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Progesterone Inhibition of Neuronal Calcium Signaling Underlies Aspects of Progesterone-Mediated Neuroprotection

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Abstract

Progesterone is being utilized as a therapeutic means to ameliorate neuron loss and cognitive dysfunction following traumatic brain injury. Although there have been numerous attempts to determine the means by which progesterone exerts neuroprotective effects, studies describing the underlying molecular mechanisms are lacking. What has become clear, however, is the notion that progesterone can thwart several physiological processes that are detrimental to neuron function and survival, including inflammation, edema, demyelination and excitotoxicity. One clue regarding the means by which progesterone has restorative value comes from the notion that these aforementioned biological processes all share the common theme of eliciting pronounced increases in intracellular calcium. Thus, we propose the hypothesis that progesterone regulation of calcium signaling underlies its ability to mitigate these cellular insults, ultimately leading to neuroprotection. Further, we describe recent findings that indicate neuroprotection is achieved via progesterone block of voltage-gated calcium channels, although additional outcomes may arise from blockade of various other ion channels and neurotransmitter receptors.

Keywords

Progesterone; Brain Injury; Neuroprotection; Ischemia; Excitotoxicity; Calcium

1. Introduction

The neuroprotective effects of progesterone have been thoroughly examined and well documented in both *in vitro* and *in vivo* paradigms. Despite the extensive evidence for the beneficial effects of progesterone following insults such as traumatic brain injury, stroke, and excitotoxicity, the exact mechanism(s) by which progesterone exerts these neuroprotective effects remain elusive. The search for an underlying source of progesterone action in these models is complicated by the sheer magnitude of cellular events that occur following neuronal insult, thus providing few limits on putative progesterone targets. Moreover, progesterone is known to affect many of the cellular events that follow injury or toxicity including inflammation, edema, myelin degradation, excitotoxicity, and intracellular signaling. Thus, an emerging hypothesis is that progesterone exerts its neuroprotective effects by regulating many of these cellular events in parallel. Indeed, one clue as to the

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identity of a putative molecular target of progesterone is that ion channel signaling is linked to many of the events that occur following neuronal injury. Work from our lab and others support the hypothesis that progesterone regulation of neuronal calcium signaling via its regulation of voltage-gated calcium channels may underlie the neuroprotective effects of therapeutic concentrations of progesterone. This review will address this hypothesis in the context of two classes of studies: 1) those in which progesterone-mediated neuroprotection is established in models such as stroke injury, traumatic brain injury, and excitotoxicity, and 2) those that address the underlying modulation of calcium signaling, which may account for the observed neuroprotective effects.

2. Neuroprotective Effects of Progesterone

While there are many *in vivo* reports of the neuroprotective effects of progesterone, the most popular paradigm for studying this phenomenon is a stroke model in which a focal ischemia is induced by middle cerebral artery occlusion (MCAO) followed by reperfusion and subsequent assessment of neuronal death. One of the earliest reports of progesterone neuroprotection in this model was a qualitative 1990 study by Betz et al. [1] demonstrating that pretreatment with progesterone decreased MCAO-induced edema. A later study quantitatively documented that progesterone decreased the ischemic area following MCAO [2]. Offering the first insights as to the specific mechanism of action were studies that, somewhat surprisingly, discovered that progesterone need not be administered prior to artery occlusion, but that it was efficacious even when administered up to 24 hours post-injury. This finding has been corroborated by other studies in models of both focal and global (four-vessel occlusion) ischemia, with progesterone administration occurring anywhere between one-half hour to two hours post-occlusion [3; 4; 5; 6; 7; 8; 9; 10; 11; 12]. Importantly, in addition to preventing cell death, progesterone also preserved neuronal function, as assayed by rodent behavioral tests [5; 7; 8; 9; 10; 12], thus highlighting the potential therapeutic utility of progesterone as a treatment for human brain injury.

