

Effect of Deep Seawater on Expression of μ -Opioid Receptor in Cultured Rat Hippocampal Neurons

Il Soo Moon² and Seong-Ho Kim^{1*}

¹Department of Internal Medicine, Inje University College of Medicine, Busan 612-030, Korea

²Department of Anatomy, Dongguk University College of Medicine, Gyeongju 780-714, Korea

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Deep seawater (DSW) generally refers to seawater at depths equal to or greater than 200 meters. DSW is rich in inorganic materials which have attracted attention for its various applications. In this study we investigated the effects of the DSW upwelled from the East Sea, offshore Yang Yang (KangWon-do, Korea), on the expression of μ -opioid receptor (MOR) of cultured rat hippocampal neurons. Neurons were grown in a minimal essential medium containing 10% (v/v) fetal bovine serum and either 25% (v/v) distilled water, or hardness (H) 800, or H 1000 DSW. Cultures grown in the presence of DSW with H 800 and H 1000 exhibited robust MOR immunoreactive signals in both neurons and astrocytes. Interestingly, the increase in MOR immunoreactive signals was more dramatic in astrocytes than in neurons. Statistical analysis revealed that the relative intensities for MOR clusters increased approximately 4-fold in astrocytes cultured in H 800 and H 1000 media. These increases were statistically very significant ($p < 0.001$). In contrast, the increase in intensities for MOR immunoreactive signals was relatively less dramatic in neurons, where only the increase in the H 1000 culture was statistically very significant ($p < 0.001$). These results indicated that DSW promotes expression of MOR in both neurons and astrocytes, and more significantly in the latter.

Key words : Deep seawater, hippocampal neuron, mu-opioid receptor, astrocytes, fibromyalgia

Introduction

Deep seawater (DSW; deep ocean water) refers to water that is extracted from the ocean, usually at depths of 200 meters or more. Sunlight does not reach at such depths. Due to lack of photosynthesis, DSW is relatively free of microorganisms such as phytoplanktons. Wealth in various minerals including indispensable trace elements in DSW has demonstrated their usefulness in various applications [6]. Some experimental evidence supporting the efficacy of DSW are available by now. DSW controls the increase of serum lipid values [total cholesterol and low density lipoprotein (LDL) cholesterol] of cholesterol-fed rabbits, and promotes the reduction of serum lipid hyperlipemia rabbits [20]. DSW is useful for the prevention of hyperlipidemia and arteriosclerosis compared to the surface seawater by reduction of the LDL cholesterol level and enhancement of GPx activity [12]. DSW could improve cardiovascular hemodynamics [7] and indices for cancer chemoprevention [15].

We have been studying the effects of the DSW upwelled

from the East Sea, off-shore YangYang (KangWon-do, Korea) on the cultured rat hippocampal neurons. Using this model system we recently tested the efficacy of DSW on morphological differentiation, and found that, although DSW had no effect on initial morphological differentiation (17 hr post-plating), low hardness (H) (0 and 200) DSW reduced dendritic branching resulting in bipolar-like morphologies in extreme cases [10]. H 600 DSW was more or less same as control groups, and H 1,000 DSW doubled the number of dendritic branches indicating that DSW with appropriate hardness ameliorates neuronal health.

Our other previous study showed the effects of the DSW on the synaptic maturation of cultured rat hippocampal neurons [9]. The examination of DIV21 showed that coupling rates of synaptic vesicle 2 and N-methyl-D-aspartate (NMDA) receptor (NR) 2B were significantly increased in neurons grown in the presence of H 800 and H 1000 DSW. That study suggests that DSW promotes the formation of excitatory postsynaptic signal transduction NMDA-associated complexes and functional synapses. NMDA and opioid receptor systems are considered central to nociception and antinociception. The relationship between these systems is not only functional but also spatial within multi-

*Corresponding author

Tel : +82-51-797-2044, Fax : +82-51-797-2046

E-mail : junjan@paik.ac.kr

ple central nervous system regions (CNS) [11]. These receptor systems are found concentrated in presynaptic and postsynaptic sites of the dorsal horn (particularly lamina II), suggesting a close functional relationship [5]. Opioids can directly and indirectly modulate NR activity and affect the NR mediated calcium influx into neurons [2,16,19,21].

In this study we investigated the effects of the DSW on the mu opioid receptor (MOR) expression of cultured rat hippocampal neurons.

