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A new use for an old method: The Woelcke myelin stain for counting degenerating neurons in the brain of mice following *status epilepticus*

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ABSTRACT

The Woelcke method is classically used for myelin staining. Degenerating neurons can be revealed histologically by hemalun and phloxin (H&P) where they appear "eosinophilic". In the first 24 h following soman-induced *status epilepticus*, we observed that the Woelcke method also revealed condensed, dark blue/black cells (W+ cells) in the gray matter of brain regions known to be sites of seizure-related brain damage, marked by the presence of eosinophilic cells. In the present study, using adjacent brain sections alternately stained with either the Woelcke or the H&P method, we show that eosinophilic cells and W+ cells are the same degenerating cells. Moreover, we show that semi-automated quantitative evaluation of W+ cells through computerized image analysis is considerably easier and faster than that of eosinophilic cells. It is therefore concluded that the Woelcke technique could be very useful, especially for quantifying acute brain cell damage following *status epilepticus*.

1. Introduction

Organophosphorus (OP) nerve agents including tabun (GA), sarin (GB), soman (GD), cyclosarin (GF) and VX, are a continuing threat to military personnel and civilian populations despite being banned under the Chemical Weapons Convention (CWC). They act as irreversible inhibitors of acetylcholinesterase (AChE) triggering a hypercholinergic crisis mostly explaining the observed broad spectrum of toxic signs. Depending on the dose, exposure can lead to hypersecretion, respiratory distress, fasciculations and muscle paralysis, cardio-vascular dysfunction, convulsive epileptic seizures, coma and death. In experimental settings, surviving animals can present irreversible seizure-related brain damage (SRBD) as well as long-term neurological deficits such as epileptogenesis and various cognitive impairments. In such a field of research, brain histology is therefore constantly needed to describe the morpho-pathological changes, to explore the mechanisms of SRBD and to evaluate neuroprotective medications. Standard hemalun–eosin (H&E) or hemalun–phloxin (H&P) stains have been and are still a widely used method for visualizing soman-induced cell damage on paraffin-embedded histological brain sections (e.g. Lemercier et al., 1983; Shih et al., 2003; Tuovinen, 2004; Baille et al., 2005; Testylier et al., 2007; Carpentier et al., 2010). Other techniques are also used such as Fluoro-Jade B staining (e.g. Myhrer et al., 2008; Dorandeu et al., 2005, 2007) or more neuron-specific immunostaining such as for the neuronal nuclei antigen (NeuN) (e.g. Collombet et al., 2006) or microtubule-associated protein 2 (MAP-2) (e.g. Kan et al., 2005; Ballough et al., 1995).

With H&E or H&P techniques, degenerating nerve cells typically show a dark blue pyknotic nucleus and a bright red or dark red acidophilic (or eosinophilic) cytoplasm. Apart from changes in staining, these traditional topographical methods also yield other precious information on the pathological condition of the surrounding tissue (e.g. edema, ventricular dilatation, vacuolization and cellular infiltrates). On H&E or H&P sections, the number of eosinophilic cells can be assessed either grossly through semi-quantitative methods (e.g. McDonough et al., 1998; Carpentier et al., 2001; Baille et al., 2005; Testylier et al., 2007; Dorandeu et al., 2007), or, more precisely, through the use of computerized image analysis systems (Collombet et al., 2006; Carpentier et al., 2010). Theoretically, image analysis programs can be customized to allow the automated or semi-automated counting of eosinophilic cells on the basis of their color components, shape and size. However, customization is not always entirely satisfactory since false negative and false positive selections can be acquired. Therefore, time-consuming secondary manual control is often needed.

In one of our recent, unpublished, pilot experiments on mice sacrificed within 24 h after soman exposure, brain sections were

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submitted to immunohistochemistry of Glial Fibrillary Acidic Protein (GFAP), a specific astrocytic marker. For better anatomical identification of the regions where the astrocytic reaction occurred, we counterstained the sections with the ancestral Woelcke method for myelin (Woelcke, 1942) (also called Woelcke's modification of the Heidenhain method or the Heidenhain–Woelcke method). This simple and reliable technique has often been used in the past in neuroanatomical and neuropathological studies (e.g. Gattass and Gross, 1981) including some devoted to soman poisoning (Petras, 1981; Lemercier et al., 1983). As expected, the method was shown to perfectly delineate the white matter. Moreover, in histological sections from intoxicated animals that previously experienced long-lasting seizures, condensed, blue/black cells were repeatedly noted in the gray matter of regions known to be sites of soman-induced SRBD. It was then suspected that these particular cells might represent a Woelcke counterpart of the eosinophilic cells detected through the H&E/H&P methods. If this hypothesis proved right, and since Woelcke positive (W+) cells were the only elements stained in the gray matter, appearing highly contrasted against a pale background, it was presumed that their counting with automated or semi-automated procedures might be easier and faster than for eosinophilic cells revealed by the H&E/H&P methods.

In the present study, this hypothesis was more accurately assessed by using adjacent histological sections, alternately stained by the H&P or Woelcke protocols, from brains of mice that previously experienced a long-lasting *status epilepticus* following soman poisoning.