The neuroprotective effects of progesterone have also been demonstrated in rodent models of traumatic brain injury (TBI). These models often involve neuronal survival assays following controlled cortical contusion injury (CCI). Roof et al. first suggested a link between progesterone and neuroprotection in these models of brain injury with their observation that pseudopregnant rats, which have high levels of circulating progesterone, were nearly spared from injury-induced cognitive deficits [13]. The same group later found that progesterone treatment could be delayed up to 24 hours post-injury without losing effectiveness [14]. Several other studies have corroborated the finding that post-injury treatment with progesterone is neuroprotective in terms of reducing neuron loss [15; 16; 17; 18; 19; 20] and in preserving cognitive outcome [16; 17; 18; 19; 20; 21; 22]. Together these data suggest that the window of opportunity for treatment with progesterone is relatively large (i.e. on the order of hours), and thus it need not be administered immediately following a brain insult.

Paralleling the effects of progesterone neuroprotection in whole animal models of injury, are results from *in vitro* neuronal models of glutamate-induced excitotoxicity. Glutamate overload is one of the key neurotoxic effects thought to occur following *in vivo* brain injury, and thus *in vitro* excitotoxicity is a tractable preparation for investigating the molecular mechanisms of neuronal death following injury. Indeed, progesterone is protective against excitotoxicity in dissociated cultured neurons [23; 24; 25; 26; 27; 28] as well as in cerebral cortical slice culture [29; 30]. Furthermore, progesterone exerts protective effects in alternative models of toxicity in which trophic starvation, MPTP, oxidative stress, and β -amyloid treatment induce neuronal death [25; 31; 32], suggesting that progesterone may directly influence an upstream process common to these various forms of neuronal insult.

These data have also been used to suggest that progesterone may be useful as a therapy in neurodegenerative disorders such as Parkinson's and Alzheimer's disease [25].

In initial findings, the neuroprotective effects of progesterone in human injury cases parallel the results from animal models. For instance, progesterone significantly reduced cognitive impairments in human patients with TBI [33; 34; 35]. To date, the administration of progesterone has been the safest and most effective therapeutic strategy for treatment following traumatic brain injury. Thus, the use of progesterone as a therapy following TBI is now in phase III clinical trials. Although there are reports of females exhibiting better recovery from brain injury compared to males [36], this enhanced efficacy of progesterone therapy is thought to be due to the higher basal level of circulating progesterone in women, as opposed to differences in progesterone efficacy between the sexes [37; 38]. Consistent with this lack of sex-specificity, the studies cited above have utilized concentrations of progesterone that far exceed normal circulating levels of progesterone (except those associated with late-term pregnancy), and that are in excess of those needed to activate that classical progesterone receptor. While the brain does engage in *de novo* progesterone synthesis [39; 40], it is currently unknown whether it achieves concentrations that contribute to neuroprotection. Furthermore, The notion that progesterone could synergize with additional neuroprotective steroid hormones that act via receptor-dependent mechanisms, such as estrogen (either endogenous or therapeutically administered), is intriguing. This possibility may depend on the specific paradigm in question as progesterone and estrogen have been shown to have both synergistic [41] and antagonistic interactions in neuroprotective paradigms [42; 43].

Based on the widespread effectiveness of progesterone in numerous traumatic brain injury paradigms, the list of putative cellular and molecular targets of progesterone action is overwhelmingly large. As mentioned above, one method of simplifying the experimental and theoretical analysis of these targets is to separately group and catalog those neuroprotective effects of progesterone that may share a common upstream mechanism. For example, at least a subset of the beneficial effects of progesterone discussed above could be accounted for by direct progesterone modulation of ion channel signaling. In this spirit, the remainder of this review will briefly summarize the neuroprotective effects of progesterone that have been shown to be ion channel-dependent, and to discuss recent data from our laboratory that directly demonstrates progesterone inhibition of neuronal ion channel signaling.

