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The effects of nitric oxide inhibition prior to kainic acid treatment on neuro- and gliogenesis in the rat dentate gyrus *in vivo* and *in vitro*

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Summary. Treatment with the nitric oxide synthase (NOS) inhibitor, L-NAME prior to the induction of seizures with kainic acid (KA) [L-NAME+KA] increases the expression of activity-dependent neuroprotective protein (ADNP) in cells in the subgranular zone (SGZ) of the rat dentate gyrus 3-days after seizure induction (Cosgrave et al., 2009). Using the incorporation of BrdU we found that this protocol [L-NAME+KA] stimulates neuro- and gliogenesis. By comparison, L-NAME or KA alone produced smaller effects. Doublecortin$^+$ (BrdU negative) neuroblasts in the SGZ also significantly increased with L-NAME+KA treatment, suggesting that L-NAME+KA cause more cells to differentiate into neurons.

L-NAME alone increased BrdU$^+$ astrocytes in the hilus implying that NO inhibits stem cell differentiation into astrocytes and may also influence their migration. Although NOS inhibition increased cell proliferation *in vivo* and *in vitro* it disrupted cell clustering as revealed by ADNP immunoreactivity. *In vitro* KA treatment resulted in eccentric nuclei, reduced neurite extension and branching in neurons and retracted processes of glia cells, these changes were inhibited with prior treatment of L-NAME suggesting that KA-induced NO production affects cell morphology. Consequently, this data suggests an important role for NO in regulating stem cell proliferation and their fate in the SGZ.

Key words: Neurogenesis, Epilepsy, Nitric oxide synthase inhibitor, Cell proliferation, Neuroprotection

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Introduction

Increased neurogenesis and gliogenesis in the dentate gyrus (DG) has been reported in human and animal models of epilepsy (Eriksson et al., 1998; Kralic et al., 2005; Ledergerber et al., 2006; Liu et al., 2008; Kuruba et al., 2009). However, this largely depends on the animal model and the severity of seizures (Nitta et al., 2008; Rao et al., 2008; Hattiangady et al., 2008; Sharma et al., 2008; Yang et al., 2008). Animal models of temporal lobe epilepsy (TLE) have shown that prolonged seizures lead to an increase in the proliferation of granule cell progenitors in the DG (Bengzon et al., 1997; Parent et al., 1997; Gray and Sundstrom, 1998; Ledergerber et al., 2006; Liu et al., 2008; Kuruba et al., 2009). It is postulated that aberrant migration and integration of new neurons may contribute to epileptogenic hippocampal circuitry (Parent et al., 1997; Hattiangady and Shetty, 2008; Kuruba et al., 2009). However, reduced neurogenesis is associated with recurrent spontaneous seizures, for example, in TLE in humans (Hattiangady et al., 2004).

In the rat DG it is estimated that approximately nine thousand new cells are formed everyday (Cameron and McKay, 2001). Within the first few days after mitosis, the fate of these precursor cells is determined (Dayer et al., 2003), however the factors deciding whether they die or differentiate into mature neurons or glia have not been

Abbreviations. ADNP, activity-dependent neuroprotective protein; BrdU, 5-bromo-2-deoxyuridine; DCX, doublecortin; DG, dentate gyrus; KA, kainic acid; L-NAME, N^G-nitro-L-arginine methyl ester; NMDA, N-methy-D-aspartate; NO, nitric oxide; nNOS, neuronal NO synthase; sGC, soluble guanylyl cyclase; SG, stratum granulosum; SGZ, subgranular zone.

fully defined.

Nitric oxide (NO) production increases dramatically in response to kainic acid (KA)-induced seizures (Mulsch et al., 1994; Ben Ari, 2001; Kato et al., 2005; Cosgrave et al., 2008), but the contribution of this gas to the regulation of neuro- and gliogenesis following induction of seizures is not clearly known. It has been shown, however, that NO reduces adult neurogenesis (Packer et al., 2003) and hence, the seizure-induced increase in NO would be predicted to inhibit neurogenesis and inhibition of NO prior to seizure induction would promote neurogenesis. We have recently demonstrated that the KA-induced seizure increase in NO production in the hippocampus alters activity-dependent neuroprotective protein (ADNP) synthesis (Cosgrave et al., 2008, 2009). Both NO (Bredt and Snyder, 1994; Gibbs and Truman, 1998) and ADNP have key roles in development. In transgenic $ADNP^{-/-}$ null mice, the neural tube fails to close and they die at embryonic day 9 (Pinhasov et al., 2003). Heterozygous $ADNP^{+/-}$ mice display neuronal/glial pathology and reduced cognitive function (Viluh-Shultzman et al., 2007). In view of the importance of NO and ADNP during development, and their potential role in adult neurogenesis, we have investigated the effects of NO synthase (NOS) inhibition on neuro- and gliogenesis in the DG. Stem cell proliferation is stimulated by seizure activity using the KA-rodent model of epilepsy during the early stages of epileptogenesis. A range of cell markers were used to identify early differentiated neurons, progenitor cells and glia cells, combined with the cell proliferation marker, 5-Bromo-2-deoxyuridine (BrdU), to identify newly produced cells. In cultures of acutely dissociated DG cells we have further investigated some of the cellular morphological changes produced by KA and NOS inhibition that may give further insight into the *in vivo* behavior of newly formed and early differentiated cells.

Materials and methods

Young adult (35-40 day old) male Wistar rats were used for *in vivo* experiments and postnatal day 6-7 rat pups for *in vitro* studies. Animals were kept under controlled environmental conditions (19-23°C, 12 h light, 12 h dark) with food and water available *ad libitum*. All experiments were subject to University of Liverpool ethical review and conducted under appropriate UK Home Office project and personal licenses.

In vivo BrdU incorporation, NOS inhibition and kainic acid (KA) induction of seizure and tissue collection

The following drugs were used: Cell proliferation marker, 5-Bromo-2-deoxyuridine (BrdU, Sigma); NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, Sigma) and kainic acid (KA, Tocris Cookson, UK). These were all dissolved in distilled water (DW) which was used as vehicle control. The effective drug doses were derived from previous studies (Mulsch et al., 1994; Gabriel et al., 2000; Bagetta et al., 2002; Catania et al., 2003; Gupta and Dettbarn, 2003; Thippeswamy et al., 2007a, b).

All the animals in a group of 16 adult male Wistar rats were treated with BrdU (100 mg/kg, i.p.) twice daily through the five days of the experimental protocol. These were divided into two equal groups receiving three doses of L-NAME (50 mg/kg i.p /dose) or vehicle administered over a 24 h period starting a day after the start of BrdU treatment. Four animals from each of these two groups then received a single injection of KA (10 mg/kg i.p.) 48 hrs after the start of BrdU treatment. These four treatment groups were designated as: vehicle control, L-NAME only, KA only and L-NAME + KA. All KA-treated rats were injected with diazepam (10 mg/kg, i.p.) after the onset of the first full generalized seizure (stage 5 Racine scale, maximum of 3 h after receiving KA) to prevent development of status epilepticus. Note: Since not all rats injected with KA achieve stage 5 seizure, we used more animals and selected only those (a minimum of 4 animals per group) that showed stage 5 seizures in all experimental groups. This is to minimize the variability in the severity of seizure on neuro- and gliogenesis. The other group that did not receive KA was also treated with diazepam for comparison since diazepam also suppresses NO production (Wilms et al., 2003). The amount of seizure activity the animals experienced was standardized as far as possible by permitting the animals to only experience a maximum of two stage 5 seizures, each lasting for about 10-15s, which represents a mild insult. Further details on seizure patterns and its modulation by L-NAME and the rationale are discussed elsewhere (Cosgrave et al., 2008). Three days after seizure induction animals were deeply anaesthetized and fixed by vascular perfusion with 4% paraformaldehyde (PFA) in PBS. Brain tissue harvested from these animals was processed for immunohistochemistry using a standard procedure as previously described (Cosgrave et al., 2008, 2009).

In vitro: Dentate gyrus cell isolation and culture, BrdU incorporation, NOS inhibition and KA treatment of cultures

Under aseptic conditions, the brains of 6-7 day old male rat pups were collected (a minimum of 8 pups/ experiment) in Gey's balanced salt solution (Sigma, UK) containing 4.5 mM glucose. Hippocampi were removed and sliced into 1 mm thick transverse coronal slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., UK). The dentate gyrus was dissected from the rest of the hippocampus and collected on ice in Petri dishes containing Neurobasal-A medium supplemented with 2% B27 (NB+B27) + papain (2 mg/ml, Sigma, UK) for 30 min at 37°C (agitating every 10 min) to aid enzymatic digestion for cell dissociation.