2. Materials and methods

2.1. Animals

Male Swiss mice (30–40 g; Janvier, France) were used. They were housed in a controlled environment (21 ± 2 °C; 12 dark/light cycle with light provided between 7 a.m. and 7 p.m.) and were given food and water ad libitum. All the procedures were in accordance with the regulations regarding the "protection of animals used for experimental and other scientific purposes" from the relevant Directives of the European Community (86/609/CEE). Study protocols were approved by the Ethical Committee of our Institute.

2.2. Drugs

Soman (>97% pure as assessed by gas chromatography) was supplied by the Centre DGA Maîtrise NRBC (Vert-Le-Petit, France). Solutions were freshly prepared by diluting the initial stock solution (2 mg/mL in isopropanol) in ice-cold 0.9% (w/v) saline. The oxime HI-6 dichloride was generously provided by DRDC Suffield (Canada).

2.3. Experimental design

Mice were pretreated with HI-6 (50 mg/kg; i.p.; injection volume 200 μL in saline) 5 min prior to the administration of a convulsive dose of soman (172 μg/kg; s.c.; injection volume 200 μL; 1.6 LD_{50} in the absence of pretreatment). HI-6 is a re-activator of soman-inhibited cholinesterase (ChE). It predominantly acts peripherally thus allowing a longer survival time without interfering with soman-induced central ChE inhibition or preventing seizures and SRBD. In this paradigm, soman was previously shown to induce long-lasting convulsions (several hours in duration), electroencephalographic seizures and SRBD in more than 75% of the intoxicated mice among which less than 30% died within 24 h (e.g. Baille et al., 2005; Carpentier et al., 2010). For the present histological analysis, 21 surviving intoxicated mice, most of them having displayed convulsions, were sacrificed either 1 h (n = 2), 3 h (n = 1), 12 h (n = 2) or 24 h (n = 16) post-challenge.

Table 1
H&P technique.

Preparation of solutions and reagents	
Hemalun	- Dissolve 50 g aluminum potassium sulfate dodecahydrate (Jansen Chimica, Geel, Belgium) in 1 L distilled water - Boil for 5 min and cool the solution - Add 3 g hematein (Merck, Darmstadt, Germany) - Boil for 5 min, cool, filtrate the solution - Add 18 mL glacial acetic acid Filtrate before use
Phloxin	- Dissolve 3 g phloxin-B (Merck, Darmstadt, Germany) in 100 mL distilled water - Filtrate before use
Acid–alcohol	- Add 0.375 mL HCl (7.68 N) to 250 mL 95% ethanol
Method	
- Wash the deparaffinized and rehydrated sections in tap water - Immerse for 2–5 min in the hemalun solution and wash in tap water - Differentiate several seconds in acid–alcohol and wash in tap water - Immerse in phloxin for 5–20 s and wash in tap water - Dehydrate and mount in Histolaque® (LMR, Paris, France)	

2.4. Histology

At the selected time points, mice were deeply anesthetized (sodium pentobarbital, 80 mg/kg; i.p.) and transcardially perfused with 25 mL of saline with heparin (5 UI/mL) followed by a fixative solution made of formaldehyde (4%) and acetic acid (3%) in saline. Once perfused, the animals were kept at least 1 h at 4 °C to allow complete fixation and to avoid the appearance of any artifactual cell damage (e.g. "dark" cells) due to post-mortem manipulation (Cammermeyer, 1978; Jortner, 2006). Afterwards, the brain was removed and post-fixed 6 h at 4 °C in 4% formaldehyde in saline. One coronal block of brain, centered 1.7–2.2 mm posterior to bregma (coordinates from Paxinos and Franklin, 1997) was embedded in paraffin. Histological sections (7 μm thick) were then serially cut, collected in pairs, and mounted on slides. The two sections present on one slide were thus separated from those on the next slide by either 7, 14 or 21 μm. After being deparaffinized and rehydrated, the first of two adjacent slides was devoted to H&P staining (described in Table 1) while the second one was processed for the Woelcke method (described in Table 2). The sections chosen for analysis were situated approximately 2.06 mm posterior to bregma (Paxinos and Franklin, 1997). They contained several areas known to be damaged following soman-induced *status epilepticus* (hippocampus, thalamus, amygdala and piriform cortex).

2.5. Image analysis

For brain histology, high resolution (1.25 M pixels, 48 bit colors) digital images were acquired using an Axio Imager Z1 microscope equipped with an Axiocam MR2 camera (Zeiss, Germany). Some microphotographs were acquired with the 40× objective to describe the detailed aspects of the Woelcke-positive cells (W+ cells). Comparisons between H&P- and Woelcke-stained sections were performed on pictures acquired at 20×: for a given brain and a given region, one microphotograph was acquired on a H&P-stained section. With the help of clearly identified anatomical landmarks (microvessels, myelinic fibers, etc.), an equivalent area was photographed on one of the Woelcke-stained sections of the contiguous slide. Pairs of matching H&P and Woelcke microphotographs of the CA1 sector (n = 21), the piriform/amygdala region (n = 19), the dorsolateral thalamus (n = 9) and the auditory cortex (n = 9) were then constituted and served for qualitative and quantitative comparison.

