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Progesterone and allopregnanolone improve stroke outcome in male mice via distinct mechanisms but neither promotes neurogenesis

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Abstract

Based on the outcome of a number of experimental studies, progesterone holds promise as a new therapy for stroke. To understand more about the mechanisms involved we administered progesterone (or the major metabolite, allopregnanolone), intra-peritoneally, for a period of 24 h after transient middle cerebral artery occlusion to male mice variably expressing intracellular progesterone receptors A/B. Effects on infarct volume and neurogenesis were then assessed up to one month later. Predictably, infarct volume in wild-type mice receiving either drug was significantly smaller. However, mice heterozygous for intracellular progesterone receptors A/B showed protection by allopregnanolone but not by progesterone. There was robust amplification of cell division in the wall of the lateral ventricle on the injured side of the brain, these cells migrated into the striatum and lateral cortex, and a significant number survived for at least three weeks. However, very few doublecortin-positive cells emerged from the subventricular zone and subsequent expression of NeuN in these newborn neurons was extremely rare. Neither progesterone nor allopregnanolone amplified the rate of neurogenesis, suggesting that the long term benefits of acute drug administration results from tissue preservation.

Keywords

progesterone; allopregnanolone; stroke; neurogenesis; intracellular progesterone receptor

The annual costs of stroke will increase substantially over the next 20 years, placing increased emphasis on prevention, acute care and rehabilitation (Ovbiagele et al., 2013). Tissue plasminogen activator (tPA) can dissolve an obstructive clot and restore blood flow, but only a minority of stroke patients are eligible for treatment and there is risk of haemorrhage. Nothing has, as yet, followed tPA into the stroke clinic (Sena et al., 2007; Crossley et al., 2008).

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Compared with men of the same age, pre-menopausal women have a lower risk of stroke; however, the incidence of stroke then increases rapidly, coincident with diminished circulating levels of estrogen and progesterone (Appleros et al., 2009; Haast et al., 2012; Gibson, 2013). Experimental studies of our own and others suggest that administration of progesterone (PROG) after focal cerebral ischemia in a variety of species confers both anatomic and functional benefits (Gibson et al., 2008; Wong et al., 2013). While the mechanisms are incompletely known, male mice $^{+/-}$ and $^{-/-}$ for the classic intracellular progesterone receptor (iPR) have larger infarcts and are no longer protected by PROG (Liu et al., 2012).

Neural stem cells in the two germinal zones of the brain increase rate of proliferation post-stroke and apparently generate neuroblasts (Lindvall and Kokaia, 2011; Kahle and Bix, 2013); interference with neurogenesis is reported to negatively influence stroke outcome (Wang et al., 2012). In our initial report of the benefits of PROG after ischemic stroke in mice, we showed that a course of administration over the first 24 hours resulted in functional benefits evident many weeks later (Gibson and Murphy, 2004). As PROG (Zhang et al., 2010a,b) and one product of its metabolism, allopregnanolone (ALLO) (Wang et al., 2008), are reported to promote survival of newly born neurons, we administered these drugs to wild-type and iPR-deficient male mice after transient middle cerebral artery (MCA) occlusion and assessed effects on neurogenesis up to one month later.

Materials and methods

Primary antibodies: BrdU (Novus NB500-169, 1:500); DCX (Santa Cruz sc-8066, 1:250); NeuN (Upstate ABN90, 1:250). Secondary antibodies (Jackson ImmunoResearch): Cy3 anti-rat (1:250); DyLight 649 anti-goat (1:250); Alexa Fluor 647 anti-guinea pig (1:250). DAPI (1:50,000) was from Invitrogen and ALLO (AS04 041S) from Agrisera. All routine chemicals, including PROG (P-0130), finasteride (FIN, F1293), (2-Hydroxypropyl)- β -cyclodextrin (HOP, H5784), and 2, 3, 5-triphenyltetrazolium chloride (TTC), were from Sigma. The C57bl/6 mice were purchased from Jackson Labs.

Animals

The breeding colony was derived from male $^{-/-}$ PR^{lacZ} mice provided by Baylor College of Medicine (Ismail et al., 2002). Males were paired with C57bl/6 females and the offspring genotyped by PCR. Heterozygote (PR $^{+/-}$) breeding pairs were established so as to generate PR $^{+/+}$, PR $^{+/-}$ and PR $^{-/-}$ littermates.

