the level of GFP  
12 expression and thus the number of infected neuron detected (Figure 7A). Interestingly,  
13 immunodetection of the P-protein of rCVS-N2c-M-GFP with the RABV anti-P monoclonal antibody  
14 (Figure 7B) also reveals higher numbers of infected neurons, confirming that the level of GFP  
15 expression underestimates the degree of viral infection, and, more importantly, shows greater details  
16 of their dendritic arborization, such as visualization of spines, which could be due to the different  
17 expression levels and localization of the viral P and M proteins. It can be then concluded that detection  
18 issues rather than infectivity account for the poorer direct labeling obtained rCVS-N2c-M-GFP.

19  
20 Besides avoiding the step of immunodetection of CVS, rCVS-N2c-P-mCherry and rCVS-N2c-M-GFP  
21 have been engineered with the aim to address an additional complexity level in the brain networks'  
22 connectivity diagrams by the combination of viral tracers with similar strain properties in single  
23 animals. For example, by injecting one of the recombinant viruses in one target of a given brain  
24 structure and the other in another target of the same structure, we aimed at investigating the issue of  
25 parallel or integrated information processing in subcircuits within the network, by the absence or  
26 presence of dually-stained primary and/or higher-order traced neurons. Regarding the basal ganglia  
27 model system, it is recognized that the striatonigral and striatopallidal neuronal populations are highly  
28 segregated. However, it remains unknown whether the inputs they receive from identified populations  
29 of striatal interneurons or from extrinsic sources arise from the same neurons or from segregated  
30 subpopulations. Successful application of such dual transneuronal tracing requires that the two viruses  
31 infect a neuron within a narrow time window (few hours) due to virus interference that decreases the  
32 efficiency of the second infection in a time dependent manner (Ohara et al., 2009). The failure of  
33 rCVS-N2c-M-GFP to provide satisfactory staining per se did not allow us to achieve such an  
34 objective. Recently, multiplex G-deleted RABV tracing has been developed to simultaneously label  
35 and characterize multiple circuits in single animals (Suzuki et al., 2020).

### 36 37 **3.4. CTB versus RABV labeling**

38  
39 The injection of the fluorescent retrograde tracer CTB in the SN shows a large population of labeled  
40 striatal neurons and very few cortical neurons. Even though the staining is intense, it is restricted to the  
41 cell body cytoplasm (Figure 5F), contrasting with the Golgi-like labeling resulting from RABV  
42 infection, as illustrated for rCVS-N2c-P-mCherry (Figure 5C) or CVS-11 (Figure 5F) at 42 h post  
43 injection. However, for a similar volume of injection, the number of striatal CTB-positive neurons is  
44 always greater than the number of RABV-infected neurons, consistent with wider diffusion of CTB  
45 than RABV in the injection site, as already reported in previous studies (Ugolini, 2010).

### 46 47 **3.5. Golgi-like staining with RABV**

48  
49 Since the publication of the seminal work by Santiago Ramón y Cajal (Cajal, 1893), Golgi  
50 impregnation remains one of the most reliable histological techniques enabling the detailed  
51 visualization of the entire morphology of neurons, including dendritic spines. However, stains based  
52 on the Golgi method do not allow distinguishing neurons with quite identical morphology, such as the  
53 two populations of striatal projection neurons, and can hardly be combined with other labeling  
54 methods. Together with the randomness of staining, these are major limitations for mapping neuronal  
55 circuits. Advances in the tools allowing a Golgi-staining like labeling include intracellular labeling

1 methods, the development of various retrograde tracers and of virus vectors and the generation of  
2 transgenic mice, such as the *Thy1*-XFP mice (in which XFP stands for any fluorescent protein) in  
3 which projection neurons are labeled in many parts of the nervous system, notably deep cortical  
4 projection neurons. The Golgi-like labeling in *Thy1*-XFP mice is used in numerous studies to visualize  
5 dendritic spines and explore their plasticity (Feng et al., 2000) and has been combined with tract-  
6 tracing techniques (Porrero et al., 2010).

7  
8 Transneuronal retrograde tracing methods using RABV can offer the advantage to associate charting  
9 of serially connected neurons and powerful detection of the dendritic structure. The fluorescent  
10 RABV-recombinant rCVS-N2c-P-mCherry leads to an impressive Golgi-like labeling of primary-  
11 infected striatonigral neurons, as did SADΔG.eGFP, but also of second-order corticostriatal projection  
12 neurons, as also seen by immunodetection of CVS-11. Figure 8 illustrates the morphology of a striatal  
13 MSN and of a cortical pyramidal neuron revealed by the classical Golgi impregnation method (Figure  
14 8A) and by the viral tracing approach using rCVS-N2c-P-mCherry (Figure 8B). The resolution is quite  
15 comparable, allowing detailed visualization of thin dendrites and dendritic spines, such as the spines  
16 seating on the secondary branches of the extended apical dendrite of the pyramidal cortical neurons as  
17 also observed in basal dendrites. However, the density of detectable spines appears to be higher in the  
18 Golgi-impregnated material. This could be due to methodological limitations, such as the penetration  
19 of the antibody for the detection of the RABV P-protein using CVS-11, and/or to heterogeneous  
20 spreading of the targeted RABV protein within the neuron. RABV tracing can nicely reveal the  
21 different morphologies of the distinct neuronal populations innervating a given brain region. Among  
22 the populations connecting the SN, first-order infected neurons in the subthalamic nucleus and the  
23 parafascicular thalamic nucleus are also illustrated (Figure 9A and 9B, respectively). One can note that  
24 these neurons are poorly spiny in comparison to striatonigral neurons even though their dendritic  
25 processes are well visualized, and that the subthalamic neurons are poorly branched compared to the  
26 rich arborization of parafascicular neurons.

27  
28 The Golgi-like RABV tracing and its combination with other anatomical tools is an asset to reveal the  
29 levels of complexity in the anatomofunctional organisation of the studied circuitry and its remodelling  
30 in physiopathological conditions. For example, the striatum is organized into histochemically defined  
31 compartments termed matrix and striosomes (Graybiel and Ragsdale, 1978), whose specific  
32 connections and functions are still under scrutiny (Brimblecombe and Cragg, 2017; Smith et al.,  
33 2016). Previous work has examined the disposition of the dendritic processes of Golgi-stained neurons  
34 laying in either compartment with regards to the compartment boundaries in cat (Bolam et al., 1988).  
35 Here, using immunodetection of the mu opioid receptor to visualize the striosomes, we provide  
36 illustrations of the dendritic arbors of RABV-infected striatonigral neurons laying in either  
37 compartment in mice. In these examples, the arborizations of the neurons are markedly influenced by  
38 the compartment boundaries: the arborizations of the neurons in the matrix avoid the striosome, while  
39 the dendritic trees of the neurons in the striosomes remain mostly confined to this compartment  
40 (Figure 10), as also described in the rat nucleus accumbens after juxtacellular labeling (van Dongen et  
41 al., 2008). Quantitative analyses of such material, for example in terms of proportions of neurons  
42 identified from their target in either compartment, the respective spine densities of these neurons or the  
43 compartmentalization of their dendritic tree, could provide new insights onto organizational principles  
44 underlying striatal functions. In the same vein, the combination of retrograde RABV tracing with the  
45 anterograde tracing of striatal input systems (López et al., 2010; Salin et al., 2008) and the use of the  
46 transsynaptic spread of the virus combined with neurochemical identification of the interneurons  
47 connecting the primary-infected projection neurons have demonstrated their added value to explore the  
48 striatal microcircuits (Salin et al., 2009). On the one hand, the definition of the postsynaptic element  
49 achieved by the Golgi-like labeling of identified striatofugal neurons with RABV enabled the analysis  
50 at confocal microscopy level of the potential contacts they receive from anterogradely-labeled cortical  
51 and thalamic afferents. On the other hand, the unequivocal characterization of second-order  
52 interneurons showed that striatonigral and striatopallidal neurons are connected by equivalent numbers  
53 of parvalbumin interneurons, the same holding true for cholinergic interneurons. Recently, an  
54 approach combining the use of a newly developed anterograde RABV ΔG variant and of a retrograde  
55 RABV ΔG to address reciprocal connectivity through long ranging pathways has been reported to