After 30 min the medium was removed by a brief centrifugation and the cell pellet was washed twice with NB+B27. The pellet was then pipetted a few times until a homogenous cell suspension was achieved and the cell suspension was then applied gently to the top of 2-step density OptiPrep gradient (10% and 20%) and centrifuged for 15 min at room temperature at 1100 rpm to separate cells from debris. The fraction between the gradient containing the cells was gently pipetted out and diluted into 2 ml NB+B27 and centrifuged for 2 min at RT at 1100 rpm. The supernatant was removed and the cell pellet was re-suspended in 4 ml NB+B27. Viable cells were counted by trypan blue exclusion and the remaining cell suspension was diluted to have a cell density of 10^5 cells/ml, subsequently 500 μl was added to each well of a 24 well plate that had been previously coated with poly-D-lysine (10 μg/ml in ice cold PBS for 2 h at 37°C; Sigma, UK) and laminin [10 μg/ml in Dulbecco's modified Eagle's medium (DMEM), overnight at 37°C; Invitrogen, UK]. Cells were incubated at 37°C in humidified air with 5% CO_2 and a medium change was carried out the next day to remove any cell debris and non-adherent cells. The plating medium contained; 10% FCS (Gibco, UK), 500 μM L-Glutamine (Sigma, UK) and antibiotic/ antimycotic 250 μM (Pencillin-Streptamycin and Fungizone, Gibco, UK) in NB+B27. After three days FCS was excluded from the medium to promote neuronal differentiation, and thereafter a medium change was carried out every 3 days. In view of our aim of identifying the direct effects of KA and/or L-NAME on neuronal or glial differentiation, no other supplements (such as fibroblast growth factor) were added to the medium.

Detection of proliferating cells in vitro

Cells were grown for seven days *in vitro* (DIV) prior to drug treatment. In order to study the effects of NO inhibition and KA treatment on cell proliferation and differentiation all cultures were treated with BrdU (10 μM) and either; vehicle control, KA (10 μM), L-NAME (500 μM) or L-NAME+KA. The effective drug dose for cultures was determined by cell toxicity (MTT) assay and/or nitrite assay as described previously (Thippeswamy et al., 2005). Cells were incubated for 3 days post treatment, fixed in 4% PFA and processed for immunostaining with BrdU and cell markers. Some BrdU-treated cultures without L-NAME or KA served as a control for basal level cell proliferation. To understand the short-term effects of drug treatment on cell proliferation, some cultures were fixed 6 h post-KA treatment.

BrdU immunostaining and co-staining with other cell markers

Since BrdU is a thymidine analogue, it is incorporated into all cells in S-phase of mitosis, and antibodies specific to BrdU can therefore, be used to detect proliferating cells. The 4% PFA fixed brains were cryostat sectioned, and sections were thaw mounted on slides which were stored at -40°C until further use. For cultures, the medium was removed and cells were washed with 0.1 M Phosphate buffered saline (PBS, pH 7.4), then fixed in 4% PFA for 30 min at room temperature (RT). Prior to staining, cells/tissue sections were washed with three changes of PBS to remove unbound fixative. To denature the DNA for BrdU detection, they were incubated sequentially with: 50% formamide in citrate solution (SCC: 0.3 M NaCl and 0.03 M sodium citrate, BDH) 65°C, 90 min; SCC, 5 min; 2N HCl, 37°C, 30 min; 0.1 M boric acid (pH 8.5) RT, 10 min; 5 washes in PBS at RT. They were incubated with 10% donkey serum, 1h, RT and then at 4°C overnight with primary anti-BrdU (1:100, raised in mouse, Invitrogen, UK). As BrdU is confined to the nucleus, sequential staining with other primary antibodies raised in the mouse, against cytoplasmic cell markers was employed when suitable antibodies raised in other species could not be obtained. The following primary antibodies were used: ADNP (rabbit raised, 1:500; Chemicon, UK), doublecortin (goat raised, 1:1000, Chemicon), nestin (raised in mouse, 1:1000, Chemicon), GFAP, NG2 (both raised rabbit 1:1000; Chemicon, UK), nNOS (raised in sheep, 1:1000, kind gift from Dr PC Emson, Cambridge, UK), and NeuN (raised in mouse 1:100; Chemicon, UK). Specificity of these antibodies has been described elsewhere (Thippeswamy and Morris, 2001; Thippeswamy et al., 2007b; Arora et al., 2007; Cosgrave et al., 2008, 2009). Immunostaining was carried out simultaneously on control and drug-treated sections using the same reagents and antibodies. Primary antibody omission was routinely used as a negative control and, for some antibodies, a tissue section from a known positive control was also used in parallel [for example, axotomised dorsal root ganglion for nNOS (neurons) and ADNP (glia) - Thippeswamy and Morris, 2001; Thippeswamy et al., 2007b]. Depending on subsequent double or triple immunostaining, appropriate biotinylated and fluorescently labeled antibodies were used (Jackson ImmunoResearch, USA). Sections were covered with VectaShield (Vector Laboratories), cover slipped and viewed with a Nikon inverted microscope (Nikon, UK). Images were captured by scanning sections using the appropriate filters for each fluorochrome and merged using IPL laboratory software (Nikon, UK).

Cell quantification

From each of the four animals per treatment group, nine sections from approximately the same levels (three each from cranial/middle/caudal) of the DG were selected for counting. The total number of $BrdU^+$ cells was counted in the different layers of the DG {stratum granulosum (SG), subgranular zone (SGZ), hilus, and outer molecular layer (OML)}. Cell counts were also

made to determine the extent of co-localization of BrdU with ADNP, doublecortin and NeuN. Immunopositive cells from cultures were counted in a minimum area of 6 mm^2 from three different wells and repeated on a minimum of four samples. To calculate the percentage of cells with abnormal morphology, such as vacuolated cytoplasm and thick/retractile cell processes, abnormal cell counts were expressed as a percentage of total cells in that area. The average cell numbers per group of sections/cultures were calculated for animal or culture group, the SEM calculated for each treatment group and 'p' values obtained using ANOVA (Graphpad software Inc, USA) by comparing drug treated vs. appropriate controls. A 'p' value less than 0.05 was considered to be significant.

Results

The effects of KA, L-NAME and L-NAME+KA on cell proliferation and ADNP expression in vivo and in vitro

No increase in the numbers of $BrdU^+$ nuclei was seen in the SGZ three days post-KA when compared to vehicle treated controls (Figs. 1i,ii, 2). In contrast, NOS inhibition by L-NAME (Figs. 1iii, 2) and L-NAME treatment prior to induction of seizures by KA (Fig. 1iv) caused a marked and statistically significant increase in the number of $BrdU^+$ nuclei (Fig. 2). Many of the $BrdU^+$ SGZ cells in L-NAME+KA treated animals also expressed ADNP (Figs. 1viii, 2). L-NAME alone also increased the number of $BrdU^+$ cells in the hilus and in the outer molecular layer, however, their colocalization with ADNP did not change. This suggests that the basal level endogenous production of NO and that stimulated by seizures inhibits stem cell proliferation and migration.

The interaction of NO-ADNP was examined further in DG cultures. L-NAME alone (Figs. 3vii,viii, 4) or L-NAME+KA (Figs. 3ix,x, 4) increased cell proliferation, as shown by the number of $BrdU^+$ nuclei, many of which also expressed ADNP when compared to appropriate controls (Figs. 3i-iv, 4). KA alone (Fig. 3v,vi) did not increase cell proliferation when compared to vehicle control (Figs. 3i,ii, 4). Interestingly, both L-NAME (Fig. 3vii,viii) and KA (Fig. 3v,vi) disrupted the organization of $ADNP^+$ cell clusters and chain-like patterns compared to vehicle control (Fig. 3i-iv). $ADNP^+$ cells were usually spindle shaped with processes extending at both ends (Fig. 3iii,iv). In contrast, $ADNP^+$ cells containing $BrdU^+$ nuclei in KA, L-NAME and L-NAME+KA treated cultures were spherical with eccentric nuclei (Fig. 3vi,viii,x). This suggests that altering the concentrations of NO in culture changes the morphology of $ADNP^+$ cells.

Identification of cell types containing BrdU by colocalization with doublecortin, nestin and GFAP following KA, L-NAME and LNAME+KA treatment in vivo

In order to identify the cell types affected by KA and/or L-NAME, cell markers were used in combination with BrdU. Doublecortin (DCX) and nestin were used to label neuronal lineage cells, while GFAP and NG2 were used to mark glial lineage cells. Cells marked with the neuronal lineage markers nestin or DCX, but not expressing BrdU, allowed the investigation of the effects of NO and/or KA on neurons formed prior to BrdU labeling.

