

## DELTA OPIOID PEPTIDE (D-ALA 2, D-LEU 5) ENKEPHALIN: LINKING HIBERNATION AND NEUROPROTECTION

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### 1. ABSTRACT

Hibernation is a potential protective strategy for the peripheral, as well as for the central nervous system. A protein factor termed hibernation induction trigger (HIT) was found to induce hibernation in summer-active ground squirrels. Purification of HIT yielded an 88-kD peptide that is enriched in winter hibernators. Partial sequence of the 88-kD protein indicates that it may be related to the inhibitor of metalloproteinase. Using opioid receptor antagonists to elucidate the mechanisms of HIT, it was found that HIT targeted the delta opioid receptors. Indeed, delta opioid (D-Ala 2, D-Leu 5) enkephalin (DADLE) was shown to induce hibernation. Specifically, HIT and DADLE were found to prolong survival of peripheral organs, such as the lung, the heart, liver, and kidney preserved en bloc or as a single preparation. In addition, DADLE has been recently demonstrated to promote survival of neurons in the central nervous system. Exposure to DADLE dose-dependently enhanced cell viability of cultured primary rat fetal dopaminergic cells. Subsequent transplantation of these DADLE-treated dopaminergic cells into the Parkinsonian rat brain resulted in a two-fold increase in surviving grafted cells. Interestingly, delivery of DADLE alone protected against dopaminergic depletion in a rodent model of Parkinson's disease. Similarly, DADLE blocked and reversed the dopaminergic terminal damage induced by methamphetamine (METH). Such neuroprotective effects of DADLE against METH neurotoxicity was accompanied by attenuation of mRNA expressions of a tumor necrosis factor p53 and an immediate early gene c-fos. In parallel to these beneficial effects of DADLE on the dopaminergic system, DADLE also ameliorated the neuronal damage induced by ischemia-reperfusion following a transient middle cerebral artery occlusion. In vitro replication of this ischemia cell death by serum-deprivation of PC12 cells revealed that DADLE exerted neuroprotection in a naltrexone-sensitive manner. These results taken together suggest that DADLE stands as a novel therapeutic agent. In this review paper, we present laboratory evidence supporting the use of DADLE for protection of peripheral and central nervous system.

### 2. INTRODUCTION

Hibernation exemplifies a natural model of tolerance to oxygen-, blood-, or energy-depleting injuries. Profound physiological changes including respiratory depression, hypothermia, bradycardia, hypophagia, analgesia, and a cessation of renal output are life-threatening processes that occur in hibernating animals. Despite these otherwise detrimental physiological alterations, the resulting reduction in metabolic rate allows the hibernators, like the ground squirrel, woodchuck, the brown cave bat, the European hedgehog, and the black bear, to survive winters when food supplies are rare.

In view of this phenomenal survival of hibernators, it is of interest to extend such protective effects of hibernation in diseased states characterized by similar deleterious pathophysiological conditions. In order to advance hibernation protective therapy, the molecular components involved in the hibernation process itself must be unveiled. In the late 60s, laboratory studies implicated certain factor(s) in the plasma of winter hibernating animals as critical for inducing hibernation (Table 1). Dawe and Spurrier demonstrated that the transfusion of the plasma of hibernating thirteen-lined ground squirrels into either summer-active ground squirrels or woodchucks induced hibernation (1). The next logical step is to purify this hibernation-inducing substance; unfortunately, to date identification of such a molecule remains elusive.

Oeltgen and colleagues, however, were able to partially purify a protein factor that comigrates with serum albumin and causes hibernation when injected into summer-active ground squirrels (2, 3). Subsequent purification of the hibernation induction trigger (HIT) has yielded an 88-kD peptide that is enriched in the plasma of the winter hibernators but not the summer-active animals (4). Of interest, partial sequencing of the 88-kD peptide indicates that it has a high homology with a protein identified as human 1B-glycoprotein (5). Although the function of 1B-glycoprotein is yet to be determined, a metalloproteinase inhibitor homologous to 1B-glycoprotein

