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The effect of the NMDA NR2B subunit antagonist, ifenprodil, on precursor cell proliferation in the hippocampus

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Abstract:

The N-methyl-D-aspartate (NMDA) receptor, one of the ionotropic glutamate receptor, plays important physiological and pathological roles in learning and memory, neuronal development, acute and chronic neurological diseases and neurogenesis. This work aimed to examine the contribution of the NR2B NMDA receptor subunit to adult neurogenesis/cell proliferation under physiological conditions and following an excitotoxic insult. We have previously shown *in vitro* that a discrete NMDA-induced, excitotoxic injury to the hippocampus results in an increase in neurogenesis within the dentate gyrus. Here we have characterised adult neurogenesis or proliferation, using BrdU, in an *in vivo* model of excitotoxic injury to the CA1 subfield of the hippocampus. We demonstrate a peak in neural stem cell proliferation/neurogenesis between 6-9 days after the excitotoxic insult. Treatment with ifenprodil, an NR2B subunit specific NMDA receptor antagonist, without prior injury induction, also increased the number of BrdU-positive cells within the DG and posterior periventricle, indicating that ifenprodil itself could modulate the rate of proliferation. Interestingly though, the increased level of cell proliferation did not change significantly when ifenprodil was administered following an excitotoxic insult. In conclusion, our results suggest and add to growing evidence that NR2B-subunit containing NMDA receptors play a role in neural stem cell proliferation.

Key Words: Adult Neurogenesis; Dentate Gyrus; BrdU; excitotoxicity

Introduction:

In the mammalian brain, neurogenesis occurs throughout life (Altman and Das, 1965, Cameron and McKay, 2001). This neurogenic activity is centred on two regions of the adult brain: the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and subventricular zone (SVZ) of the lateral ventricle (Doetsch et al., 1997, Seri et al., 2004). The neuronal progenitor cells in the SGZ migrate a short distance into the granule cell layer where they mature, extend axonal projections and integrate into local networks (Stanfield and Trice, 1988, Altman and Bayer, 1990). A large number of cells are produced in the SGZ but the majority die as a result of apoptosis. However, once through this critical period, they demonstrate long-lasting integration with the granule cell layer network (Kempermann and Gage, 1999).

There are many factors with demonstrated regulatory influence on adult hippocampal neurogenesis. These include particular growth factors such as epidermal growth factor and fibroblast growth factor-2 (Kuhn et al., 1997), and conditions, such as enriched environments (Kempermann et al., 1997) and physical activity (van Praag et al., 1999). Another influence on hippocampal neurogenesis is glutamatergic neurotransmission, primarily mediated through the NMDA receptor (Gould et al., 1994, Cameron et al., 1995). Despite extensive study of glutamatergic neurotransmission, the role of glutamate in adult neurogenesis is far from clear. Some studies have shown that NMDA receptor blockade increases neurogenesis suggesting that glutamatergic input to the dentate gyrus is involved in suppression of neurogenesis (Gould et al., 1994, Cameron et al., 1995, Nacher et al., 2003). However, work in ischaemia models and seizure models have shown that increased glutamate signalling

caused an increase in hippocampal neurogenesis and that NMDA receptor blockade resulted in a reduction of neuronal stem cell production (Parent et al., 1997, Bernabeu and Sharp, 2000, Arvidsson et al., 2001). While these effects seem contradictory, one explanation for this discrepancy may be that the resulting cell death, rather than glutamate receptors directly, stimulates neurogenesis. However, it is also possible that different NMDA receptor subtypes produce different effects.

The majority of the studies, to date, examining the role of the NMDA receptor in neurogenesis, have used non-selective antagonists, such as MK-801, APV or memantine (Kluska et al., 2005, Poulsen et al., 2005, Maekawa et al., 2009). One study which examined the role of NR2B containing NMDA receptors *in vitro*, suggested that these receptors mediate both survival and death signalling within a single neuron (Martel et al., 2009). It is of interest therefore to examine, *in vivo*, the role of the NMDA NR2B-subunit in the regulation of adult neurogenesis.

We have previously established an *in vivo* model of excitotoxic injury in the mouse (Concannon et al., 2008). In this study we aimed to characterise *in vivo* the effect of a discrete excitotoxic injury in the hippocampal CA1 subfield on neurogenesis, and the long-term survival of these cells in the dentate gyrus and the posterior periventricle as this has recently been shown to be a neurogenic zone (Chechneva et al., 2005). Finally, using this model, the effects of the NMDA receptor NR2B subunit selective antagonist, ifenprodil, on proliferation and survival were evaluated.

Materials and Methods:

2.1 *Animals:*

Male C57BL/6 mice (9-weeks old), obtained from Harlan UK Ltd., were housed 5 per cage, with food and water available *ad libitum*. A 12-hour light-dark cycle was maintained (light hours 07.00–19.00 h). All animal experiments were carried out under licence from the Department of Health and Children (Ireland), in accordance with the European Communities Council Directive 86/609/EC and all procedures were approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland. All mice were 10-weeks old at the start of the experimental procedures.

2.2 *Surgery:*

The mice (n=6-9 per group) were anaesthetised by injection of 'Avertin' (i.p.) at a dose of 10 ml/kg (Avertin concentrate consists of 100 g of 2,2,2-tri-bromoethanol dissolved in 62 ml of *tert*-amyl alcohol: 5 ml of this concentrate are added to 20 ml absolute alcohol and 250 ml of 0.9% saline). The head was secured in a stereotaxic frame (Stoelting, USA) and an incision made in the scalp to expose the skull. If necessary, the position of the incisor bar was adjusted to ensure that bregma and lambda were in the same horizontal plane. The body temperature of the mice was maintained at a normal level using a feedback controlled homeothermic blanket.