A clear increase in DCX^+ cells with their soma in the SGZ was seen in the L-NAME+KA treated group (Fig. 5iv,vi, Table 1) many of which were also $BrdU^+$ demonstrating that they were newly formed neuroblasts. L-NAME or KA alone and L-NAME+KA treatment also increased the overall numbers of DCX^+ cells (that did not contain $BrdU^+$ nuclei) suggesting that more cells produced prior to the start of BrdU treatment differentiate into neurons (Fig. 5, Table 1). However, in these treatment groups, none of these DCX^+ cells were found to contain nestin (data not shown).

In the vehicle treated control group (Fig. 6i) astrocytes were distributed throughout the hilus and SGZ with relatively low numbers being $BrdU^+$ in the SGZ suggesting that ongoing stem cell activity in this region generates new astrocytes. Induction of seizures (KA treated) causes a marked increase in GFAP expression (antibody concentrations and parameters for image capture were kept constant) revealing that the astrocytes have become reactive although relatively few of these contain BrdU (Fig. 6ii). In marked contrast, NOS inhibition by L-NAME produces limited astrocytes activation but many astrocytes in the hilus were $BrdU^+$

Table 1. The effects of L-NAME and/or KA on cells that express various markers of neuro- and gliogenesis *in vivo*.

Treatment group	Doublecortin (DCX)		Nestin		GFAP	
	BrdU positive	BrdU negative	BrdU positive	BrdU negative	BrdU positive	BrdU negative
Vehicle control	3±2	24±6	5±2	16±8	3±1	22±9
L-NAME	7±3	$44±7^+$	9±3	$37±6^+$	$12±3^+$	$8±4^+$
Vehicle + KA	6±4	$42±5^+$	4±2	21±5	4±1	27±6
L-NAME + KA	$16±3^Δ$	$76±11^Δ$	8±4	$33±5^Δ$	$17±5^Δ$	$38±4^Δ$

The numbers represent the average ± SEM per animal counted from the area between the two limbs of the dentate gyrus, including the subgranular zone, and from the start of the CA3 area (15±3 mm^2). Comparison was made between vehicle+KA vs. L-NAME+KA (^{Δ}: $p<0.05$); vehicle control vs. L-NAME or KA ($^+$: $p<0.01$), all n=4.

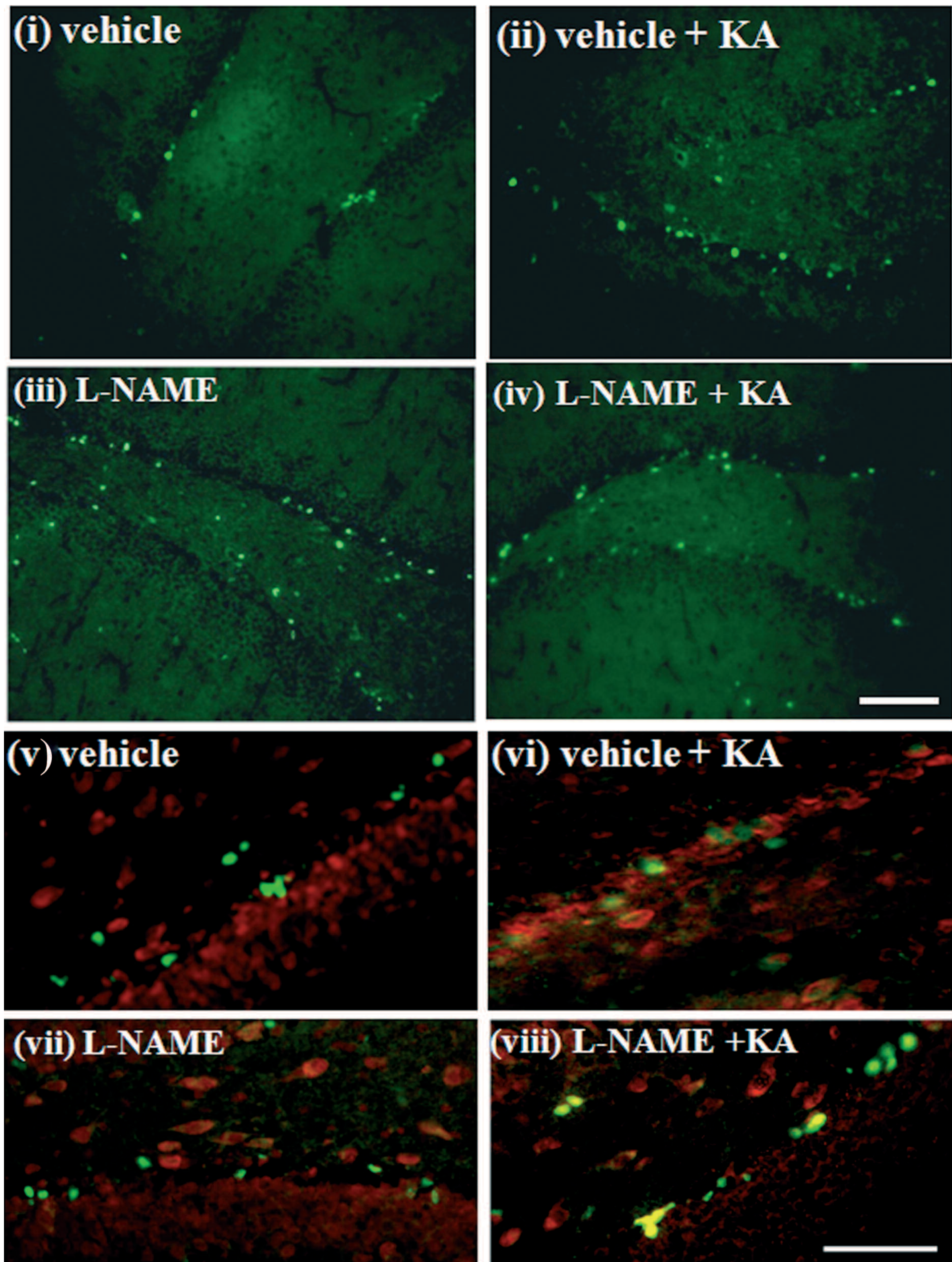

Fig. 1. The effects of KA, L-NAME and L-NAME+KA on cell proliferation and ADNP expression in the dentate gyrus *in vivo.* BrdU **(i-iv)** and BrdU +ADNP **(v-viii)** immunostaining. More BrdU$^+$ nuclei were present in the subgranular zone (SGZ) in the L-NAME **(iii)** and L-NAME+KA **(iv)** compared to vehicle control **(i)**. KA alone had no significant effect on the number of BrdU$^+$ cells in the SGZ **(ii)**. The number of BrdU$^+$ nuclei that were co-localised with ADNP$^+$ cells in L-NAME+KA **(viii)** was more compared with the L-NAME **(vii)** or KA **(vi)** on its own. L-NAME on its own also increased the number of BrdU$^+$ cells in the hilus and the outer molecular layer. Scale bar: 100 μm.

suggesting that NO also inhibits stem cell proliferation and differentiation into astrocytes (Fig. 6iii). NO inhibition may also favour astrocytes migration into the hilus as some GFAP BrdU+ cells were seen in the SGZ of L-NAME treated animals. When NO is inhibited prior to seizures (L-NAME+KA) the astrocytes stain more intensely for GFAP and many also contained BrdU. This suggests that NO inhibition permits stem cell production of astrocytes (Fig. 6iv) and that both the newly formed astrocytes and those produced prior to seizures can become reactive.

The effects of KA, L-NAME and L-NAME+KA on cell morphology in dentate gyrus cultures

This *in vivo* data infers an interaction between seizures and NO, which may have effects not only in stem cell proliferation but also in differentiation, cell fate and migration. To further discern these processes, studies investigating the effects of these treatments were carried out *in vitro* where more details of cell type, morphology and behaviour can be observed.

KA treatment produced signs of cell stress indicated by retraction of cytoplasmic processes by astrocytes (Fig. 7ii) and NG2 cells (Fig. 7viii) accompanied by cytoplasmic vacuolations in some cells. This was prevented by prior treatment with L-NAME (Fig. 7i,vii). Newly formed (BrdU+ nuclei) nestin+ cells had extensive branching processes in L-NAME+KA (Fig. 7iii) which were clearly affected by KA in the absence of L-NAME (Fig. 7ii). As these neuronal lineage cells become more differentiated, revealed by co-immunostaining of nestin and DCX, their dendritic processes become more elaborate with finer branches. KA treatment caused these to become thicker and/or retracted (Fig. 7vi,x) with fewer neurite branches and this effect was blocked by L-NAME pre-treatment in cultures (Fig. 7v,ix). This effect produced by KA was also shown with another marker of immature neurons, Tuj-I (Fig. 7xii) and this was also inhibited with prior treatment of L-NAME (Fig. 7xi).