The quantitative study was performed using Axiovision software (vs 4.6.3.0.). On the whole microphotographs, a first, entirely manual count was performed on either H&P- or Woelcke-stained tissue by simply clicking on each of either the visually

Table 2
Woelcke technique.

Preparation of solutions and reagents	
Iron alum 5% (prepare just before use)	- Dissolve 25 g ferric ammonium sulfate dodecahydrate (Sigma–Aldrich, St. Louis, MO, USA) in 500 mL distilled water
Hematoxylin stock solution (10%)	- Dissolve 10 g hematoxylin monohydrate (Merck, Darmstadt, Germany) in 100 mL 95% ethanol - Mature the solution for about 3 months
Weigert hematoxylin (prepare just before use)	- Add 30 mL hematoxylin stock solution to 270 mL distilled water - Add 21 mL aqueous saturated lithium carbonate (Sigma Chemical Co., St. Louis, MO, USA)
Method	
- Immerse the deparaffinized and rehydrated sections in iron alum overnight - Wash quickly but energetically twice in distilled water - Immerse in freshly prepared Weigert hematoxylin at room temperature for 0.5–4 h (depending on the maturation of hematoxylin) to obtain bright blue myelin and gray matter as pale as possible - Rinse twice in tap water - Differentiate in 80% ethanol - Dehydrate and mount in Histolaque® (LMR, Paris, France)	

determined eosinophilic cells (H&P+ cells) or W+ cells. This counting method established the "exact" number of damaged cells in a given area to precisely compare the figures obtained in matching H&P and Woelcke sections.

Semi-automated counting was also performed on the same photographs. A first program was set to tentatively identify and count H&P+ cells automatically, on the basis of their color components, shape and size. A second program was similarly set to count the W+ cells. Both programs allowed the operator to manually eliminate false positive selections or to add false negative selections identified in the primary automatic phase. After exercising several times, the time necessary for the semi-automatic counting of either H&P+ or W+ cells was measured on each picture using a standard chronometer. The chronometer was started at the beginning of the automatic phase and stopped at the end of the secondary sequence of manual correction. For a given series of microphotographs, the four counts (i.e. manual HP+, manual W+, semi-automatic HP+, semi-automatic W+) were performed separately over several days, blind to the results of one another, to prevent biased evaluation.

2.6. Immunohistochemistry of astrocytes and Woelcke counterstaining

As mentioned in Section 1, counterstaining GFAP-treated sections with the Woelcke staining allowed the double labeling of reactive astrocytes and putative damaged cells (W+ cells). To illustrate this unpublished observation, supplementary brain sections were treated for GFAP immunohistochemistry (Baille et al., 2005): after being deparaffinized and hydrated, some sections were incubated for 10 min in Tris–HCl 0.1 M/H_2O_2 0.3%, rinsed in Tris–HCl, incubated for 60 min in Tris–HCl 0.1 M/bovine serum albumin (BSA) 1%, and then incubated overnight at 4 °C with the primary antibody, rabbit anti-cow GFAP1/2000 (DAKO, Trappes, France). After washing in Tris–HCl, the sections were incubated for 30 min at room temperature with a biotinylated secondary antibody (goat anti-rabbit IgG 1/200) and the signal amplified for 30 min with the avidine-biotin–HRP complex, using the Vectastin ABC® kit according to the indications of the manufacturer (Vector, Burlingam, CA, USA). Immunoreactivity was revealed with 3,3′-diaminobenzidine (Sigma Fast™ DAB tablet set, D4293, Sigma–Aldrich, St. Louis, MO, USA). The sections were then processed for the Woelcke method as described in Table 2.

3. Results

3.1. Qualitative comparison of H&P- and W-stained sections

On H&P-stained sections from intoxicated but non-convulsive mice, no eosinophilic cells were ever detected (Fig. 1a). On Woelcke-stained sections from the same non-convulsive animals, the tissue showed bright blue myelin fibers while the gray matter appeared almost uniformly light blue-gray (Fig. 1b). On this clear background, the cells were hardly visible except the nucleoli appearing as small black spots.