A small hole was drilled to the right of the midline at the appropriate coordinates (anterior-posterior = -1.9mm from Bregma; Medial-lateral = +1.5mm from the midline; dorsal-ventral

= -1.2 mm from brain surface; injection volume 0.05 µl). An infusion of 10 mg/ml *N*-methyl D-aspartic acid (NMDA) solution [in phosphate buffered saline (PBS)] or vehicle was made. Injections were made using a 34-gauge stainless steel cannula (Cooper's Needle Works, Birmingham, UK) mounted on a 5 µl microsyringe (Scientific Glass Engineering, Milton Keynes, UK). Each infusion was administered at a rate of 0.1 µl/min and the cannula remained in place for 1 minute following infusion to allow for diffusion into the surrounding tissue. These injection coordinates and volume were chosen based on our previous work (Concannon et al., 2008) and preliminary studies to ensure only the CA1 subfield was affected and not the dentate gyrus. Finally, the scalp was sutured and the animals were closely monitored, in a temperature-controlled environment until recovery from the anaesthetic.

2.3 *BrdU Administration:*

In order to visualise dividing cells in the dentate gyrus, BrdU (5-bromo-2- deoxyuridine) was administered by intraperitoneal (i.p.) injection on days 6-9. BrdU was prepared at 10 mg/ml (65 mM) in sterile saline (0.9 %) and injected i.p. at 5 ml/kg to achieve an effective dose of 50 mg/kg as previously described (Gage et al., 1995)(Figure 1).

2.4 *Ifenprodil administration:*

Where appropriate to the experiment, ifenprodil (5 mg/kg, in 0.9% sterile saline (Sigma-Aldrich, Ireland)) was administered by the i.p. route on days 4-9 following stereotaxic

surgery, to ensure the primary cell death processes had ceased and to cover the peak neurogenesis time (Figure 1C)

2.5 *Histology:*

After the appropriate survival times, animals were sacrificed by terminal anaesthesia with sodium pentobarbitone, (200 space mg/kg, i.p.) and transcardially perfused, at a rate of 4 ml/min, with either 40 ml phosphate buffered saline (PBS) or 20 ml PBS followed by 20 ml of ice cold 4 % paraformaldehyde (PFA). Following perfusion, PBS treated brains were removed, snapfrozen in iso-pentane and stored at -80°C . PBS and 4 % PFA perfused brains were removed and post-fixed in 4 % PFA for 12 hours at 4°C . These brains were then transferred into 30 % sucrose/PBS until they were equilibrated and then frozen at -20°C before being stored at -80°C until further processing.

In preparation for histology, the brains were sectioned, coronally, at $10\ \mu\text{m}$, using a cryostat (Leica, Germany). Collection of the sections commenced at an anterior–posterior coordinate of -1.4mm (from Bregma according to the Mouse Brain in Stereotaxic Coordinates, Paxinos and Franklin (Paxinos, 2001)). Sets of four consecutive sections were collected every $40\ \mu\text{m}$ up to an anterior–posterior coordinate of -2.4mm (from Bregma).

2.6 *Tissue preparation:*

This was carried out using established protocols, published elsewhere (Bunk et al., 2010a) but described in brief here.

2.6.1 BrdU labelling, BrdU/NeuN and BrdU/GFAP double-labelling:

Brain slices were washed in PBS, DNA was denatured with 2 N HCl for 20 min at 37°C, and neutralised with 0.1M borax buffer (pH 9) for 20 min at room temperature. After a washing step, the tissue was incubated with 10% fetal calf serum for 1 h at room temperature. Primary antibodies (rat anti-BrdU, AbD Serotec, UK, 1/300; mouse anti-NeuN, Chemicon, Ireland, 1/500 or mouse anti-BrdU, BD Bioscience, UK, 1/100; rabbit anti-GFAP, 1/500, Sigma-Aldrich, Ireland) were diluted in blocking solution and applied overnight at 4 °C. After washing, the respective secondary antibodies were applied for 2 h at room temperature.

2.6.2 Doublecortin labelling:

Brain slices were washed in PBS before incubation with 0.1% Triton X-100/PBS for 15 min on ice. After washing in PBS, slices were blocked in 5% horse serum and 0.3% Triton X-100/PBS for 30 min at room temperature. After further washing in PBS, goat anti-DCX antibody (Sant Cruz, 1/100, Santa Cruz Biotech, Heidelberg, Germany) was applied in blocking solution overnight at 4°C. After washing the secondary antibody, donkey-anti-goat rhodamine conjugated (Jackson ImmunoResearch, Plymouth, PA, USA, 1/1000) was applied for 2 h at room temperature.

2.6.3 BrdU and doublecortin double-labelling:

For this step, a combination of the protocols described above was used. First, brain slices were stained for DCX using goat anti-DCX and donkey-anti-goat fluorescein conjugated. Following washing, slices were then prepared for BrdU labelling and mouse anti-BrdU (BD Bioscience, 1/100) was used as primary antibody and goat anti-mouse 568 (Molecular probes, 1/1000) was used as secondary antibody. Slices were embedded in DAPI-containing mounting media or incubated with Hoechst 33342, and then washed and mounted in FluorSave Reagent (Calbiochem, Merck Bioscience, Nottingham, UK). Control experiments were performed by incubation with secondary antibodies only and no unspecific staining was observed.