Materials and Methods

DSW

The DSW was pumped up from a depth of 1,100 m off YangYang (Kang Won-do, Korea) was desalinated and concentrated by reverse osmosis. The desalinated water was defined as hardness (H) zero (H 0). The mineral ingredient content of the H 4,000 DSW, which was manufactured by dilution of the DSW, is shown elsewhere [15], and obtained from Waterbis Co., Ltd (Ansan, Korea).

Antibodies

The following antibodies were used. MOR (1:5,000; rabbit polyclonal, Millipore, Billerica, MA, USA). Anti-Glial fibrillary acidic protein (GFAP) (1:500; Millipore, Billerica, MA, USA). Postsynaptic density protein (PSD) 95 (1:1,000; chicken polyclonal UCT-C1, a kind gift from Dr. Randall Walikonis, University of Connecticut, CT, USA).

Neuronal culture

Embryonic day 18 (E19) rat hippocampal cells were dissociated by triturating trypsin-treated tissues, and were plated on poly-DL-lysine-coated coverslips (1,000~1,500 cells/mm²) in the minimal essential medium (MEM; Gibco, #11700-077) containing 10% (v/v) fetal bovine serum (Gibco # 26140-079), 25% (v/v) DSW of various H, and 25 μ M glutamate, as previously described [1,4]. One third of the culture media was replaced with the same fresh one but glutamate every 3 days.

Immunocytochemistry

Cells were fixed through the PFA/MeOH fixation method [13]. Coverslips were rinsed briefly in phosphate buffered saline (PBS) and with 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 10 min. Coverslips were rinsed in PBS and then incubated in -20°C methanol at -20°C for

20 min. The cells were then rinsed once with PBS and blocked overnight at 4°C in preblocking buffer [5% normal goat serum, 0.05% Triton X-100 in H-PBS (450 mM NaCl and 20 mM phosphate buffer, pH 7.4)]. Primary antibodies were diluted in preblocking buffer (250 μ l per well of 24-well culture plate), replaced with preblocking buffer, and incubated overnight at 4°C. Coverslips were rinsed (15 min \times 3) in preblocking buffer, and incubated with secondary antibodies [Alexa Fluor 488-conjugated goat anti-mouse, Alexa Fluor 568 conjugated-goat anti-rabbit, and Alexa Fluor 647-conjugated goat anti-chicken IgG (each diluted 1:1,000 in blocking buffer; Invitrogen)] at RT for 1-2 hr. Coverslips were rinsed once in preblocking buffer for 15 min, twice in PBS, and mounted on slides with 4% n-propylgallate in 90% glycerol and 10% sodium carbonate buffer (pH 8.7).

Fluorescence light microscopy

A Leica Research Microscope DM IRE2 (Leica Microsystems AG, Wetzlar, Germany) equipped with filter systems I3 S, N2.1 S and Y5 was used to capture light and fluorescent microscopic images. Digital images were acquired with low power (10X) and a HCX PL FL 100X oil-immersion lens and a high-resolution CoolSNAPTM CCD camera (Photometrics Inc., Germany) under the control of a computer equipped with FW4000 (Leica) software. Images (1388 \times 1039 pixels) were processed with the use of Photoshop 5.0 (Adobe Systems).

Histograms and surface plots

Using an Adobe PhotoShop software (version 5.0), fluorescence images were converted into a gray scale mode (value from 0 to 255), and inverted so that IR signals become dark. Histograms and surface plots (wireframe mode) were produced using a Scion Image software (Beta 4.03; Scion Corp., Frederick, MD, USA) after removing backgrounds through the 2D rolling ball mode.

Measurement of IR signal densities

To compare the relative strength of IR signals, images were converted to a gray scale mode (0~255 scale), inverted, and backgrounds were removed through the 2D rolling ball mode. A small circular area including one IR puncta was selected and the mean density of the selected area was measured using a Scion Image software. The circle was dragged to other punctae and the densities were measured.

Analysis

For relative intensities of MOR IR signals, mean densities of about 100~150 clusters from 3-5 dendritic segments were measured and expressed as mean±SD. Multiple pairwise comparisons for statistical significance were evaluated by Kruskal-Wallis one-way ANOVA, and the differences between groups by Mann-Whitney *U*-test. The *p* values of less than 0.05 and 0.01 were considered to be significant and very significant, respectively.