Transient middle cerebral artery occlusion

Only male offspring were subject to MCAo surgery (25–32 g body weight, ~12 weeks of age) and genotype was identified post-mortem to ensure randomization and blinding. Surgery was performed as described in Jayadev et al. (2011) with approval from the Institutional Animal Care and Use Committee of the University of Washington. Regional cerebral blood flow was routinely determined in all mice by laser Doppler flowmetry through the intact skull. Vehicle (HOP, final 2% in PBS), PROG (8 mg/kg), or ALLO (10 mg/kg) was administered ip immediately upon reperfusion, and again 6 and 24 h later. This

regimen recapitulated our primary study (Gibson and Murphy, 2004). Groups of mice were killed 48 h after MCAo, the brains cut into 2 mm sections, and slices incubated in TTC (1 mg/ml saline) at room temperature for 30 min. Digital photographs were taken and infarct area calculated by at least two observers blinded to treatment, correcting for edema.

The remaining mice were injected with bromodeoxyuridine (BrdU; 50 mg/kg, ip twice each day, 8 h apart) on days 6 and 7 post-surgery, to coincide with the peak neurogenic response following MCAo (Zhu et al., 2003; Luo et al., 2005). Some mice were killed by intra-cardiac perfusion with 4% paraformaldehyde on day 11 to monitor BrdU incorporation, but the majority were maintained until day 28. Excised brains were post-fixed for 24 h and then placed in 30% sucrose, which was changed three times. Trimmed brains were mounted in optimal cutting temperature compound on dri-ice and stored at -20°C until sectioning by cryostat.

Immunohistochemistry

Coronal sections of brain (25 μm) were blocked and permeabilized in 10 % donkey serum/0.5% Triton-X for 60 min before addition of primary antibody (overnight at 4°C). After a thorough wash in PBS, sections were exposed to secondary antibodies overnight at 4°C . After further washing, slides were incubated in 4',6-diamidino-2-phenylindole (DAPI) for 10 min and cover-slipped in gelvatol. The protocol for BrdU was that of Pan et al. (2012), which involved two HCl steps and neutralization in sodium borate.

Imaging

Images were collected via a confocal microscope (FV-1000 Olympus). The DAPI signal was excited with a 405 nm laser, Cy3 with a 561 nm solid state diode laser, and Alexa 647 with a Helium-Neon laser. Positive cells were recorded in a randomly chosen field (512 μm square), within the lateral cortex, striatum, sub-ventricular zone (SVZ), dentate gyrus (DG) and CA1, in both ipsi- and contra-lateral brain, in one tissue section from each of the eight slides selected at intervals through the brain (a 1:20 series). A total of 28 optical sections of 0.5 μm thickness were collected in the z-axis from a single field using a 20x objective lens under fixed gain, laser power, pinhole, and PMT settings. Images were captured and presented to two or three observers, who counted cells in a blinded fashion.

Statistical analysis

Data are summarized as mean $\pm$ standard error. Mean values were compared by ANOVA or Student's 't' test, as appropriate. *P*-values < 0.05 were considered statistically significant.

Results

To determine whether metabolism was important for the neuroprotective effects of PROG, one group was injected with finasteride (FIN; 10 mg/kg, ip), an inhibitor of 5 α -reductase. Groups of wild-type C57bl/6 males were subject to 1 h of MCAo followed by administration of PROG, FIN prior to PROG, ALLO or vehicle. Compared with vehicle, the average infarct volume of mice receiving PROG and ALLO was significantly smaller (37% and 65%, respectively). In the group of six mice receiving FIN prior to PROG, the average infarct

volume was not significantly different from either vehicle or PROG-only mice; notably, there was increased variability in the PROG/FIN group (Figure 1).

To produce a more definitive answer regarding metabolism in the neuroprotective effects of PROG, we assessed infarct volume in mice genetically variable in iPR expression. We focused on heterozygous males because these mice have been shown to have reduced responsiveness to PROG (Liu et al., 2012), and they were the largest constituency as dictated by our breeding strategy. For these experiments, the duration of MCA occlusion was reduced from 60 min to 45 min, in case loss of iPR removed endogenous neuroprotection and risked animal survival. For comparison, we measured average infarct volumes in $^{+/+}$ and $iPR^{-/-}$ mice 48-h after MCAo and noted these were not significantly different (Figure 2A), confirming the findings of Liu et al. (2012). In $iPR^{+/-}$ mice, the administration of PROG after MCA occlusion no longer resulted in neuroprotection while the benefits of exogenous ALLO were unaffected (Figure 2B). Post-surgical survival of mice was unaffected by treatment.