1 enable the high-resolution reconstruction of key neuronal morphological features (dendrites, spines,  
2 boutons) (Haberl et al., 2017). The level of resolution of the RABV-based tracing approaches also  
3 allows addressing the plasticity of the neural networks. For example, evidence has been previously  
4 provided for a reorganization of the connections between striatal cholinergic interneurons and the two  
5 populations of striatal projection neurons in a rat model of Parkinson's disease (Salin et al., 2009). As  
6 another example, it is known that striatal spiny neurons undergo pruning and remodelling in patients  
7 with Parkinson's disease and animal models but data regarding the population affected, D2 and/or D1  
8 dopamine receptor expressing neurons are controversial (for review see Villalba & Smith, 2018).  
9 Integrating the dimension of striatal compartmentalization and associated input specificity might help  
10 to reconcile such discrepant observations. Using monosynaptic RABV tracing, adaptations in dendritic  
11 spine density at different stages of cocaine administration and withdrawal have been evidenced in  
12 distinct brain areas projecting to the subpopulations of D1- and D2-expressing neurons in the nucleus  
13 accumbens (Barrientos et al., 2018). Developmental issues have been also addressed as illustrated in a  
14 report showing default of pruning within intracortical circuits in mice deficient for candidate  
15 molecules involved in activity-regulated process needed for proper circuit sculpting (Adelson et al.,  
16 2016).

17

### 18 **3.6. Advantages and limitations of RABV tract-tracing**

19

20 Beside its Golgi-like staining property and compatibility with other tracing tools, RABV has many  
21 advantages over other retrograde neuronal tracers. Most *in vivo* studies report strict preference of Lab-  
22 adapted RABV for neurons compared to other CNS cells, in contrast to PRV, while glia infection has  
23 occasionally been noted with street RABV. Recent work evidenced astrocyte infection *in vivo* by field  
24 RABVs independent of the inoculation route (intramuscular or intracerebral) and for Lab-adapted  
25 RABV depending on strain and route (astrocyte infection frequency of 13% after intracerebral rCVS-  
26 11, while 0% with two other lab RABV strains) (Potratz et al., 2020). However, this analysis has been  
27 performed in clinically-diseased animals at a late stage of infection (up to 21 days post-inoculation),  
28 which is outside the time window post-inoculation usually used for transsynaptic tracing purpose.  
29 RABV infects neurons only via synapses without uptake contamination of the passing fibers crossing  
30 the injection site. RABV is considered to move exclusively backwards (retrograde) and to propagate  
31 between interconnected neurons without inducing apparent inflammation or cell death up to 4 days  
32 post-injection (Ugolini, 2010). Evidence has been provided for morphological alteration or  
33 degeneration of specific neuronal types, but within several days after injection of the unmodified  
34 CVS-11 (Ruigrok et al., 2016), and after 16 days for SADΔG-EGFP (Wickersham et al., 2007a).  
35 Single cell RNA sequencing and immunodetection of glial markers provided evidence that RABV-  
36 ΔG-EGFP can lead within 2 weeks to alterations in gene expression, mainly genes associated with the  
37 immune response, as well as microglial reaction at the injection site and in retrogradely labeled  
38 structures (Sun et al., 2019). However, as illustrated here, intracerebral RABV inoculation is able to  
39 reveal neurons connected to primary infected neurons (second-order) in less than 2 days, and even  
40 higher-order neurons within 3 days, providing an extended safe exploration window for depicting  
41 multisynaptic brain circuits. The rapid uptake of RABV is a factor limiting its spreading and thus the  
42 number of neurons infected from the injection site, as compared to conventional retrograde tracers,  
43 such as CTB. A major limitation in the use of transsynaptic tracers is the ambiguity in the  
44 determination of monosynaptic connections due to their incapacity to distinguish weak direct  
45 connections from strong indirect ones, which is overcome with the approaches using monosynaptic  
46 restriction of transsynaptic tracing. Other limitations have been stressed regarding the use of RABV to  
47 identify chains of connected neurons. It has been reported that RABV is unable to infect neurons via  
48 electrical synapses (gap junctions) (Tang et al., 1999), showing that the virus propagates only at  
49 chemical synapses. Moreover, several studies have reported failure of primary infection or of  
50 transneuronal transfer for identified neuronal populations (Albisetti et al., 2017; Ruigrok et al., 2016).  
51 In the basal ganglia, we also reported that striatal interneurons expressing neuronal nitric oxide  
52 synthase that are known to co-express somatostatin and neuropeptide Y are not second-order labeled  
53 after RABV infection of striatal efferent neurons (Salin et al., 2009). Finally, a recent study shows that  
54 retrograde viral tracers, including RABV and PRV, exhibit different patterns of neurotropism,

1 outlining the importance of the appropriate virus selection for the neural networks to be dissected (Sun  
2 et al., 2019).

3  
4 **In conclusion**, the pitfalls in the use of RABV as a transsynaptic retrograde tracer are balanced by its  
5 remarkable advantages to study morphology and connectivity of large populations of neurons  
6 identified from their anatomical target. In our experimental conditions and study model, RABV is  
7 more potent than the Bartha strain of PRV. Among the RABV strains and variants tested, the poorest  
8 labeling, both numerically and qualitatively, is offered by rCVS-N2c-M-GFP. CVS-11, rCVS-N2c-P-  
9 mCherry and SADΔG.eGFP all provide impressive Golgi-like staining of primary infected neurons,  
10 the greatest numbers of neurons being detected with rCVS-N2c-P-mCherry, followed by CVS-11 and  
11 then SADΔG. rCVS-N2c-P-mCherry thus represents a new powerful transsynaptic fluorescent tracer  
12 for deciphering neuronal networks, with the limitations and advantages inherent to its transsynaptic  
13 properties.

#### 14 **Author contributions**

15  
16  
17 PC and DB provided viral materials and tools for detection. P.S. and P.C. performed the experiments.  
18 PS and L.K-L.G. conceptualized the study and prepared data and figures. All authors contributed to  
19 the paper writing.

#### 20 **Declaration of Competing Interest**

21  
22  
23 None.