3. Mechanisms for progesterone-dependent neuroprotection

3.1 Inflammation and edema

Inflammation is often triggered by stroke or TBI, and subsequently induces cerebral edema, which can lead to substantial cell death. Calcium signaling is an upstream mediator of inflammation and edema both in peripheral tissues and in the brain. As such, calcium channel blockers (specifically those that preferentially block L-type calcium channels) have been widely used to treat cardiopulmonary disease due to their ability to inhibit inflammation. Diltiazem, a non-dihydropyridine L-type calcium channel blocker, inhibits cardiopulmonary bypass induced inflammation in humans by reducing cytokine production [44]. Additionally, lercanidipine, a dihydropyridine sensitive L-type calcium-channel blocker, inhibits inflammation in patients with hypertension [45]. Finally, studies from rats have shown that dihydropyridines reduce formalin-induced inflammation in the rat paw [46].

Studies in the brain parallel these results from peripheral tissue in which numerous calcium channel blockers have been shown to reduce and/or prevent edema. For instance, rats treated

with the dihydropyridine benidipine showed reduced edema [47], while the dihydropyridine calcium channel blockers, azelnidipin or amlodipine, reduced edema and neuronal loss following focal ischemia in rats [48]. The novel blockers of both calcium and sodium channels, NS-1 and T-477, were found to be neuroprotective and edema-reducing in rats that underwent focal ischemia, suggesting that inhibition of sodium channels may also play an important role [49; 50]. Finally, (S)-emopamil, a phenylalkylamine-based calcium channel blocker, reduces both edema and memory dysfunction following focal brain injury in rats, again suggesting that blocking calcium signaling during/following injury may have cognitive benefits [51].

Given the overwhelming evidence for the fact that progesterone decreases edema and inflammation in the brain [15; 20; 52; 53; 54; 55], and the fact that inflammation and subsequent edema has been shown to depend on calcium channel signaling in both peripheral tissues as well as the brain, it is reasonable to suggest that progesterone inhibition of calcium signaling may be a mechanism by which progesterone reduces inflammation and subsequent edema following injury. Consistent with this hypothesis, progesterone-mediated inhibition of inflammation and edema occur via a progesterone-dependent reduction of cytokine and inflammatory metabolite production [11; 56; 57; 58; 59], which themselves depend on calcium signaling [44]. This does not, however, rule out the hypothesis that progesterone may inhibit cytokine production in a calcium-independent manner as well.

3.2 Preservation of myelination

Severe nervous system insults often result in the degradation of myelin, the protective sheath that protects the axoplasm and facilitates salutatory conduction along axons. Progesterone has been shown to preserve myelination following spinal cord injury [60; 61; 62; 63]. Consistent with the hypothesis that progesterone influences many cellular processes via inhibition of ion channel signaling, one report [64] found that blockers of voltage-gated calcium channels prevented the loss of myelination in the spinal cords of mice with inflammatory demyelinating disease. Similarly, Winkler et al. [65] showed that treatment with the dihydropyridine nimodipine significantly reduced the degradation of myelin following spinal cord injury in rats. Although these findings collectively suggest that progesterone may prevent demyelination through inhibition of calcium channels, this hypothesis requires further testing.

3.3 Excitotoxicity

Glutamate-induced excitotoxicity, whereby injured neurons release and are inundated by massive amounts of the neurotransmitter glutamate, is widely accepted as being a key event in the initiation of neuron death following many types of neuronal insult [23; 66; 67]. Excitotoxic death of neurons is a complex process that has been shown to occur through a variety of mechanisms that are not yet fully understood. However, it is thought that release of this neurotransmitter into the extracellular space occurs sometime shortly after insult, leading to the activation of both ionotropic and metabotropic glutamate receptor-mediated signaling pathways. Subsequent intracellular calcium elevations and calcium-dependent signaling pathways induced by this sharp increase in extracellular glutamate is a major cause of injury-induced neuron death [68; 69; 70; 71]. While progesterone is protective against excitotoxicity in dissociated cultured neurons [23; 24; 25; 26; 27; 28] as well as in cerebral cortical slice culture [29; 30], there is a lack of consensus surrounding the molecular mechanisms by which this occurs. Since excitotoxicity often leads to calcium-dependent death [72] and progesterone is neuroprotective, it has been well reasoned that progesterone prevents excitotoxic neuronal death by preventing the resulting calcium overload.

