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Metallic gold treatment reduces proliferation of inflammatory cells, increases expression of VEGF and FGF, and stimulates cell proliferation in the subventricular zone following experimental traumatic brain injury

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Summary. Traumatic brain injury represents a leading cause of morbidity in young individuals and there is an imperative need for neuroprotective treatments limiting the neurologic impairment following such injury. It has recently been demonstrated that bio-liberated gold ions liberated from small metallic gold implants reduce inflammation and neuronal apoptosis, while generating an increased neuronal stem cell response following focal brain damage. In this study mice were subjected to a unilateral traumatic cryo-lesion with concomitant injection of 25-45 μm gold particles near the lesion. Placebo-treated mice subjected to cryo-lesion served as controls. The effects of gold-treatment were investigated by examining gold-induced growth factor expression (VEGF and FGF) in the first two weeks after the insult, and the extent of the neurostimulatory effect of gold was explored by comparing cell proliferation in the subventricular zone as judged by immunohistochemical staining for CDC47. Vimentin staining revealed a decrease in activated microglia and a transient astrogliosis in response to the gold liberation. Moreover, gold ions significantly increase the expression of VEGF and FGF following trauma and a significant increase in cell proliferation in both the ipsilateral and the contralateral subventricular zone was found in response to gold-treatment. In conclusion: we confirmed the previously demonstrated anti-inflammatory effect of bio-liberated gold ions, and further show that metallic gold increases growth factor expression and adult neurogenesis.

Key words: Dissolucytosis, Growth factor, Metallic gold, Regeneration, Traumatic brain injury

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Introduction

The global annual incidence of head trauma is about 106 per 100,000 (Hyder et al., 2007) and in the western world alone, at least 11.5 million people are currently living with traumatic brain injury (TBI)-related disabilities (Bramlett et al., 2004; Schouten, 2007), which result in personal suffering and high costs to society.

Most adult human tissues retain a reservoir of self-renewing, multipotent stem cells that can generate new cells. Until recently, the brain was thought to represent an exception to this general rule, as neurogenesis (the process of generating functionally integrated neurons from progenitor cells) was believed only to occur during embryonic development in the mammalian central nervous system (CNS), and the predominant repair mechanism in the adult CNS was generally regarded as being restricted to post mitotic sprouting of axon terminals and synaptic reorganization (Ming and Song,

Abbreviations. AMG: Autometallography; CNS: Central nervous system; GFAP: Glial fibrillary acidic protein; FGF: Fibroblast growth factor; IL: Interleukin; NSC: Neural stem cells; ROS: Reactive oxygen species; SVZ: Subventricular zone; TBI: Traumatic brain injury; VEGF: Vascular endothelial growth factor

2005). However, there is now abundant evidence that neural stem cells (NSC) of astrocytic lineage persist in the adult brain, and that they can differentiate into astrocytes, oligodendrocytes and neurons, thus supporting ongoing neurogenesis in the CNS (Alvarez-Buylla et al., 2001; Simard and Rivest, 2004; Ihrie and Alvarez-Buylla, 2008). In most mammals, active neurogenesis has been shown to occur throughout life in a few restricted areas of the CNS, namely the subventricular zone (SVZ) of the lateral ventricle and in the sub granular zone of the dentate gyrus in the hippocampus (Gage, 2002; Simard and Rivest, 2004; Ihrie and Alvarez-Buylla, 2008). Neurogenesis outside these two regions appears to be extremely limited, but may occur after pathological stimulation, such as brain insults (Ming and Song, 2005). The demonstration of active adult neurogenesis provides an exciting possibility for repairing the adult CNS after injury or degenerative neurological diseases by means of stimulating the endogenous population of stem cells.

In TBI the primary damage to the CNS elicits a characteristic inflammatory response (Quintana et al., 2005; Penkowa, 2006; Werner and Engelhard, 2007), which is characterized by activation of resident microglia and blood-derived monocytes, as well as astrocytes showing hypertrophy and reactive astrogliosis (Penkowa et al., 1999a-c; Giralt et al., 2002; Quintana et al., 2005). The inflammatory cascade is an attempt by the system to eliminate the challenge imposed on the brain, remove dead and damaged neurons, and rescue the normal functioning of this vital organ. However, during the inflammatory process pro-inflammatory cytotoxic mediators, such as tumor necrosis factor alpha (TNFα) and interleukin (IL)-1α/ß are generated, along with tissue-damaging reactive oxygen species (ROS), and an uncontrolled immune response has also been shown to impair NSC survival and proliferation, as well as blocking tissue repair processes (for a review see Das and Basu, 2008).

Outside the CNS, gold compounds have been used for treating the autoimmune condition *rheumatoid arthritis* for more than 70 years (Yanni et al., 1994; Yoshida et al., 1999; Zhang and Lippard, 2003) as gold ions exhibit anti-inflammatory properties, such as inhibition of the lysosomal enzymes of phagocytic cells in inflamed tissues (Griem and Gleichmann, 1996). Gold ions have also been shown to inhibit nuclear factor-kappa B (NF-κB) DNA-binding activity which indirectly results in decreased synthesis of pro-inflammatory cytokines (Yanni et al., 1994; Traber et al., 1999; Yoshida et al., 1999; Eisler, 2003). Despite the immunomodulatory properties of gold ions, the use of gold compounds has been limited in the later years as the systemic spread of gold ions from such gold salts results in adverse effects, especially nephrotoxicity (Schiff and Whelton, 2000; Eisler, 2003).

In the brain, gold implants have been shown to release gold ions (Danscher, 2002) by means of macrophage-induced dissolucytosis (Larsen et al., 2007, 2008), whereupon the gold is engulfed and accumulated in the lysosomes of microglia, astrocytes and neurons near the implant (Danscher, 2002). Dissolucytosis is an extracellular liberation of gold ions taking place in an ultrathin membrane or biofilm, the dissolucytosis membrane, located between the macrophages and the gold surface. This membrane, at least partly secreted by the macrophages, generates a controlled chemical environment in which a very slow liberation of gold is seen (Larsen et al., 2007). Dissolucytosis also takes place outside the CNS and constitutes a localized and thus safe way of administrating gold ions (Danscher, 2002). The effects of bio-released gold ions from metallic gold implants have recently been investigated with regard to its potential for alleviating inflammation following brain injury in mice (Larsen et al., 2008). In this study gold implants were shown to dampen the activation of microglia/macrophages and induce transient astrogliosis (as judged by immunohistochemical staining for Glial fibrillary acidic protein (GFAP) and Lectin. Furthermore, gold-treatment reduced apoptotic neuronal cell death (as judged by TUNEL staining), and increased the number of Frizzled-9 positive neuronal stem cells (NSCs) (Larsen et al., 2008). The aim of the present study was to further investigate and characterize gold's potential to stimulate regenerative processes following cryo-lesion to the neocortex of mice, i.e. the expression of growth factors (vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)) known to have a vital role in stem cell survival, proliferation, migration and differentiation.

Materials and methods

Animals

Thirty-two 7 week old female C57b16 mice (Taconic M&B, Ry, DK) were used for the study. The animals were housed under standard conditions i.e. a 12-hour light/dark cycle, at 22°C, given free access to food and water. All experimental procedures were performed in accordance with Danish law. The animals were divided into two groups, each consisting of 16 animals. The gold-treated group was injected with metallic gold suspended in sodium hyaluronate (463 mg gold in 0.85 ml hyaluronic acid) intra-cranially before applying the cryo lesion to the neocortex. The control group received only vehicle (sodium hyaluronate injection) prior to the cryo lesion.