In contrast, H&P-stained sections from mice that experienced long-lasting convulsions showed a number of H&P+ cells in multiple brain regions known to be susceptible to soman-induced SRBD (Fig. 1c and e). These damaged cells typically showed shrunken perikarya, red (acidophilic) cytoplasm and dark blue (basophilic) pyknotic nuclei. At higher magnification (not shown) and depending on the stage of degeneration, cell shrinkage and condensation could be more or less pronounced and could lead to virtual disappearance of the cytoplasm. At 24 h, the occasional presence of formless elements evoked cell debris from complete cell destruction. In matching brain areas of the Woelcke-stained sections, W+ cells, in variable numbers, could be detected, neatly contrasted against the pale background (Fig. 1d and f). As shown in Fig. 2, these W+ cells could present various aspects: light blue cytoplasm and patches of condensed chromatin in the nuclei (type 1; Fig. 2c); deeper blue coloration of the cytoplasm and entirely black nuclei (type 2; Fig. 2d); entirely black cells in which the cytoplasm was no longer distinguishable (type 3; Fig. 2e). All these cells appeared more or less shrunken. A fourth type (type 4; Fig. 2f) resembled "ghost" cells with a shapeless aspect, homogenous dense gray-blue stain and indiscernible nuclei. Sometimes, small dark blue spots could be seen inside these cells and were interpreted as being either nucleoli or nuclear remnants. All these types of W+ cells could be present, in varying proportions, in the damaged cerebral areas of convulsing mice sacrificed 24 h post-challenge. At earlier times, a smaller number of types 1 and 2 could equally be seen as early as 1 h and 3 h post-challenge while type 3 was more readily detectable at 12 h. Type 4 was almost exclusively seen at the 24 h time point. These observations suggest a continuum, from type 1 to type 4 over time.

As shown in Fig. 1, the localization and the numbers of the W+ cells apparently matched those of the H&P+ cells detected in the contiguous sections. However, despite the closeness of sections, we did not succeed in exactly matching the various subtypes of W+ cells to the different forms of HP+ cells.

3.2. Quantitative comparison of H&P+ and W+ cells

The manual counting of H&P+ and W+ cells, an especially tedious and lengthy task, was performed on 58 pairs of microphotographs. Confirming our qualitative observation, the number of W+ cells in a given brain area of a given mouse appeared very close or sometimes identical to that of H&P+ cells in the matching region. Subsequently, the total (per region and all regions confounded) of W+ cells closely approached that of H&P+ cells (Table 3).