2.6.4 TUNEL staining:

To analyse apoptotic cell death, specifically nuclear DNA fragmentation, brain sections were labelled using the DeadEnd™ Fluorimetric TUNEL System (Promega). Briefly, after washing in PBS, the brain sections were permeabilised in 3 % Triton X-100 / PBS for 20 min, again washed with 0.1 % Triton X-100 / PBS and incubated with equilibration buffer for 15 min at room temperature. After equilibration the TUNEL solution containing 90µl equilibration buffer, 5 µl nucleotide mix and 1 µl rTdT (terminal deoxynucleotidyl transferase) per microscope slide was applied to the slices for 90 min at 37 °C. The enzymatic reaction was terminated with 2 x SSC (sodium chloride-sodium citrate buffer) for 15 min at room temperature. The slices were washed 3 x 5 min in 0.1 % Triton X-100 / PBS, 2 x 5 min in PBS and 1 x 5 min in dH2O and mounted with DAPI containing medium.

2.7 *Data quantification and statistical analysis:*

Cell counting was performed using an Eclipse TE 300 inverted microscope (Nikon) and a 40x oil objective. BrdU, DCX, or TUNEL-positive cells or cells double positive for BrdU and DCX or NeuN were counted in the DG of the hippocampus. Only cells located within the SGZ and the GCL were included; cells without direct contact to the DG (located within the hilus) were excluded from counting. All counts were performed in a blinded manner and data are given as mean \pm S.E.M per 10 μ m coronal section. Additionally, cell counts within the posterior periventricle were performed. This area was defined as a two to three cell layer-comprising structure along the hippocampal cornu ammonis and leading into the lateral ventricle. Data were tested for normal distribution using the Shapiro-Wilk test of normality. Statistical significance of differences between means was evaluated, with SPSS version 15 & 18, using different statistical tests depending on the situation and the parameters being evaluated. The tests utilised were, for parametric data, the student's t-test, one-way ANOVA and *post hoc* Tukey's test or two-way ANOVA with *post hoc* Newman Keuls. For non-parametric data the Mann–Whitney U-test with a Bonferroni correction was used.

Results:

3.1 Cell proliferation peaks in the DG, CA1 and pPV 6-9 days after NMDA-induced CA1 injury: We used an *in vivo* model of discrete excitotoxic injury to the CA1-region of the hippocampus and determined neurogenesis up to 65-days following the lesion (Figure 1). As can be seen in Figure 2 A&B, stereotaxic administration of NMDA into the hippocampus produced a selective excitotoxic lesion to the CA1 hippocampal subfield (Concannon et al., 2008). In contrast, injection of saline produced no detectable hippocampal injury (Figure 2A).

It is well established that proliferation is frequently increased after injury to the brain. To examine if this proliferation was up-regulated following NMDA-induced excitotoxicity, animals received daily BrdU injections (50 mg/kg, ip) at different time points following intrahippocampal NMDA or saline administration (day 0 – 3, 6 – 9 or 12 – 15). The animals were sacrificed 24 hours after the last BrdU injection and the brains examined. BrdU-positive cell counts within the DG and the posterior periventricle (pPV) revealed that proliferation was significantly up-regulated at 6 – 9 days following a discrete lesion of the hippocampus (DG, control 16.9 ± 2.7 vs. NMDA 32.2 ± 3.3 $p < 0.05$; pPV, control 18.2 ± 2.4 vs. NMDA 28.5 ± 3.2 $p < 0.05$; values represent numbers of BrdU-positive cells/10 μm brain section \pm standard error of the mean; control groups: $n = 4$ animals; NMDA treatment groups: $n = 6 - 11$ animals). Interestingly, while BrdU-positive cells were absent in the CA1

subfield of control animals, their number significantly increased within the CA1 subfield of NMDA injected animals at all time-points analysed (Figure 2C). It can be concluded therefore that inducing injury to the CA1 subfield of the hippocampus results in increased proliferation within the DG (and also in CA1 and pPV) which is significant at days 6 – 9 following injury. Subsequently, BrdU treatment was performed at this time point for all following experiments.

In order to determine the phenotypic origin of the BrdU-positive cells co-staining with the astrocytic marker glial fibrillary acidic protein (GFAP), the neuronal progenitor marker, doublecortin (DCX) and the neuronal marker Neuronal Nuclei (NeuN) was performed. NeuN expression commences in early post-mitotic stages of immature neurons and remains within fully differentiated cells. GFAP-positive cells within the DG not only account for astrocytes but also for radial glia-like stem cells (type I cells) (Steiner et al., 2004). BrdU administration 6 – 9 days after NMDA-induced injury and analysis 24 hours after the last BrdU injection revealed that BrdU-positive cells were present in the SGZ of the DG (Figure 2D). Additionally, GFAP-positive cells were located in and near the SGZ (Figure 2E). However, the staining did not overlap with BrdU, indicating that BrdU-positive cells did not have properties of astrocytes or radial glia-like cells. Additionally, it was observed that GFAP staining was increased at the site of injury, indicating the activation of astrocytes in response to excitotoxicity. DCX-positive cells were located along the SGZ of the DG and BrdU-positive cells, located within the pool of DCX-positive progenitor cells, also expressed DCX, indicating a neuronal progenitor phenotype of the newly generated cells (Figure 2F) and thereby demonstrating increased proliferation. While mature neurons of the GCL expressed NeuN, the majority of BrdU-positive cells were negative for NeuN expression, indicating that

the newly generated cells had not yet matured into neurons. Interestingly, in the NMDA treated mice, 13% of the BrdU positive cells already expressed NeuN, 24 hours after 4 days of BrdU injection, indicating that some cells had matured into neurons over this time period (Figure 3 A&B).