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38

## 1 **Figure legends**

2 Figure 1. Schematic history of the major strains of rabies virus and of their variants used in laboratory  
3 settings for neural tract-tracing (A) and representation of the full-length cDNA recombinant plasmid  
4 prCVS-N2c (B). A. The cartoon depicts the major milestones (timeline not to scale) in the generation  
5 of the variants and recombinants of the two main rabies virus fixed strains lab-adapted from the street  
6 Challenge Virus Standard (CVS, upper panel) and Street-Alabama-Dufferin (SAD) virus (lower  
7 panel). B. The plasmid prCVS-N2c –P-mCherry (P-mch) differs from the parental genomic plasmid  
8 prCVS-N2c by fusion of the mch gene at the 3' end of the P gene. The plasmid prCVS-N2c –M-GFP  
9 differs from prCVS-N2c by insertion of the EGFP gene within the M gene after the residue 78. The  
10 sets of two restriction enzymes used to insert these modified genes in the genomic plasmid are  
11 indicated.

12 Figure 2. Transsynaptic retrograde tracing within the cortico-striatal circuitry after CVS injection in  
13 the substantia nigra (SN). A. Illustration of the labeling at the injection site in the SN 42h after  
14 injection of CVS-11. The lower panel is a zoomed image of the boxed area (dashed line rectangle) in  
15 the upper image in which the SN is delineated by full line. The viral P-protein is detected by  
16 immunofluorescence. No immunolabeled cell body or neuritic network is seen; only limited,  
17 presumably nonspecific, labeling along the injection tract is observed. B. Schematic representation of  
18 two-neuron chains investigated. At 24 h first-order striatonigral neurons would be faintly stained. At  
19 42 h, the virus would self-amplify in the primary infected neurons and propagate to the neurons  
20 innervating them: the striatal staining would mostly include numerous intensely labeled first-order  
21 striatonigral neurons and few faintly labeled second-order interneurons, while the cortical staining  
22 would mostly represent second-order corticostriatal projection neurons. The very few first-order  
23 corticonigral neurons are not represented in the scheme. C and D. Illustrative images of the stains in  
24 the cerebral cortex (C) and the striatum (D) at 42 h post-injection of the rCVS-N2c-P-mCherry  
25 recombinant.

26 Figure 3. Images exemplifying the staining in the striatum (A) and the cerebral cortex (B) at 24 (A1,  
27 A2,B1), 42 (A3, B2) and 72 h (A4, B3) after injection of unmodified CVS-11 in the substantia nigra  
28 and immunodetection of the viral P protein. At 24 h, the staining is mainly limited to the cell bodies and  
29 primary dendrites of the first-order neurons in both structures and associated with Negri bodies. At 42  
30 h most of the infected striatal neuronal and few of the infected cortical neurons exhibit extensive  
31 staining of their dendritic trees. At 72 h, the labeling in the striatum is so huge that the neurons can no  
32 more be individualized, and in the cortex neurons in all the layers are infected. cc: corpus callosum;  
33 Cx: cerebral cortex; St: striatum.

34 Figure 4. Visualization of striatonigral (A) and striatopallidal (B) neurons at 42 h after injection of  
35 CVS-11 in their respective target in *Drd1a*-tdTomato line 6 mice. The striatal projection neurons are  
36 identified by immunofluorescence detection of the viral P-protein (pseudocolor: green; A1 and B1)  
37 and the D1-expressing striatal neurons are visualized by td-Tomato fluorescence (pseudocolor: red; A2  
38 and B2). Merge images are shown in A3 and B3. As expected, the great majority of neurons traced  
39 from the substantia nigra are D1-positive, whereas those traced from the globus pallidus are D1-  
40 negative. Arrow in A shows one of the few infected neurons that does not express D1 dopaminergic  
41 receptors. Arrow in B indicates one of the rare D1-expressing striatopallidal neurons observed. Scale  
42 bars: 50  $\mu$ m

43 Figure 5. Illustrative images of the high resolution detection of the dendritic arborization of striatal  
44 medium-sized spiny neuron obtained 42 h after intranigral injection of different first-order or  
45 multisynaptic retrograde tracers. A. Bartha GFP-pseudorabies virus (pseudocolor: green). B.  
46 SADAG.eGFP (pseudocolor: green). C. rCVS-N2c-P-mCherry (pseudocolor: red). D. rCVS-N2c-M-  
47 GFP (pseudocolor: green), E. unmodified CVS-11 (immunodetection of the phosphoprotein;  
48 pseudocolor: green, Alexa Fluor 488). F. Alexa Fluor conjugate of cholera toxin B (pseudocolor: red,

1 Alexa Fluor 594). Arrow in C indicates a faintly labeled second-order infected neurons. Scale bars: 20  
2  $\mu\text{m}$

3 Figure 6. Images illustrating the relative quantities of infected striatonigral neurons visualized 42 after  
4 intranigral injection of SAD $\Delta$ G.eGFP (A), CVS-11 (B) and rCVS-N2c-P-mCherry (C). Striatal fields  
5 have been chosen after examination of all the injected animals showing substantial labeling in the  
6 dorsolateral striatum to illustrate the average numbers of infected neurons detected for each RABV  
7 vector. Scale bars: 50  $\mu\text{m}$

8 Figure 7. Enhanced staining of striatonigral neurons after intranigral injection of rCVS-N2c-M-GFP  
9 by immunodetection of the fusion-GFP or by immunodetection of the viral P protein. The native GFP  
10 fluorescence of the recombinant CVS shows only few and lightly stained neurons (pseudocolor: green;  
11 A1, B1). Amplification by immunodetection of the GFP enhances the number of neurons well  
12 observable (pseudocolor: red; A2), while immunodetection of the viral P protein reveals the dendritic  
13 arbors in a Golgi staining-like fashion (pseudocolor: red; B2), as nicely shown in the merge images  
14 (A3, B3). Scale bars: 50  $\mu\text{m}$

15 Figure 8. Images illustrating the resolution provided by viral tracing using rCVS-N2c-P-mCherry (B)  
16 (pseudocolor: white), as compared to Golgi impregnation staining (A) for the visualization of striatal  
17 medium-sized spiny neurons (A1, B1), with magnification of a portion of dendritic arbors (A2, B2),  
18 and of cortical pyramidal neurons (A3, B3). The illustrations in B come from sections obtained 42 h  
19 after intranigral injection of the CVS recombinant.