Gold treatment

The gold treatment has been described in detail in (Larsen et al., 2008). In brief, 463 mg of 25-45 μm spherical gold particles 99% pure metallic gold (Alfa-Aesar®, Karlsruhe, Ge) was mixed with 0.85 ml of sodium hyaluronate (10 mg/ml, PROVISC®, Alcon, Se). After anesthetization with a mixture of Narcoxyl-vet™

(Xylazin) and Ketominal-vet™ (0.1ml per 10 grams), each animal was placed in a stereotaxic instrument (Benchmark, myNeuroLab.com, St. Louis, MO, USA) and injected with 2x5 μl gold-sodium hyaluronate mixture or pure sodium hyaluronate. The injections were made with a 50 μl Hamilton syringe (700 LT, Hamilton Bonaduz, Switzerland) equipped with a 27 gauge needle using the stereotaxic instrument. The injections were placed in the depth of the neocortex, 1.2 mm from the surface, at the following coordinates 2 mm lateral, 1 mm posterior to Bregma and 2 mm lateral, 3 mm posterior to Bregma, respectively (Paxinos and Franklin, 1997).

Brain injury

Immediately after the injection of gold /sodium hyaluronate or sodium hyaluronate, a small pencil-shaped piece of dry ice (2x2 mm, -78°C) was placed on the extracranial surface of the skull for 30 seconds. The dry ice was placed in the middle between the 2 drill holes.

Our group has applied this model of traumatic brain injury for more than a decade producing consistent and reproducible results (for detailed descriptions see Penkowa et al., 1999c, 2000, 2003).

Fixation and tissue processing

After freeze-lesioning, the animals were returned to their cages for one or two weeks, after which they were anesthetized (Mebumal®, pentobarbital, 50 mg/ml, SAD, DK) and killed by transcardial perfusion. Zamboni´s fixative (2% paraformaldehyde, 15% picric acid in 0.1 M sodium phosphate buffer with pH 7.4) was used for transcardial perfusion of animals destined for histopathological analyses, but at each time point two animals from each group were perfused with 3% glutaraldehyde (Merck 4239 supplied by VWR, Albertslund, DK) to obtain optimal conditions for gold-visualisation (autometallography, AMG). All brains were allowed to post fixate in the same fixative for 24 hours.

For histopathological analyses the brains were dehydrated using standard procedures, embedded in paraffin and cut in serial 5 μm frontal sections.

For light microscopical AMG analyses, the fixated brains were dehydrated, paraffin-embedded and cut in 10 μm frontal sections which were then de-paraffinated, AMG developed (see below) and counterstained with a 0.1% aqueous toluidine blue (pH 4.0), before being embedded in DePex and mounted with a cover glass.

For electron microscopical gold visualization, 100 μm thick vibratome sections were cut and subsequently AMG developed. The areas of interest were cut out and embedded in Epon. 3 μm thick Epon sections were cut and counterstained with toluidine blue. After light microscopical analysis, selected sections were re-embedded on top of a new Epon block from which ultra thin sections were cut, placed on a grid and counterstained with uranyl citrate and lead acetate (Danscher and Stoltenberg, 2006).

Autometallography

One hundred ml of AMG developer was made of 60 ml gum Arabic solution (Bidinger, Aarhus, DK) to which was added 10 ml sodium citrate buffer (25.5 g of citric acid (Merck, 0244 supplied by VWR, Albertslund, DK), 23,5 g sodium citrate (Merck 6448, VWR, DK)) to 100 ml distilled H_2O, 15 ml reducing agent (0.85 g of hydroquinone (Merck 4610, VWR, DK) dissolved in 15 ml distilled water at 40°C), and 15 ml solution containing silver ions (0.12 g silver lactate (Fluka 85210 supplied by Sigma-Aldrich, Vallensbæk, DK) in 15 ml distilled water at 40°C). The AMG developer was prepared immediately before use, thoroughly stirred and placed in a jar in a 26°C water bath. Tissue slices were placed in the jar under a dark hood and developed for 60 min.

The AMG development was stopped by replacing the developer with a 5% thio-sulphate solution for 10 min and the sections rinsed several times in distilled water (for details see Danscher, 2002; Danscher and Stoltenberg, 2006).

Histopathological analyses

Paraffin sections were rehydrated according to standard procedures (3x5 minutes in xylene, 2x5 minutes in 99% alcohol, 1x2 minutes in 96% alcohol and 1x2 minutes in 70% alcohol) before undergoing heat-induced epitope retrieval (HIER) in Tris-base EGTA (TEG) buffer with pH 9.1 for 10 (FGF) or 30 (CDC47, VEGF) minutes. Sections were then incubated in 1.5% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl; with 0.01% Nonidet P-40) (Sigma-Aldrich, St. Louis, MO, USA, code N-6507) for 30 min at room temperature to quench endogenous peroxidase followed by incubation in 10% goat serum (In Vitro, Fredensborg, DK, code 04009-1B) in TBS/Nonidet for 30 min at room temperature to block nonspecific binding. Sections prepared for incubation with monoclonal mouse-derived antibodies (CDC47) were incubated with Blocking Solutions A+B from HistoMouse-SP Kit (Zymed Lab. Inc., Carlsbad, CA, USA, cat. no. 50-300) to quench endogenous mouse IgG. After these steps, sections were ready for immunohistochemistry.

Immunohistochemistry

Sections were incubated overnight at 4°C with the following primary antibodies: Rabbit anti-vimentin (a marker of stem cells, myelo-monocytes and reactive astrocytes) (Azumi and Battifora, 1987) prediluted (ready-to-use) by the manufacturer (Abcam, Cambridge, USA, cat. no.: Ab8545); Rabbit anti-human FGF-2/b-FGF (a marker for fibroblast growth factor) (Rifkin and

Moscatelli, 1989) diluted 1:100 (Santa Cruz, Santa Cruz, CA, USA, cat. no.: sc-79); Rabbit anti-human VEGF (a marker for vascular endothelial growth factor) (Ferrara et al., 1991) diluted 1:50 (Neomarkers, cat. no.: RB-222-P1); and Mouse anti-CDC47 (a marker for cell proliferation) (Suzuki et al., 1998) diluted 1:200 (Neomarkers, Fremont, CA, USA, cat. no.: MS-862-P).

On the second day the primary anti-body was detected using biotinylated secondary antibodies (Anti-mouse IgG, Sigma-Aldrich, St. Louis, MO, USA, cat. no.: 8774, diluted 1:200 for CDC47 staining, and Anti-rabbit IgG, Sigma-Aldrich, St. Louis, MO, USA, cat. no.: 3275, diluted 1:400 for Vimentin, VEGF and FGF staining), incubating for 30 min at room temperature. The staining was then enhanced using streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dako, Glostrup, DK, code K377) and tyramide signal amplification (TSA)-kit (NEN, Life Science Products, Waltham, MA, USA, code NEL700A), which were both applied following the manufacturer's recommendations. Finally, the immunoreactions were visualized by using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature.