Results

Hippocampal cultures were double-labeled with antibodies against MOR and either PSD 95 or GFAP, specific markers for neurons or astrocytes, respectively. Fluorescent microscopic images of MOR/PSD 95 double-labeling of hippocampal neurons grown in control media were shown in Fig. 1A. MOR immunoreactive signals were distributed throughout somatodendritic domains, and formed clusters

Control media

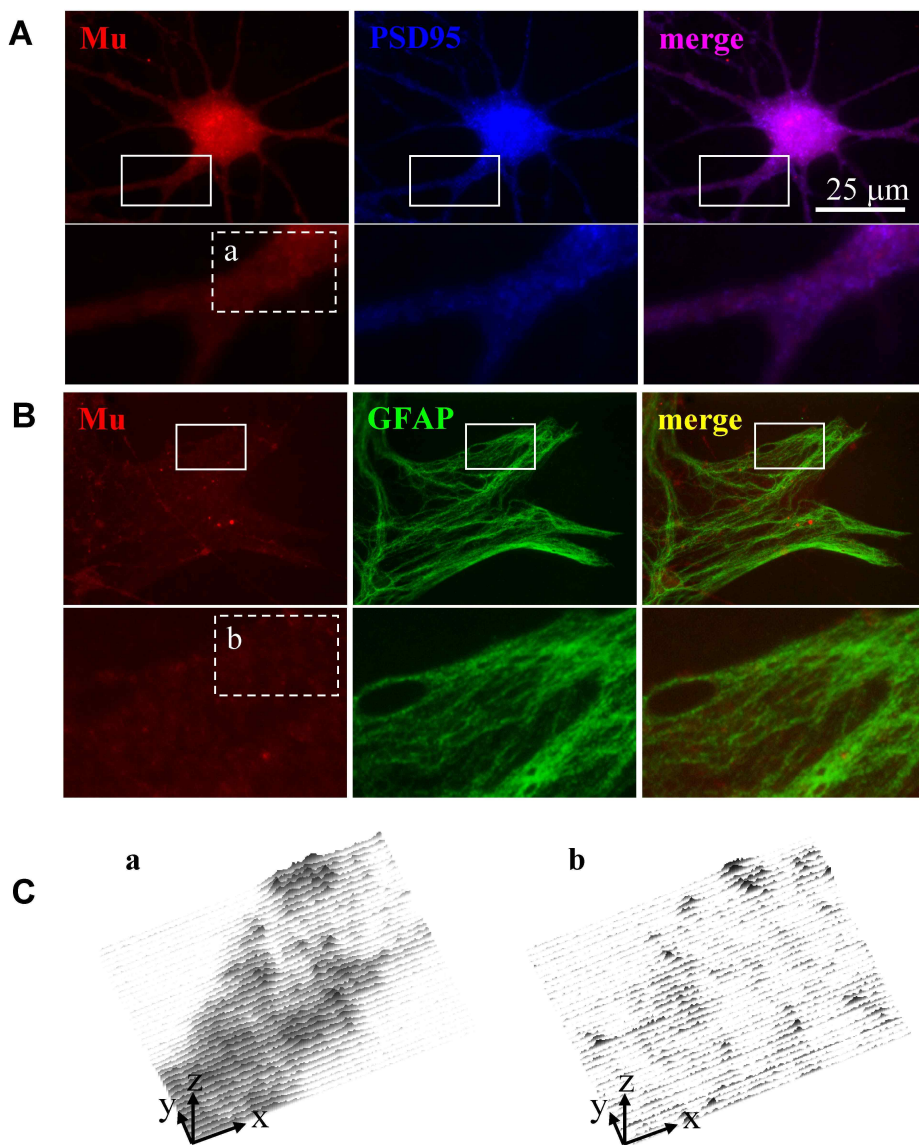


Fig. 1. Expression of Mu-opioid receptors (MOR) in the control media. Cultured cells were triple-labeled with antibodies against MOR, PSD95, and GFAP. Fluorescence micrographs of a neuron (A) and an astrocyte (B) were shown in single and merge images. Boxed areas were shown enlarged in insets. The surface plots of the boxed areas a and b of each inset for MOR expressions were shown in panel C. Scale bar; 25 μ m.