20 Figure 9. Images of first-order neurons detected in the subthalamic nucleus (A) and the parafascicular  
21 thalamic nucleus (B) at 42 h after intranigral injection of the CVS recombinant rCVS-N2c-P-mCherry  
22 (pseudocolor: red). Scale bars: 20  $\mu\text{m}$

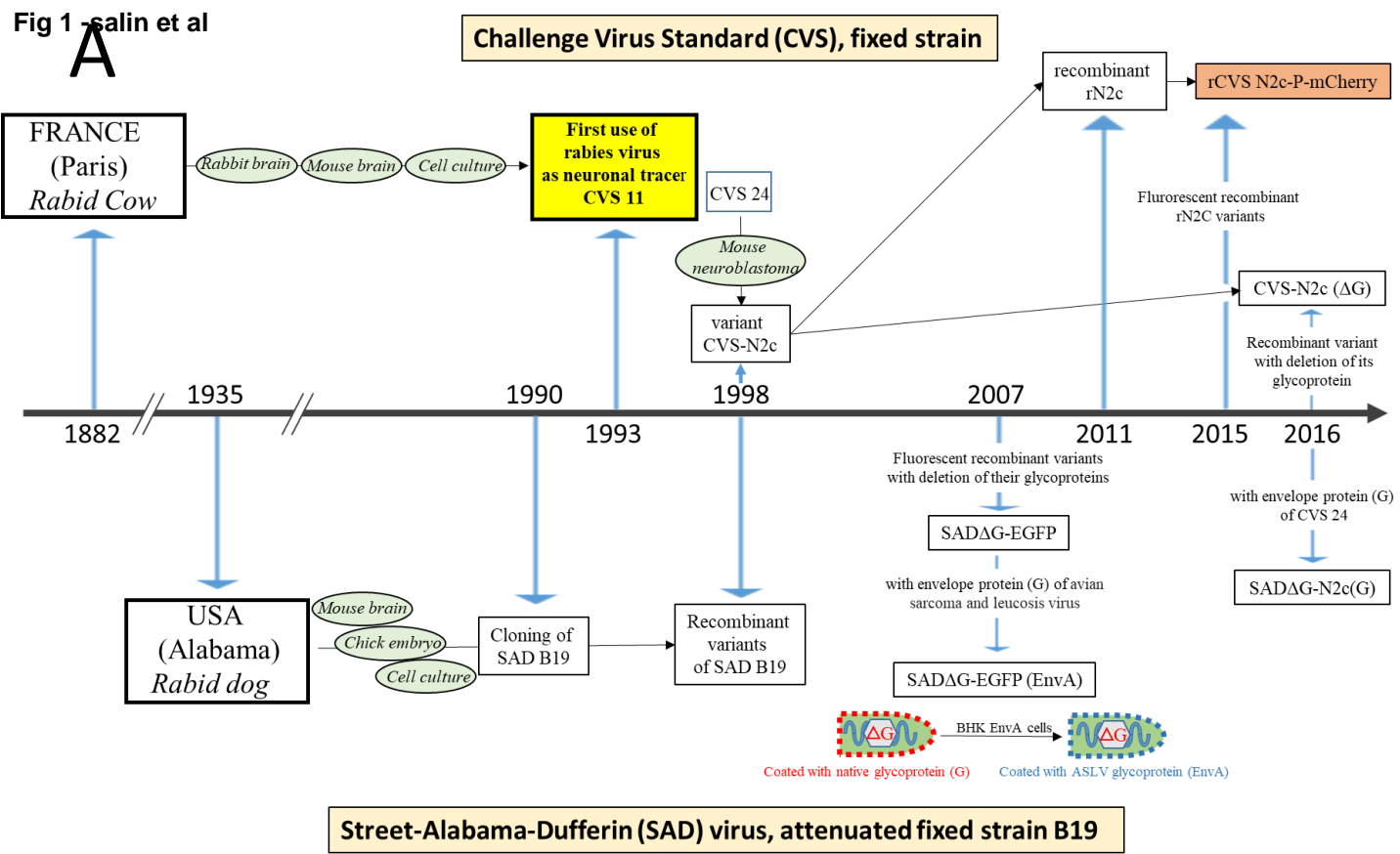
23 Figure 10. Disposition of the dendritic arborizations of striatonigral neurons in relationship with the  
24 striosome-matrix compartmentalization. The detection of striatonigral neurons by mCherry  
25 fluorescence (pseudocolor: magenta) at 42 h after intranigral injection of rCVS-N2c-P-mCherry is  
26 combined with immunostaining of the mu opioid receptor (pseudocolor: green) for visualizing the  
27 striosomes (delineated areas). A and B illustrate neurons whose dendritic arbors are mostly confined  
28 within the matrix or the striosomes, and then tend to respect the compartment boundaries. Scale bars:  
29 20  $\mu\text{m}$