Fluorescence labelling

In order to distinguish in what cell types FGF and VEGF were expressed in the lesion area, double staining for VEGF and FGF with the neuronal marker NeuN and Glial fibrillary acidic protein (GFAP) was carried out. Sections were incubated overnight at 4°C with Rabbit anti-human FGF-2/b-FGF diluted 1:100 (Santa Cruz, Santa Cruz, CA, USA, cat. no.: sc-79); Rabbit anti-human VEGF diluted 1:50 (Neomarkers, cat. no.: RB-222-P1); Mouse anti-NeuN diluted 1:100 linked with Alexa Fluor 488 (Chemicon, cat. no. MAB 377X); and/or Rat anti-bovine GFAP diluted 1:100 (Zymed, cat. no. 13-0300). On the second day, VEGF and FGF were detected by using Goat anti-rabbit IgG 1:30 linked with Texas red (TXRD) (Jackson ImmunoResearch Lab., Inc., West Grove, PA, Code 111-075-144), while GFAP was detected using Anti-rat IgG 1:20 linked with fluorescein (FITC) (Calbiochem, Calbiochem-Novabiochem Corp., La Jolla, CA, cat. no. 401414). Since the primary antibody for NeuN was linked with Alexa Fluor 488, no secondary antibody was needed for its detection. The secondary antibodies were used simultaneously for 30 min at room temperature. The sections were then embedded in fluorescent mounting (Dakopatts, Code S3023) and kept in darkness at 4°C.

For the examination and recording of the stainings, a Zeiss Axioplan2 light microscope equipped with a tripleband (FITC/TXRD/AMCA) filter was used.

Morphological analysis of the tissue response to injury

Vimentin stained tissue sections were used for the morphological evaluation of astrocytes and the presence of activated microglia/macrophages. Reactive microglia were defined as clearly stained round cells without processes in the lesion area, while reactive astrocytes were defined as hypertrophic cells with clear astrocytic morphology and clearly distinct cellular processes. In addition, the NSCs were defined as CDC47 positively stained cells in the SVZ that were round and without visible processes (i.e. the cells were differentiated from astrocytes in that they lacked processes, and were larger than reactive microglia seen in the lesion area). Moreover, NSCs migrating from the SVZ towards the lesion area were defined as CDC47 positive cells that matched the before mentioned criteria, but were localized outside the SVZ between the upper lateral corner of the ventricle and the lesion area.

Cell counts and statistical analysis

In addition to the morphological evaluation, cell counts of the variables analyhzed were carried out in the 5 μm brain sections. For growth factors these quantifications were carried out in 0.16 mm^2 matched areas of the upper at the borderzone between the lesioned and unlesioned tissue in the centre of the lesion, as described in Penkowa et al., 2000. In the sections immunohistochemically stained for CDC47, cell counts were performed in the SVZ at 0.16 mm^2 matched areas of the upper lateral corner, as well as along a 0.45 mm section of the middle part of the lateral wall of the lateral ventricles. All cell counts were performed in a blinded manner, with positively stained cells defined as cells with positive staining of the cell soma. For each parameter analyzed, brain sections from at least 3 mice (n=3-6) of each group were used and a mean value of the positively stained cells was calculated. Group means of gold-treated and control animals were compared using Student's t-test. Results were considered statistically significant with a 2P-value < 0.05.

Results

Localized gold liberation from the injected gold particles

AMG development revealed a sparse gold liberation seen as very fine AMG grains near the gold deposits (Fig. 1a,b) and the neighbouring lesion area. The AMG grains i.e. silver-enhanced gold nano particles were seen in glial cells (arrow in Fig. 1a) and neurons (Fig. 1b). The gold accumulation was purely cytoplasmatic and electron micrographs confirmed their location within lysosome-like organelles (Fig. 1c). All control sections were void of AMG staining.

Gold-treatment reduces signs of inflammation following traumatic brain injury

Vimentin staining of the lesioned area revealed a marked difference between the gold-exposed and the placebo-treated control animals one week post injury. At this time point, the gold-treated animals displayed a

clearly visible reduction in the number of activated microglia, as well as a visible increase in reactive astrogliosis (Fig. 2b,d), as compared to the control group (Fig. 2a,c). Two weeks post injury the extent of the lesion was smaller in both groups of animals, and there were fewer proliferating cells in the lesion area in both groups, as compared to the animals at 1 week. Moreover, at this point the majority of the proliferating cells had the morphological characteristics of reactive and hypertrophied astrocytes, while there were only very few visible activated, round microglia surrounding the lesion (Fig. 2e-h).

Bio-released gold ions increase the expression of growth factors in the lesion area following experimental traumatic brain injury

As depicted in Table 1 and Figs. 3-5, gold-treated animals showed a statistically significant increase in the expression of VEGF (2P = 0.0080) (Figs. 3, 5) at 1 week post lesion, compared to controls. There was no significant increase in FGF expression at this point (Figs. 4, 5). At two weeks post lesion, however, the gold-treated animals showed a significantly higher expression of both growth factors compared to controls (2P<0.05, Table 1).

Gold-induced proliferation and migration of neural stem cells in the SVZ

The stem cells of the SVZ were investigated by immunohistochemical staining for CDC47, which is a known marker of mitosis, staining proliferating cells, including NSCs. In the ipsilateral hemisphere, gold-treated animals showed a statistically significant increase in the number of CDC47 positive cells in the lateral wall of the lateral ventricle (107% increase) as well as in the upper lateral corner of the ventricle (83% increase) as early as one week post lesion (Table 1, Figs. 6, 7). A significant 77% increase in CDC47 positive cells in the lateral wall of the ventricle was still seen 2 weeks post lesion (Table 1). Furthermore, at this time point the gold-

