

Stress Effects on Neuropathic Pain

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Stress may exacerbate neuropathic pain by increasing inflammation in the nervous system. Peripheral nerve injury produces neuropathic pain and is accompanied by neuroinflammation, therefore, we hypothesized that acute stress exposure immediately prior to nerve injury would increase neuropathic pain. Specifically, we examined the effect of 60 min of restraint administered immediately prior to spared nerve injury (SNI) on neuropathic pain in adult female mice. Mechanical allodynia was assessed before and 24 hours after a single period of restraint and was found to be similar between stressed and non-stressed mice. However, 1, 3, 5, and 7 days following SNI, mechanical allodynia (as assessed by von Frey monofilament testing) in the hindpaw ipsilateral to the injury was significantly enhanced in mice stressed immediately prior to SNI as compared to non-stressed mice. The physiological mechanism underlying the exacerbated pain response in stressed mice involves corticosterone (CORT) acting via glucocorticoid receptors. In the absence of stress, exogenously administered CORT reproduced the stress-induced increase in allodynia. Administration of RU486, a glucocorticoid receptor antagonist, prior to restraint prevented the stress-induced increase in allodynia. Because stress has been shown to prime microglia, we evaluated morphological indicators of microglial activation in the spinal cord dorsal horn. Although SNI significantly increased microglial activation in the dorsal horn ipsilateral to the injury; the level of immunoreactivity was not affected by stress. Taken together, these data suggest that stressful conditions can exacerbate neuropathic pain, such as that caused by nerve injury, via a mechanism that involves CORT acting at glucocorticoid receptors.

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Degenerative diseases such as Alzheimer's and Parkinson's disease show marked neuroinflammation that may contribute to cell death in the hippocampus and substantia nigra. Anti-inflammatory substances are being explored as therapeutic responses to degenerative processes. Antagonism of adenosine receptors has been recently described as having anti-inflammatory properties, although the mechanism by which this occurs is still undefined. We investigated the proposed anti-inflammatory role of an adenosine antagonist, caffeine, in a model of chronic neuroinflammation and in aged male rats. The slow infusion of lipopolysaccharide (LPS) into the 4th ventricle induces pronounced microglial activation. Young rats (3 months) were implanted with a cannula into the 4th ventricle that was connected to an osmotic minipump (model 2004) filled with either LPS (250 ng/hr) or the vehicle (aCSF). LPS and aCSF treated young rats and untreated aged rats (24 months) received daily injections of caffeine (40 mg/kg, i.p.) or vehicle (saline, 1 ml/kg) for two weeks. Biomarkers of inflammation, e.g. activated microglia and astrocytes, and the density of A1 and A2a adenosine receptors were determined in the hippocampus and substantia nigra. Caffeine diminished the number of MHCII positive microglia in the hippocampus of young LPS injected animals, but did not change the number of activated microglia in aged rats. Our results support epidemiological evidence of a negative correlation between caffeine consumption and the prevalence of selected neurodegenerative diseases. Supported by RO1 AG10546

Keywords: Neuroinflammation, adenosine, caffeine, Alzheimer's, Parkinson's, aging

PRESYNAPTIC FUNCTION IS ALTERED IN ACID-SENSING ION CHANNEL1 KNOCKOUT HIPPOCAMPAL NEURONS IN MICROISLAND CULTURE

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Acid-sensing ion channels (ASICs) are proton-gated ion channels, which produce transient cation currents in response to acid. They are expressed widely throughout the brain where they play a role in learning, memory, and fear conditioning. Evidence from ASIC1 knockout mice suggests that ASICs impact synaptic plasticity. However, the cellular and molecular mechanisms of ASICs role in synaptic transmission have not yet been described. We chose to analyze the role of ASICs in microisland cultures of hippocampal neurons from wild-type, ASIC1, and ASIC2 knockout mice. Neurons in microisland culture from wild-type and ASIC2 KO mice displayed proton-gated currents. Neurons from ASIC1 KO mice did not show proton-gated currents with the application of pH 6 solutions. We compared whole-cell excitatory and inhibitory postsynaptic currents (EPSC and IPSC) using the whole-cell patch clamp technique and found no dramatic differences between the three genotypes. Readily-releasable pool size and refilling rate were not altered in ASIC1 KO neurons. However, paired-pulse ratio of AMPA EPSC was reduced significantly in ASIC1 KO neurons. In quantal analyses, the frequency of miniature EPSC was increased in ASIC1 KO neurons whereas there was no difference in the amplitude of miniature EPSC suggesting that synaptic transmission of ASIC1 KO neurons is altered presynaptically. Furthermore, the inhibition rate of NMDA EPSC by MK801 was faster in ASIC1 KO neurons suggesting that probability of neurotransmitter release is increased. Our results indicate that ASIC1 may regulate synaptic transmission by modulating presynaptic function.