**Table 1.** Progress in DADLE Research

Milestone discoveries	Reference
1. Isolation of certain factors in the plasma of winter hibernating animals as critical for inducing hibernation	1
2. Partial purification of a protein factor that comigrates with serum albumin and causes hibernation when injected into summer-active ground squirrels	2-3
3. Purification of the hibernation induction trigger HIT, yielding an 88-kD peptide that is enriched in the plasma of the winter hibernators	4
4. 88-kD peptide possibly represents a metalloproteinase inhibitor	6-8
5. HIT-induced hibernation in ground squirrels blocked by universal opioid receptor antagonist naloxone, but HIT-induced depression of electrically induced twitches of guinea pig ileal myenteric plexus preparation not reversed by naloxone	2
6. Endogenous opioids and associated receptors actively participate in achieving hibernation; opioid-mediated hibernation highly specific- mu and kappa opioid receptor agonists less potent than delta opioid receptor agonists	3 9
7. Morphine and morphiceptin, Dynorphin and U-69593, display low efficacy in inducing hibernation, but these non-hibernation inducing agents when co-administered with HIT can block the HIT-induced hibernation	3
8. HIT and DADLE enhance organ survival and preserve organ function in multiorgan preservation preparation en bloc	10
9. DADLE enhances survival in isolated organ preservation of lungs	13
10. HIT and DADLE pretreatment of rabbit heart enhances functional recovery, selective delta-1opioid DPDPE promotes protective effects	14 15 16
11. DADLE completely blocks DAT loss induced by METH, DADLE post-treatment after METH administration restores DAT to normal level, effect of DADLE against METH-induced DAT loss mediated by opioid receptor and by DADLE's novel free radical scavenging and antioxidative stress properties	19
12. DADLE blocks METH-induced elevation of brain mRNA levels of immediate early gene c-fos and gene of a tumor necrosis factor p53	21 22
13. Parkinson's disease rats pre-treated with DADLE prior to dopamine-depleting neurotoxin 6-hydroxydopamine lesion displayed significant sparing of tyrosine hydroxylase immunoreactive cells, pretreatment with DADLE dose-dependently enhanced cell viability of cultured primary rat fetal mesencephalic cells	23 24
14. DADLE enhances survival of serum-deprived PC12 cells, DADLE-treated PC12 cells appear morphologically similar to cells treated with nerve growth factor, principle pathway of DADLE neuroprotection via participation of opioid receptor	25
15. DADLE enhances viability and functionality of fetal dopaminergic cell grafts transplanted for Parkinson's disease	26 27
16. DADLE protects against ischemia-reperfusion-induced brain damage after transient middle cerebral artery occlusion, naltrexone only transiently blocks early phase of reperfusion-induced behavioral deficit but fails to block prolonged protective effect of DADLE	28

has been identified and can be used to speculate some possible roles for this protein. For example, it is known that the European hedgehog, a hibernator, is resistant to the metalloproteinase present in the venom of the European viper (6). Based on this observation, 1B-glycoprotein, or at least one of its factors, can be thought of as a metalloproteinase inhibitor. Additional studies are needed to determine whether the 88-kD peptide represents a metalloproteinase inhibitor. Moreover, the hibernation induction property of the 88-kD peptide has not been demonstrated in summer-active hibernators. Recent studies have shown that aberrant metalloproteinase activity appears to exacerbate the pathological conditions of ischemia, increasing the likelihood of hemorrhagic transformation (7,8). Accordingly, the demonstration of metalloproteinase inhibition activity following treatment with HIT and its

peptides should provide a possible mechanistic link between hibernation and protection. Alternatively, compounds known to inhibit metalloproteinase activity can be examined for their ability to trigger hibernation, which should also offer insights into molecular pathways underlying hibernation protective effects.

## 3. HIBERNATION AND OPIOIDS

Many physiological features seen in hibernation mimic those produced by opioids, including analgesia and respiratory depression. Moreover, endogenous opioid peptides are found in the brain. Accordingly, it has been speculated that HIT may act like an opioid. Indeed, HIT-induced hibernation in ground squirrels was blocked by a universal opioid receptor antagonist naloxone (2).