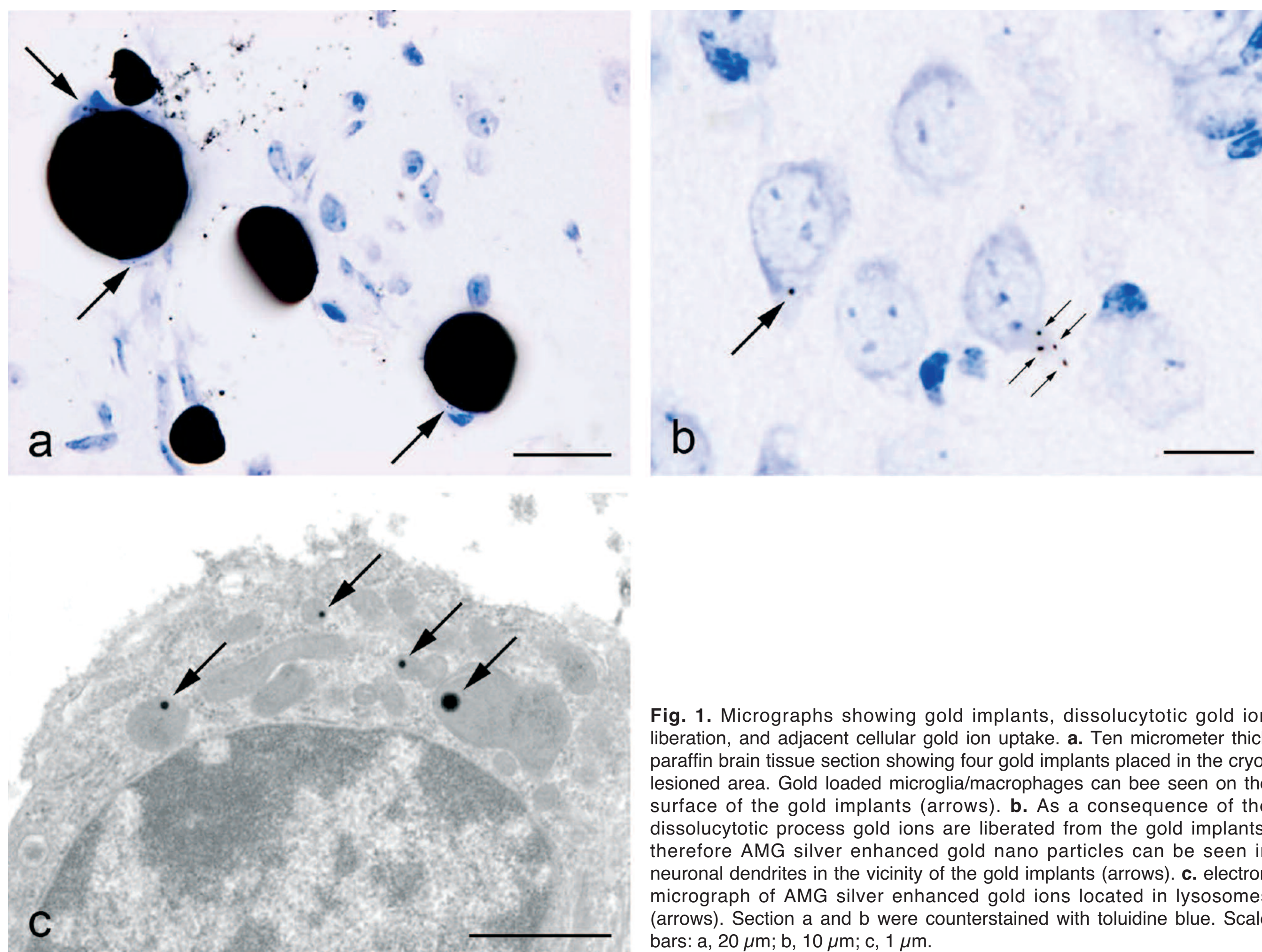

Fig. 1. Micrographs showing gold implants, dissolucytotic gold ion liberation, and adjacent cellular gold ion uptake. **a.** Ten micrometer thick paraffin brain tissue section showing four gold implants placed in the cryo-lesioned area. Gold loaded microglia/macrophages can bee seen on the surface of the gold implants (arrows). **b.** As a consequence of the dissolucytotic process gold ions are liberated from the gold implants; therefore AMG silver enhanced gold nano particles can be seen in neuronal dendrites in the vicinity of the gold implants (arrows). **c.** electron micrograph of AMG silver enhanced gold ions located in lysosomes (arrows). Section a and b were counterstained with toluidine blue. Scale bars: a, 20 μm; b, 10 μm; c, 1 μm.

Table 1. Statistical information regarding the cell counts.

Staining type	Days Post lesion	Control		AU		P-value
		Mean	SEM	Mean	SEM	
bFGF/FGF-2	1 week (n=3)	4.333	2.186	14.00	9.018	0.3564
	2 weeks (n=5)	9.40	3.487	32.60	6.779	0.0160 (*)
VEGF	1 week (n=6)	34.17	9.235	79,83	10.280	0.0080 (**)
	2 weeks (n=5)	22.60	9.474	64.40	10.220	0.0171 (*)
CDC47 Ipsilateral corner	1 week (n=5)	24.80	5.361	51.40	7.194	0.0180 (*)
	2 weeks (n=4)	9.75	2.175	12.50	0.957	0.2911
CDC47 Ipsilateral wall	1 week (n=5)	20.20	2.615	37.00	5.612	0.0265 (*)
	2 weeks (n=5)	10.60	0.927	18.80	2.709	0.0210 (*)
CDC47 Contralat corner	1 week (n=5)	48.60	10.520	44.80	7.921	0.7802
	2 weeks (n=3)	2.33	2.333	12.00	2.517	0.0480 (*)
CDC47 Contralat wall	1 week (n=5)	25.20	3.200	30.60	2.088	0.1953
	2 weeks (n=3)	5.33	2.906	14.00	0.577	0.0430 (*)

treated animals also displayed a clear increase in the migration of positively stained cells from the corner area of the ipsilateral ventricle towards the lesion site (Fig. 7). Contra-laterally, stem cell proliferation had increased both in the corner areas (515% increase) and lateral wall (262% increase) of the SVZ two weeks post lesion when comparing gold-treated animals to controls.

Discussion

The discovery of neural stem cells (NSCs) *in vivo* in the adult human brain has revolutionized the field of neuroscience and has provided new possibilities for treating neurodegenerative diseases. However, even though TBI has been shown to stimulate neurogenesis, the inflammatory response initiated by the trauma have detrimental effects on the NSCs, since several microglia-derived pro-inflammatory mediators have been shown to impair neurogenesis (Das and Basu, 2008; Ekdahl et al., 2003; Simard and Rivest, 2004). Because of this, and the small number and low mitotic activity of the NSCs, the endogenous capacity of the intact mammalian brain to regenerate and replace damaged tissue is limited (Ming and Song, 2005); however, the demonstration of adult neurogenesis is still exciting, since it provides a possibility for repairing brain tissue either by transplantation or by means of endogenous stimulation.

We have recently reported a gold- induced increase in FRIZZELED-9 positive neuronal progenitor cells (Larsen et al., 2008) and our present study confirms that the presence of gold-liberating implants within a traumatized brain area results in a gold-induced neurogenesis within the SVZ. This was confirmed by an increase in CDC47 positive cells with NSC-like morphology seen within this region.

Based on the sparse and highly localized gold liberation seen when implanting gold particles (Fig. 1; Danscher, 2002; Larsen et al., 2007, 2008) and the considerable distance between the traumatized cortical area and the SVZ we hypothesized that gold-induced neurogenesis involves the release of diffusible factors from cells near the center of the trauma. This hypothesis is supported by our findings of a contra-lateral effect on the NSCs, in that the effects were seen as early as 1 week post lesion on the ipsilateral side, spreading out to the contralateral side 2 weeks after the lesion. A vast number of studies have demonstrated the importance of the growth factors VEGF and FGF for the survival, proliferation and migration of NSCs, and it is well known that the expression of VEGF and FGF increase following TBI (Mocchetti and Wrathall, 1995; Zachary, 2005; Agasse et al., 2007). VEGF is known to exhibit neurotrophic properties acting directly on neurons, astrocytes, and NSCs (Jin et al., 2002; Zachary, 2005). Furthermore, intraventricular infusion of VEGF has been shown to promote NSC survival, proliferation and differentiation (Schänzer et al., 2004; Shen et al., 2004; Meng et al., 2006), as well as exhibiting chemotactic effects on NSCs (Zhang et al., 2003), thus making the gold-induced increase in VEGF expression we see in the gold-treated animals (Fig. 3) a likely mechanism involved in gold-induced neurogenesis. Thus, it could be hypothesized that the gold-mediated increase in VEGF expression in the lesion area could result in secretion of VEGF from synthesizing cells, whereupon the VEGF released through diffusion could stimulate the NSCs in the SVZ. Thus, the gold-mediated increase in VEGF, in addition to reducing neuronal apoptosis, might serve as a guidance cue for the increased migration of NSCs towards the lesion site seen in this study. As well as the gold-induced increase in VEGF, the present study showed an increase in the expression of FGF in gold-treated animals, as compared to controls (Fig. 5). This finding is well in accordance with the effects of gold on the NSCs, as some reports have suggested that the stimulatory effect of VEGF on NSCs proliferation and migration is FGF dependent (Zhang et al., 2003; Xiao et al., 2007), just as FGF has been shown to enhance both