INTERACTIONS OF NONCOMPETITIVE ANTAGONISTS WITH A3B4 NEURONAL NICOTINIC RECEPTORS: MODELING OF A NEGATIVE ALLOSTERIC BINDING SITE.

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Neuronal nicotinic acetylcholine receptors (nAChRs) have been involved in several pathological conditions including nicotine addiction. Noncompetitive sites have been described as promising targets for more effective drugs to treat some diseases associated with nAChRs. As a novel approach for neuronal nAChR drug discovery, our laboratory is targeting novel sites (noncompetitive sites, noncompetitive allosteric binding sites) located on nAChRs. nAChRs contain several sites at which negative allosteric modulators bind. Our laboratory has developed a library of analogs of methyllycaconitine (MLA) that act as noncompetitive inhibitors (NCIs) of native $\alpha 3\beta 4^*$ nAChRs. Initially, we generated a pharmacophore and 3D-QSAR model for a negative allosteric binding site based on functional data of 68 compounds. We found this model to be predictive when data-mining yielded commercially available compounds with NCI activity. Whether these compounds are actually interacting only on the nAChRs can not be determined from these initial studies. For this reason, we also investigated and compared the functional effects of these compounds on native and recombinant $\alpha 3\beta 4$ nAChRs. The functional approaches used were nAChR- and non nAChR-mediated neurosecretion, and nAChR-mediated calcium influx. We grouped our compounds based on their non nAChR-mediated activity. Group I compounds selectively inhibited native and recombinant nAChR-mediated function (no effects on non nAChR-related activity), suggesting that these molecules act at a single negative allosteric binding site. Group II compounds inhibited native and recombinant nAChR-mediated function, along with non nAChR-related function. These data support an additional site of action of this group of molecules distal to membrane depolarization in the stimulus-secretion coupling pathway. These data will help us target potentially novel sites on nAChRs and develop predictive models for the synthesis of useful drugs to treat diseases associated with nAChRs.

HERKINORIN DERIVATIVES EVALUATED FOR MU OPIOID RECEPTOR REGULATION AND SIGNALING

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The mu opioid receptor (MOR) is subject to regulation which can determine biochemical and physiological responses to different opiate agonists. Mice lacking β -arrestin2, a MOR regulatory protein, display enhanced and prolonged morphine-induced analgesia without the development of tolerance. Thus, a MOR agonist that does not promote β -arrestin2-MOR interactions may promote enhanced analgesia and reduced tolerance. We have recently described a derivative of the natural product, salvinorin A, termed herkinorin, which has affinity for the MOR, and therefore may represent a novel compound in the treatment of pain. Herkinorin does not lead to robust β -arrestin2 recruitment or MOR internalization, yet it induces MOR-mediated ERK activation. While herkinorin is a promising prototypic compound, chemical derivatives are being synthesized to improve solubility, stability, bioavailability, and receptor selectivity. Herein, we evaluate whether the desired cellular effects of herkinorin are maintained in four of these new derivatives, termed p-methoxy-, p-bromo-, p-nitro-, and herkamide. MOR signaling is maintained in all four herkinorin derivatives, as each can dose-dependently increase ERK activation with comparable efficacy and potency to herkinorin. One derivative (p-nitro), however, appears to be less potent, consistent with a decreased binding affinity seen for this compound. MOR regulation was assessed by β -arrestin2 recruitment and MOR internalization. Three of the herkinorin derivatives retain the inability to induce robust β -arrestin2 recruitment and MOR internalization, similar to herkinorin itself. One derivative (herkamide), however, does induce robust β -arrestin2 recruitment and MOR internalization. The chemical structure of herkamide, in comparison to herkinorin, may shed light on the receptor-ligand interactions that can dictate β -arrestin mediated receptor regulation, and hence may improve our targeted drug development efforts to devise agonists that activate receptors without recruiting β -arrestins. NIDA grants DA14600, DA18860 (LMB) and DA18151 (TEP)