However, HIT-induced depression of the electrically induced twitches of the guinea pig ileal myenteric plexus preparation was not reversed by naloxone (2). These observations suggest that HIT is not an opioid itself, but is a potent releaser of endogenous opioid peptides. An alternative explanation is that the opioid-like substance in HIT may be present in a negligible amount that cannot be detected by the guinea pig ileum bioassay (i.e., the naloxone reversibility cannot be detected or it was masked by other twitch-depressing substances). Moreover, the 88-kD peptide has not been tested in the guinea pig ileum bioassay. Nonetheless, the induction of hibernation by HIT and the reversal of the HIT by naloxone suggest that endogenous opioids and associated receptors actively participate in achieving hibernation. Indeed, direct infusion of opioids into summer-active ground squirrels induced hibernation. This opioid-mediated hibernation is highly specific to a group of opioids since different classes of opioids produced different profiles. It appears that mu and kappa opioid receptor agonists are less potent than delta opioid receptor agonists in promoting hibernation. For example, both morphine and morphiceptin, relatively selective for mu opioid receptors, displayed low efficacy in inducing hibernation (3). In addition, Dynorphin and U-69593, two selective kappa opioids, exhibited minimal efficacy in inducing hibernation in summer-active ground squirrels (3,9). Of note, although shown as non-hibernation inducing agents, morphine, morphiceptin, dynorphin, or U-69593, when co-administered with HIT, can block the HIT-induced hibernation (3). On the other hand, DADLE, a selective ligand for delta opioid receptors, did not block the hibernation induced by HIT, and when administered alone was demonstrated to be highly effective in inducing hibernation (3). Such discrete effects of opioids suggest that endogenous delta opioids and delta opioid receptors are likely involved in the entry phase of hibernation, while mu and kappa opioids and their receptors are primarily engaged in the arousal phase of hibernation. Because of DADLE's potent ability to induce hibernation, studies on hibernation protective therapy have focused on investigating the beneficial effects of DADLE treatment in both peripheral and central nervous systems.

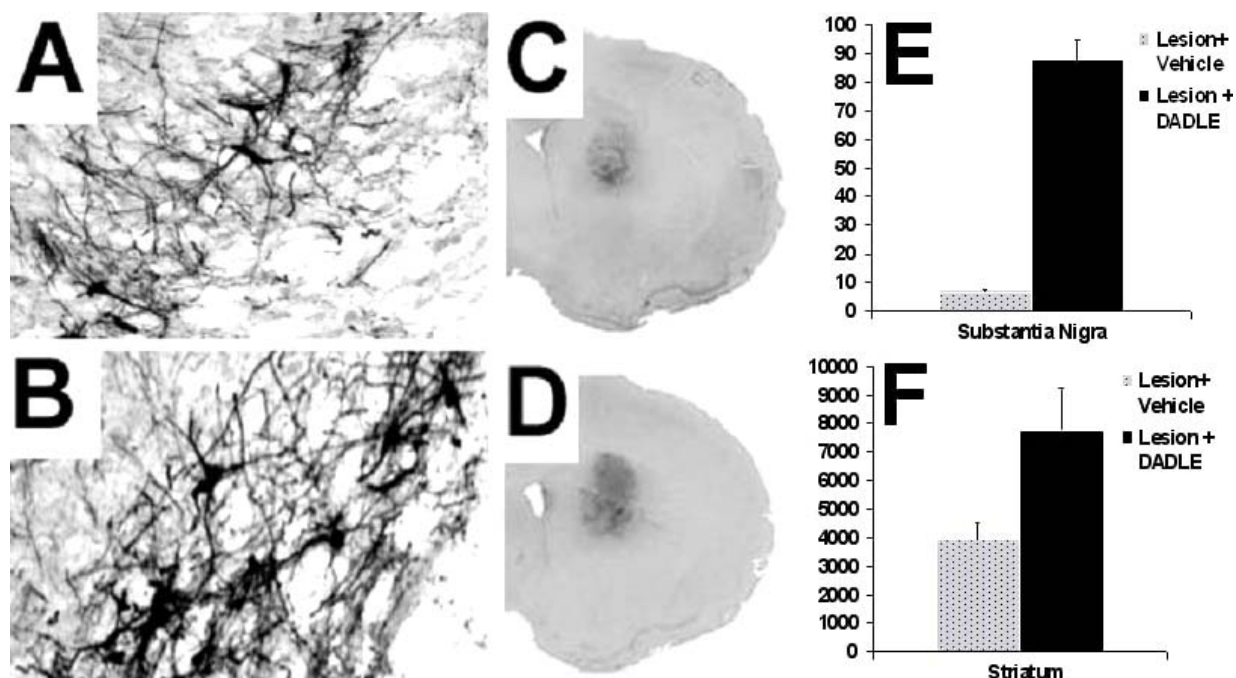
#### 4. DADLE: PROTECTION OF PERIPHERAL ORGANS

