Mini Review

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Are the endocannabinoid-like compounds \( N \)-acyl aminoacids neuroprotective after traumatic brain injury?

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Abstract: In recent years, a library of approx. 70 \( N \)-acyl aminoacids (NAAAs) was discovered in the rat brain. A particular member of this family of compounds is arachidonoyl serine (AraS), which has generated special interest as a potential therapy for traumatic brain injury (TBI). This is due to its structural similarity to the endocannabinoid (eCB) 2-arachidonoyl glycerol (2-AG), which was previously shown to be beneficial in the recovery in a closed head injury model of TBI. Indeed, AraS exerted eCB-mediated neuroprotection, which was evident in numerous aspects related to the secondary damage characterizing TBI. These findings promoted broadening of the research to additional compounds of the NAAA family that share a structural similarity to AraS, namely, palmitoyl serine (PalmS) and oleoyl serine. The latter did not exhibit any improvement in recovery, whereas the former displayed some neuroprotection, albeit inferior to 2-AG and AraS, via unknown mechanisms. Interestingly, when a combined treatment of 2-AG, AraS and PalmS was tested, the overall effect on the severity score was inferior to their individual effects, suggesting not only a lack of direct or indirect synergism, but also possibly some spatial hindrance. Taken together, the complexity of the damage caused by TBI and the many open questions concerning the role of the eCB system in health and disease, the findings so far may serve as a small trace to the understanding of the eCB system, as well as of the mechanisms underlying TBI.

Keywords: endocannabinoids; neuroprotection; traumatic brain injury.

Introduction

The endocannabinoid (eCB) system consists of ligands, such as anandamide and 2-arachidonoyl glycerol (2-AG); receptors (CB1, CB2, TRPV1, GPR55 and possibly more); and transporters and enzymes, which are responsible for the synthesis (\( N \)-acyl phosphatidylethanolamine phospholipase D and diacylglycerol lipase) and degradation of these lipid mediators (fatty acid amide hydrolase and monoacylglycerol lipase) \([1, 2]\). Unlike “classical” neurotransmitters, eCBs are not stored in pre-synaptic vesicles, rather, they are produced “on demand” from membrane lipid precursors only when and where they are needed \([1]\). The in vivo bioavailability of eCB is tightly regulated by the activation of the enzymes responsible for its synthesis and degradation. In recent years, many additional fatty acid amides of ethanol amines and amino acids \( [N \)-acyl aminoacids (NAAAs)] have been found \([3]\). They do not bind to the classical cannabinoid receptors; however, few of these compounds were found to exert their actions via cannabinoid receptor-mediated pathways (for a recent review, see Ref. \([4]\)). Recently, Raboune et al. \([5]\) have identified 20 novel \( N \)-acyl amides that collectively activate (stimulating or inhibiting) TRPV1-4. The levels of at least half of these compounds were elevated in the brain in a model of neuroinflammation, indicating that they are regulated “on demand” in the brain after central nervous system (CNS) injury. In addition, their signaling at ion channels may point, at least in part, to their neuroprotective properties \([5]\).
Arachidonoyl serine (AraS), which shares a structural similarity to anandamide, and 2-AG, palmitoyl serine (PalmS) and oleoyl serine (OleoS) are members of the NAAA family that display high chemical stability. Although binding studies of these compounds revealed that they do not bind to one of the known CB receptors (CB1, CB2, TRPV1 or GPCR55), they are also considered to be components of the eCB family. Although the eCB system exerts its manifold activities in both the periphery and the brain, the present review will focus on the cerebral role of this system, with special emphasis on traumatic brain injury (TBI). Several studies have demonstrated the role of eCB compounds in the control of excessive neuronal activity in the brain and in the modulation of neurotransmission [6]. This process is thought to occur via retrograde synaptic suppression, through the CB1 receptor found at presynaptic terminals, which is associated with the inhibition of neurotransmitter release [7] (for a review, see Ref. [8]). As the synthesis of eCB is activated in response to pathogenic events, and CB receptor agonists activate signaling pathways that lead to neuronal repair, it has been suggested that the eCB system is part of the brain’s compensatory repair mechanism, mediated via CB receptor signaling [9].