NOVEL ALLOSTERIC MODULATORS OF NICOTINIC RECEPTORS: STRUCTURE-ACTIVITY STUDIES

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Neuronal nicotinic acetylcholine receptors (nAChRs) are part of a family of pentameric ligand-gated ion channels which contain substantial differentiation concerning the subunit composition (α 2- α 10, β 2- β 4). Neuronal nAChRs exist in different locations throughout the peripheral and central nervous systems and have been implicated as playing a role in various neurological disorders such as Alzheimer's disease, Parkinson's disease, depression, and schizophrenia. In addition, nAChRs can be attributed to cognitive functions such as learning and memory and are also responsible for the addiction to controlled substances such as nicotine and alcohol. For these reasons, it has become increasingly important to discover not only therapeutic drugs for nAChRs, but also drugs that are subtype selective. In pursuit of this goal, our laboratory has synthesized over 100 different drug analogs based upon the compound, methyllycaconitine (MLA). The purpose of these studies is to determine the structural features that are important for binding to nAChRs. For these studies, our laboratory used cell models expressing native and recombinant nAChRs to assess pharmacologic activity and three assays to collect functional data and to determine the potency of the synthesized compounds. Analysis of the functional data has led to the discovery of many structural components that modify the analogs' activity. For instance, increasing the analogs' carbon chain length (in respect to a piperidine ring), results in an observed increase in the inhibitory potency. In addition, increasing the compounds' electron donating ability via addition or modification to the aromatic rings also increases inhibitory potency. Interestingly, these same modifications increase cross-reactivity of the analogs with additional sites in the neurosecretory pathway. These results demonstrate the effectiveness of structure-activity relationship studies and may lead to the identification of novel therapeutic drugs that target nAChRs.

" Tryptophan hydroxylase 2 (TPH2) haplotypes predict levels of TPH2 mRNA expression in human pons."

Dysfunction of serotonergic neurotransmission has been implicated in a variety of mental illnesses, including depression, anxiety disorders, schizophrenia, alcoholism, drug abuse, aggression and suicidal behavior. Most of the serotonin in the brain is produced by serotonergic neurons located in the raphe nuclei in the brain stem. These cells express tryptophan hydroxylase 2 (TPH2), which catalyzes the rate-limiting step in the synthesis of serotonin. Levels of serotonin in the brain may therefore be controlled by levels of expression of the *TPH2* gene. We hypothesize that genetic variants that decrease TPH2 mRNA expression TPH2 increase the risk of depression or suicide.

To identify functional polymorphisms that influence the expression of the *TPH2* gene, we first measured expression of *TPH2* mRNA in an allele-specific manner in autopsy sections of human pons containing the dorsal and median raphe nuclei. Differences in the expression of one allele over the other indicates the presence of *cis*-acting elements that differentially affect transcription and/or mRNA processing and turnover. To make these measurements, we selected two SNPs with high heterozygosity for use as allele-specific mRNA markers: rs7305115 (located in exon 7) and rs4290270 (located in exon 9). Using these SNPs, we detected allelic expression imbalance (AEI) ranging from 1.2 to 2.5-fold in 19 out of the 27 samples, suggesting the existence of *cis*-acting polymorphisms that differentially affect *TPH2* mRNA levels in pons. AEI results from individuals heterozygous for both marker SNPs were closely correlated ($r = 0.93$), validating the AEI analysis. AEI is tightly associated with the exon 7 marker SNP in 17 of 18 subjects. In each sample, the minor A-allele yielded higher levels of TPH2 mRNA expression than the G-major allele. Since the G-allele is the apparent ancestor allele, the presence of the A-allele in a significant fraction of the population suggests possible positive-selection for this (or another tightly linked) "gain-of-function" allele. By genotyping twenty additional *TPH2* SNPs, we identified a haplotype block comprising five tightly linked SNPs for which heterozygosity is highly correlated with AEI. We are currently attempting to identify the functional genetic variant that regulates levels of TRPH2 mRNA expression. Future studies will focus on the possible role of low-expressing TPH2 alleles as a susceptibility factors for depression and suicide and high-expressing TPH2 alleles as a possible protective factors against these disorders.