We have previously shown that KA induced seizures reduce the expression of ADNP in a subset of hilar neurons in the dentate gyrus (Cosgrave et al., 2009). This suggests that as these neurons become stressed this neuroprotective protein synthesis is inhibited. In order to investigate whether this occurs in newly formed neurons, DG cultures were treated with KA with and without the addition of L-NAME; these were then immunostained for ADNP, nestin or DCX and/or BrdU (Fig. 8). A number BrdU+ cells that contained both nestin and ADNP was evident in L-NAME+KA (Fig. 8i). ADNP was seen in relatively few stem cells as indicated by BrdU co-immunostaining with nestin in KA treated cultures (Fig. 8ii,iv) compared to L-NAME+KA treated cultures (Fig. 8i,iii). However, the morphology of cells that expressed ADNP, even in KA treated cultures, was 'normal' (red cells in Fig. 8iv). As the neurons differentiate and express DCX, nestin expression is down regulated and ADNP expression up-regulated in the cell bodies. KA treatment caused a marked reduction in dendritic branching in DCX+ neurons (Fig. 8vi) and a

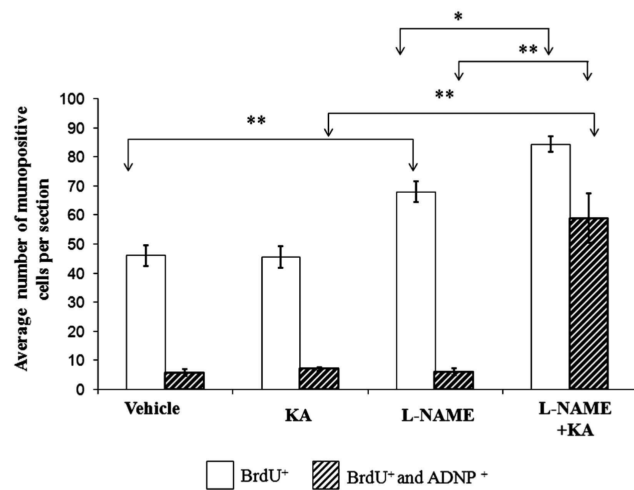

Fig. 2. The effects of KA, L-NAME and L-NAME+KA on cell proliferation and ADNP expression in the dentate gyrus *in vivo*. Quantification of BrdU+ and ADNP+ cells and their co localization. Comparison was made between vehicle vs. L-NAME (p<0.5), KA vs. L-NAME+KA (p<0.001) and L-NAME vs. L-NAME+KA (**: p<0.001, *: p<0.5), all n=4.

Table 2. Comparison of abnormal morphology of various cell types in dentate gyrus culture (in percent).

Treatment group	Doublecortin % abnormal	Tuj-1 % abnormal	Nestin % abnormal	GFAP % abnormal	NG2 % abnormal	ADNP % abnormal
Vehicle control	9±2	12±4	8±3	6±2	3±2	7±2
L-NAME	11±4	10±7	4±2	5±3	4±3	14±8
Vehicle +KA	40±6$^{\Delta}$	72±19$^{\Delta}$	62±13$^{\Delta}$	37±9$^{\Delta}$	21±7$^{\Delta}$	46±7$^{\Delta}$
L-NAME + KA	12±5	16±8	20±9	14±7	8±5	19±9

For neuronal lineage cells, neurites growth cones vs retractile bulbs, the extent of neurites branching vs thick neurites without collaterals, and with or without cytoplasmic vacuoles were considered. For glial lineage cells, thick cell processes without branching pattern vs well defined fine processes with or without cytoplasmic vacuoles were taken into account. A few examples are shown in Fig. 7. Overall L-NAME treatment prior to KA decreases the number of abnormal cells in culture suggesting the effect of KA-induced NO toxicity on early differentiated neuronal or glial lineage cells. Comparison was made between vehicle+KA versus L-NAME+KA ($^{\Delta}$: p<0.001), all n=6.

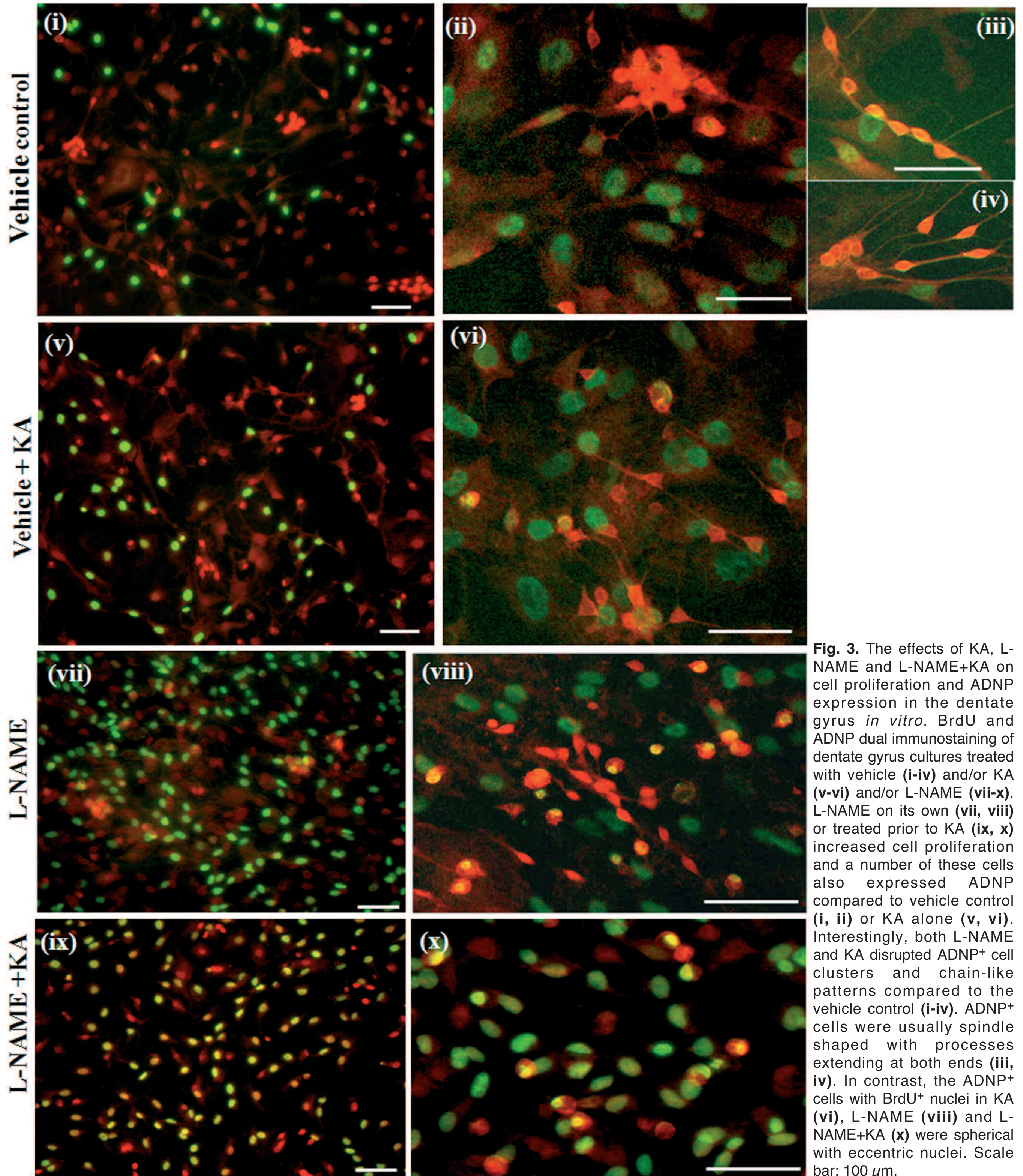

Fig. 3. The effects of KA, L-NAME and L-NAME+KA on cell proliferation and ADNP expression in the dentate gyrus *in vitro*. BrdU and ADNP dual immunostaining of dentate gyrus cultures treated with vehicle **(i-iv)** and/or KA **(v-vi)** and/or L-NAME **(vii-x)**. L-NAME on its own **(vii, viii)** or treated prior to KA **(ix, x)** increased cell proliferation and a number of these cells also expressed ADNP compared to vehicle control **(i, ii)** or KA alone **(v, vi)**. Interestingly, both L-NAME and KA disrupted ADNP$^+$ cell clusters and chain-like patterns compared to the vehicle control **(i-iv)**. ADNP$^+$ cells were usually spindle shaped with processes extending at both ends **(iii, iv)**. In contrast, the ADNP$^+$ cells with BrdU$^+$ nuclei in KA **(vi)**, L-NAME **(viii)** and L-NAME+KA **(x)** were spherical with eccentric nuclei. Scale bar: 100 μm.

reduction in ADNP expression, this was prevented by L-NAME treatment (Fig. 8v). The percentage of abnormal cells of neuronal and glial lineage in cultures in response to KA with or without L-NAME is shown in table 2. Overall, these findings further support the view that the release of an excessive amount of NO by KA is causing marked changes in cell morphology and that ADNP may counter these effects.