In the brain, the level of endogenous 2-AG is generally a few orders of magnitude higher than that of anandamide. The pioneering study in the field [10] revealed that, 4 h after TBI, there is a tenfold increase in 2-AG in the injured hemisphere. We have then shown that treatment with synthetic 2-AG attenuated edema formation, infarct volume, blood-brain barrier permeability, neuronal cell loss at the CA3 hippocampal region, and neuroinflammation [11, 12]. Moreover, greater recovery of the neurobehavioral function was noted for up to 3 months after the treatment of TBI in mice with 2-AG (Cohen-Yeshurun, unpublished data). Further studies, by us and other authors, which focused on the neuroprotective role of the eCB system, corroborate the role of 2-AG in the resolution of secondary injury after TBI and in neurodegenerative disorders [13].

In the present review, we will focus on the neuroprotective properties of the serine-fatty acid derivatives AraS, PalmS and OleoS.

**Traumatic brain injury**

TBI is the leading cause of death and disability for people under the age of 45 years [14], and posttraumatic epilepsy represents 20% of all epilepsy cases in the general populations [15]. It triggers a cascade of events characterized by the activation of molecular and cellular responses, mostly harmful, leading to secondary injury [16, 17]. The evolution of the secondary injury in the area surrounding the site of trauma is an active process in which many biochemical pathways have been identified. These include early (minutes to hours) impairment of brain ionic homeostasis and intracellular calcium accumulation, release of glutamate and excitotoxicity, oxidative stress, and neuroinflammation. In the later phase (hours to days), the development of apoptosis, axonal injury and neuronal cell death is associated with neurological, cognitive and emotional deficits, which are the long-term outcomes of TBI. Almost half of all TBI patients suffer from long-term disabilities, including neurological disorders such as epilepsy and sleep disorders, neurodegenerative diseases, neuroendocrine disorders, psychiatric diseases and even non-neurological disorders. In a closed head injury (CHI) model for TBI [18], we used functional recovery as the most fundamental parameter to define “neuroprotection” using a set of 10 neurobehavioral tasks [neurological severity score (NSS)] that examine reflexes, alertness, coordination, motor abilities and balancing [19]. Failure to perform a task scores 1 point and a success scores 0. Hence, the NSS of normal healthy mice is 0, whereas a score of 10 reflects maximal neurological impairment. The first NSS, which is obtained at 1 h after TBI, reflects the initial severity of injury and is predictive of mortality, morbidity and the extent of damage seen on magnetic resonance imaging [20]. We chose to induce a moderate degree of injury, with an initial NSS of 6–7. The extent of recovery during the post-TBI period was calculated as the difference between the NSS at 1 h and that at any subsequent time point (ΔNSS). Following TBI, a moderate spontaneous recovery, which is mediated by naturally occurring endeminc neuroprotective processes, is thought to include, among other mechanisms, the induction of the endocannabinoid system [13]. “Neuroprotection” is represented by an increase in ΔNSS, which is greater than spontaneous, and is brought about by a tested drug (e.g. Refs. [10, 19]) or by other manipulations [21]. The formation and accumulation of eCB in response to injury, along with their multipotent properties such as anti-oxidants, vasodilators, anti-inflammatory agents and inhibitors of excitotoxicity, as well as their role in neurogenesis, suggest that the formation of eCB may represent a “self-neuroprotective” and neuro-regenerative response.

**AraS is neuroprotective after TBI**

Based on our earlier findings with 2-AG, our group [22, 23] have explored the possible neuroprotective properties
of AraS. Although it does not bind directly to any of the known CB receptors, it was found to improve neurobehavioral and cognitive functions after TBI. At 24 h post injury, AraS had a minor, albeit insignificant, effect as compared to vehicle-treated mice. The effect was significantly inferior to that of 2-AG (Figure 1A). From 48 h on, recovery of the AraS-treated mice was accelerated and ΔNSS levels became significantly higher as compared with control, reaching at day 28 a level of 3.4±0.6 U, similar to that of 2-AG (Figure 1B). AraS also reduced lesion volume from 14.39±2.35% in the vehicle-treated mice to 8.00±0.87% and water content from 82.77±0.25% to 81.82±0.16%. Interestingly, these effects were abolished by co-treatment with SR144528, a CB2 antagonist [24], or with capsazepine, a TRPV1 antagonist [25], but not with SR141716A (rimonabant), a CB1 antagonist [26], suggesting the involvement of these receptors in mediating AraS-induced neuroprotection. The cellular mechanisms by which AraS mediates neuroprotection after TBI were also investigated by our group [22]. We found an induction of a pro-survival and