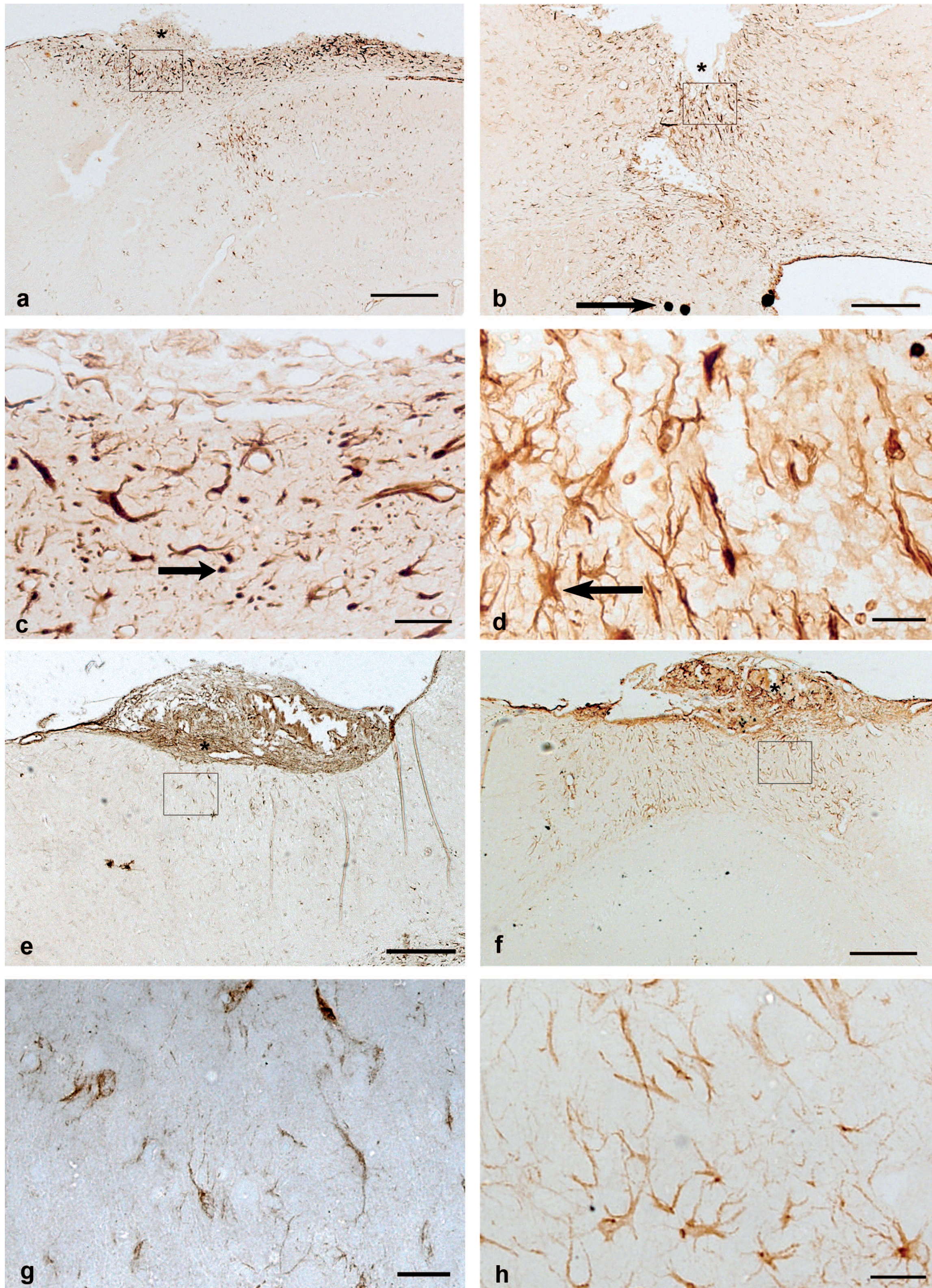

Fig. 2. Immunostaining for Vimentin at 1 week **(a-d)** and 2 weeks **(e-f)** post lesion. **a.** At 1 week, placebo-treated mice showed a small increase in reactive astrogliosis and a marked increase in activated microglia in the lesion area (asterisk), compared with gold-treated mice **(b)**, which showed a marked increased in reactive astrogliosis, while only a few reactive microglia were seen. Note the injected gold particles seen close to the lesion area (arrow) seen in **(b)**. **c.** Higher magnification of the framed area in **(a)**. The black arrow points toward one of the many reactive microglia cells seen in the lesion area. d: Higher magnification of the framed area in **(b)**. The black arrow denotes one of the reactive astrocytes seen in the gold-treated animals. **e-f.** At 2 weeks post lesion control mice **(e)** and gold-treated mice **(f)** showed a lesion (asterisk) surrounded mainly by reactive astrocytes. **g-h.** Higher magnifications of the framed areas in e and f, respectively. Scale bar: a, b, e, f, 250 μm; c, d, g, h, 25 μm.

proliferation and survival of NSCs on its own (Sun et al., 2002; Monfils et al., 2006).

The present study also confirms the previously found anti-inflammatory effect of gold implants, as a clearly visible decrease in the number of activated microglia is seen in the gold-treated animals at 1 week post lesion, as compared to hyaluronate(placebo)-treated controls (Fig. 2a-d). Extensive research results show that microglia-derived inflammatory mediators (including cytokines, ROS and complement) contribute to both acute and chronic tissue damage following TBI (for a review see Lucas et al., 2006). Moreover, along with this activation of microglia and their production of pro-inflammatory cytokines, astrocytes become activated