Several earlier studies have proposed that BrdU can not only be incorporated into the DNA of dividing cells but also during DNA repair mechanisms of dying cells (Kuan et al., 2004). However, recent evidence suggests that this is rarely the case (Bauer and Patterson, 2005, Czaja et al., 2008). Therefore, to test whether this was the case here, the brain sections were stained for the apoptotic marker TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labelling) and for BrdU by immunohistochemistry. As expected, BrdU-positive cells were present in the SGZ of the DG as well as in the CA1 subfield. However, only low levels of TUNEL-positive cells were observed in the CA1 subfield. More importantly, there was no co-localisation of the two markers in the DG, indicating that BrdU was not taken up by apoptotic cells (Figure 2G).

3.2 Long-term survival of newly-generated cells:

While there is clearly an upregulation in neurogenesis following excitotoxic injury, it has been demonstrated that the majority of newly generated cell do not survive, with apoptotic cell death accounting for the fate of 60 – 80 % of the cells (Biebl et al., 2000). To examine the long term survival of the newly generated cells in this experimental model, BrdU was administered from days 6-9 following NMDA administration and the animals allowed to survive 24 hours, 28 days or 56 days after the last BrdU injection. Other cell proliferation markers (for example Ki67, pH3) are effective indicators of cell proliferation, but are not

always applicable for tracking the cell fate (Kee et al., 2002). As expected and seen above, twenty-four hours after the last BrdU injection the number of BrdU-positive cells was significantly up-regulated in all areas analysed. However, after longer survival periods of 28 or 56 days the number of cells was greatly reduced in DG, CA1 and pPV, indicating that the newly generated cells did not survive longterm (Figure 4). Importantly, there was no difference between control animals and those that received intrahippocampal NMDA injections, indicating that even the increased number of cells generated following excitotoxicity could not survive.

It was of interest to assess the phenotypic fate of the cells that survived for 28 or 56 days. Co-staining for BrdU and the neuronal marker NeuN was performed. After 28 days (Figure 5 (A&B)) and 56 days (Figure 5 (C&D)), the majority of BrdU-positive cells were positive for the mature neuronal marker NeuN in both control and NMDA-treated animals. Survival of the animals for 28 or 56 days following the last BrdU injection resulted in maturation of 70 – 83 % of the BrdU-positive cells into a neuronal phenotype. However, there was no difference between NMDA treated and control animals (28 days: control (70%) vs NMDA (83%), $p>0.05$; 56 days: control (80%) vs NMDA (72%), $p>0.05$).

3.3 Ifenprodil increases adult neurogenesis/cell proliferation in the dentate gyrus:

To examine the role of NMDA receptor modulation on neurogenesis, the polyamine/NR2B antagonist, ifenprodil, was assessed. In addition to the administration of BrdU following intrahippocampal NMDA, the animals received intraperitoneal injections of ifenprodil on days 4-9 following NMDA-induced excitotoxicity. In order to analyse the effect of ifenprodil

on injury-induced proliferation, one group of animals was sacrificed 24 hours after the last BrdU and ifenprodil injection.

As expected from the earlier experiments, in the animals sacrificed 24 hours after BrdU administration, there was a significant increase in the number of BrdU-positive cells in the DG of NMDA injected mice ($F_{1,21} = 5.855$, $p < 0.05$; Figure 6A). There was also a significant effect of ifenprodil treatment ($F_{1,21} = 6.008$, $p < 0.05$), though there was no interaction ($F_{1,21} = 1.627$, $p > 0.05$). Interestingly, in control animals that did not receive NMDA, but were treated with ifenprodil, proliferation was similarly increased (saline/ifenprodil 18.9 ± 2.3 ($n = 8$), $p < 0.05$ vs saline/saline group). Additionally, an increase was also observed when animals received NMDA and subsequently ifenprodil 4 – 9 days afterwards (NMDA/ifenprodil 21.5 ± 1.7 ($n = 10$ animals), with $p < 0.01$ versus control saline/saline group). However, the increase in BrdU-positive cells within the NMDA/ifenprodil animals was similar compared to NMDA/saline or saline/ifenprodil animals.

In the pPV the effect of NMDA was significant ($F_{1,23} = 11.783$, $p < 0.01$), meaning NMDA injection resulted in an increase in BrdU-positive cells. However, the effect of ifenprodil did not reach statistical significance, though there was a trend towards increased BrdU-positive cells (Figure 6B). However, NMDA-induced excitotoxicity alone (NMDA/saline) or together with ifenprodil (NMDA/ifenprodil) resulted in an increase of BrdU-positive cells within the pPV 24 hours after the last BrdU and ifenprodil injection (pPV (24 h), saline/saline 19.5 ± 3.3 ($n = 6$) vs. NMDA/saline 39.6 ± 3.7 ($n = 10$ animals), $p < 0.01$ and vs. NMDA/ifenprodil

35.9 ± 3.3 (n = 10) , $p < 0.01$, values represent numbers of BrdU-positive cells/10 μm brain section). In addition, there was no difference between the NMDA/ifenprodil and NMDA/saline groups.

Finally we were interested in studying the long-term survival of newly-generated cells 28 or 56 days after the last BrdU injection. Only a small number of newly generated cells remained within the DG and there was no difference in cell numbers between the different treatment groups, suggesting that the administration of ifenprodil during the period of neurogenesis had no influence on long-term survival of the newly generated cells. However, phenotypic analysis, through double staining with NeuN, showed that 70-83% of the surviving cells in the DG displayed a neuronal phenotype.