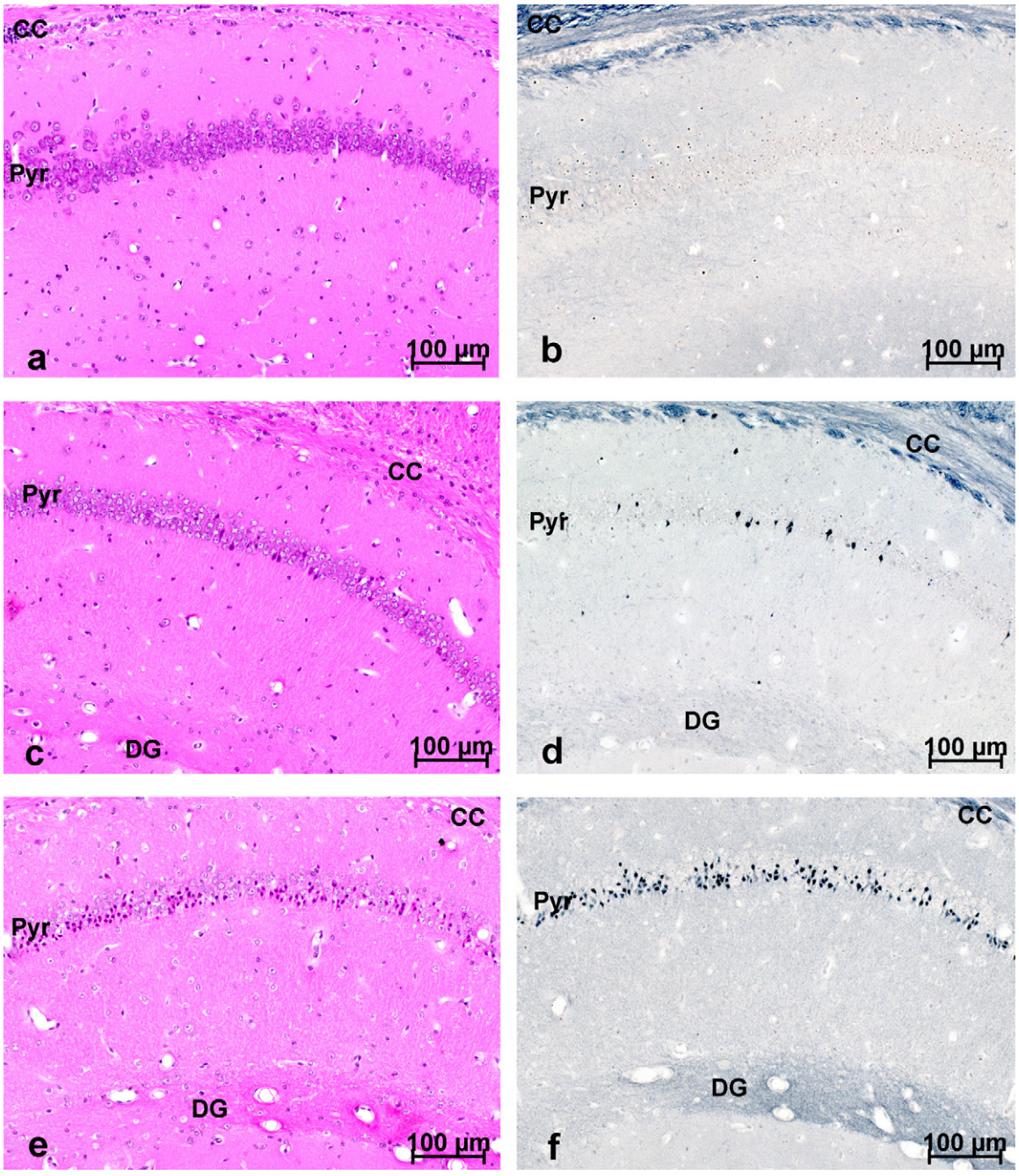

Fig. 1. Qualitative comparison between contiguous H&P and Woelcke-stained sections of the CA1 hippocampal sector (20×). H&P-stained sections (left column) show various aspects of the histological response to soman poisoning in three different mice: without seizures and without any damage (a); with short-lasting convulsions and a few eosinophilic cells (H&P+ cells) appearing in red within the pyramidal layer (c); with long-lasting convulsions and numerous eosinophilic cells (e). In the Woelcke-stained counterparts of the same regions (b, d and f), note that the localization and number of W+ cells, appearing in dark blue, are similar to those of the H&P+ cells. CC, corpus callosum; Pyr, pyramidal layer; DG, dentate gyrus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

After the manual counting, the same microphotographs were reused for independent sessions of semi-automatic counting. An automatic program was then set up for counting H&P+ cells. This task was rather arduous, the difficulty stemming from the great informative richness of the H&P method which stains almost every tissue element, and also from the large variety of shapes, sizes and colors of the H&P+ cells. Therefore, although advanced functions of the Axiovision software were used, the probability of selecting objects other than the H&P+ cells (false positives) or of not detecting them (false negatives) was increased. In spite of this, a final version of the program was considered satisfactory after numerous preliminary tests. With this program, the number of automatically selected H&P+ cells generally largely exceeded that previously established through manual counting. Manual correction of the automatic selections produced figures that closely matched those obtained with the entirely manual, "exact" counting (Table 3). However, the entire process (i.e. automatic phase followed by manual corrections) was excessively time-consuming (more than 5 min per photograph: Table 3).

The program for counting W+ cells was much easier to set up as these cells were well contrasted and virtually the only cellular elements revealed by the staining in the gray matter. The automatic process applied to the Woelcke-stained sections immediately gave a number of W+ cells that approached that of