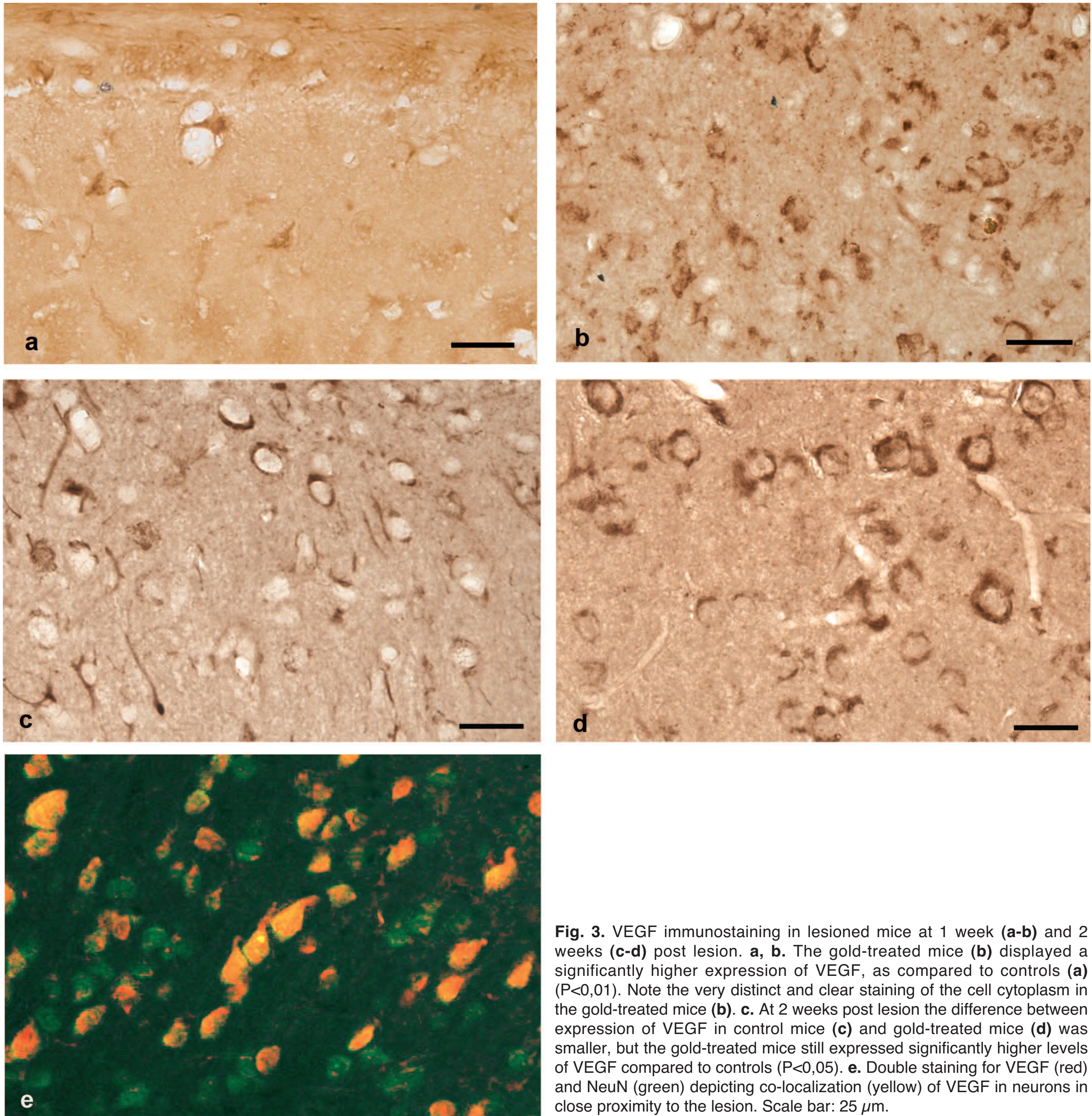

Fig. 3. VEGF immunostaining in lesioned mice at 1 week **(a-b)** and 2 weeks **(c-d)** post lesion. **a, b.** The gold-treated mice **(b)** displayed a significantly higher expression of VEGF, as compared to controls **(a)** ($P<0,01$). Note the very distinct and clear staining of the cell cytoplasm in the gold-treated mice **(b)**. **c.** At 2 weeks post lesion the difference between expression of VEGF in control mice **(c)** and gold-treated mice **(d)** was smaller, but the gold-treated mice still expressed significantly higher levels of VEGF compared to controls ($P<0,05$). **e.** Double staining for VEGF (red) and NeuN (green) depicting co-localization (yellow) of VEGF in neurons in close proximity to the lesion. Scale bar: 25 μm.

and induce protective and tissue sparing mechanisms, including increased expression of several proteins important to neuroprotection and regeneration, such as granulocyte-macrophage colony-stimulating factor, FGF, VEGF and several transcription factors (Nakagawa et al., 2005). Prevention of this reactive astrogliosis has been shown to reduce scar formation, increase the magnitude and duration of inflammation and increase neural cell loss, degeneration and tissue damage following TBI (Faulkner et al., 2004; Sofroniew, 2005; Laird et al., 2008). This highlights a unique feature of the gold-treatment, an increased astrogliosis occurring alongside the decreased microgliosis in the early phase of trauma-induced inflammation (Fig. 2; Larsen et al.,

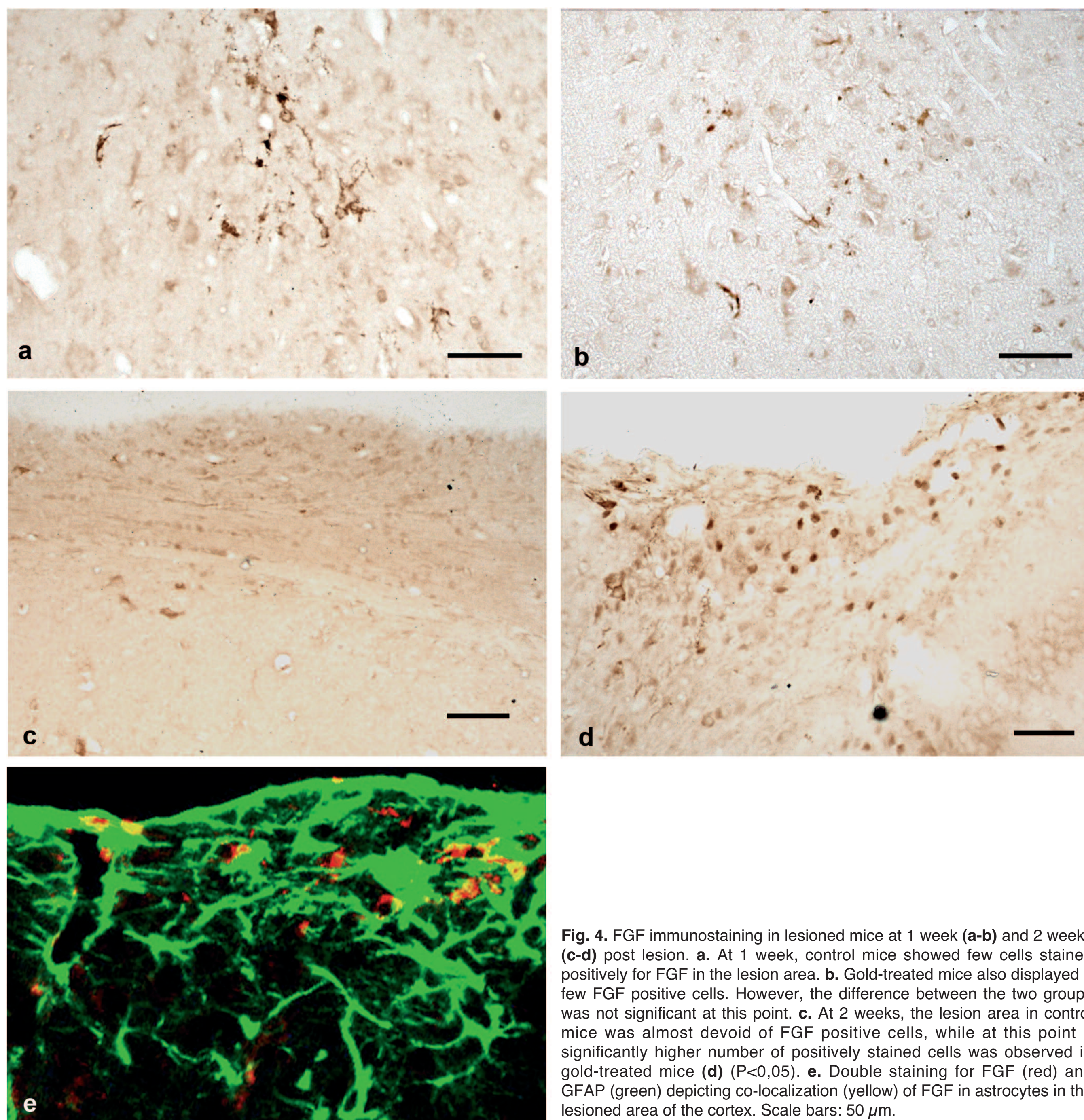

Fig. 4. FGF immunostaining in lesioned mice at 1 week **(a-b)** and 2 weeks **(c-d)** post lesion. **a.** At 1 week, control mice showed few cells stained positively for FGF in the lesion area. **b.** Gold-treated mice also displayed a few FGF positive cells. However, the difference between the two groups was not significant at this point. **c.** At 2 weeks, the lesion area in control mice was almost devoid of FGF positive cells, while at this point a significantly higher number of positively stained cells was observed in gold-treated mice **(d)** (P<0,05). **e.** Double staining for FGF (red) and GFAP (green) depicting co-localization (yellow) of FGF in astrocytes in the lesioned area of the cortex. Scale bars: 50 μm.

2008). Thus, the gold-treatment not only decreases the detrimental aspects of the inflammatory response (namely the activation of microglia), but simultaneously increases the protective aspects of inflammation by increasing the reactive astrogliosis, further limiting tissue loss.