Discussion

These results primarily suggest an important role for NO as an inhibitor of stem cell proliferation, subsequent cell fate determination and possibly cell migration in the SGZ of the dentate gyrus during the early stage of epileptogenesis. Secondly, the production of NO following the induction of seizures with KA may reduce the stimulatory effect of seizures on stem cell proliferation. Thirdly, the release of NO by KA causes cytotoxic effects on stem cells differentiating into neurons and glia. Furthermore, the data suggest that ADNP has a neuroprotective function in seizures.

NO has been implicated in neural development (Bredt and Snyder, 1994; Contestabile, 2000), cell division (Garg and Hassid, 1990; Lepoivre et al., 1990), neurogenesis (Moreno-López et al., 2004), transcription factor regulation and gene expression (Riccio et al., 2006; Zhu et al., 2006; Arora et al., 2007). It is an ideal local signaling molecule able to freely diffuse across cell membranes, having a short half-life, which is produced on demand (Garthwaite, 2008). These properties would make this gaseous molecule a very suitable agent for regulation of cell signaling in stem cell niches. At low concentrations its actions tend to be cytoprotective and it acts principally via the induction of cGMP and neurotrophic factors (Thippeswamy et al., 2005). However, it is also well established that as NO levels increase its actions become cytotoxic primarily through the formation of peroxynitrite when it combines with superoxide. KA-induced seizure increases NO release in the rodent hippocampus (Catania et al., 2003; Kato et al., 2005; Chuang et al., 2007; Liu et al., 2008; Cosgrave et al., 2008) and hence, its actions on stem cells could be potentially complex depending on the NO concentration reached. NOS inhibition with either L-NAME or 7-NI increases SVZ cell proliferation (Packer et al., 2003; Yang et al., 2008) indicating that resting levels of NO release tend to inhibit stem cell proliferation.

BrdU was used to label mitotic cells in S-phase (Cameron and McKay, 2001; van Praag et al., 2005) before- and at the time of seizure induction and immediately afterwards. Unlike in embryonic or neonatal rats, the dose of BrdU used in this study did not cause any cytotoxic effect on the proliferating and differentiating cells (Hancock et al., 2009). Although changes were seen in the numbers of BrdU$^+$ cells following NOS inhibition or NOS inhibition prior to seizure induction, seizures alone were not found to increase cell proliferation in the present study. However, in this study seizures induced by KA were controlled by the administration of diazepam. As a result the animals experienced only one or two full generalized seizures for a short duration. In animals subjected to more severe seizures, changes in stem cell proliferation have been reported (Kralic et al., 2005; Ledergerber et al., 2006; Yang et al., 2008), and these occur quite rapidly within 24 h following seizure induction (Steiner et al., 2008). Factors such as the severity of seizures, the route of administration of seizure- inducing drugs and the duration of seizures, all appear to influence stem cell activity and cell fate, i.e. gliogenesis vs. neurogenesis (Kralic et al., 2005; Ledergerber et al., 2006; Yang et al., 2008). The effect of NOS inhibition on the numbers of BrdU$^+$ cells that were co-localized with stem cell marker, nestin clearly demonstrates that the basal level of NO is an important regulator of stem cell proliferation. The hilus and the SGZ contain many interneurons that express the neuronal isoform of NOS (nNOS) and these have axons ramifying in the granule cell layer (Jinno and Kosaka, 2004). In these animals, which have not experienced seizures, the inducible form of NOS is very low (Cosgrave et al 2008), although blood vessel endothelial cells will contain eNOS. It has been shown that eNOS deficient mice have a significant reduction in neuronal progenitor cell proliferation in the DG compared to wild type litter mates (Reif et al., 2004). In the present case it seems probable, in view of the role of peptides released from interneurons in regulating SGZ stem cells (Jinno and Kosaka, 2004) that the interneurons containing nNOS are the source of the basal levels of NO that inhibit SGZ stem cell proliferation. The combination of NOS inhibition, by L-NAME, with seizures resulted in a further increase in the number of BrdU$^+$ cells more than the number of BrdU$^+$

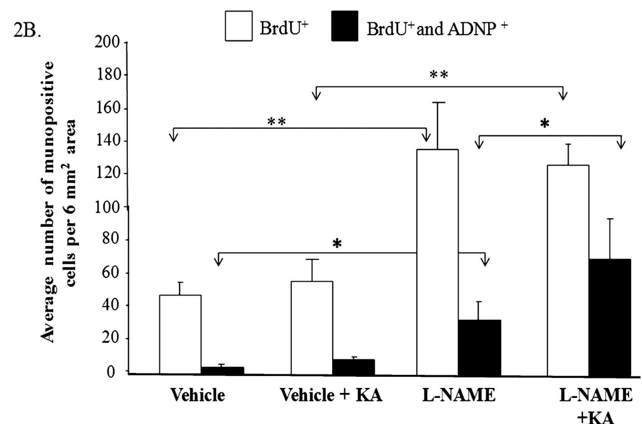

Fig. 4. The effects of KA, L-NAME and L-NAME+KA on cell proliferation and ADNP expression in the dentate gyrus *in vitro*. Quantification of BrdU and ADNP$^+$ cells and the cells that expressed both in the dentate gyrus *in vitro*. Comparison was made between vehicle vs L-NAME ($p<0.5$), vehicle+KA vs L-NAME+KA (*: $p<0.01$, **: $p<0.005$) and L-NAME vs L-NAME+KA (*: $p<0.5$), all n=6.

cells with L-NAME alone. This suggests that some further release of NO during seizures is also contributing to an inhibition of stem cell proliferation. The synchronous release of many different transmitters and signaling molecules by the paradoxical discharge of many neurons during a seizure will create a complex extracellular milieu around the SGZ stem cells producing not only abnormal patterns of cell proliferation but also influencing their fate, differentiation and survival. The complex changes in cell morphology produced in neurons formed around the time of seizure induction have recently been elegantly documented (Jessberger et al., 2007). In the present study in addition to the changes in the numbers of BrdU$^+$ cells observed, differences were also found in their distribution. Small numbers of BrdU$^+$ cells were found outside the SGZ suggesting that NOS inhibition could be influencing the migration of early differentiated cells