Cannabinoid receptor stimulation is anti-inflammatory and improves memory in old rats

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The number of activated microglia increase during normal aging. Stimulation of endocannabinoid receptors can reduce the number of activated microglia, particularly in the hippocampus, of young rats infused chronically with lipopolysaccharide (LPS). In the current study we demonstrate that endocannabinoid receptor stimulation by administration of WIN-55212-2 (2 mg/kg/day) can reduce the number of activated microglia detected using immunohistochemistry in hippocampus of aged rats (45% reduction in the CA3 region, $p < 0.05$) and attenuate the spatial memory impairment in the water pool task (Day 2, 3 and 4, $p < 0.05$ compared to untreated aged animals). Our results suggest that the action of WIN-55212-2 does not depend upon a direct effect upon microglia or astrocytes, as no colocalization was found between CB1 and GFAP (astrocytes), OX-6 (activated microglia) or OX-42 (resting microglia), but seems dependent upon stimulation of neuronal cannabinoid receptors, as a strong colocalization was found with NeuN (neuronal marker) and NMDA-R1 (neuronal glutamate receptor). Aging significantly reduced cannabinoid type 1 receptor binding (about 33% reduction in the hippocampus, $p < 0.05$) but had no effect on cannabinoid receptor protein levels in the hippocampus. We are currently exploring which subtype of cannabinoid receptor is involved in this anti-inflammatory effect. Overall, stimulation of cannabinoid receptors

may provide clinical benefits in age-related diseases that are associated with brain inflammation, such as Alzheimer's disease.

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Morphine-Induced Physical Dependence and Inhibition of Gastrointestinal Transit in GRK6 Knockout Mice

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Opiates, such as morphine, are useful for the treatment of pain. However, they also produce several undesirable side effects including physical dependence and constipation. The pharmacological effects of morphine are mediated through activation of the mu-opioid receptor which is a G protein-coupled receptor (GPCR). GPCRs are subject to regulation by several proteins, including G protein receptor kinase 6 (GKR6). Cell culture studies have shown that GKR6 regulates the sensitivity of several GPCRs and animal studies have shown that it can affect GPCR responsiveness *in vivo* as well. Mice lacking GRK6 display increased coupling of the D2 dopamine receptor to G proteins as well as enhanced locomotor activity in response to psychostimulants when compared to their wild-type littermates. In this study, we evaluated the role of GRK6 in regulating mu-opioid receptor responsiveness by assessing morphine-induced physical dependence and constipation in GRK6 knockout (GRK6-KO) mice. Physical dependence was assessed by measuring several naloxone-precipitated withdrawal signs, including the number of jumps, wet dog shakes, paw tremors, and the presence or absence of diarrhea and mastication in the GRK6-KO mice and their WT littermates after chronic morphine treatment. When assessed collectively, the overall signs of withdrawal suggested that there is no difference in the onset of physical dependence in the GRK6-KO mice in response to morphine. We also evaluated the role of GRK6 in morphine-induced constipation directly by measuring the production of fecal boli in response to acute morphine. In this assay, the GRK6-KO mice produce less fecal boli than their WT counterparts, indicating a greater degree of constipation. Collectively, our findings suggest that GRK6 plays an important role in morphine-induced constipation.