30

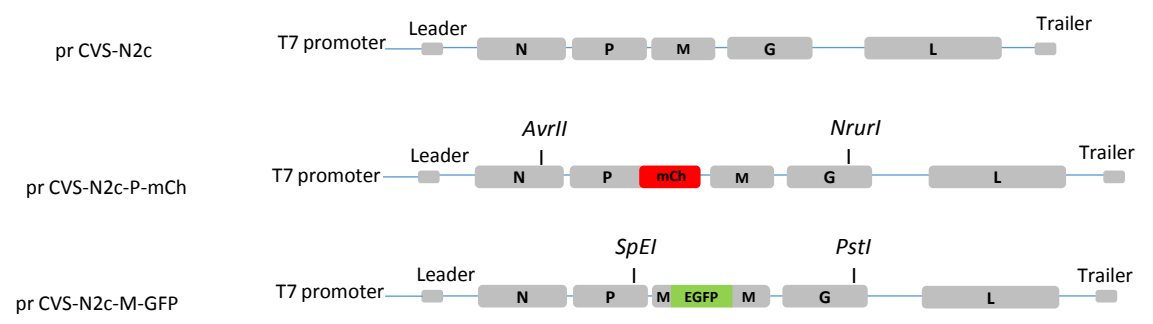
31

Fig 1 Galin et al

**A**



**B**



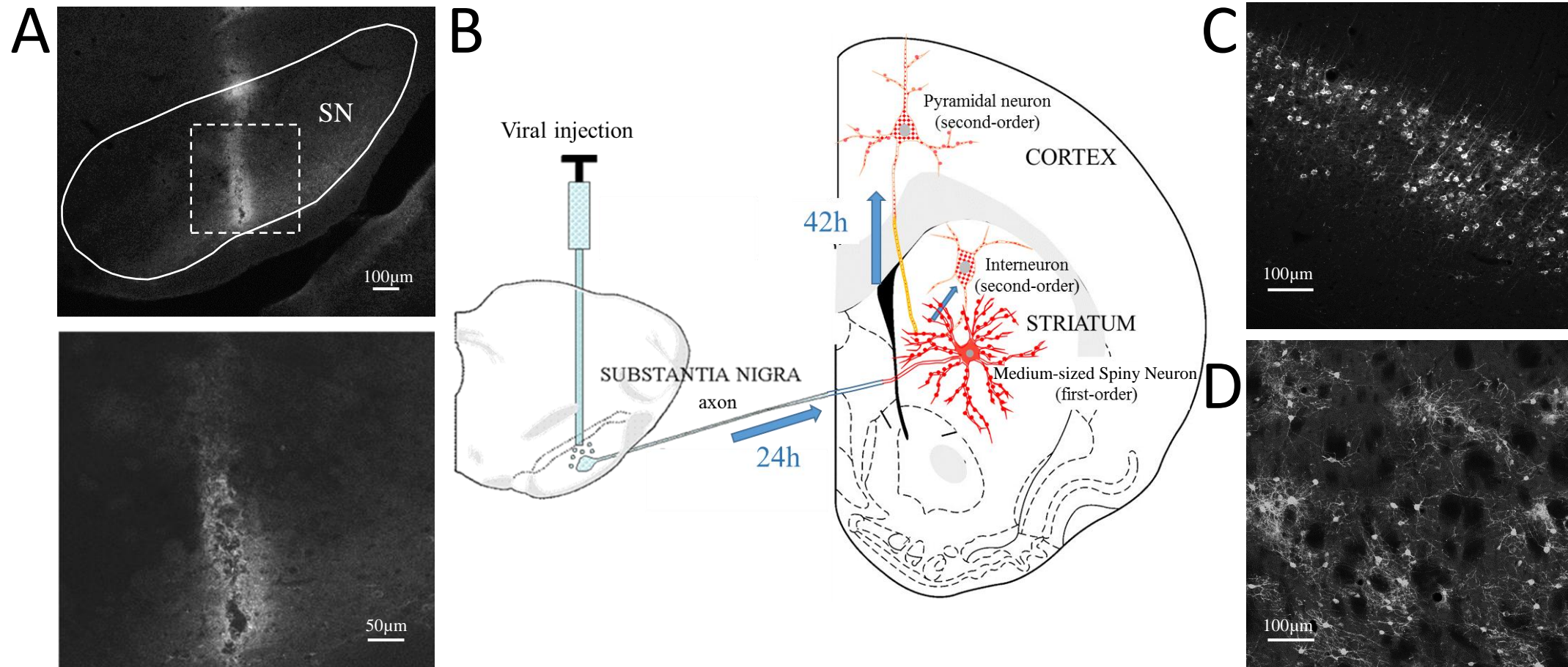


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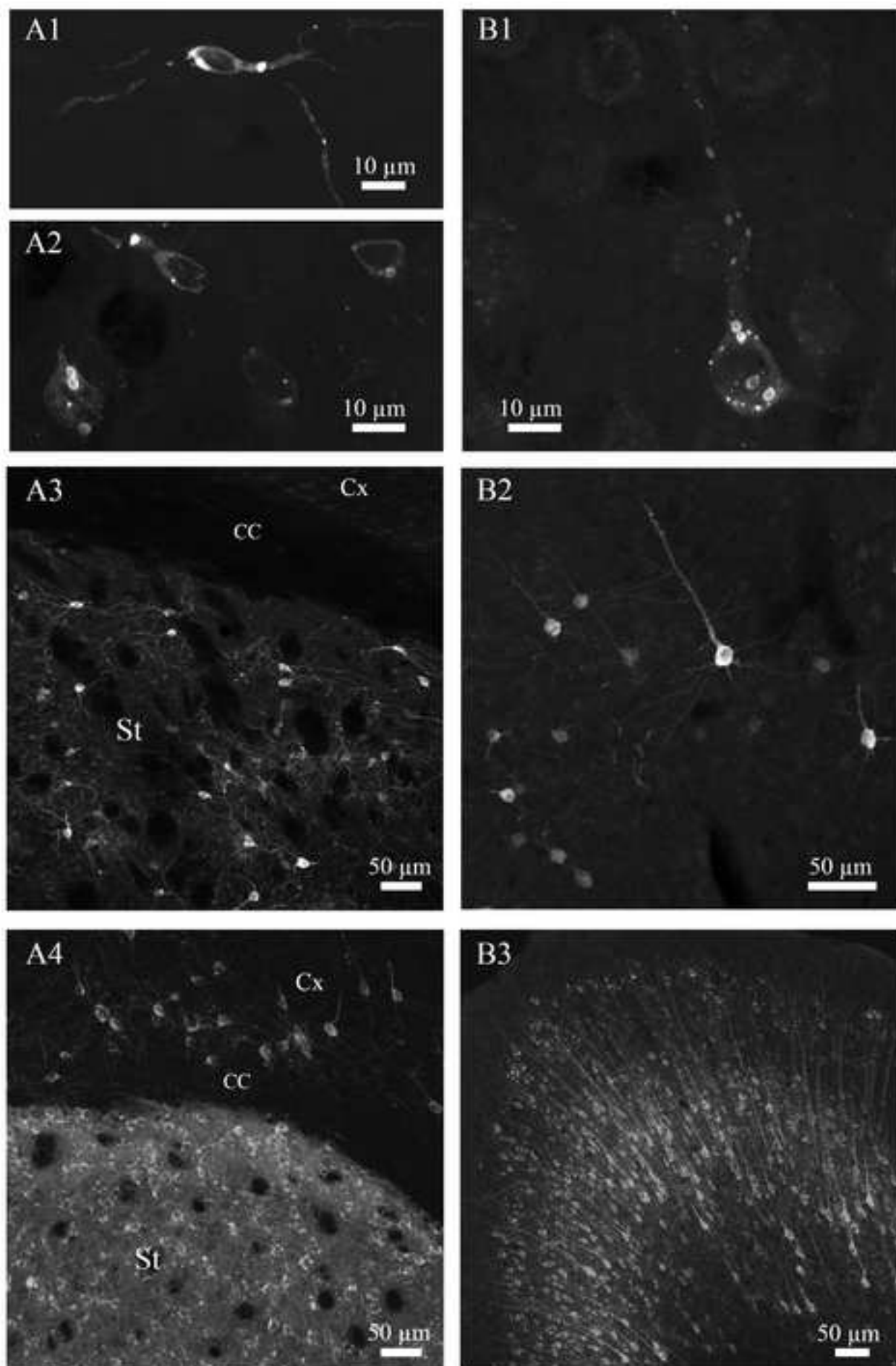
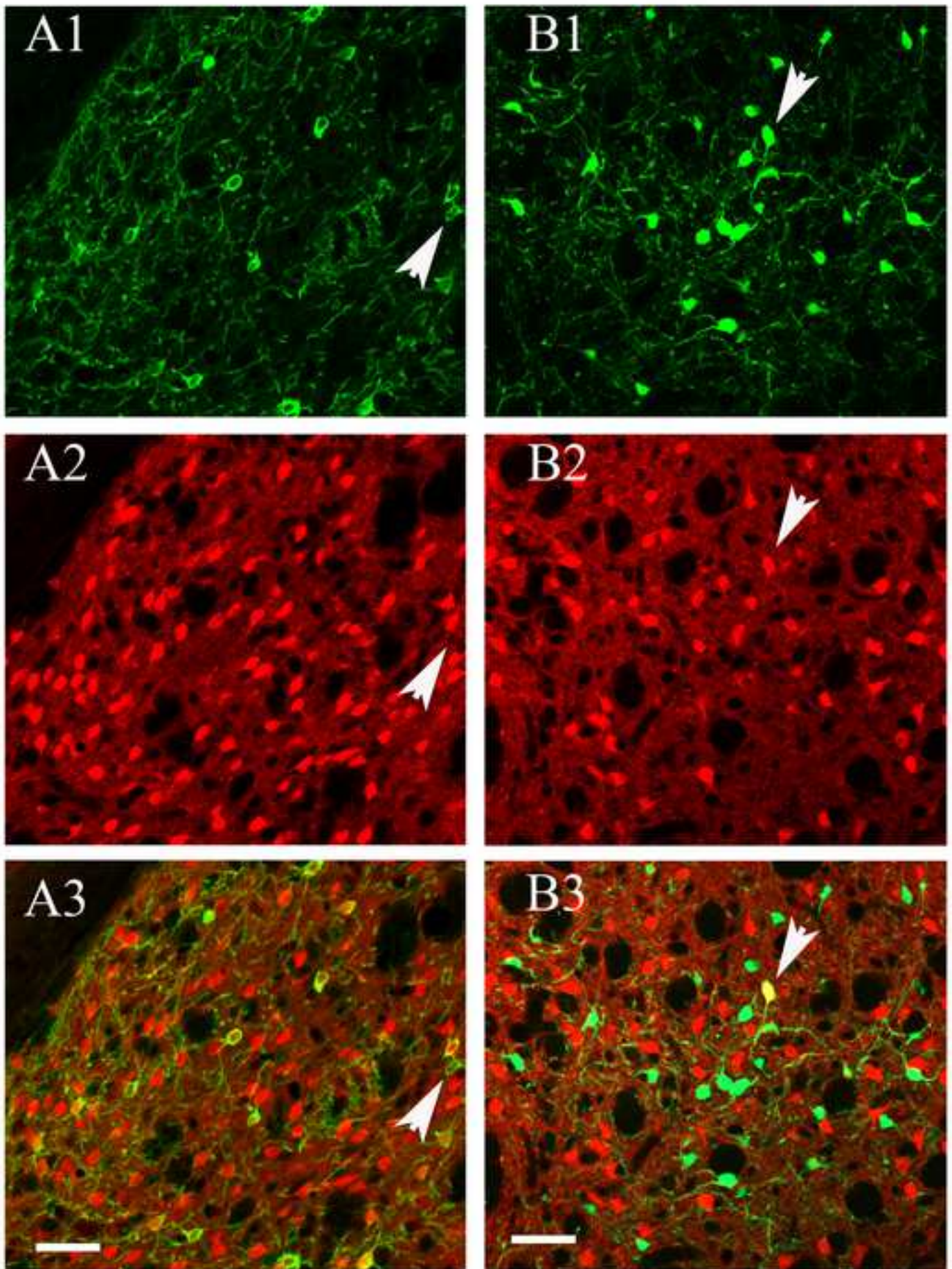