The first line of evidence suggesting that hibernation may be exploited as a therapeutic strategy comes from natural observations of robust survival of hibernators despite the long duration of hibernation which lasts usually about 5–8 months. Further examination of these hibernating animals revealed that they do not show any sign of damage to their internal organs after arousing from hibernation. The maintenance of healthy and normal functioning organs is phenomenal considering that hibernation entails survival-limiting conditions, including a near zero degree body temperature as well as hypoxia for such a long duration of time. The typical body temperature during hibernation is about 7°C, and the respiratory rate is about two respirations per minute (2, 3). Because certain diseased states, such as ischemia, are similarly characterized by reduced blood or oxygen flow, it will be

of utmost therapeutic benefit if one can induce hibernation during these deleterious periods to prevent organ failure. However, it is highly unlikely that non-hibernating animals will be able to tolerate hibernation (e.g., hypoxia and hypothermia). Perhaps one strategy to circumvent these extreme conditions associated with hibernation but still induce the protective mechanisms associated with hibernation is to explore drugs that trigger hibernation. To this end, accumulating evidence demonstrates that HIT and DADLE can induce hibernation, as well as enhance organ survival.

Pioneering studies on HIT and DADLE as organ survival-enhancing agents were conducted by Chien and colleagues (10). These researchers used a multiorgan preservation preparation that has been shown as optimal for prolonged organ survival. This “en bloc” method of organ preservation involves dissection of the internal organs such as the heart, lungs, liver, spleen, jejunum, and kidneys and retaining the veins and arteries connecting the organs (10). In this study, the whole organs were preserved as a block and maintained in a preservation bath (10). With this multiorgan preservation preparation, organ survival time was about 2 h up to an average of 8 h. Even hard to preserve organs, such as heart and liver, showed good survival using this en bloc preparation. Of interest, treatment with HIT, by injection into the multiorgan preservation preparation via veins, extended the survival time of organs from 8 to 44 h (10). Moreover, treatment with DADLE to the multiorgan preservation preparation (1 mg/kg based on original body weight; every 2 h, i.v.) prolonged the organ survival time up to 46 h. This enhanced survival window is the longest in the history of the preservation of the heart and the liver (11). Furthermore, not only was prolonged survival documented, but normal functioning of the lungs, preserved en bloc with HIT treatment, was demonstrated following transplantation into a host animal (12). These observations indicate that treatment with hibernation inducing agents HIT and DADLE resulted in enhanced survival of preserved multiorgans and also translated into maintenance of normal functions of these organs following transplantation.

Encouraged by the positive results of multiorgan survival and maintained functions with HIT and DADLE, Wu and colleagues (13) next examined whether the same hibernation inducing agents can prolong organ survival even when each organ is preserved as an individual isolated unit. To further demonstrate a stringent parameter of therapeutic efficacy of HIT and DADLE, hard to preserve organs were isolated for single organ preservation preparation. For example, because of its delicate texture, the lung is one of the most difficult organs to preserve as a single organ. Accordingly, using conventional preservation methods, isolated lungs usually developed severe pulmonary edema, hemorrhage, and occlusive vascular resistance. In contrast, when isolated rat lungs were stored in a 24-hour hypothermic preservation bath treated with DADLE, enhanced survival was achieved, coupled with the preserved lungs exhibiting good air flow, almost normal vascular resistance, good oxygenation, and normal tissue wet/dry weight ratio (13). This is the first indication that