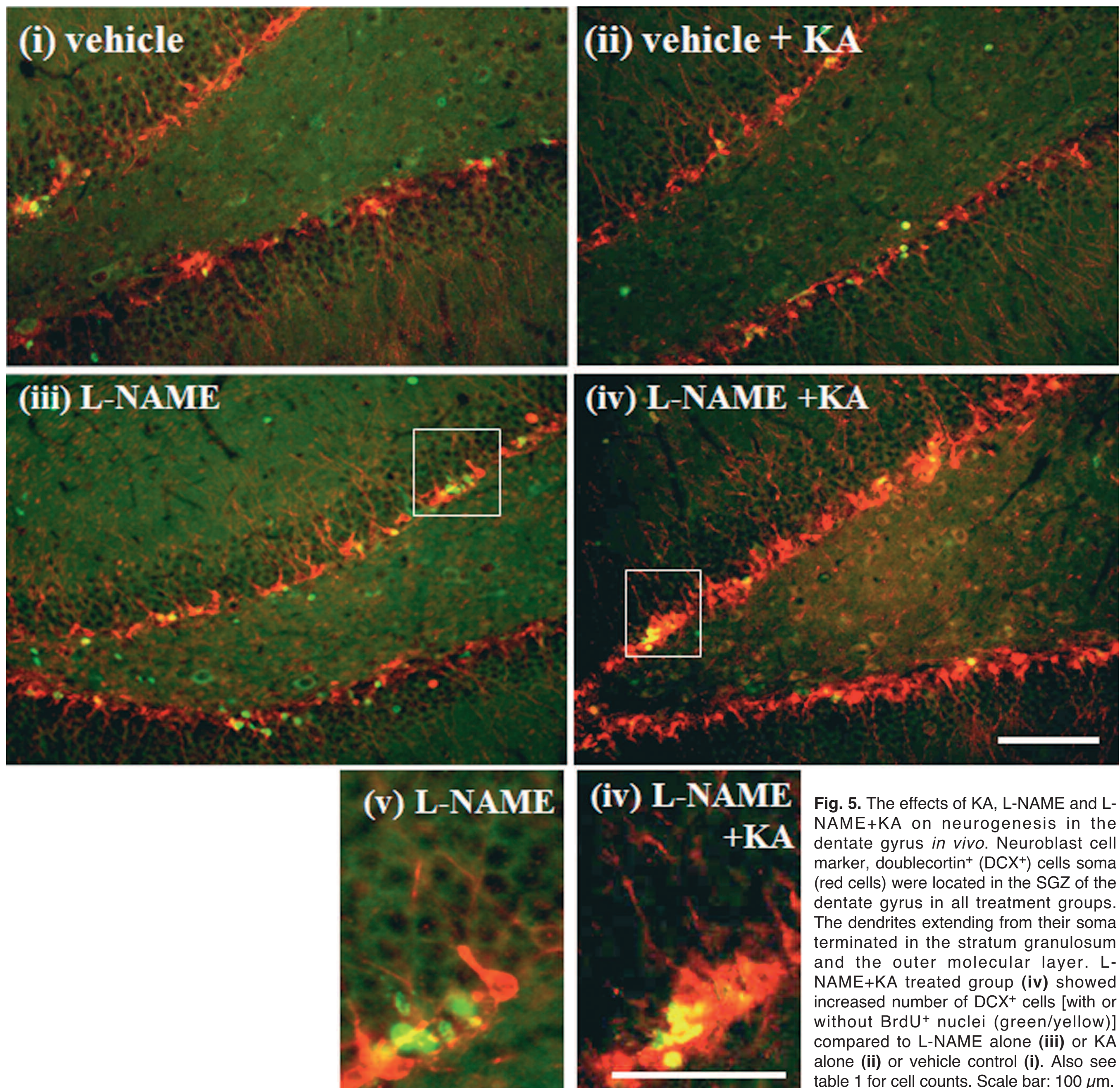

Fig. 5. The effects of KA, L-NAME and L-NAME+KA on neurogenesis in the dentate gyrus *in vivo*. Neuroblast cell marker, doublecortin$^+$ (DCX$^+$) cells soma (red cells) were located in the SGZ of the dentate gyrus in all treatment groups. The dendrites extending from their soma terminated in the stratum granulosum and the outer molecular layer. L-NAME+KA treated group **(iv)** showed increased number of DCX$^+$ cells [with or without BrdU$^+$ nuclei (green/yellow)] compared to L-NAME alone **(iii)** or KA alone **(ii)** or vehicle control **(i)**. Also see table 1 for cell counts. Scale bar: 100 μm.

(Moreno-López et al., 2000; Gibbs, 2003; Gutiérrez-Mecinas et al., 2007). However, as these cells might represent dividing astrocytes, microglia or cells infiltrating the CNS, as proposed by Lee et al. (2003) and Ledergerber et al. (2006), further cell phenotype analysis was undertaken.

Stem cells in the DG can proliferate and differentiate into neurons or glia and changes in BrdU cell numbers expressing neuronal or glia markers reveal either changes in cell fate determination or cell survival. Inhibition of endogenous NO production resulted in an increase in $BrdU^+$ astrocytes suggesting that in addition to NO suppressing stem cell proliferation it also selectively reduces their entry to the glia cell route. In contrast, Covacu et al. (2006) have shown that treating neural stem cell cultures with an NO donor increases astrogliogenesis implying that NO concentration and the extracellular milieu may also influence cell fate. It is also plausible that some of these early differentiated astrocytes could re-differentiate into neurons (Seri et al., 2001). In our *in vivo* model, NO may inhibit astrocytes migration from the SGZ stem cell niches into the hilus as more $BrdU^+$ astrocytes were found in the hilus when NOS was inhibited with L-NAME. In contrast, when NOS was inhibited prior to the production of seizures greater numbers of $BrdU^+$ cells expressed the early neuronal marker DCX. Kim et al. (2006) have also shown that NOS inhibition increases the number of DCX^+ and $BrdU/DCX^+$ cells in the SVZ and DG. These observations suggest that NO also acts to block stem cell

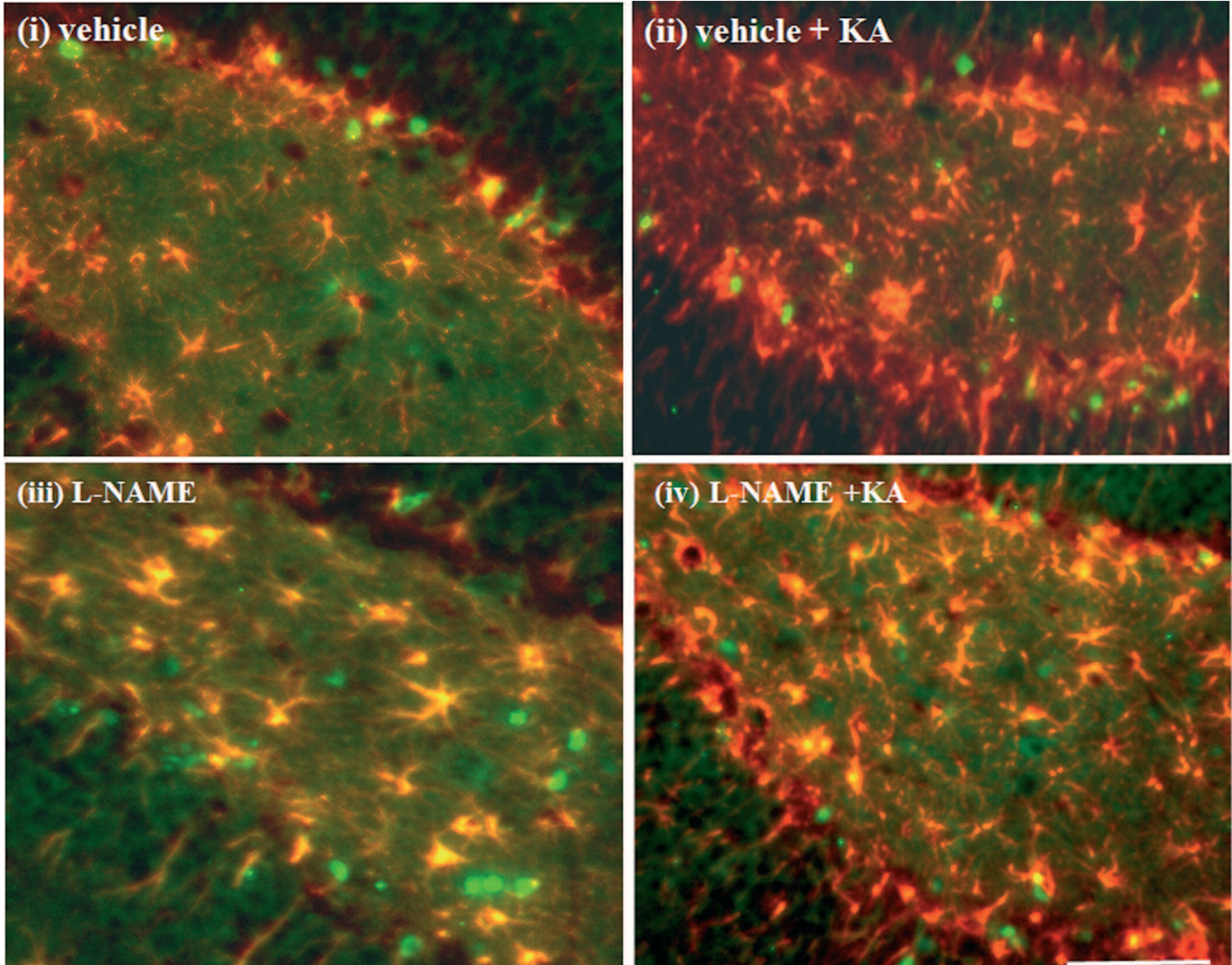

Fig. 6. The effects of KA, L-NAME and L-NAME+KA on astrogliogenesis in the dentate gyrus *in vivo*. L- NAME+KA **(iv)** or KA **(ii)** on its own increases GFAP (red) expression in the SGZ and the hilus, and more number of astrocytes ($GFAP^+$ cells) were co-labeled with $BrdU^+$ nuclei (green/orange) in the L-NAME pre-treated **(iv)** than in KA alone **(ii)**. Also see table 1 for cell counts. In L-NAME only treated group, the SGZ cells did not show GFAP staining **(iii)** when compared to vehicle **(i)** or KA-treated groups **(ii, iv)**. Scale bar: 100 μm.

proliferation and differentiation into neurons by a complex interaction with other regulators of stem cell proliferation and fate.