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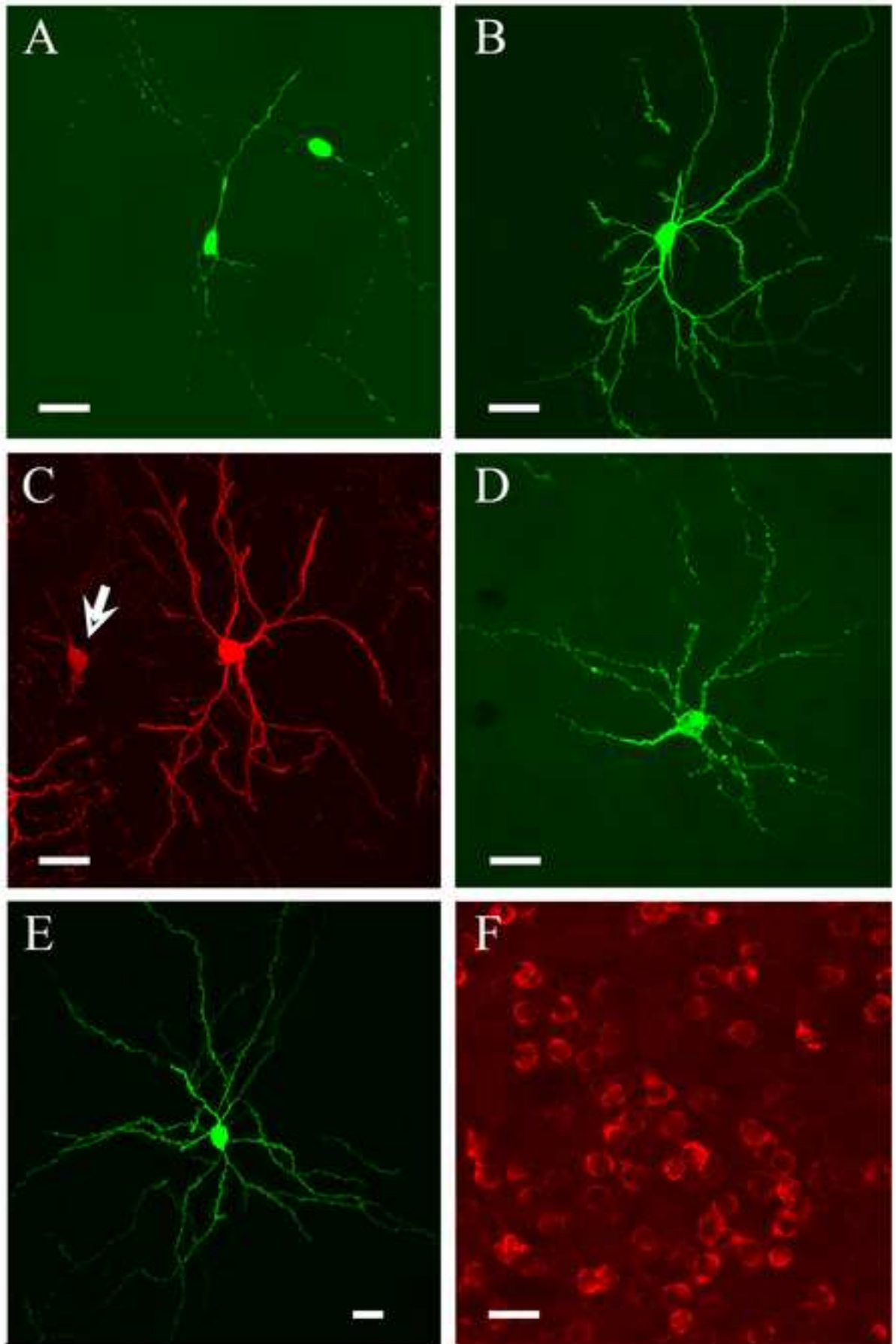