It has previously been shown that gold-treatment diminishes neuronal cell death (as judged by TUNEL) in gold-treated animals during the first weeks following an experimental TBI (Larsen et al., 2008). This could be caused by the anti-inflammatory effects of bio-liberated gold ions (Fig.2; Larsen et al., 2008), but could also be explained by the increased synthesis of VEGF demonstrated in this study (Fig. 3). VEGF has been shown to improve survival in neurons by directly inhibiting programmed cell death via activation of the phosphatidyloinositol 3-kinase/Akt pathway signal transduction pathway, which, in consequence, leads to the inhibition of programmed cell death by activation of anti-apoptotic proteins and inhibition of pro-apoptotic signaling by Bad, caspase-9 and caspase-3 (Zachary, 2005; Kilic et al., 2006). This is well in accordance with the multi-factorial role of VEGF in the central nervous system, where neuroregenerative and neuroprotective effects seem to be at least as important as the angiogenic effects of VEGF. The double-stainings performed in the present study showed that VEGF is primarily expressed in neurons (Fig. 3), while FGF to a larger extent was expressed by astrocytes (Fig. 4) in the lesioned area. This could indicate that gold ions acted differently on different cell types and might be the reason for the somewhat delayed increase in FGF expression, as compared to VEGF, observed in this study.

Based on the present findings, we hypothesize that gold implants i.e. non-phagozytosable gold particles (>20μm) with vast surfaces, injected in a mixture with

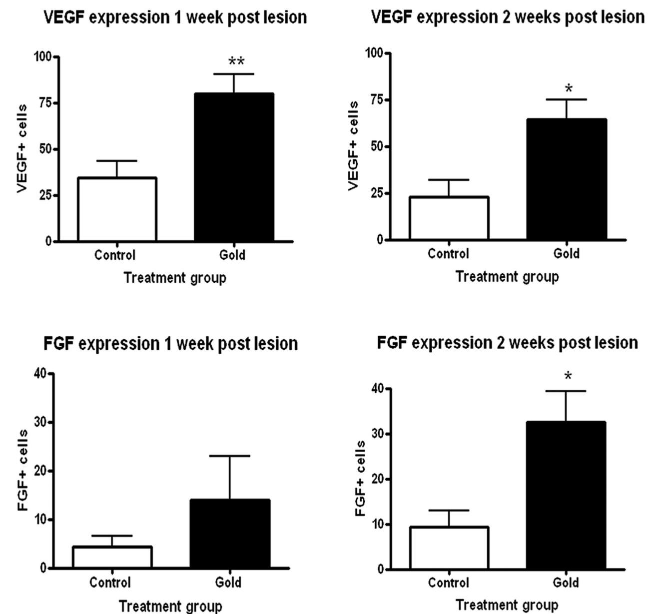

Fig. 5. Box plots depicting the expression of VEGF and FGF (cell counts, mean ± SEM) seen at the lesion site in gold-treated mice and controls at 1 and 2 week(s) post lesion. *: indicates statistically significant difference, 2P<0.05; **: indicates statistically significant difference, 2P<0.01.

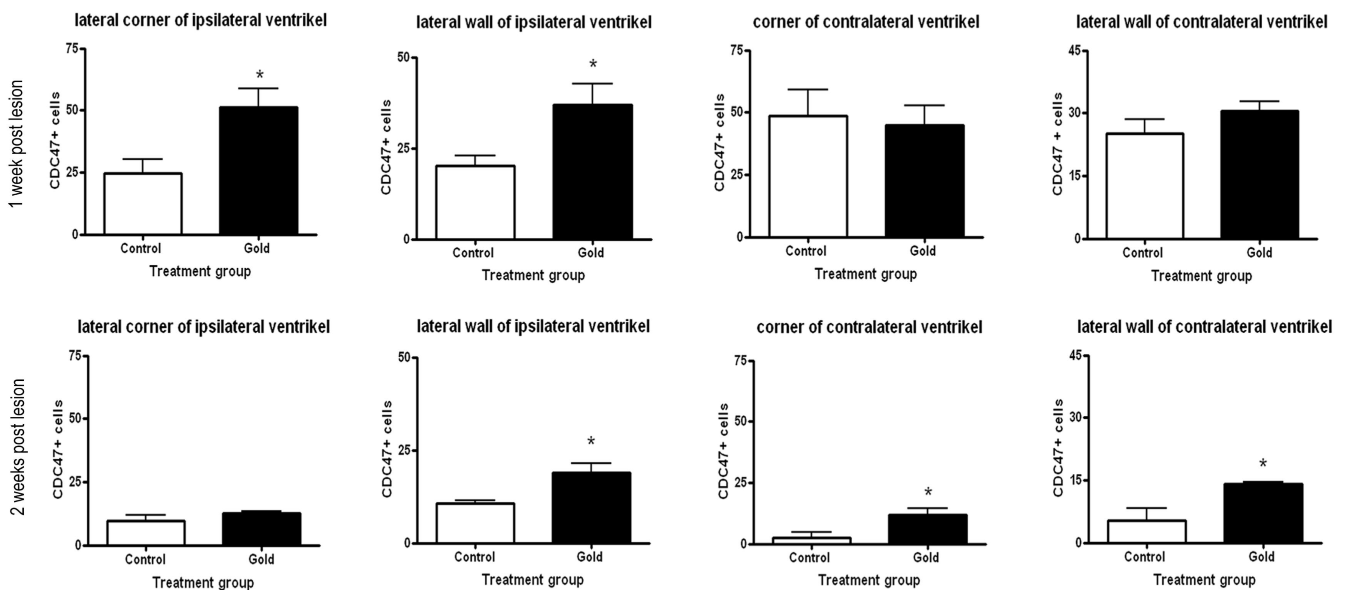

Fig. 6. Box plots depicting the expression of CDC47 (cell counts, mean ± SEM) in the SVZ of the corner and lateral wall of the lateral ventricles. Cell counts are shown for both ipsilateral and contralateral hemispheres 1 week and 2 weeks post lesion in gold-treated animals and controls, respectively. *: indicates statistically significant difference, 2P<0.05.