Newly-born cells ($BrdU^{+}$), and immature (DCX^{+}) and mature ($NeuN^{+}$) neurons were identified at various times post-stroke, by indirect immunofluorescence using specific probes, in various brain regions of mice $^{+/+}$ and $^{-/-}$ for iPR (Figure 3A, 3B). In mice that had been pulse-labeled with BrdU on days 6/7 after MCAo we noted, on day 11 (six mice), very large increases in the number of $BrdU^{+}$ cells in the lateral cortex and striatum on the injured, as compared to the contralateral side ($>100:1$). There were large numbers of DCX^{+} (>100), $BrdU^{+}$ (50–100) and double-labelled cells (>100) in the SVZ (many fewer in the DG) on day 11, but few DCX^{+} cells (<50) had migrated from the SVZ into the lateral cortex or striatum.

A considerable proportion of the $BrdU^{+}$ cells seen on day 11 persisted until day 28 (six mice), particularly in the striatum ($\sim 80\%$) but less so in the lateral cortex ($<50\%$), and $BrdU^{+}$ cells remained scattered throughout the SVZ and DG. None of the $BrdU^{+}$ cells in the injured striatum and lateral cortex were positive for DCX^{+} and the co-expression of BrdU and $NeuN^{+}$ was extremely rare. Acute treatment with PROG or ALLO has been reported to suppress post-stroke inflammation and to promote neurogenesis *in vitro*. However, we found that neither drug enhanced neurogenesis at 28 days post-stroke; the only significant effect we noted was a 50% reduction in the number of $BrdU^{+}$ cells remaining in the striatum of mice ($iPR^{+/+}$ and $^{-/-}$) that had PROG administered (VEHICLE: 190 ± 29 vs. PROG: 100 ± 13 , $n = 6$ and 7 ; $p < 0.02$). This was not seen in mice receiving ALLO (201 ± 29 , $n = 7$).

Discussion

Both PROG and ALLO are neuroprotective when administered acutely in animal models of acute brain injury, resulting in the presumption that the effects of PROG are secondary to its metabolism by Type II steroid 5α -reductase in the brain (Mahendroo et al., 1996). Despite our equivocal findings in wild-type mice with a 5α -reductase inhibitor (FIN), the definitive outcomes we see in $iPR^{+/-}$ mice confirm the recent proposal (Liu et al., 2012) that PROG and ALLO achieve neuroprotection through different signaling pathways. The potential

mechanisms involved have been exhaustively reviewed (Hosie et al., 2006; Schumacher et al., 2007, 2008). One interpretation regarding the effects of PROG is that established neurons are more likely to survive cerebral ischemia because the inflammatory response (elevated interleukin-1 β , tumor necrosis factor α , inducible nitric oxide synthase) is heavily suppressed by the drug (Grossman et al., 2004; Gibson et al., 2005). As GABA_A receptors are important in stroke recovery (Clarkson et al., 2010), and ALLO is well-known to modulate these receptors (Hosie et al., 2006; Knight et al., 2012), this could represent an additional mechanism.

Amplification of neurogenesis after stroke is well-documented, both in rodent and in human brain (Lledo et al., 2006; Macas et al., 2006; Murphy and Corbett, 2009). Though the functional significance and roles of these newly born cells are very far from clear, the time scale of this phenomenon correlates with the recovery of function from stroke. Furthermore, conditional depletion of DCX-immunopositive cells, indicative of immature neurons, has been reported to inhibit long-term recovery in mice (Wang et al., 2012). These new cells arise in the two persisting germinal regions of the brain, DG and SVZ, and the latter migrate to sub-cortical and (to a lesser extent) cortical structures.

In mice injected with BrdU one week after MCAo we observed, a few days later, very large increases in the number of BrdU⁺ cells in the lateral cortex and striatum on the injured, as compared to the contralateral side. While there were large numbers of DCX⁺ and DCX⁺/BrdU⁺ cells in the SVZ (fewer in the DG) at 11 days after stroke, few had migrated out into the surrounding tissue. A significant proportion of this population of newborn cells persisted, particularly in the striatum. Even after four weeks we found numerous BrdU⁺ cells still in the striatum (fewer in the lateral cortex) ipsilateral to injury, but BrdU⁺ cells co-expressing either DCX or NeuN were extremely rare! The absence of newborn neurons in this study is notable, but it is worth remembering that we could trace only the fate of those cells born on days 6 and 7 post-injury.