Figure 1: Effect on severity score as reflected by ΔNSS.
(A) Most rapid effect on severity score as reflected by ΔNSS 24 h after trauma. It is evident that 2-AG produces the highest effect for the shortest amount of time examined: 0.75 (±0.31). PalmS displayed some efficiency [0.1 (±0.1)], but to a lesser extent than AraS [0.25 (±0.16)] and 2-AG. However, neither OleoS nor any of the therapeutic combinations showed any NSS improvement 24 h post trauma, suggesting their lack of efficiency. (B) Maximal potential effect on severity score as reflected by ΔNSS on day 28 after trauma. It is evident that the overall therapeutic potential is dependent on the nature of the fatty acid connected to the amino acid; thus, AraS and 2-AG display the highest efficacy. AraS exerted an improvement in NSS of 3.88 (±0.44) on day 63 and 2-AG displayed 4.25 (±0.31) on day 42, while PalmS reached 2.6 (±0.34). OleoS did not present a significant improvement in the NSS values compared with the controls. In addition, it is apparent that no entourage effect was generated by the combined therapy. The values were expressed as mean±SEM; n=8–29. *p<0.05, **p<0.001, using the Tukey-Kramer multiple comparisons test.
anti-apoptotic cascade in the contused hemisphere in the AraS-treated mice. Increased phosphorylation of Akt and ERK, followed by increased levels of the anti-apoptotic protein Bcl-xL, and reduced activity of the pro-apoptotic enzyme caspase-3, supports the notion that, at least in part, the neuroprotective effects exerted by AraS include activation of pro-survival signaling, which is mainly CB2, but not CB1, mediated.

At different embryonic and postnatal stages of brain development, the eCB system is involved in the regulation of neural progenitor (NP) differentiation, which occurs in parallel to CB1 receptor expression [27, 28]. CB2 is present in progenitor cells from embryo origin and from adult brain, so it is assumed that CB2 mediates the acceleration of neurogenesis and stimulates NP proliferation [29, 30]. To examine the effect of AraS on the proliferation and migration of multipotent neural precursor cells (NPCs), cortical neurospheres, consisting of NPCs, were isolated from E15 mouse brains and grown for evaluation of the proliferation and migration for 4 and 5 days, respectively. When different doses of AraS were added to the medium culture, the size of the neurospheres was increased in a dose-dependent manner, along with the increase in cell number, implying a proliferative effect. Moreover, NPCs treated with AraS migrated during 5 days in culture to a distance 1.7-fold greater from the core of the neurosphere compared with the vehicle-treated spheres (235.35±26.91 and 140.67±26.47 μm, respectively) (Figure 2). Interestingly, the antagonists of CB1 (SR141716A; rimonabant), CB2 (SR144528) and TRPV1 (capsazepine) abolished the proliferative effect induced by AraS in vitro [22]. Staining for different neuronal and glial markers revealed a reduction in the expression of the astrocytic marker glial fibrillary acidic protein, together with the neuronal marker TUJ1, in NPCs treated with AraS, compared with the vehicle. In contrast, a 2.5-fold increase in the expression of the NPC marker nestin was detected in the treated culture compared with the control. Staining for the marker for oligodendrocytes, galactocerebroside, did not show any differentiation to these cells. Taken together, these results indicate that AraS maintains NPCs in an undifferentiated state, increases their migration abilities (Cohen-Yeshurun, unpublished data) and reduces their terminal differentiation in vitro [23].

PalmS is neuroprotective after TBI

Following the results obtained with AraS, we examined the therapeutic potential of PalmS and OleoS injected 1 h after TBI [31]. The recovery of motor and cognitive functions was evaluated over the course of 28 days post injury. In addition, edema formation, cytokine levels, molecular signaling and lesion volume were evaluated at different time points after TBI. At 24 h post injury, no effect of PalmS on ΔNSS was found (Figure 1A), and only from 48 h on did the effect increase, reaching its maximal level at 22–28 days. The effect was inferior to that of AraS and 2-AG (Figure 1B), and displayed a bell-shaped dose-dependent pattern, with maximal efficacy at 1 mg/kg. Interestingly, this effect was attenuated when PalmS was given, along with the classical eCBR antagonists of CB1, CB2 or TRPV1. Moreover, in CB2-/- mice, PalmS was ineffective as compared to wild-type (WT) controls. Unexpectedly, treatment with PalmS did not affect the typical neuropathology following TBI, i.e. cerebral edema formation, lesion volume and neuroinflammation markers (TNFα and IL1β). The anti-apoptotic mechanisms, which involve Akt phosphorylation and its down-stream effectors, were not affected by the treatment with PalmS. Thus, although PalmS did not bind directly to the known CB receptors, it caused a significant improvement in the neurobehavioral outcome (albeit inferior to AraS and 2-AG), in a cannabinoid receptor-dependent manner, as has been shown using genetic and pharmacological interventions [31]. However, despite having similar effects on recovery after CHI, PalmS and AraS do not share the effects on the pathological and molecular mechanisms. Mann et al. [31] have proposed...
that PalmS contributes to the activity of the receptors by taking part in a palmitoylation process, a posttranslational modification, with rapid turnover, which provides proteins with additional function and regulatory control beyond genomic information [32–34]. It plays a pivotal role in protein trafficking and in the function of membrane-bound proteins, such as G protein-coupled receptors, and allows them to shuttle between intracellular compartments upon extracellular signals [32, 35, 36]. This hypothesis may explain the contradictory results and needs to be further explored.