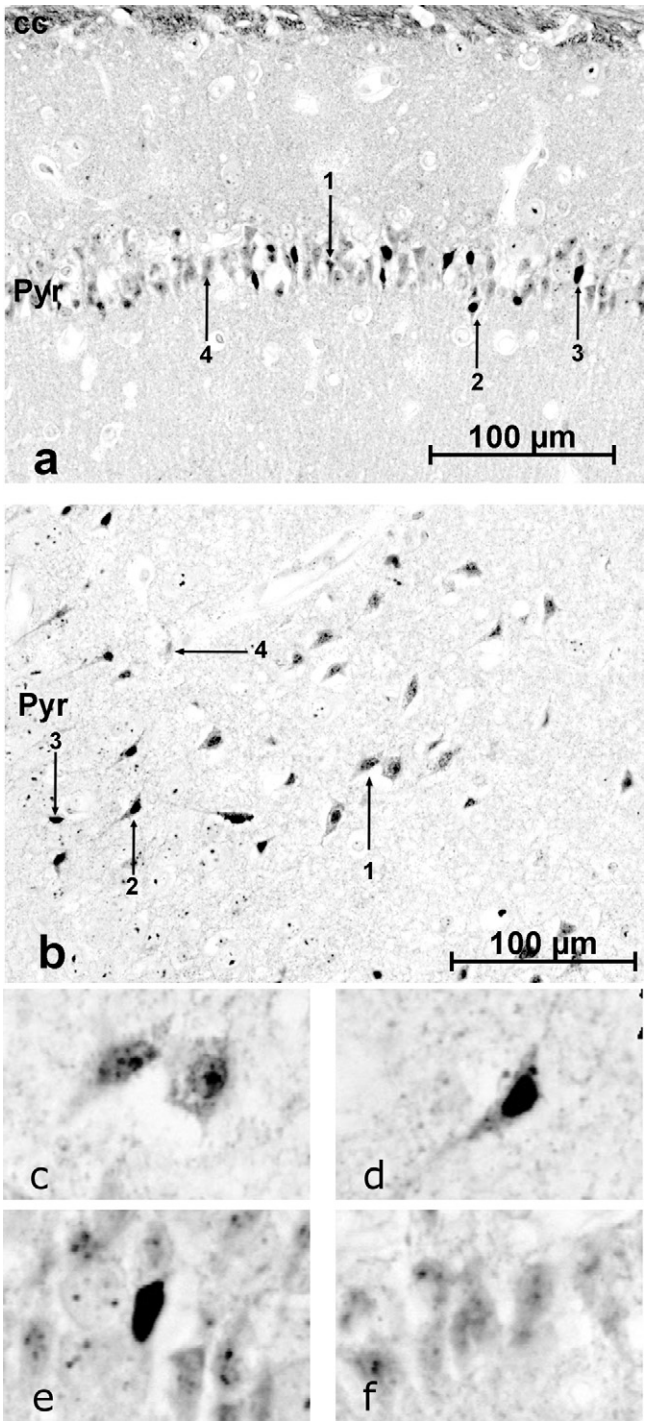

Fig. 2. Detailed aspects of Woelcke-positive (W+) cells (40×). (a and b) Hippocampal CA1 sector (a) and piriform cortex (b) from soman-intoxicated mice that experienced long-lasting convulsions. The various morphopathological aspects of W+-cells are numbered from type 1 to type 4 (numbers + arrows). (c–f) *Electronically magnified W+ cells.* (c) Type 1: shrunken perikarya with patches of compact chromatin in the nuclei and slightly condensed cytoplasm. (d) Type 2: shrunken perikaryon with highly condensed nucleus and slightly condensed cytoplasm. (e) Type 3: entirely and highly condensed cells with no distinguishable cytoplasm. (f) Type 4: weakly condensed "ghost" cells with no discernable intracellular components. CC, corpus callosum; Pyr, pyramidal cells of either CA1 (a) or piriform cortex (b).

the "exact" manual counting. Only a few manual corrections (mainly, separation of some aggregated contiguous cells counted as one) were required before obtaining figures that were comparable to those obtained by manual counting (Table 3).

Table 3
Manual and semi-automatic counting of H&P+ and W+ cells in matching pairs of contiguous sections.

Manual counting of cell numbers		
Total all regions (mean ± SEM per photograph)	H&P+	2882 (49.6 ± 6.1)
	W+	2913 (50.2 ± 6.1)
Semi-automatic counting of cell numbers		
Total all regions (mean ± SEM per photograph)	H&P+	2961 (51.0 ± 6.1)
	W+	2933 (50.5 ± 6.1)
Time (min) required for the semi-automatic counting		
Total all regions (mean ± SEM per photograph)	H&P+	335.8 (5.7 ± 0.4)
	W+	100.4 (1.7 ± 0.1)

H&P+ and W+ cells were counted on 58 pairs of microphotographs (20×) of topographically matching cerebral areas from contiguous sections stained with either H&P or the Woelcke method; brain regions included the CA1 sector of the dorsal hippocampus (21 pairs of photographs), the piriform/amygdala region (19 pairs of photographs), the auditory cortex (9 pairs of photographs), and the dorsolateral nucleus of the thalamus (9 pairs of photographs). In CA1, the counting was restricted to the pyramidal layer, only while in other regions it was performed on the entire surface of the microphotographs.

The entire process of counting on Woelcke-stained sections was 3–4 times quicker than the equivalent counting on the H&P-stained sections (less than 2 min per photograph: Table 3).

3.3. Other advantages of the Woelcke staining

3.3.1. Reduced impact of poor tissue fixation

When dissecting mouse brains, we incidentally found some small pinkish patches at the surface of the cortex. This macroscopic observation was rare but suggested that, at least in some limited zones, perfusion could occasionally fail to entirely drain blood from brain vasculature. Incomplete fixation was therefore expected. Indeed, straight below these zones, exploration of H&P-stained sections invariably revealed more or less numerous cells appearing almost uniformly hyperchromatic, showing a monotonous blue-red or violet tint and often indistinct nuclei in deformed perikarya (Fig. 3a). These abnormal cells more than probably represented poorly fixed neurons with features that differed from those of truly eosinophilic neurons marked by intense acidophilia (red stain) of the cytoplasm and clear basophilia (dark blue stain) of the nucleus. Among these cells, it was extremely difficult to identify those which are really dying. Semi-automatic counting of H&P+ cells was therefore virtually impossible. In contrast, in the same badly fixed areas, the various types of W+ cells in Woelcke-stained sections (Fig. 3b) were still unambiguously recognizable while light-gray cells that were never detected in adequately fixed tissue probably represented ill-fixed but not pathologically damaged cells. Spotting and counting the real W+ cells in such badly fixed tissue was therefore still possible.

3.3.2. Staining of red blood cells and detection of extravasation

Clusters of highly contrasted small round cells, stained in dark blue, were occasionally detected on the Woelcke-stained sections of the brain of convulsing mice (Fig. 3d). By comparing with H&P counterparts, these cells unambiguously appeared to be extravasated erythrocytes in parenchymal or subarachnoidal microhemorrhagic zones which, due to the high contrast of the hematoxylin-loaded red blood cells, were much easier to detect with the Woelcke method than with the H&P method.