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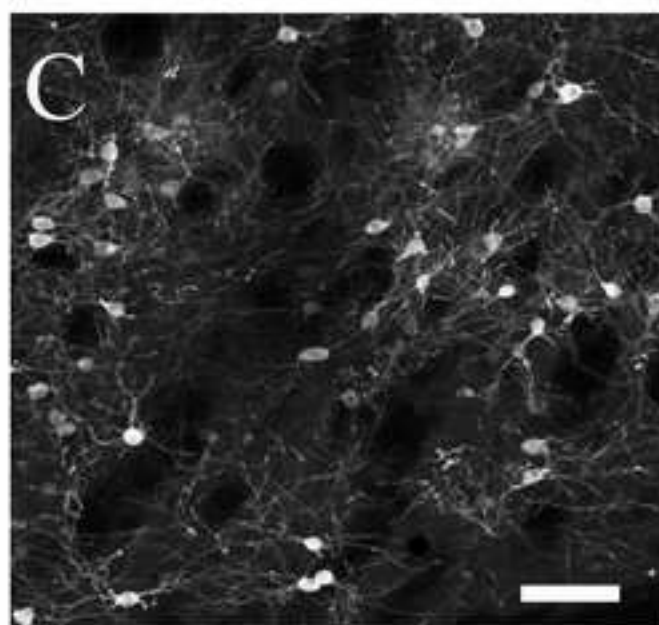
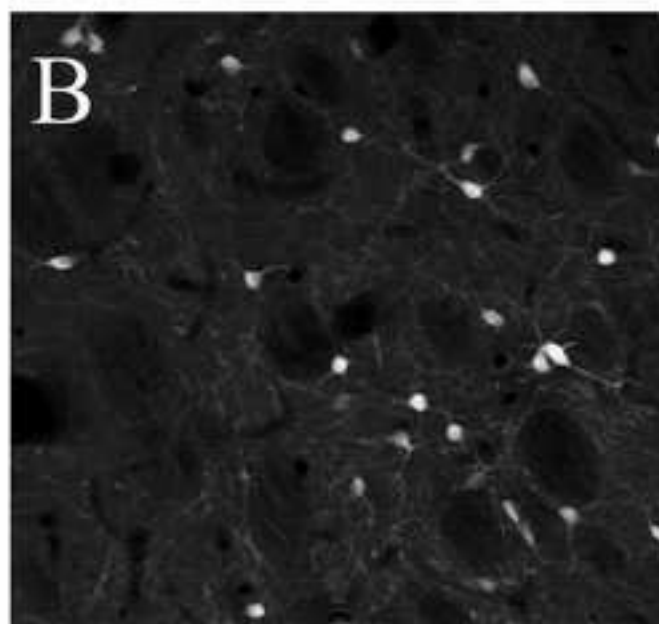
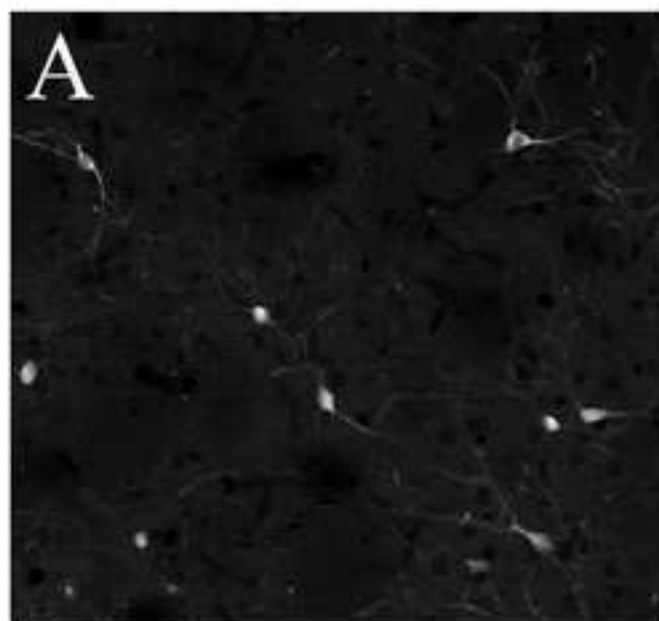


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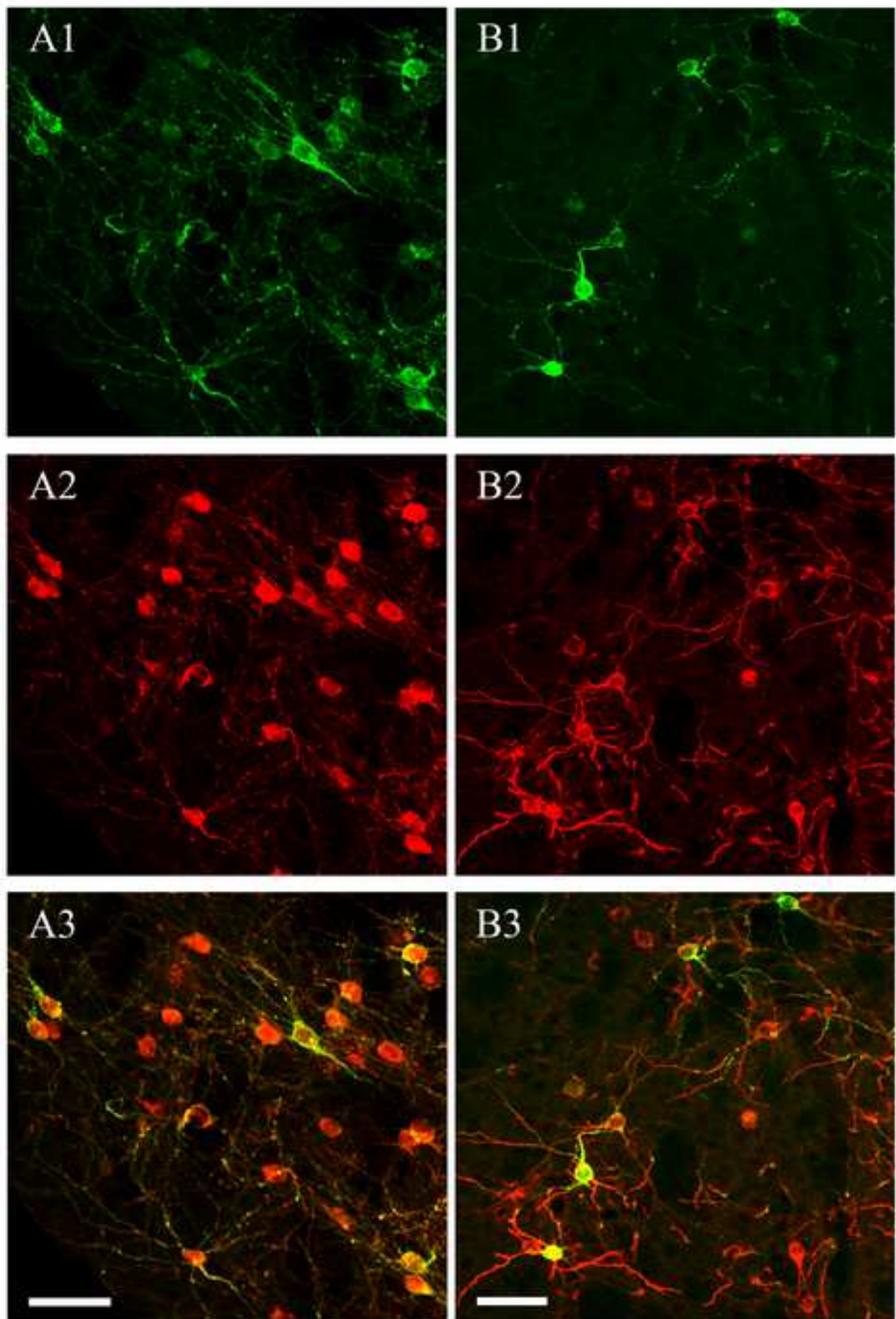