In the pPV, BrdU-positive cells were still significantly elevated 28 days following BrdU and ifenprodil treatment in NMDA-injected mice, suggesting that these cells had survived longer compared to control (pPV (28 d), saline/saline 3.5 ± 1.1 (n = 6) vs. NMDA/ifenprodil 10.4 ± 2.3 (n = 10), $p < 0.05$). In the NMDA/saline group there was a trend towards increased BrdU positive cells, though this did not reach significance ($p=0.08$). However, after 56 days survival there was a further decrease in the number of BrdU positive cells and only cells within the NMDA/Saline group were significantly increased compared to controls (pPV (56 d), saline/saline 2.5 ± 0.6 (n = 6) vs. NMDA/saline 5.7 ± 1.2 (n = 10), $p < 0.05$).

In addition, the effect of ifenprodil on the number of BrdU-positive cells within the CA1 subfield after NMDA-induced excitotoxicity was examined. Similar to the results from the model characterisation (Figure 4B), control animals which had not received NMDA treatment (saline/saline) did not display BrdU-positive cells in the hippocampal CA1 subfield at any time point analysed. Likewise, control animals, receiving intraperitoneal ifenprodil injections (saline/ifenprodil), did not show BrdU-positive cells within the CA1. Both the saline/ifenprodil and the saline/saline groups showed an average of <1 BrdU positive cell per 10 μm section at the three time points. This suggested that ifenprodil alone had no effect on the number of BrdU-positive cells in this area, in contrast to the observed effect within the DG and pPV.

Survival for 28 days resulted in similar numbers of BrdU-positive cells within the NMDA/Saline and NMDA/ifenprodil treatment groups when compared to each other (NMDA/saline vs NMDA/ifenprodil; 11.9 ± 5.2 vs 13.9 ± 9 cells/10 μm section, $p > 0.8$). BrdU-positive cells were almost completely absent in the CA1 subfield after survival of the animals for 56 days.

4. Discussion:

Injury to the brain, for example cerebral ischemia, status epilepticus or traumatic brain injury results in the excessive release of glutamate and over-activity of glutamatergic receptors producing apoptotic and necrotic cell death in the affected areas (Choi, 1994, Henshall, 2007, Besancon et al., 2008). However, these types of injuries also result in increased neurogenesis within the SGZ of the hippocampal dentate gyrus (Parent et al., 1997, Arvidsson et al., 2001). The aim of this study was to further characterise adult neurogenesis/cell proliferation in an *in vivo* model of NMDA-induced excitotoxicity, specifically targeting the hippocampal CA1, which is known to be susceptible to excitotoxic injury and selectively vulnerable in models of global cerebral ischaemia (Kirby and Shaw, 2004, Zhang and Chen, 2008). In addition, the influence of the NMDA receptor subunit, NR2B, was examined in these processes, through the use of the competitive antagonist to NR2B-subunit containing receptor complexes, ifenprodil (Williams, 2001). Our study shows that there is up-regulation of proliferation in the dentate gyrus and the posterior periventricle following the excitotoxic insult and that the peak of proliferation (represented by BrdU incorporation) occurs approximately 6 - 9 days after the insult. This is in line with previous *in vitro* work from our lab using organotypic hippocampal cultures that also showed increased proliferation 6 - 9 days after NMDA exposure (Bunk et al., 2010b). Furthermore, our result is in agreement with other literature, which shows there is a latent period between occurrence of the injury and subsequent increase in proliferation (Parent et al., 1997, Takagi et al., 1999). Takagi *et al.* (1999) demonstrated an up-regulation of neurogenesis 4 – 9 days after transient forebrain ischemia, which was significantly decreased thereafter (14 – 16 days). Parent *et al.* (1997) showed up-regulation of neurogenesis for a more prolonged period of up to 13 days following status

epilepticus. However, the likelihood is that different insults to the brain induce proliferation within slightly altered timeframes.

As other groups have previously demonstrated, the cell proliferation was confirmed using double-labelling with BrdU and the immature neuronal marker, DCx (Rao et al., 2008). DCx expression has been shown to occur very soon after cell birth in neuronally destined cells (Kempermann et al., 2003). The phenotypic fate of the neural stem cells was also examined through the use of double labelling with BrdU and the early post-mitotic neuronal marker, NeuN. Expression of NeuN commences as early as 1 or 2 days following the last cell division and subsequently BrdU/NeuN-positive cells can be detected one day following a single BrdU injection (Brandt et al., 2003). Indeed, 24 hours after the last BrdU injection some BrdU-positive cells were found to express NeuN indicating that those cells had left the cell cycle and developed a neuronal phenotype. In addition, co-labelling for GFAP was carried out to determine the effect on the two types of GFAP expressing cells in the dentate gyrus, namely radial glia-like stem cells and horizontal astrocytes (Steiner et al., 2004). However, the excitotoxic insult did not result in increase in BrdU/GFAP-positive co-labelling in the dentate gyrus, nor was there an increase in GFAP expression alone in the dentate gyrus. Coupled with the lack of BrdU labelling of horizontal astrocytes, this suggests that gliosis did not play a major role in the dentate gyrus in this injury model. This is in line with previous work by Tureyen and co-workers who showed that middle cerebral artery occlusion induced up-regulated BrdU incorporation into DG cells, but 21 days following injury those cells had developed into neurons, while no BrdU cells were found positive for GFAP (Tureyen et al., 2004).

There is some debate as to whether BrdU could be incorporated into dying cells during DNA repair mechanisms (Kuan et al., 2004), though there is evidence to suggest that this is not actually the case (Palmer et al., 2000, Bauer and Patterson, 2005, Czaja et al., 2008). In our study, no co-staining of the two markers was observed either in the DG or in the hippocampal CA1 subfield, strongly suggesting that BrdU incorporation was not a result of repair mechanisms of dying cells, but rather due to DNA replication during cell division.