OleoS is not neuroprotective after TBI

The effect of OleoS on the neurobehavioral recovery after CHI was also examined over a 4-week period, and in contrast to the robust effect of AraS and to the milder one of PalmS, no significant effect of OleoS on NSS nor on the cognitive function was noted at any time. Interestingly, the most efficacious dose was 1 mg/kg, which was significantly more effective than 0.1, 3 or 10 mg/kg, yet even this dose did not reach significance vs. vehicle (Figure 1B). Moreover, when CB2-/− mice were treated with OleoS, no difference from the WT controls was noted, attesting to the irrelevance of CB2 in OleoS function in this context. Taken together, our findings support the notion that each of the NAAAs tested exerts a different mode, dynamics and extent of neuroprotection after TBI.

Combined (entourage) effect of NAAA

An “entourage effect” was previously noted in several behavioral and binding assays for anandamide and 2-AG. Namely, when administered together with other compounds, they presented a greater effect than when given alone [37, 38]. We therefore examined whether PalmS, given together with 2-AG or with 2-AG+AraS, would display an entourage effect. To test this possibility, mice were divided into three treatment groups after TBI and received a single injection of either one of the following treatment combinations: (i) 2-AG+PalmS, (ii) 2-AG+AraS and (iii) 2-AG+PalmS+AraS (2-AG, 5 mg/kg; AraS, 3 mg/kg; and PalmS, 1 mg/kg). NSS was compared with that of mice treated with any of the compounds alone. Surprisingly, not only was there no evidence of an entourage effect following the combination treatments, but also they were all inferior to each of the single-compound treatments (Figure 1). This suggests that each of the three NAAAs tested is able to abolish the effect of 2-AG and that there is no direct or indirect synergism between them. It is most likely that the presence of higher-than-innate levels of the other cannabinoid and cannabinoïd-like compounds hinders the binding of 2-AG and probably also of each other.

Summary

From the library of more than 70 N-acyl amides described by Tan et al. [3] and Raboune et al. [5], we selected AraS, PalmS and OleoS for testing as possible neuroprotectants in our TBI model. Table 1 summarizes the role of 2-AG and 3 of the NAAAs in neuroprotection after TBI. While similar functional and pathological effects were seen after the treatment with 2-AG and AraS, namely, improved functional outcome and reduced lesion volume and edema, they did not share the mechanism(s) by which these effects were achieved. Thus, CB1 is involved in 2-AG-, but not in AraS-, mediated neuroprotection, which is CB2 and TRPV1 dependent. PalmS, AraS and OleoS share a structural similarity, but the last one does not afford recovery of the impaired neurobehavioral function, whereas the first two compounds are both neuroprotective; however, they do not share signaling cascades.