3.3.3. Compatible with immunohistochemistry

As illustrated in Fig. 3c, Woelcke counterstaining of GFAP-treated sections proved to be helpful, by outlining myelin fibers, thus allowing better localization of the astrocytic reaction. Furthermore, the double labeling allowed the simultaneous

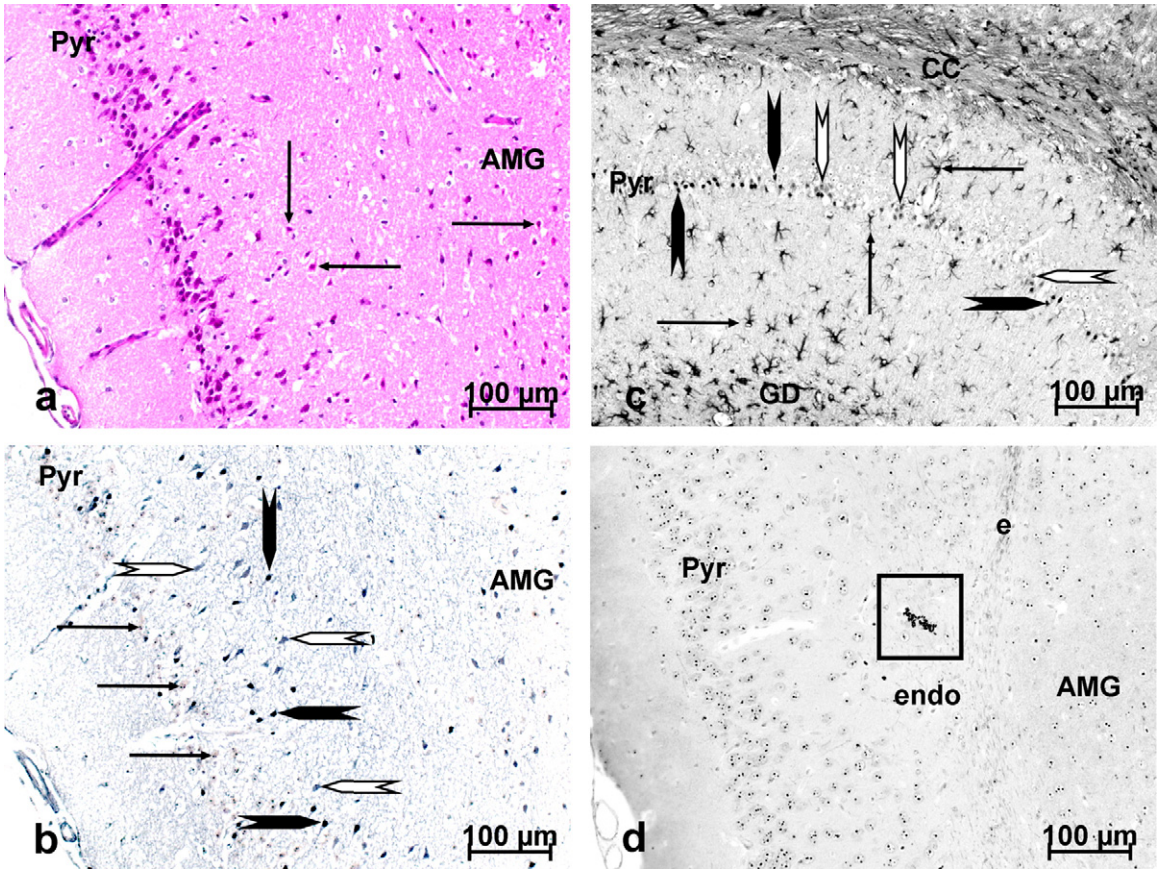

Fig. 3. Other advantages of Woelcke staining (20×). *H&P (a) and Woelcke (b) staining in poorly fixed piriform cortex 24 h post-soman.* In the H&P-stained section (a), hyperchromatic, uniformly purple cells, often with indistinguishable nuclei, are abundant in the pyramidal layer (Pyr); among these cells it is very difficult to detect those which are pathologically damaged. For comparison, unambiguously eosinophilic cells (black arrows) are also present on the same section and show the typical characteristics (red acidophilic cytoplasm, dark blue basophilic pyknotic nuclei) of pathologically degenerating cells. (b) The contiguous Woelcke-stained section: in spite of poor fixation, types 2–3 (black arrowheads) and type 4 (white arrowhead) W+ cells are still easily detectable. Light-gray cells (black arrows) can be seen in the pyramidal layer (Pyr) which probably represent non-pathological, poorly-fixed cells. *GFAP immunohistochemistry and Woelcke couterstain in the hippocampus CA1 sector 24 h post-soman (c).* GFAP-positive astrocytes (thin black arrows) were well individualized by their morphology and brown color; the Woelcke counterstain, by revealing the white matter, helps to precisely identify the observed region and allows the concurrent observation of types 2–3 (thick black arrowheads) and type 4 (thick white arrowheads) W+ cells. *Microhemorrhages in the endopiriform cortex 24 h post soman (d).* Evidencing parenchymal microhemorrhage, a cluster of extravasated erythrocytes (black-framed) can be seen in the otherwise intact region (no detectable W+ cells; however, the thalamus of this particular mouse appeared severely damaged). Subarachnoid microhemorrhages have also been detected in the brains of other mice (not shown). AMG, amygdala; CC, corpus callosum; Pyr, pyramidal cells of either CA1 (c) or piriform cortex (a, b and d); GD, dentate gyrus; endo, endopiriform cortex; e, external capsule. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

observation of reactive astrocytes and damaged cells on the same section.