4. Progesterone inhibition of calcium signaling

The influence of progesterone on calcium signaling (progesterone-calcium signaling hypothesis) may account for some of the therapeutic benefits of progesterone, including reducing inflammation and edema, preventing myelin degradation, and reducing excitotoxic neuronal death. A recent report from our laboratory directly tested the hypothesis that progesterone influence of calcium signaling underlies progesterone inhibition of excitotoxic neuron death [28]. In neurons, excitotoxic death occurs when glutamate overload sufficiently depolarizes the neuronal membrane potential to activate voltage-gated calcium channels (See Figure 1). Subsequent calcium influx activates a cascade of intracellular signaling pathways and transcription factors that initiate neuronal apoptosis. Thus, progesterone inhibition of calcium signaling and this subsequent neuronal death could occur via progesterone interaction with primary (glutamate receptor) or secondary (calcium channel) sources of calcium influx. To experimentally isolate these mechanisms, we utilized cultured striatal neurons, which lack glutamatergic input but are sensitive to external glutamate application. Additionally, progesterone has been shown to be neuroprotective in the intact striatum [73].

In support of the progesterone-calcium signaling hypothesis, we found that in cultured striatal neurons progesterone completely blocked voltage-gated calcium channel-mediated neuronal death (See Figure 1). Calcium imaging and electrophysiological approaches revealed that this progesterone-mediated neuroprotection occurs via direct inhibition of voltage-gated calcium channel function. These data are consistent with studies that have implicated calcium channels in glutamate-induced cell death [74; 75; 76]. Since the concentrations of progesterone needed to achieve inhibition of L-type channels ($IC_{50} = 27 \mu M$) are far in excess of those needed to activate the progestin receptor [77], expression of the receptor is likely not necessary for these observed effects of progesterone. Receptor-independent actions of progesterone are consistent with the idea that progesterone neuroprotection solely depends on progesterone concentration, rather than receptor availability. Importantly, progesterone was without effect on both glutamate-induced calcium influx, as well as glutamate receptor-mediated ionic currents, demonstrating that progesterone inhibits excitotoxic death exclusively via direct inhibition of voltage-gated calcium channels. The fact that progesterone also inhibited voltage-gated (L-type) calcium channel-mediated activation of the transcription factors CREB and NFAT sheds light onto the downstream mechanisms that may contribute to excitotoxic neuronal death.

In all of the above experiments, neither the progesterone precursor pregnenolone nor the progesterone metabolite allopregnanolone exerted any inhibitory effects. Interestingly, these neurosteroids have been shown to be neuroprotective in other paradigms [78; 79; 80; 81; 82; 83], suggesting the need for further study.

While characterizing progesterone block of L-type calcium channels, it became clear that micromolar concentrations of the hormone could also influence the conductance of other voltage-gated calcium channels. For example, dose-response experiments indicated that at $100 \mu M$, progesterone completely abolished the whole-cell calcium current. While this concentration was higher than that required for neuroprotection (i.e. $50 \mu M$), there was no clear delineation among the progesterone concentrations used that appeared to isolate one calcium channel conductance from another. Thus, it remains unclear whether progesterone inhibition of non L-type calcium channels is neuroprotective, or represents a “supplementary” inhibition.

5. Progesterone-dependent modulation of additional ion-channels

Recent work from our laboratory suggests that this supplementary inhibition extends beyond voltage-gated calcium channels to voltage-gated potassium channels, voltage-gated sodium channels, and GABA_A receptors [84]. While these additional effects may underlie the anesthetic properties of progesterone [85; 86], it is currently unclear how inhibition of these ion channels contributes to neuroprotection. There is, however, precedent in the literature for neuroprotective drugs indiscriminately influencing multiple voltage-gated ion channels. As mentioned, NS-1 or T-477, which blocks both calcium and sodium channels, reduced edema and neuron loss following ischemia [49; 50]. These results are in accordance with additional studies demonstrating that specific sodium channel blockers (tetrodotoxin, riluzole, mexiletine, or phenytoin) reduce ischemia-induced edema and cell death [87; 88], and that broad-spectrum inhibitor of cation channels reduces lesion volume in rats with MCAO-induced ischemia [89].