**Figure 1.** The neurotoxin 6-OHDA produced dopaminergic depletion in the substantia nigra of an adult rat pretreated with saline (A), whereas pretreatment with DADLE blocked such neurotoxicity (B, E). Similarly, fetal ventral mesencephalic cells that were exposed to DADLE (D) prior to transplantation increased graft survival in the striatum of adult rats compared to cells exposed to saline (C, F).

hibernation inducing agents like DADLE can render enhanced hypothermic preservation of organs as an isolated unit. This beneficial effect was demonstrated even with hard to preserve organs such as the lungs.

Another hard to preserve organ is the heart. Bolling and colleagues (14-16) studied isolated rabbit hearts initially prepared in a standard preservation buffer. Global ischemia insults to these preserved hearts in a routine cardioplegic solution usually leads to only a 30% functional recovery of the heart. Of note, functional recovery of the heart increased to an average of 70% when pretreated with HIT or DADLE for 15 min at 37°C before 18 h of global ischemic storage at 4°C (14-16). In this study, parameters of functional recovery of the heart, including isovolumic-developed pressure, maximal positive and negative dP/dt, coronary flow, and myocardial oxygen consumption, were compared as a percentage of prestorage values versus 45 min after removal from storage and reperfusion. The results revealed that DPDPE, a selective delta-1 opioid, promoted protective effects on the developed pressure, positive and negative derivatives of left ventricular pressure, but not on the coronary flow and the myocardial oxygen consumption (14-16). This observed partial functional recovery of the heart following DPDPE treatment suggests that the myocardial protective effect produced by DADLE (a nonselective peptide for delta-1 and delta-2 opioid receptors) is probably mediated via delta-1 and delta-2 opioid receptors. Supporting such selective opioid effects on the preserved heart is the observation that TAN-67, a delta-1 alkaloid opioid, elicited a cardioprotective effect via delta-1 opioid receptors that was blocked by a selective delta-1 receptor antagonist (17).

## 5. DADLE: PROTECTION OF THE CENTRAL NERVOUS SYSTEM

The significant overlaps in pathophysiologic mechanisms of cell death between peripheral and central organs have prompted investigations into the protective effects of hibernation inducing agents on the central nervous system. The overall premise here is that the endogenous delta opioid system may represent one of nature's protective mechanisms against cell death. It is interesting to note that endogenous opioid systems, specifically the delta opioids, have been implicated in the survival of animals against hypoxic shock (18).

Tsao and colleagues (19) initially investigated the effects of DADLE against the dopamine neurotoxicity produced by methamphetamine (METH), which is a known drug of abuse. METH has been shown to produce long-term loss of striatal dopaminergic terminals after a high dose of single administration or a prolonged use at medium doses. In this study, systemic (intraperitoneal) treatment with DADLE at 30 min before METH administration was found to completely block the dopamine transporter (DAT) loss induced by METH (19). Moreover, DADLE posttreatment at 2 weeks after METH administration when the DAT had been reduced by about 30% restored the DAT to a normal level (20). Subsequent studies revealed that the effect of DADLE against METH-induced DAT loss was partially mediated by the opioid receptor, as well as through DADLE's novel free radical scavenging and antioxidative stress properties (19). Furthermore, DADLE blocked the METH-induced elevation of brain mRNA levels of an immediate early gene *c-fos* and the gene of a

tumor necrosis factor p53 (21,22). These results provide convincing evidence that DADLE is a potent free radical scavenger and antioxidant, and is actively neuroprotective at the genomic level (19).