It is well established from ischaemia models and kainic acid induced damage, that damage to the CA1 results in increased proliferation in the DG (Arvidsson et al., 2001, Wang et al., 2005). Furthermore, work has also demonstrated re-population of cells in the CA1 following an ischaemic insult, with the origin of these cells being the posterior periventricle (pPV)(Nakatomi et al., 2002). Similarly, Bendel and co-workers showed regeneration of cells in the CA1 following an ischaemic insult, in line with restoration of cognitive function, with the origin of the cells likely to be the periventricular region (Bendel et al., 2005). Through similar experimental means, research has shown the important role of these newly generated cells in learning and memory. If there is a reduction in neurogenesis, through drug-induced ablation of neurogenesis, impairment in pattern recognition has been seen (Clelland et al., 2009). Similarly, other hippocampal dependent tasks and long term spatial memory are impaired if there is a reduction in neurogenesis (Shors et al., 2001, Deng et al., 2009, Jessberger et al., 2009). These studies were more concerned with increased proliferation in the DG, but the work by Nakatomi et al looked at the pPV (Nakatomi et al., 2002). Further work has shown that the pPV generated progenitors have the ability to migrate to the peri-injured cortex following traumatic brain injury (Yi et al., 2013). Even with this evidence, there is still debate as to the functional and clinical importance of neurogenesis and hippocampal (or pPV) generated progenitor cells.

Furthermore, despite significant research effort, the exact signalling mechanism behind these effects is still to be fully elucidated. However, there is some evidence to implicate different growth factors in the neurogenesis. Nakatomi et al demonstrated a role for epidermal growth factor and fibroblast growth factor-2 (Nakatomi et al., 2002) and Activin A, a member of the transforming growth factor- β superfamily (Abdipranoto-Cowley et al., 2009). While the experimental protocols are different between these studies and ours, it is reasonable to suggest that they may have a role in the increased proliferation seen in our study.

Following long-term survival, there was a marked decrease in the number of BrdU positive cells. However, based on the literature, this was to be expected as other groups have seen this, albeit in naïve animals (Kempermann et al., 1997, Dayer et al., 2003). Two main reasons have been proposed for this decrease in numbers over time; dilution of BrdU to undetectable levels through cell division (Hayes and Nowakowski, 2002) and death of the newly generated cells (Biebl et al., 2000). It is thought that cell death accounts for the majority of this decrease as it has been suggested that while neurogenic regions show increased cell proliferation, they also have higher rates of cell death (Biebl et al., 2000). The death of the newly generated cells seems to cease at approximately 4 weeks and this is the time that corresponds to the development of mature neuronal features (Dayer et al., 2003). This is mirrored in our study which shows a large decrease in BrdU positive cells up to 28days, but the decrease from 28 to 56 days survival is only modest. The death of these newly generated cells is most likely through programmed cell death/apoptosis (Biebl et al., 2000). The likelihood is that the pro- and anti-apoptotic Bcl-2 protein family play an important role in this as it has been demonstrated that Bcl-2 expression is retained in neurogenic regions of the adult brain and that Bcl-2 overexpression reduces death of

adult/newly-generated cells (Merry et al., 1994, Kuhn et al., 2005). Furthermore, it has been demonstrated that knock-out of the pro-apoptotic protein Bax (Sun et al., 2004) or the pro-apoptotic BH3-only proteins BIM and PUMA (Bunk et al., 2010a) significantly reduces apoptosis in the dentate gyrus.

Of these surviving BrdU-positive cells, the majority expressed a neuronal phenotype. Following 28 or 56 days survival between 70 and 83 % of the remaining BrdU-positive cells showed NeuN expression and there was no difference between control and NMDA treated. This result is consistent with other studies on naïve animals (Biebl et al., 2000, Brandt et al., 2003, Dayer et al., 2003) as well as after brain injury such as transient forebrain ischemia (Kawai et al., 2004), demonstrating that the majority of newly generated cells develops a neuronal phenotype within the DG. In addition, the work by Nakatomi et al showed that newly re-generated pyramidal cells in the CA1 became integrated in the hippocampal circuitry for at least the period of their study (6 months) and similarly the work by Bendel et al demonstrated that the newly regenerated cells expressed a mature neuronal phenotype and indicated by NeuN staining (Nakatomi et al., 2002, Bendel et al., 2005).

It is well established that the NMDA receptor has a role in the regulation of adult neurogenesis; however, it is not clear if this is a facilitatory or inhibitory role (Cameron et al., 1995, Arvidsson et al., 2001, Nacher et al., 2003). Ifenprodil was used to antagonise NR2B-subunit containing NMDA receptor activity following an excitotoxic insult and determine the effect on neurogenesis.

In our study, it was demonstrated that following ifenprodil administration, proliferation was significantly increased in the dentate gyrus not only after NMDA treatment but also in control animals that had received ifenprodil alone. As ifenprodil alone is unlikely to cause cell death, this result shows that the effect seen is due to ifenprodil, particularly as this is also seen in areas not strongly or directly affected by the cell death. This finding suggests that reduction of NR1/NR2B NMDA receptor activity (Amico-Ruvio et al., 2012), through ifenprodil treatment, can promote neuronal precursor cell proliferation. While the result for the control and ifenprodil treated animals did not reach statistical significance in the pPV, there was a trend showing an increase in BrdU⁺ labelling. In addition, the increase in proliferation that we have seen, cannot be simply linked to cells undergoing cell death as we would have therefore seen a decrease rather than increase in BrdU⁺ labelling when comparing the NMDA and NMDA/ifenprodil groups (Mishra et al., 2011). Of note, ifenprodil has been shown to exert effects through other receptor systems, such as the 5-HT₃ receptors (McCool and Lovinger, 1995), sodium channels (Tanahashi et al., 2007), calcium channels (Bath et al., 1996) and the Na⁺/Ca²⁺ exchanger (Brittain et al., 2012).