The ability of micromolar range concentrations of progesterone to inhibit multiple classes of ion channels parallel the effects of similarly high concentrations of nifedipine, a dihydropyridine sensitive L-type calcium channel blocker that also non-selectively inhibits voltage-gated sodium channels, potassium channels, and GABA_A [84; 90; 91; 92]. The results from several other studies corroborate the finding that in addition to L-type calcium channels, dihydropyridines inhibit voltage-gated sodium channels, voltage-gated potassium channels, and GABA_A receptors [90; 91; 92; 93]. Since progesterone and dihydropyridines share neuroprotective [94; 95] and ion channel modulatory properties, they may share a similar mechanism of receptor binding and inhibition. Given these striking similarities, scientists and clinicians utilizing progesterone for therapeutic purposes would be wise to keep in mind that some neuroprotective concentrations of dihydropyridines result in unpleasant and undesired side effects [96; 97]. Thus, a comprehensive molecular understanding of the specific concentrations of and mechanisms by which progesterone inhibits specific ion channels can inform an efficacious and productive analysis of progesterone treatment for TBI.

Conclusion

While progesterone has been shown to be a highly effective means of treating TBI in both the laboratory and clinic, the molecular mechanisms by which this occurs remain elusive. In fact, progesterone has been shown to influence a variety of neuronal parameters that occur as a result of traumatic injury or toxicity including inflammation, edema, myelin degradation, excitotoxicity, and intracellular signaling. In this review, we have developed the hypothesis that progesterone influence of calcium signaling via direct inhibition of voltage-gated calcium channels underlies the neuroprotective effects of progesterone. Furthermore, we presented recent data from our lab demonstrating these inhibitory actions are not limited to calcium channels, but also occur at several voltage- and ligand-gated ion channels, similar to the actions of equimolar concentrations of dihydropyridines. Thus, future investigators are challenged to elucidate the specific conditions and mechanisms by which progesterone exerts these inhibitory effects so that we may fully exploit the therapeutic potential of progesterone.

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Figure 1. Putative mechanism for progesterone mediated neuroprotection

A) Excitotoxic insult is characterized by the influx of both sodium and calcium-ions through glutamate receptors leading to depolarization-induced opening of L-type calcium channels. Calcium flux through L-type calcium channels contributes to calcium overload and subsequent activation of various calcium-mediated death pathways. B) Through an unknown mechanism, extracellular application of progesterone (P) inhibits the influx of calcium ions through L-type calcium channels, protecting the neuron from excitotoxic death.

Table 1
Neuroprotective effects of progesterone: A summary of key findings

Key studies that demonstrate the neuroprotective effects of progesterone are organized here by the condition or disease studied. Further organization by the method of neuron insult and the animal model used; middle cerebral artery occlusion (MCAO), four-vessel occlusion (FVO), traumatic brain injury (TBI), controlled cortical impact (CCI), spinal cord contusion (SCC), amyloid-beta (A β), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

Disease or Condition Studied	Insult	Model	Reference
<i>Stroke</i>	MCAO	Rat	1,3,6,7,9,10,11,3,6,7,9,10,12
	FVO	Rat	2,4
	MCAO	Mouse	5,11
<i>Traumatic Injury</i>	TBI	Human	33,34,35
	CCI	Rat	15,16,17,19,21
	CCI	Mouse	20
	SCC	Rat	22
<i>Toxicity</i>	Glutamate	Rat Neurons-Dissociated Culture	23,24,25,26,27,28,
	Glutamate	Mouse Organotypic Slice Culture	29,30
	Chronic Depolarization	Rat Neurons-Dissociated Culture	28
	A β or FeSO ₄	Rat Neurons-Dissociated Culture	25
	Trophic Deprivation MPTP	PC12 Cultured Neurons	31
		Mouse Neurons-Dissociated Culture	32