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Compartmental Neural Simulations with Spatial Adaptivity

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Neural simulation packages like NEURON and GENESIS are seeing more widespread use in the neuroscience community to provide insight into complex neurophysiological phenomena. Models of reconstructed neurons can be used in these programs to conduct “virtual” experiments. During these simulations, the software numerically solves a set of differential equations for the membrane voltage of the cell throughout the entire morphology. The numerical methods used are both efficient and stable, but these programs typically solve the equations in all parts of the cell (or network of cells) simultaneously, regardless of whether or not all of the cell is active. This approach does not allow for the focusing of computational effort on those locations of the cell that are most active. The result is an unnecessary slow-down in neural simulations where electrical activity is localized to a small region of the cell.

We extend the numerical scheme used in packages like NEURON by making the computations local to individual branches rather than entire cells. Once the calculation is reduced to the level of branches instead of cells, spatial adaptivity can be implemented in a straight-forward manner: the active regions of the cell are detected and computational effort is focused there, while saving computations in other regions of the cell that are at or near rest. We use the algorithm on realistic neural simulations and demonstrate its improved efficiency over non-adaptive methods. We find that the computational cost of the method scales with the amount of activity present in a particular simulation, rather than with the physical size of the system being simulated. For certain problems spatial adaptivity reduces the computation time by up to 80%.

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DISTINCT INTRASPINAL MACROPHAGE ACTIVATION PROTOCOLS DIFFERENTIALLY INFLUENCE OLIGODENDROCYTE GENESIS

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Oligodendrocytes (OLs) are vulnerable to a variety of CNS insults including trauma, multiple sclerosis, ischemia, and excitotoxicity. Their death leads to deficits in myelination and axonal conductance, which contribute to neurological and functional deficits. Replacing this cell population requires proliferation and differentiation of OL progenitor cells, a process that can be potently influenced by activated macrophages which are present in most CNS pathologies. To examine the relationship between macrophage activation and OL genesis, an *in vivo* microinjection model was used in which microglia and macrophages were activated by different ligands injected into the intact spinal cord. The ligands used included lysolecithin (a demyelinating agent known to induce OL genesis), lipopolysaccharide (LPS), zymosan or vehicle (sterile PBS). During the first two weeks after injections, LPS created an environment favorable for new OL generation marked by a significant rise in proliferating progenitor cells and a dramatic increase in new OLs exceeding that found in vehicle or lysolecithin injections. In contrast, zymosan-activated macrophages produced total OL loss without stimulating progenitor proliferation, migration or new OL genesis. Zymosan injection also evoked delayed primary demyelination of previously intact myelination axons surrounding the injection site. These results reveal the dichotomous potential of macrophages in influencing progenitor cell proliferation and maturation and offer a possible explanation for why OL replacement is restricted to specific domains following CNS trauma.

Neuropeptides Potentiate Acid-Sensing Ion Channel 1a

During stroke, acidosis contributes to neuronal death by activating the acid-sensing ion channels (ASICs). Prevention of ASIC1a activation reduces neuronal death incurred in mouse stroke models, which suggests that ASIC1a could be a target for pharmacological treatment to limit brain damage following stroke. However, more information is necessary regarding the mechanism of ASIC1a-induced neuronal death. Steady-state desensitization of ASIC1a is a natural biophysical property of ASICs that would limit acidotoxicity, and compounds which modulate this property affect both acid-induced neuronal death and the severity of injury following stroke in mice. RFamide-related neuropeptides inhibit steady-state desensitization of mouse ASIC1a. In this project, we asked whether human ASIC1a was similarly affected by RFamide-related neuropeptides using the two-electrode voltage-clamp technique in an oocyte expression system. We discovered that endogenous neuropeptides robustly potentiate ASIC current by preventing steady-state desensitization. Furthermore, human ASIC1a was sensitive to a larger array of neuropeptides, and differentially affected compared to mouse ASIC1a. Our results indicate that neuropeptide modulation of ASIC1a is not limited to members of the RFamide-related neuropeptide family. We have discovered that an opioid peptide similarly affects ASIC1a steady-state desensitization. We predict that endogenous neuropeptides will enhance acid-induced neuronal death both *in vitro* and *in vivo*, and preventing this interaction with ASICs may be a novel approach to prevent or limit brain damage following stroke. To this end, we have demonstrated that neuropeptide effects on steady-state desensitization can be attenuated by competition with an exogenous peptide.