However, our results are in agreement with a recent study which showed that only the selective NR2B blocker Ro 25-6981, and not the non-subunit selective open channel blocker MK-801, could increase neuronal stem cell proliferation *in vitro* (Hu et al., 2008). Given that a certain level of NMDA receptor activity is essential for cell survival (Hwang et al., 1999), it could be that blocking the NR2B containing NMDA receptors, and leaving the activity of NR2A containing receptors unaffected, enhanced NR2A-dependent signal transduction was responsible for the increase in cell proliferation in the dentate gyrus, though this would need to be studied further.

Ro 25-6981 has been shown to inhibit the downstream enzymatic activity of neuronal nitric oxide synthase (nNOS) through an action on the NMDA receptor (Hu et al., 2008). Therefore, one possible explanation is that neuronal precursor cell proliferation is regulated in a negative manner via the NR2B subunit of the NMDA receptor, for example by regulating downstream activation of nNOS. However, MK-801 will also inhibit nNOS and yet Hu and colleagues did not see an increase in proliferation from MK-801 treatment. This suggests that the mechanisms of neurogenesis need to be further examined, particularly as recent work showed that the source of NO has a differential effect on neurogenesis, either stimulation or inhibition (Luo et al., 2010). In addition, ifenprodil interacts in a negative allosteric manner with the polyamine binding site on the NMDA receptor (Reynolds and Miller, 1989, Han et al., 2008), and since the polyamines may have a role in neural stem cell proliferation and differentiation (Cohen, 1998), this may further contribute to an effect of ifenprodil on neurogenesis.

Our study has shown that in the long-term survival groups, following ifenprodil administration, there was no difference in the ability of the cells to survive between the different treatment groups, in both the DG and the proposed neurogenic area, pPV. However, ifenprodil was administered only for a relatively short period of time (4 – 9 days following NMDA-induced injury) and it is possible that for ifenprodil to have an effect it would need to be administered at different time points throughout the survival period. Therefore, one cannot exclude an effect of ifenprodil on cell survival. However, a study by Chen *et al.* (2007) showed that expression of BDNF, which was shown to be involved in survival of newly generated cells, was independent of NR2B/NMDA receptor inactivation by ifenprodil, but was induced by NR2A/NMDA receptor activity (Chen et al., 2007).

This study has provided further evidence that administration of NMDA to the CA1 subfield of the hippocampus can produce a discrete lesion that results in an increase in proliferation in the dentate gyrus and posterior periventricle, thus allowing this process and the long-term survival of the newly-generated cells to be studied. Using this model it was shown that ifenprodil increased precursor cell proliferation even under control conditions. While further molecular mechanistic studies are needed to fully define the role of the NMDA receptors, this work suggests that NR2B/NMDA receptors may fulfil a negative regulatory role during this process of neurogenesis.

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Figure Legends:

Figure 1:

Diagrammatic representation of the experimental protocols used in this study

A: Summary of the experimental set-up to assess the peak time point of neurogenesis after NMDA-induced excitotoxic cell death. Animals received intrahippocampal NMDA or saline at day zero. BrdU was administered i.p. at different time points (days 0 – 3, 6 – 9 or 12 – 15) followed by transcardial perfusion 24 hours after the last BrdU injection.

B: Summary of the experimental set-up for analysis of long-term survival of newly generated cells after NMDA-induced excitotoxicity. Mice received intrahippocampal NMDA injections at day zero, i.p. injections of BrdU at days 6 – 9 and were sacrificed by transcardial perfusion 24 hours, 28 days or 56 days after the last BrdU injection.

C: Summary of the experimental set-up for analysis of the effect of ifenprodil on neurogenesis and survival of cells newly generated after NMDA-induced excitotoxicity. The same set-up as described in B above was used. Additionally, animals received Ifenprodil or vehicle solution at days 4 – 9 after intrahippocampal NMDA injection.

Figure 2:

Discrete NMDA-induced injury to the CA1 subfield of the hippocampus.

NMDA (10 mg/ml) was administered to the hippocampus and induced cell death localised to the CA1 region (**A, A'** – control; **B, B'** – NMDA-induced excitotoxic injury) and indicated by the arrows (hematoxylin stain). **Scale bar = 500 μ m (A & A'); 100 μ m (B & B')** **C:** Quantification of BrdU-positive cells (cell proliferation) in DG, CA1 subfield and pPV after NMDA-induced excitotoxicity. Following NMDA administration, animals were treated with daily BrdU (ip) injections at 0 – 3 (control: n = 4; NMDA treatment: n = 8 animals), 6 – 9 (control: n = 4; NMDA treatment: n = 11 animals) and 12 – 15 (control: n = 4; NMDA treatment: n = 6 animals) days after NMDA treatment. Transcardial perfusion occurred 24 hours after the last BrdU injection. BrdU-positive cells were counted in the DG, CA1 subfield and pPV and numbers were compared between NMDA treated and control animals. The peak time for BrdU-positive cells was 6-9 days after NMDA injury. DAI, days after injury; DG, dentate gyrus; CA1, cornu ammonis; pPV, posterior periventricle. Error bars represent SEM. * $p < 0.05$.