Interestingly, greater numbers of $BrdU^+$ cells in L-NAME+KA treated animals were also $ADNP^+$. As detailed in the introduction, ADNP has important developmental roles and is essential for neuronal differentiation and axonal growth (Chen and Charness, 2008). It is also present in the adult nervous system where it is implicated in neuroprotection (Pascual and Guerri, 2007). We have recently shown that the expression of ADNP is differentially regulated in the CA3 and DG by NO (Cosgrave et al., 2008, 2009) and L-NAME+KA caused a significant increase in the numbers of $ADNP^+$ cells in the SGZ three days after KA treatment (Cosgrave et al., 2009). From the present study it is clear that it is the combination of L-NAME+KA that causes induction of ADNP in the SGZ stem cells, and changes in ADNP produced by L-NAME and KA on their own are in other cells. In controls very few $BrdU^+$ cells contained ADNP and this may indicate that during the ongoing production of new cells this protein has a limited function in cell fate and survival. When they are stressed by seizure activity in the presence of the NOS inhibitor they strongly express ADNP in the nucleus. This suggests a complex interaction between NO and other factors released during seizures on the expression of ADNP in stem cells. This was further examined in cultures. In control or L-NAME treated cultures, in a similar fashion to that seen *in vivo*, most of the cells with $BrdU^+$ nuclei did not contain ADNP in either their nuclei or cytoplasm, however, following combined treatment

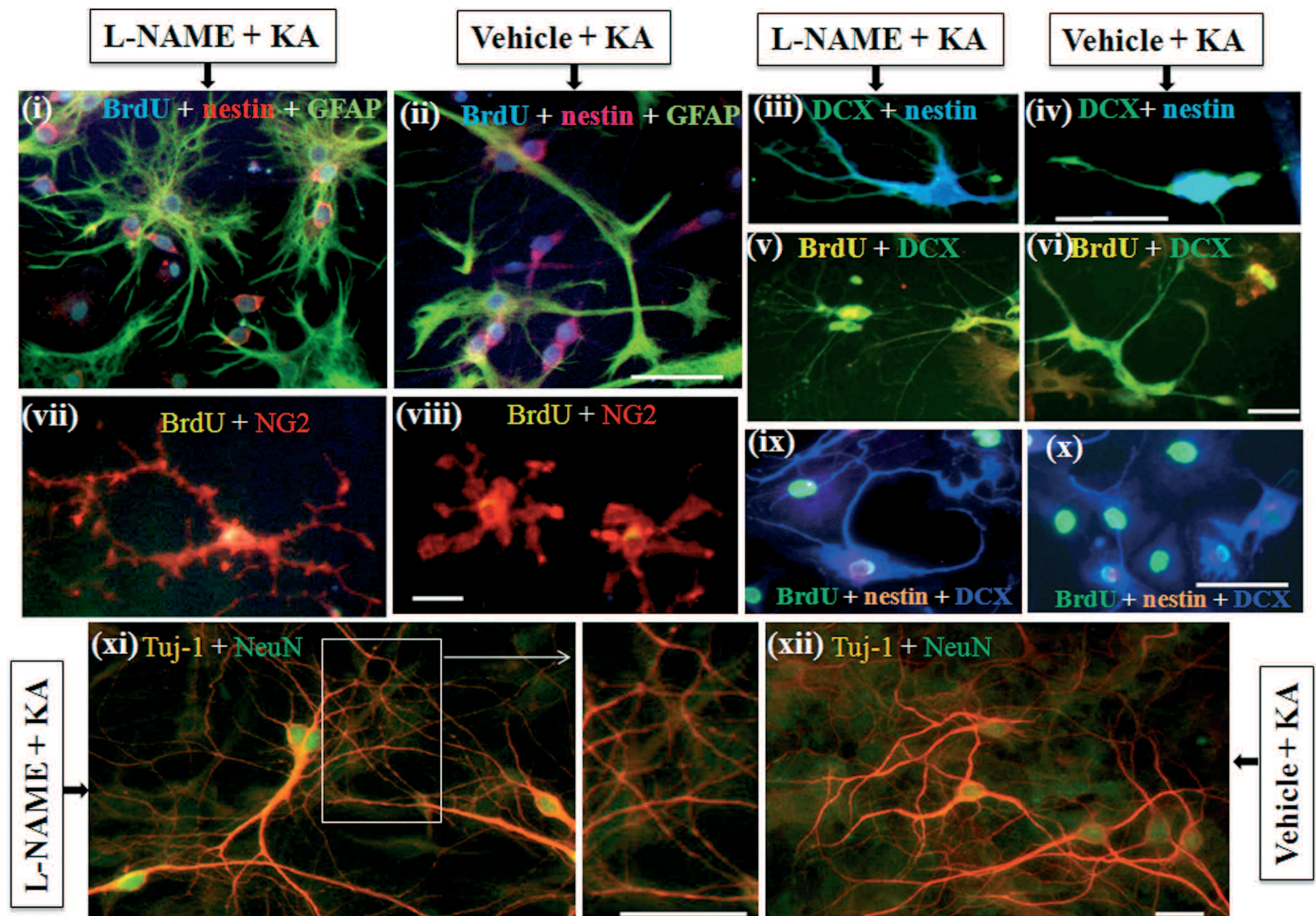

Fig. 7. Comparison of the effects of L-NAME+KA **(i, iii, v, vii, ix, xi)** versus vehicle+KA **(ii, iv, vi, viii, x, xii)** treatment on morphology of various cell types in dentate gyrus cultures. KA alone caused retraction and thickening of cytoplasmic processes of astrocytes (GFAP+ cells, green in **ii**) and NG2 cells **(viii)** compared with L-NAME pre-treated culture (**i and vii**, respectively). Red/pink cells in **(i)** and **(ii)** are $nestin^+$ cells. Nestin colocalization with GFAP did not change in either treatment. However, DCX^+ cells that contained nestin had either a fewer neurites and/or with retractile bulbs in KA treated culture **(iv)** in contrast to growth cones in L-NAME pre-treated **(iii)**. DCX and/or nestin co-labeled **(vi, x)** cells that contained $BrdU^+$ nuclei also showed similar morphological changes in response to KA alone compared to L-NAME pre-treated cultures (**ix, v**, respectively). NeuN (green labeled nuclei) co-labeled Tuj-1 neurons had thick dendritic branches in KA-treated cultures **(xii)** in contrast to fine branching of dendrites in L-NAME pre-treated cultures **(xi)**. Scale bar, 100 μm.

with L-NAME+KA many expressed ADNP in their nuclei. This suggests that NOS inhibition prior to KA translocates ADNP from the cytoplasm to the nucleus, after the induction of seizures, where it could regulate gene expression. A translocation of ADNP to the nucleus was also reported in our previous *in vivo* studies (Cosgrave et al., 2008, 2009). It has been recently shown that the ADNP-derivative, NAP protects cultured rat hippocampal neurons from kainate toxicity by interacting with microtubule-associated protein