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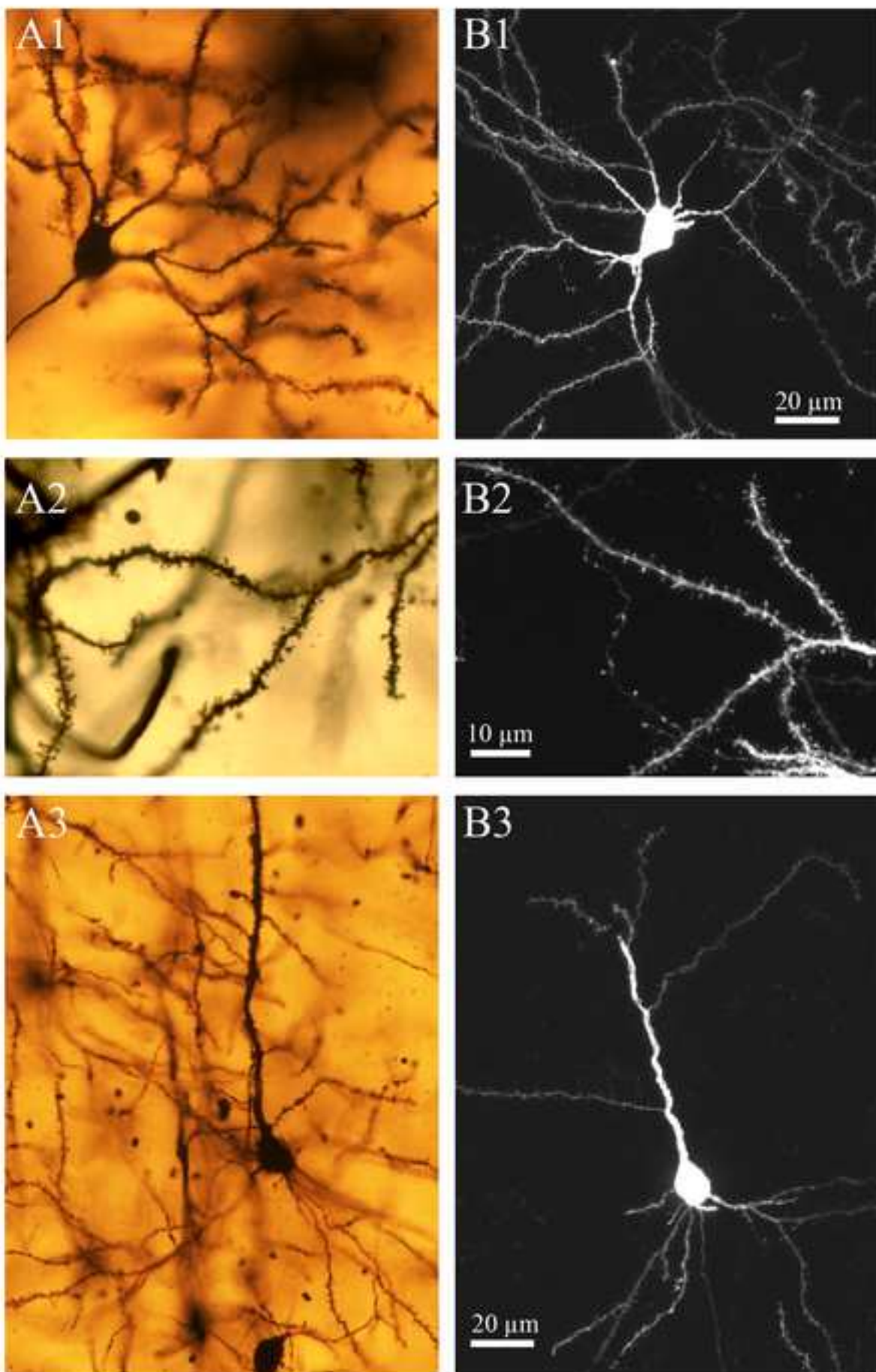




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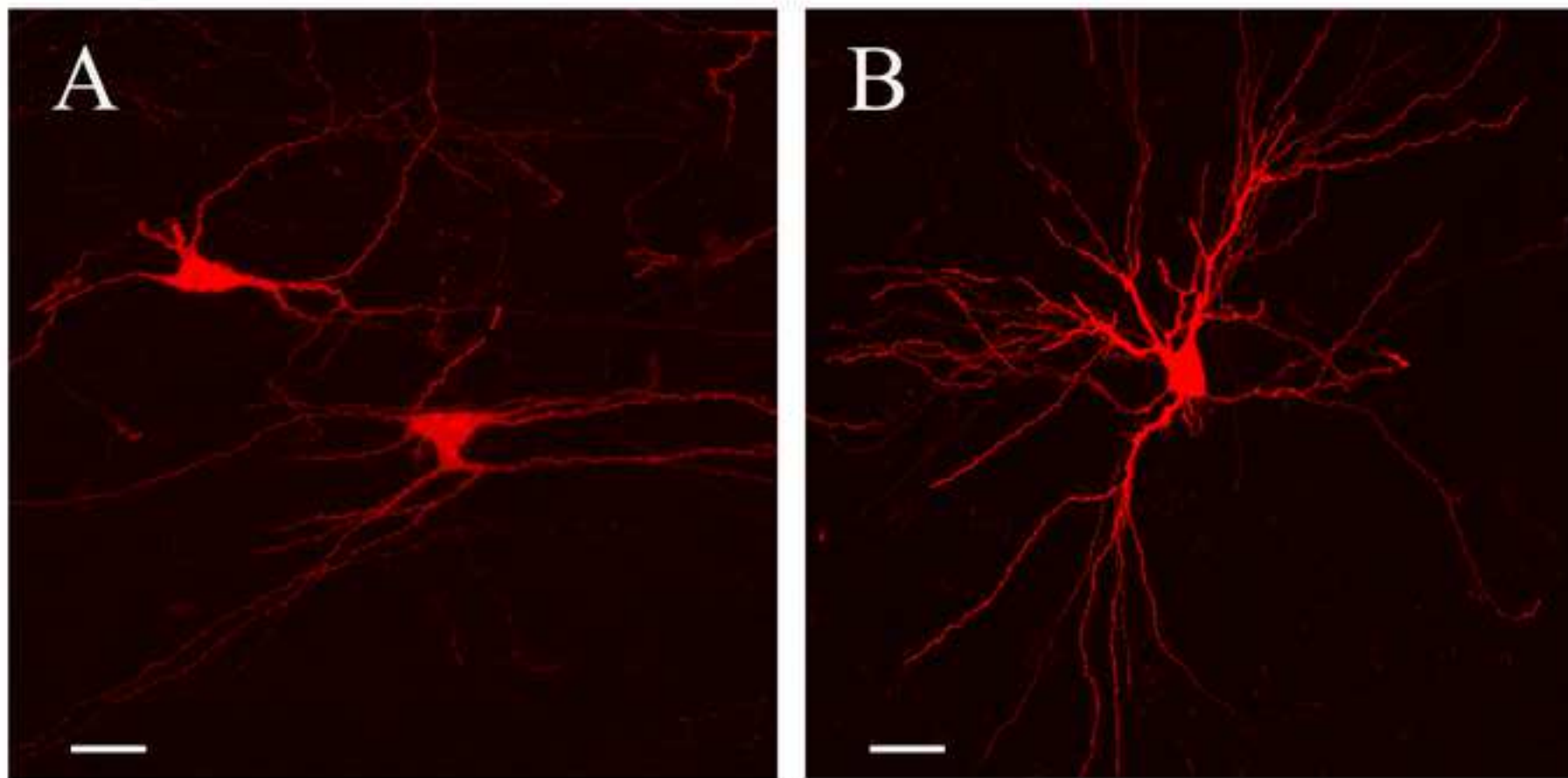


Fig 10 -salin et al  
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