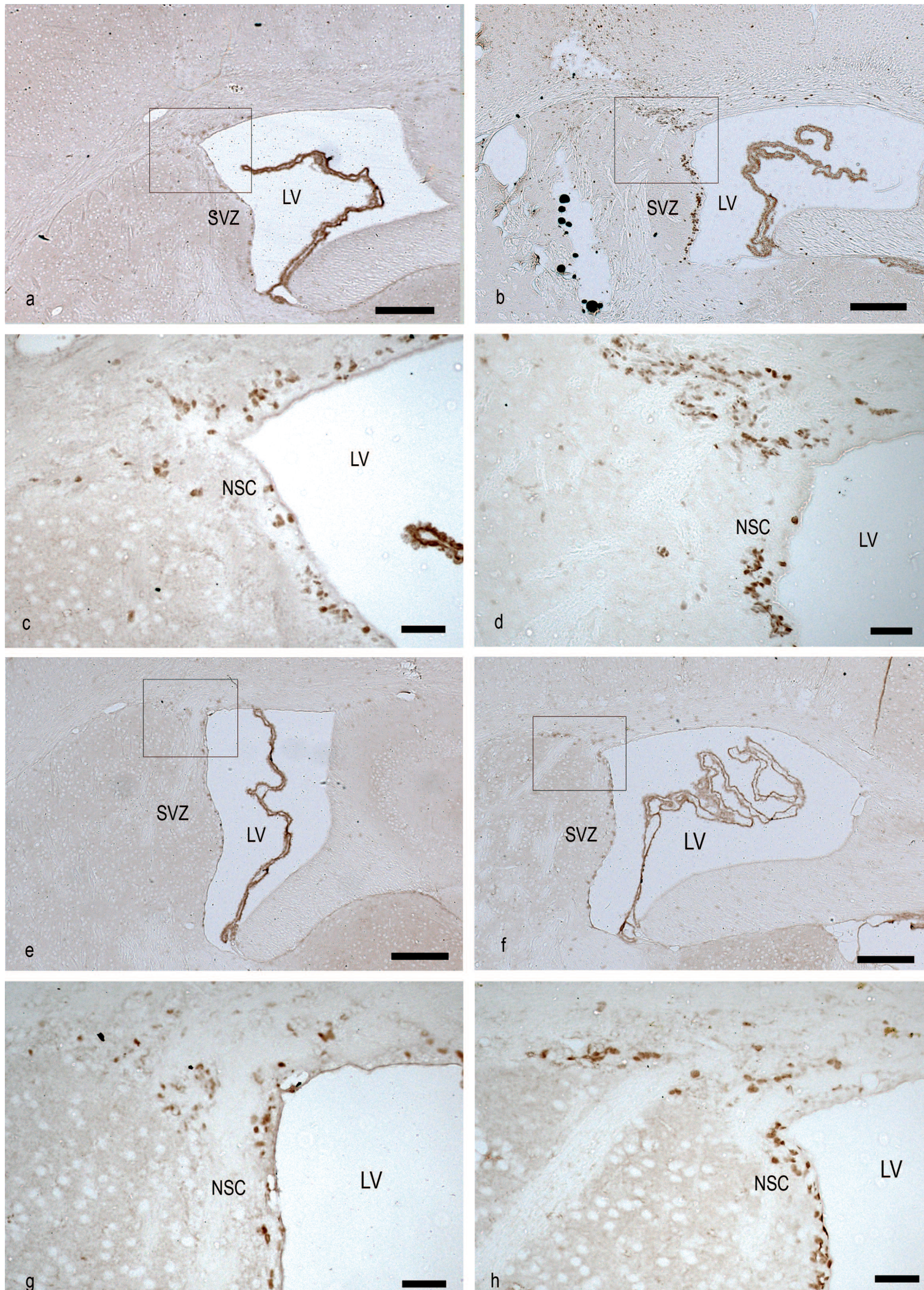

Fig. 7. CDC47 immunostainings in lesioned mice at 1 week (a-d) and 2 weeks (e-h) post lesion. **a, b.** The photos show the lateral ventricle (LV) containing the subventricular zone (SVZ), where the neural stem cells (NSC) are generated. c,d: Higher magnification of the framed areas in (**a and b**), which shows CDC47+ cells in the SVZ of placebo-treated mice **(c)** and gold-treated mice **(d)**, respectively. In both groups, NSCs are found in and adjacent to the SVZ, however, at 1 week post lesion, the gold-treated mice displayed a significantly higher number of CDC47+ cells in the SVZ of both the corner and the lateral wall areas, as compared to controls (P<0,05). Note also the clearly visible increase in cells migrating towards the lesioned area in the gold-treated mice **(d)**. **e, f.** Shows the area corresponding to photo (a) and (b) at 2 weeks post lesion. Many NSCs were detected in the SVZ of animals receiving placebo **(e)** and/or gold **(f)**. However, the gold-treated animals displayed a significantly higher number of CDC47+ cells in the SVZ in both the lesioned and unlesioned cortex, as compared to controls (P<0,05), and also showed an increase in cells migrating towards the lesioned area. **g, h.** Higher magnification of e and f, respectively. Scale bars: a, b, e, f, 250 μm; c, d, g, h, 50 μm.

hyaluronic acid could have clinical potential due to its anti-inflammatory, neuroprotective and neuro-regenerative effects in the CNS. Hyaloronic acid is currently used in ophthalmic surgery and was chosen as a vehicle in the present study due to its availability as a sterile compound well tolerated by the human body, as well as its ability to carry the weight of the heavy gold particles, hereby enabling gold treatment by a minimal invasive technique i.e. injections. It should however be noted that hyaluronic acid can itself act as a biological active compound influencing tissue inflammation (Bot et al., 2008; Heyworth et al., 2008; Thibeault and Duflo, 2008), and based on the present experiment alone we cannot exclude a positive or negative interaction between gold and hyaluronic acid in this respect; however, the overall positive effects of this form of gold treatment are beyond dispute. Unlike traditional gold compounds, the here presented form of gold treatment furthermore has the advantage of being a localized treatment, as gold ion liberation is sparse, slow and restricted to area surrounding the implants (Fig. 1; Danscher, 2002; Larsen et al., 2007, 2008) with very limited systemic spread. This means that gold implant treatment should be safe for the patients even in the early days following a neurological trauma during which impaired kidney function might occur.

As the principle of dissolucytotic gold treatment i.e. limited *in situ* gold ion liberation by dissolucytotic cells such as macrophages is quite new (Larsen et al., 2007, 2008) further studies are warranted to identify both additional positive effects of gold liberation, and to elucidate the mechanisms behind the gold-induced alterations in the *in vivo* tissue response to traumatic brain injury. In several studies, VEGF and FGF have been shown to increase following trauma due to the inflammatory response (Mocchetti and Wrathall, 1995; Zachary, 2005; Agasse et al., 2007; Morgan et al., 2007). Accordingly, traditional anti-inflammatory gold compounds have been found to inhibit the production of VEGF in cultured synoviocytes and blood from patients with rheumatoid arthritis (Nagashima et al., 1999, 2000). It could thus be expected that treatment with an anti-inflammatory agent, such as gold, might diminish the expression of these growth factors, but paradoxically our findings show that metallic gold enhances the expression of VEGF, as well as FGF, in the injured brain. We hypothesize that the unique anti-inflammatory and neuroprotective effects of bio-liberated gold ions; i.e. the transient increase in astrogliosis occurring alongside a markedly reduced microgliosis (Larsen et al., 2008) is capable of inducing a beneficial increase in the production of VEGF and FGF by neurons and activated astrocytes, as well as reducing the inflammatory response. Several studies have reported successful attempts at limiting trauma-induced neurological damage by reducing the load of pro-inflammatory cytokines involved in the primary inflammatory response (Toulmond and Rothwell, 1995; Tehranian et al., 2002; Lloyd et al., 2008), emphasizing the clinical potential of ameliorating acute inflammation following trauma. Further studies are needed in order to unravel exactly how even a highly localized and limited gold liberation and uptake in neural tissue can result in the marked effects seen, but given the central role of macrophages/microglia in the liberation process i.e. dissolucytosis (Larsen et al., 2007), alterations in microglia function and cell signaling are likely an upstream event in this process. It is however likely that gold-liberation and uptake in glial cells and neurons (Fig 1; Larsen et al., 2008) will influence the expression of other cytokines, in this way orchestrating the glial cell response, a cytokine capable of increasing the expression of FGF in folliculostellate cells *in vitro* (Kabir et al., 2005).

In conclusion: the findings of the present study support the recently established role of metallic gold as an anti-inflammatory compound, and provide evidence that gold ions contribute to a significant increase in the expression of VEGF and FGF following TBI, as well as significantly increasing the proliferation of NSCs in the SVZ. Thus, by decreasing inflammation and simultaneously increasing the expression of these vital growth factors, gold ions can stimulate all aspects of neurogenesis; survival, proliferation and migration. Given the sparse, localized and thus safe liberation of gold ions we propose that implanting gold particles could constitute a safe way of inducing neuronal protection and enhancing regeneration following TBI, as well as in many other neurodegenerative conditions where neuroinflammation is a major contributor to the loss of neuronal tissue.

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