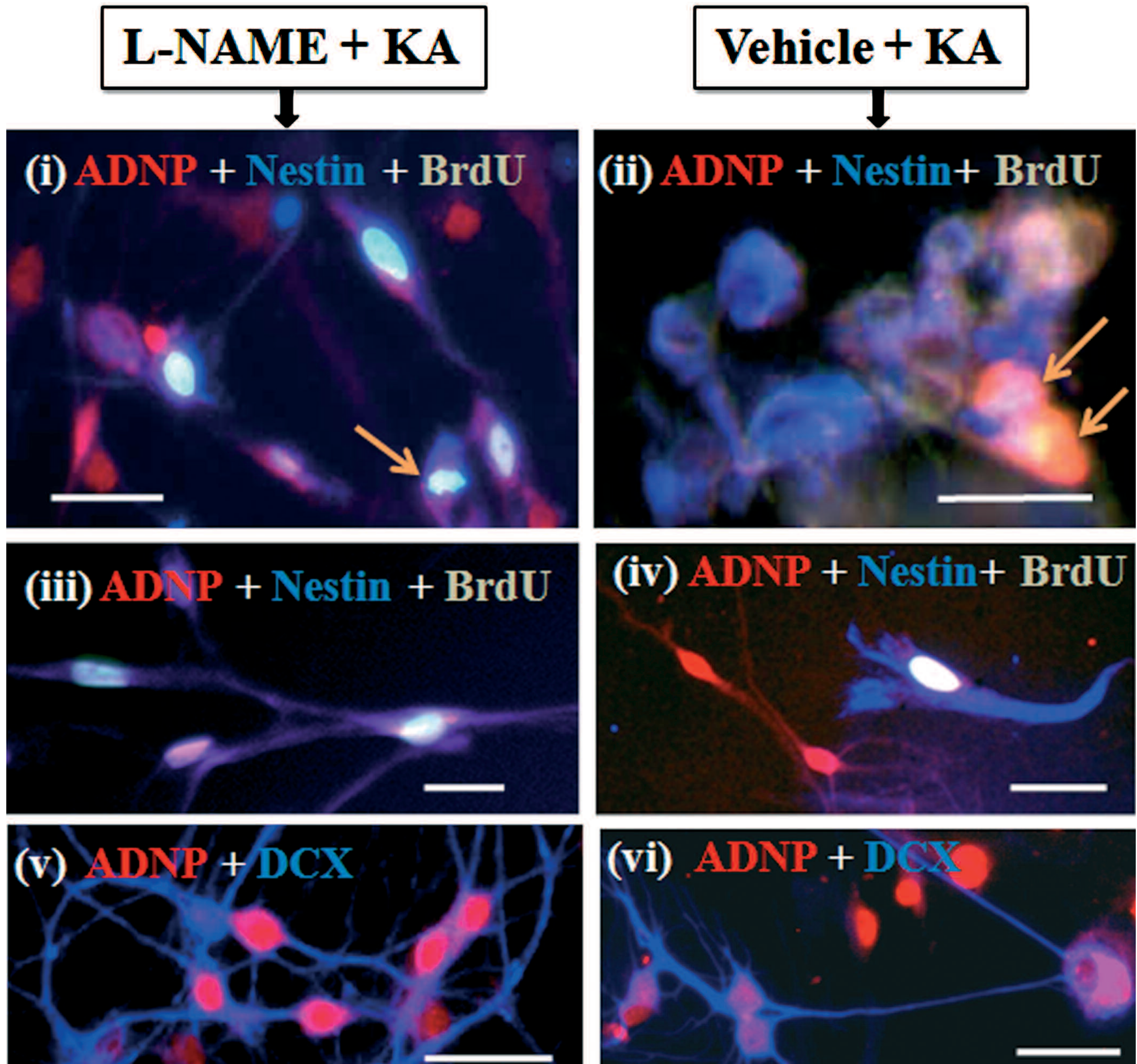

Fig. 8. Dentate gyrus cultures showing the effects of L-NAME treatment prior to KA on the morphology of ADNP+ cells (red/pink labeled cells in all) that also express either nestin (blue labeled cells in **i-iv**) or DCX (blue labeled cells in **v, vi**). KA alone affects the cell processes of both pre-existing nestin+ (blue cells in **ii**) and newly formed nestin+ cells (blue labeled cell with BrdU+ nucleus in **iv**). In contrast, in L-NAME pre-treated cultures **(i, iii)** nestin+ cells (blue/purple) had well defined processes with spindle shaped soma that resembled typical ADNP+ cell morphology as in the vehicle control cultures (Fig. 3, **iii, iv**). These nestin+ cells also contained ADNP. Newly formed ADNP+ cells that contained a BrdU+ nucleus also had no processes (red cells indicated by arrows in **ii**) in KA-treated cultures compared to pre-existing ADNP+ cells in the same culture (red cells in **iv**). A number of BrdU+ cells that contained both nestin and ADNP was evident in L-NAME+KA (an example is indicated by an arrow in **i**). DCX co-labeled ADNP+ cells had extensive neurites if cultures were treated with L-NAME prior to KA [**(v)** vs. **(vi)**] and ADNP was restricted to the cell bodies. Scale bar: 100 μm.

(Zemlyak et al., 2007). Both ADNP and NAP also modulate poly ADP-ribosylation and MAPK-PI3K/Akt pathways to promote neuronal differentiation and survival (Mandel et al., 2008; Pascual and Guerri, 2007). Mandel and Gozes (2007) have demonstrated that ADNP constitutes a novel element in the SWI/SNF chromatin remodeling complex. The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and it mediates transactivation of key neuronal specific genes, Ngn and NeuroD (Seo et al., 2005). In the present study, the majority of ADNP cells in culture that expressed the neural stem cell marker, nestin, and the marker of newly formed neurons, DCX, appeared to be healthy with a normal morphology. Hence, it is probable that the production of ADNP is part of a neuroprotective response and that if the stem cells entering the neuronal fate are stressed, by events such as seizures, they can respond by producing neuroprotective peptides. This corroborates findings in recent studies by Jessberger et al. (2007) in which neurons formed at the time of seizures often survive and form elaborate dendrites and axons, but can do so in an aberrant fashion. Recent studies using shRNA, ADNP down regulation in the pluripotent teratocarcinoma cell line P19, demonstrated that an approximately 80% reduction in ADNP led to a substantial reduction in neurodifferentiation and a significant reduction (~50%) in neurite numbers (Mandel et al., 2008). Hence, the expression of ADNP appears very important in differentiation of neurons particularly when they are stressed. This supports the view that ADNP plays a role in microtubule reorganization and changes in cell morphology, and that NO may regulate this activity. The distribution of $ADNP^+$ cells in cultures also suggests that it has an important role in cell migration. In control cultures more ADNP positive cells formed distinct clusters of chains but these were absent in cultures treated with KA or L-NAME or L-NAME+KA suggesting that NO mediates this clustering.

The dispersion of cells in culture also favoured the more detailed investigation of the effects of KA and NO on cell morphology. Tuj-1 has been used to label newly generated immature neurons (Menezes and Luskin, 1994; Doetsch et al., 1997; Parent et al., 1997; Gould et al., 2001). DCX and Tuj-1 are also co-expressed in early differentiated neurons (Brown et al., 2003; Rao and Shetty, 2004; von Bohlen Und Halbach, 2007). In the present study it was found that the Tuj-1 antibody employed gave very clear staining of neuronal cells in cultures (Fig. 7xi,xii), however, with higher background levels *in vivo* (data not shown). Treatment of cultures with KA cause a reduction in neurite branching so that they had fewer processes and the dendritic shafts appeared thicker. In the rodent KA model, during the early stages of neuronal cell death, changes can occur in dendritic structure with dendritic retraction and thickening being observed (Zeng et al., 2007).

Both *in vivo* and *in vitro* KA produced marked effects on glia. Astrocytes became reactive producing more GFAP *in vivo* in response to KA whilst *in vitro* they adopted a different shape with less branching. The changes in shape in response to KA were inhibited in vitro by L-NAME confirming that KA-induced NO production affects morphology of the astrocytic processes which play an important role in synaptic transmission and blood brain barrier function (Wang and Bordey, 2008; Koehler et al., 2009). Astrocytes are not the only glia involved in responses to KA and the role of NO in regulating both microglia and NG2 cells needs much more investigation. $NG2^+$ progenitor cells are multipotent cells that can differentiate into astrocytes, oligodendrocytes and functional neurons (Belachew et al., 2003; Zhao et al., 2009), and KA induced seizures affect their morphology (Brilli et al., 2009). In a preliminary examination, NG2 cell morphology also changes, *in vitro*, they were found to be more amoeboid after KA with fewer elongated processes and these changes were partially reversed by L-NAME.

In summary, the number of $BrdU^+$ and DCX^+ cells in the SGZ increases with L-NAME and L-NAME+KA treatment but KA alone had no effect on cell proliferation ($BrdU^+$ cells). This could be due to our mild seizure model and short duration (at day 3 post-KA). In L-NAME+KA treated groups, BrdU co-localization with DCX, Tuj-1 and ADNP increases compared to KA or L-NAME or vehicle control. L-NAME suppresses GFAP in the SGZ but increases $BrdU^+$ astrocytes in the hilus. KA alone causes abnormal morphology of glial and neuronal lineage cells in DG culture that could be reversed if they were pre-treated with L-NAME implying a critical role of NO in KA-induced morphological changes which may pre-dispose the hippocampus to pathophysiological conditions associated with epileptogenesis.

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