The efficiency of the neurogenic response following stroke is very limited, presumably because the attendant inflammation is highly detrimental (Doepfner et al., 2009; Tobin et al., 2014). Even though acute treatment with either PROG or ALLO is known to suppress post-stroke inflammation, we found that neither drug enhanced proliferation or migration. Indeed, at four weeks after stroke the only discernible difference in brain histology was a 50% reduction in the number of BrdU positive cells remaining in the striatum of mice (both ^{+/+} and ^{-/-}) that had received PROG (but not ALLO). Zhang et al. (2010a) found that PROG promoted survival of newborn DG neurons and, when administered post-stroke, suppressed proliferation but enhanced migration and improved survival of new neurons (Zhang et al., 2010b). Both PROG and ALLO are reported to promote neural progenitor proliferation *in vitro* (Wang et al., 2005) and ALLO can reverse deficits in neurogenesis in DG of a mouse model of Alzheimer's disease (Wang et al., 2010).

Post-stroke, even though cell proliferation is amplified in the ipsilateral SVZ and there is migration into the striatum and lateral cortex, it seems that these newborn cells are not destined to become (or to survive as) neurons. While it is possible that a sub-population of BrdU⁺ cells derives from bone marrow rather than SVZ, and infiltrates on the ipsilateral side

through a leaky blood-brain barrier, the massive extent of proliferation we observe in the SVZ on day 11 could easily account for the number of persisting BrdU⁺ cells on day 28. Do these surviving newborn cells have biological significance? Presumably, they could represent a source of neurotrophic factors and so participate in dynamic tissue remodeling. However, as neither PROG nor ALLO amplified proliferation or the migration of newly born cells we conclude that neurogenesis does not contribute to the long term benefits of drug administration.

Two Phase III trials of progesterone administration for head injury have completed recruitment but no results are available as yet. In the ProTECT III trial, supported by NIH/NINDS, enrolment was stopped early after the independent Data and Safety Monitoring Board determined that the data indicated it was unlikely that progesterone treatment would demonstrate better outcomes as compared to a placebo control. Progesterone and allopregnanolone have not yet been in trial for ischemic stroke.

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Abbreviations used

ALLO	allopregnanolone
BrdU	bromodeoxyuridine
DG	dentate gyrus
FIN	finasteride
HOP	2-Hydroxypropyl)- β -cyclodextrin
iPR	intracellular progesterone receptor
MCA	middle cerebral artery
PROG	progesterone
SVZ	subventricular zone
tPA	tissue plasminogen activator
TTC	2, 3, 5-triphenyltetrazolium chloride

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Figure 1.

Neuroprotective effects of PROG and ALLO. Drugs were delivered to C57bl/6 mice after 60 min MCAo and infarct volume measured 48 h later. The mice receiving PROG and ALLO had smaller infarcts than VEH, but mice treated with FIN prior to PROG were not significantly different from VEH or PROG alone. Data represented as mean $\pm$ SE (n); * p < 0.05. Two vehicle mice and one progesterone-treated died post-surgery and are not included in the study.

Figure 2.

The role of PROG and iPR in neuroprotection. **(A)** Infarct volume after 45 min MCAo and 48 h reperfusion in PR^{lacZ} mice $+/+$ and $-/-$ for PR. The apparent trend for infarct volume to increase with loss of PR was not statistically significant. One wildtype and one knockout mouse died post-surgery and are not included in the study. **(B)** Effects of drugs delivered to $PR^{+/-}$ mice after 45 min MCAo, with infarct volume measured after 48 h reperfusion. ALLO was protective in $PR^{+/-}$ mice, but not PROG. Data represented as mean $\pm$ SE (n); * $p < 0.05$. Two vehicle, three progesterone-treated mice and one allopregnanolone-treated died post-surgery and are not included in the study.

Figure 3.

Neurogenesis in the brain. (A) Coronal section of the DG 5 days after injection of BrdU. Newborn (BrdU⁺) cells arise in the sub-granular zone and migrate (DCX⁺) through the existing neuronal layers. BrdU⁺/DCX⁺ cells are indicated by white arrows. (B) Coronal section of the DG 22 days after the injection of BrdU. Cells in the sub-granular zone were NeuN⁻ and an occasional BrdU⁺ cell persisted. There were no losses of mice post-BrdU injection.