4. Discussion

Both the present qualitative (localization of cells) and quantitative (number of cells) observations concurred to demonstrate that H&P+ cells and W+ cells represent two aspects of the same degenerating process occurring within the first 24 h of soman-induced *status epilepticus*. The present study also suggests that Woelcke staining, rather than H&P staining, is of interest for rapid counting of damaged brain cells, at least during the most acute phase of soman poisoning (i.e. the first 24–48 h) before significant cell loss and subsequent reduction in normal cell density become a prominent feature (e.g. McDonough et al., 1998; Baille et al., 2005; Collombet et al., 2006).

As mentioned in Section 1, the H&P/H&E method is a widely used method to qualitatively describe the various tissue and cell changes that occur in the brain following soman-induced *status epilepticus*. However, as shown in the present study, semi-automatic counting of H&P+ cells with a computerized analysis system is not always easy to set up and can be excessively time-consuming.

In contrast, the semi-automatic counting of W+ cells proved easy and rapid and the image analysis can readily be programmed and performed by any beginner in the domain. One of the other advantages of the Woelcke method, compared to H&P, is that it allows distinction of actual degenerating cells from artifactual ones in ill-fixed tissue. In our H&P stained sections, the artifactual cells presented some of the characteristics (hyperchromatic, mostly basophilic, perikarya; often indistinct nuclei) of the "dark" neurons that are known to appear after inadequate perfusion–fixation of the brain, post-mortem manipulation of the brain, or insufficient time between perfusion and brain manipulation (e.g. Cammermeyer, 1978; Jortner, 2006 and references therein). However, not all the dark neuron features (e.g. corkscrew-shaped dendrites, separation of the affected neurons from the adjacent neuropile) could be clearly observed in our sections. This suggests that dark neurons are not the unique artifact that may appear after suboptimal fixation and that fixation-related cell changes may present various aspects. Finally, the Woelcke method also allows easy identification of microhemorrhages, an observation already noticed a long time ago (Lemercier et al., 1983), as well as at least some kinds of double labeling such as that with the astrocytic marker GFAP.

There are other methods for studying brain damage. For instance, silver impregnation techniques have been considered superior as they were thought to be very selective for degenerating neurons. However, argyrophilia can label neurons that will ultimately die or recover (Zsombok et al., 2005; Schmued et al., 2005). Moreover, the techniques are rather capricious, require intensive and time-consuming labor, and are not compatible with most multiple procedures. A significant advance within the last 15 years was the development of the Fluoro-Jade (FJ) dyes, fluorescent ligands that can stain degenerating neurons in conspicuous bright green (Schmued et al., 1997, 2005). These fluorochromes, especially the latest development, Fluoro-Jade C, possess considerable advantages as they produce high resolution labeling that is photostable and compatible with many technical conditions (e.g. fixed or unfixed tissues) and with many other histochemical or immunofluorescent markers (e.g. NeuN, GFAP, caspases). Due to their multiple qualities, FJ methods are used more and more in the field of soman poisoning (Dorandeu et al., 2005, 2007; Myhrer et al., 2005; Kan et al., 2005; Collombet et al., 2006). However, some data indicate that FJ might not be entirely specific for degenerating neurons as it could also stain activated microglia (phagocytic microglia/macrophages) and quiescent and reactive astroglia in some cases (Colombo and Puissant, 2002; Anderson et al., 2003; Saganova et al., 2006; Damjanac et al., 2007) as well as erythrocytes (Krinke et al., 2001). Moreover, FJ staining is characterized by virtually no background labeling, thus hindering the topographical identification of the brain structures where cell changes occur. Finally, in a study on soman poisoning, Kan et al. (2005) showed that the number of FJ-positive cells peaked at 12 h and, surprisingly, was considerably reduced at 24 h. This observation contrasts with that from numerous H&E/H&P studies indicating that, after soman-induced *status epilepticus*, numerous degenerating cells could be detected not only at 24/48 h when their number generally peaks but also in the following days (e.g. Lemercier et al., 1983; McDonough et al., 1998; Baille et al., 2005; Collombet et al., 2006). This difference may suggest the appearance of false negatives over time and that caution should be exercised when evaluating FJ-stained tissue of animals at longer time points. Nevertheless FJ-positive cells were still detected 7 days after soman-induced *status epilepticus* in guinea-pigs (Dorandeu et al., 2007).

5. Conclusion

In conclusion, among other available techniques, each with their own advantages and drawbacks, the Woelcke method deserves special consideration for evaluating the acute brain injury following soman-induced *status epilepticus* and probably in other cerebral pathologies: it is not perturbed by bad tissue fixation, can easily detect extravasated erythrocytes in the brain parenchyma, well delineates the tissue architecture and is compatible with GFAP immunohistochemistry. Above all, this cheap and simple method may supplement the others in quantitative studies as it may facilitate rapid counting of damaged cells on a large collection of brain sections.

Conflict of interest

There are no conflicts of interest.

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