Based on DADLE's neuroprotection against METH-induced dopamine neurotoxicity, subsequent studies investigated DADLE's therapeutic efficacy in a model of Parkinson's disease, a neurological disease characterized by dopamine depletion. Adult rats pre-treated with DADLE (4 mg/kg every 2 h, 4 injections, i.p.) just prior to the dopamine-depleting neurotoxin 6-hydroxydopamine lesion displayed a significant sparing of tyrosine hydroxylase immunoreactive cells at one month post-lesion (23, 24) (Figure 1). Similarly, pretreatment with DADLE (0.0025, 0.005, 0.01 g/ml) dose-dependently enhanced cell viability of cultured primary rat fetal mesencephalic cells (23,24). In another culture study, DADLE was found to enhance the survival of serum-deprived PC12 cells. Although data are scarce, this DADLE neuroprotection against serum deprivation-induced cell death may involve also a trophic factor mechanism because DADLE-treated PC12 cells appeared morphologically similar to the cells that were treated with nerve growth factor (25). The participation of the opioid receptor remains the principal pathway in DADLE's neuroprotective action, as revealed by naloxone blockade of DADLE-induced enhancement of PC12 cell survival (25).

Because fetal dopaminergic cells are used as a transplant source for Parkinson's disease, another study examined whether DADLE could also enhance the viability and functional effects of these cells. Grafted cells previously treated with DADLE (0.01 g/ml) exhibited about twice the number of surviving tyrosine hydroxylase-immunoreactive cells compared with those animals that received nontreated cell grafts. In addition, DADLE-treated cell grafts promoted significantly more robust behavioral recovery than control cells (26, 27). These results suggest that DADLE should be considered either as a stand alone therapy or as an adjunctive agent for neural transplantation therapy in Parkinson's disease.

The neuroprotective effects of DADLE have also been examined in stroke, another neurological disorder that closely resembles the pathologic consequences of the ischemic tissue injury in the periphery. Recent data have demonstrated that DADLE also protected against ischemia-reperfusion-induced brain damage after transient middle cerebral artery occlusion (28). In this stroke model, rats were subjected to a 60-min unilateral middle cerebral artery occlusion followed by a 24 or 72 hours of reperfusion exhibited extensive infarction in the striatum. In contrast, pretreatment with DADLE (i.p.; 4 mg/kg, 4 injections at 2-hour intervals) prior to the middle cerebral artery occlusion completely blocked the striatal infarction (28). This protective effect appears again mediated by the opioid receptor. However, naltrexone, a universal opioid antagonist, only transiently blocked the early phase of the reperfusion-induced behavioral deficit but failed to block the prolonged protective effect of DADLE (28).

Accordingly, these observations suggest that while opioid receptors participate in the initial phase of the effect of DADLE, other mechanisms such as free radical scavenging, antioxidative stress, or trophic factor release are involved in the latter phase.

These studies on the beneficial effects of DADLE on the central nervous system demonstrate that hibernation inducing agents such as opioids are able to alter the pathological consequences of neurological disorders, suggesting that hibernation, as well as the opioid system, are important avenues in the study of brain injury and protection.

## 6. PERSPECTIVE

We concur that additional studies are needed to reveal direct interaction between hibernation and neuroprotection. Despite shortcomings in elucidating the mechanism(s) of action of DADLE in protecting against organ and neuronal death, there is strong evidence to support that DADLE is a highly potent therapeutic agent for treating certain degenerative diseases, both in the central nervous system and the peripheral system. While most of the studies here are biased towards "neuroprotection", the observed "neurorestoration" seen with rescue of DA terminal damage induced by METH following DADLE posttreatment (given at 2 weeks after METH administration) (20) should lay the foundation for subsequent studies establishing DADLE as a therapeutic agent with clinical applications. A major stumbling block is identifying the active substance in the HIT. If purification and characterization of the active substance reveals a DADLE-like compound, then this should provide a major advance in linking hibernation to neuroprotection. Until then, a fertile ground to investigate hibernation-induced neuroprotective effects is the opioid system. As discussed here, DADLE consistently promotes protective effects by altering the cell death pathway, in part via the delta opioid receptor. In contrast to this delta opioid-mediated neuroprotection, morphine, a mu opioid receptor agonist, induced apoptosis (29). In the end, the pursuit of the opioid system as the key underlying factor for inducing hibernation and neuroprotection will likely reveal specific and selective participation of discrete types of opioid receptors and their associated ligands.

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