Oligodendrogenesis following spinal cord Injury: Role of CNTF.
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Demyelination and oligodendrocyte (OL) loss following spinal cord injury (SCI) are well documented. The question of whether these cells are replaced after SCI has received much less attention. Recently, we showed that accumulation of oligodendrocyte progenitor cells (OPCs) and robust oligodendrogenesis occurs in spinal cord white matter (WM) and gray matter (GM) in first 2 weeks post-injury. Current work is investigating potential mechanisms involved in this injury-induced gliogenesis. Ciliary neurotrophic factor (CNTF) is known to promote OPC proliferation and differentiation into OLs in vitro and is upregulated in a variety of central nervous system disorders. Thus, we hypothesized that CNTF expression would be increased after SCI and that CNTF contributes to the enhanced oligodendrogenesis. Using a rat spinal contusion model we quantified CNTF protein levels after SCI using Western blots. This revealed that CNTF expression continually rises between 3d and 14d post-injury (dpi). Using immunohistochemistry, we next examined tissue sections spanning the lesions at 3, 5, 7 and 14 days post-injury (dpi) to determine whether CNTF localized to spared WM, spared GM and/or lesioned tissue. We found a significant increase in CNTF expression in WM and GM at 7 and 14dpi compared to uninjured controls; CNTF was not expressed in lesion cavities. In addition, CNTF levels were significantly higher in WM compared to GM. Thus, CNTF may play a role in the oligodendrocyte genesis that occurs after SCI, particularly within the WM. To test this possibility, ongoing studies are examining the effect of down-regulating CNTF after SCI. Preliminary data verify that intraspinal CNTF siRNA administration on 1 and 3dpi successfully decreases CNTF protein by 7dpi. Studies underway will determine if preventing CNTF expression after SCI alters OPC proliferation or new oligodendrocyte formation. Collectively, these data will elucidate a potentially endogenous mechanism for CNS tissue repair that was not appreciated previously.

Abstract

Cell Proliferation Patterns in Cells of Astrocytic Lineage Following Graded Spinal Cord Contusion Injury in Mice

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Proliferation of glial and progenitor cells after spinal cord injury (SCI) contributes to anatomical and behavioral recovery. To date, however, the effects of injury severity on the proliferation and differentiation of endogenous cells is poorly understood. The purpose of this study was to examine the patterns of cell proliferation in cells of astrocytic lineage occurring the first week after either a mild, moderate, or severe mid-thoracic contusion injury in mice. Adult mice were subjected to controlled injury and proliferation was determined by incorporation of bromodeoxyuridine (BrdU) following daily intraperitoneal injections for the week following injury. The specimens were obtained at 7 weeks post-injury and spinal cord sections were stained with an antibody to BrdU. The greatest numbers of proliferating cells were found at the center of the lesion site and were not different between groups. However, there were differences in the number and distribution of proliferating cells surrounding the central canal at the rostral lesion poles. Mild injuries had more proliferating cells around the central canal than severe injuries. To follow those cells that may contribute an astrocyte lineage, double staining of BrdU and either glial fibrillary acidic protein (GFAP) or vimentin was performed on the sections. GFAP-positive astrocytes were found in spared white matter and in the area surrounding the lesion scar, but were not located in the center of the lesion in any injury level. However, vimentin-positive staining was found within the scar in all groups. Although most of this staining was extracellular, a few vimentin-positive processes associated with nuclei were found. In all groups, there were more vimentin-positive cells surrounding the central canal than GFAP-positive cells. The preliminary results of this study suggest that there are differences in cell proliferation after graded SCI, and that vimentin-positive cells reside both in the scar and the area surrounding the central canal of the spinal cord independent of GFAP. Future studies will focus on the differentiation and migration of these vimentin-positive, GFAP-negative cells, which may play a key role in repair and recovery after spinal cord injury.

Key Words: endogenous proliferation, spinal cord injury, vimentin, glial cells