**Table 1:** Summary of findings for each compound, with regard to their contribution to the recovery in the CHI model.

<table>
<thead>
<tr>
<th>Compound</th>
<th>NSS</th>
<th>Lesion volume</th>
<th>Edema</th>
<th>Pro-inflammatory cytokines decrease</th>
<th>Antagonists abolishing activity</th>
<th>Is activity affected by CB2 KO?</th>
<th>Anti-apoptotic mechanisms activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>IL1β and TNFα</td>
<td>CB1</td>
<td>N/A</td>
<td>pERK, pAKT, BclxL</td>
</tr>
<tr>
<td>AraS</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>N/A</td>
<td>CB2, TRPV1</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>PalmS</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>OleoS</td>
<td>No</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Yes, favorable effect; no, no effect; N/A, not measured.
In the studies of Panikashvili et al. [10–12] on 2-AG, only CB1 was explored as a mediator of these effects and the involvement of CB2 and TRPV1 was not addressed. However, since 2-AG activates both the CB1 and the CB2 receptors, some of its neuroprotective effects may be mediated also by signaling via CB2 [39, 40]. We further explored the role of AraS, which has been shown to cause endothelium-dependent arterial vasodilatation and to stimulate the phosphorylation of p44/42 mitogen-activated protein kinase and protein kinase B/Akt in cultured endothelial cells. These survival-related signalings are similar to those observed with the stimulation of human brain endothelial cells by 2-AG, despite the very low binding affinity of AraS to CB1 and CB2 receptors. Indeed, AraS affords neuroprotection by decreasing neurobehavioral deficits, edema and lesion volume, similar to the effects exerted by 2-AG. Activation of cell-survival signaling cascade, initiated by Akt phosphorylation, was proposed as part of its mechanism of action [22]. AraS was also shown to affect the proliferation and migration of NP cells [23]. Of interest is the finding that the in vivo and in vitro effects of AraS were abolished by antagonists to CB2 and TRPV1 receptors, but not by those to CB1, suggesting an indirect involvement of these receptors, probably via allosteric or other, yet unknown, mode of modulation of the CB2 signaling cascade. Supportive evidence on the involvement of AraS in eCB-mediated neuroprotection was published by McHugh et al. [41]. They demonstrated that N-arachidonoyl glycine (NAGly) is a highly potent pro-migratory lipid for the mouse microglia cell line BV-2 and proposed that it initiates directed microglial migration, which may represent a robust immune response in the CNS [40]. Interestingly, they also showed that NAGly-induced microglial migration is blocked by AraS, similar to the activity of the potent mediator of microglial migration: the “abnormal cannabidiol”, a synthetic isomer of the phytocannabinoid cannabidiol (CBD). These findings may serve as a lead in understanding the mechanisms via which AraS exerts its neuroprotective, anti-inflammatory actions, which may involve the GPR18 receptor and could be shared with those of CBD, perhaps as a blocker of the overactive, excessive inflammatory response.

PalmS caused a significant improvement in the neurobehavioral outcome (albeit inferior to AraS and 2-AG), in a cannabinoid receptor-dependent manner, as has been shown using genetic and pharmacological interventions. Although PalmS may share similar effects as AraS on recovery after TBI, it failed to affect any of the other neuropathological parameters and the cellular signaling pathways that were affected by AraS. The aforementioned hypothesis on the palmitoylation process, which might occur at one or more of the CB receptors, warrants further investigation.

Since it was evident at an early stage of our study that OleoS does not fulfill the basic criterion for neuroprotection, namely, neurobehavioral improvements, no further studies on its signaling pathways were conducted at this context. However, it is important to note that OleoS was shown by Smoum et al. [42] to be highly active in an osteoblast proliferation assay wherein it triggers a G1 protein-coupled receptor, as well as ERK/2. It also mitigates the number of osteoclast cells by promoting osteoclast apoptosis through the inhibition of ERK2 phosphorylation and RANKL (receptor activator of nuclear-xB ligand) expression in bone marrow stromal cells and osteoblasts. In intact mice, it moderately increases bone volume density mainly by inhibiting bone resorption. The opposing effects of OleoS on ERK in osteoblasts and osteoclasts indicate that signaling by this NAAA is cell-type dependent and, apparently, is not involved in neuroprotection after TBI.

The unexpected observation that there was no “entourage” or “ensemble” effect of the tested NAAA with 2-AG is puzzling and may point to a possibility that, by their non-direct interaction with CB1 or CB2 receptors, these compounds hinder either the binding or the signaling of 2-AG or of each other. Along the same line, an interesting observation was recently reported by Bradshaw (presented at the International Cannabinoid Research Society symposium 2015) regarding the “ensemble” effect of endogenous lipids. They compared the relative potency of combinations of N-acyl ethanolamines that activate TRPV1 receptor, predicting additive effects. Instead, they found that combinations of 2-N-acyl amides with individual activity at this receptor caused a slight decrease in potency, resembling competitive inhibition.

In conclusion, our findings reflect the complexity of the eCB pharmacology and of the role that endogenous lipids play in health and disease. The study of the family of NAAA, which acts in concert with the classical eCB system and modulates numerous cell signaling pathways and brain function, is a major challenge for understanding the mechanisms by which the brain is coping with injury and repair.

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