D: Illustration of BrdU+ cells in the dentate gyrus, the hippocampal CA1 and the posterior periventricle. Scale bar = 50 μm (DG); 25 μm (CA1 & pPV) **E:** GFAP-positive cells in DG and CA1 after NMDA-induced injury in the CA1 subfield. BrdU-positive cells (green) were present in the SGZ of the DG, indicating the generation of new cells (open arrowheads). GFAP-positive cells (red) were also found in SGZ and GCL, but staining did not co-localise with BrdU (closed arrowheads). BrdU-positive cells in the CA1 subfield were found at the site of injury. Some BrdU-positive cells stained positive for GFAP (closed arrowheads) whereas others did not (open arrowheads). DG, dentate gyrus; GCL, granule cell layer; SGZ, subgranular zone; CA1, cornu ammonis. Scale bar = 50 μm (DG); 25 μm (CA1) **F:** Doublecortin expression in BrdU-incorporated cells of the DG. The marker DCX is evident as a red marker and occasional co-localisation with BrdU-positive cells was seen (arrowhead). GCL, granule cell layer; SGZ, subgranular zone; DG, dentate gyrus; DCX, doublecortin. Scale bar = 50 μm **G:** BrdU positivity was not associated with apoptotic cell death. BrdU-positive cells (red/purple) were present in the SGZ of the DG indicating the generation of new cell. TUNEL-positive cells (green) were found in the DG, but the staining showed no co-localisation with BrdU. BrdU-positive cells appeared in the injured area of the CA1 subfield but again TUNEL staining showed no co-localisation with BrdU. DG, dentate gyrus; GCL, granule cell layer; SGZ, subgranular zone; CA1, cornu ammonis. Scale bar = 50 μm

Figure 3:

BrdU and NeuN staining in the dentate gyrus in mice sacrificed 24 hours after treatment. **A** Quantification of BrdU+/NeuN- and BrdU+/NeuN+ cells within the Dentate Gyrus revealed that 24 hours following the last BrdU injection 13 % (NMDA) – 15 % (control) of the BrdU-positive cells were already positive for NeuN. The total number of BrdU-positive cells was significantly increased in the NMDA treated animals compared to control. Control: n = 6, NMDA treatment: n = 10. Error bars represent the standard error of the mean. * $p < 0.05$ v control. **B** After 4 days of BrdU treatment, a small proportion of BrdU-positive cells were also NeuN-positive (arrow). Scale bar = 10 μm

Figure 4:

Long term survival of BrdU-positive cells in DG, CA1 subfield and pPV after NMDA-induced excitotoxic cell death in the CA1 subfield. Animals were subjected to NMDA administration and subsequent BrdU injections 6 – 9 days after NMDA and transcardial

perfusion 24 hours, 28 days or 56 days after the last BrdU injection (see Figure 1 for illustration). BrdU-positive cells were counted in DG (A), CA1 (B) subfield and pPV (C) and cell numbers were compared between NMDA treated and control animals at each survival time point. BrdU-positive cells were significantly up-regulated after NMDA treatment compared to control animals 24 hours after the last BrdU injection in all hippocampal subfields analysed. At 28 or 56 days after the last BrdU injection there was no difference between NMDA treatment and control animals. Control: n = 6, NMDA treatment: n = 10 for all survival groups. Error bars represent the standard error of the mean. * $p < 0.05$ v control/saline.

Figure 5:

NeuN-positive cells in the DG after NMDA-induced injury in the CA1 subfield following different survival periods. Animals were subjected to NMDA administration and subsequent BrdU injections 6 – 9 days after NMDA and transcardial perfusion 28 days or 56 days after the last BrdU injection. After 28 (A-B) and 56 (C-D) days BrdU-positive cells were also positive for the mature neuronal marker NeuN in control (A, C) and NMDA treated (B, D) animals. Arrows within each panel point at the same cells. Scale bar 50 μ m. Columns shown represent a merged picture, DAPI stain, BrdU stain and NeuN stain. GCL, granule cell layer.

Figure 6:

Effect of Ifenprodil on proliferation in DG and pPV after NMDA-induced excitotoxicity. Animals were subjected to intrahippocampal NMDA administration followed by i.p. BrdU injection at day 6 – 9 post NMDA treatment. Animals received 5 mg/kg (ip) Ifenprodil or saline on days 4 – 9 following NMDA. At 24 hours, 28 or 56 days after the last BrdU and Ifenprodil/saline injection animals were sacrificed by transcardial perfusion and brains were assessed for BrdU incorporation in the DG and pPV. (A) 24 hours after the last BrdU injection BrdU-positive cells increased significantly in the DG of NMDA treated animals (dark grey bar), Ifenprodil injected animals (light grey bar) and animals treated with both NMDA and ifenprodil when compared to control. At 28 or 56 days after the last BrdU injection only few cells remained and there was no difference between the different treatment groups (A). (B) In the pPV BrdU-positive cells were significantly increased in NMDA and NMDA/ifenprodil treated animals compared to control (white bar) 24 hours after the last

BrdU injection. Ifenprodil treatment alone resulted in increased BrdU-positive cell numbers compared to control animals though this did not reach significance in the pPV ($p = 0.1$). At 28 and 56 days after the last BrdU injection NMDA/Saline or NMDA/Ifenprodil treatment resulted in an increase of BrdU-positive cell survival at 28 days in the NMDA/Ifenprodil group and at 56 days in the NDMA/saline group, though the NMDA/saline group at 28 days did not reach significance ($p < 0.1$) (**B**). Saline/Saline groups: $n = 6$, Saline/Ifenprodil groups: $n = 8$, NMDA/Saline groups: $n = 10$, NMDA/Ifenprodil groups: $n = 10$ animals. Error bars represent the standard error of the mean. * $p < 0.05$ v the relevant control (saline/saline or saline/ifenprodil)