that were not overlapped with those of PSD 95 (Fig. 1A, insets). Fluorescent microscopic images for a sister culture that is double-labeled with antibodies against MOR and GFAP were shown in Fig. 1B. Similar to neuronal stainings, MOR immunoreactive signals formed clusters throughout astrocyte's cytoplasm (Fig. 1B, insets). However, as shown by the surface plots, the intensities for MOR clusters were not very strong in both neuron and astrocyte (Fig. 1C). In contrast, cultures grown in the presence of DSW with H 800 (Fig. 2) and H 1,000 (Fig. 3) exhibited robust MOR immunoreactive signals in both neurons and astrocytes. Interestingly, the increase in MOR immunoreactive signals was more dramatic in astrocytes (box b's in insets of Figs. 2 and 3) than

in neurons (box a's in insets of Figs. 2 and 3), as shown evidently in surface plots (Figs. 2C and 3C). Statistical analysis revealed that the relative intensities for MOR clusters increased approximately 4-fold in astrocytes cultured in H 800 and H 1000 media (from 13.3 ± 4.9 to 52.0 ± 15.1 and 47.3 ± 13.1 , respectively; $n=50 \sim 70$). These increases were statistically very significant ($p < 0.001$) (Fig. 4). In contrast, the increase in intensities for MOR immunoreactive signals was relatively less dramatic in neurons; from 22.0 ± 6.8 in control medium to 24.1 ± 4.6 and 34.7 ± 11.3 in H 800 and H 1000 media, respectively, where the increase in the H 1000 culture was statistically very significant ($p < 0.001$) (Fig. 4).

DSW-H 800

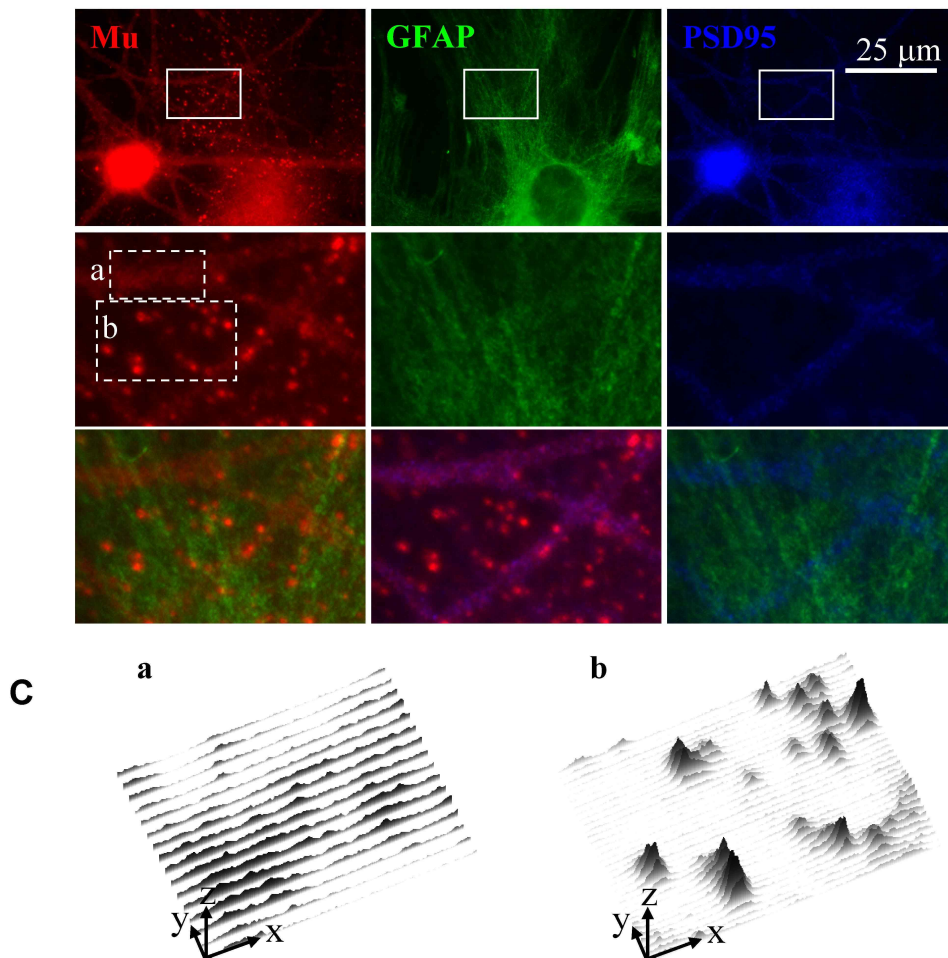


Fig. 2. Expression of Mu-opioid receptors (MOR) in the H 800 media. Cultured cells were triple-labeled with antibodies against MOR, PSD95, and GFAP. Boxed areas were shown enlarged in single channels (middle row) or in merge (bottom row). The boxed areas a and b in the middle row represents the expression of MOR in a neuron and an astrocyte, respectively, and their surface plots were shown in the panel C. Scale bar; 25 μm . Note significant increases in the MOR signal intensities in both neuron and astrocytes, being more significant in astrocytes.

DSW-H 1,000

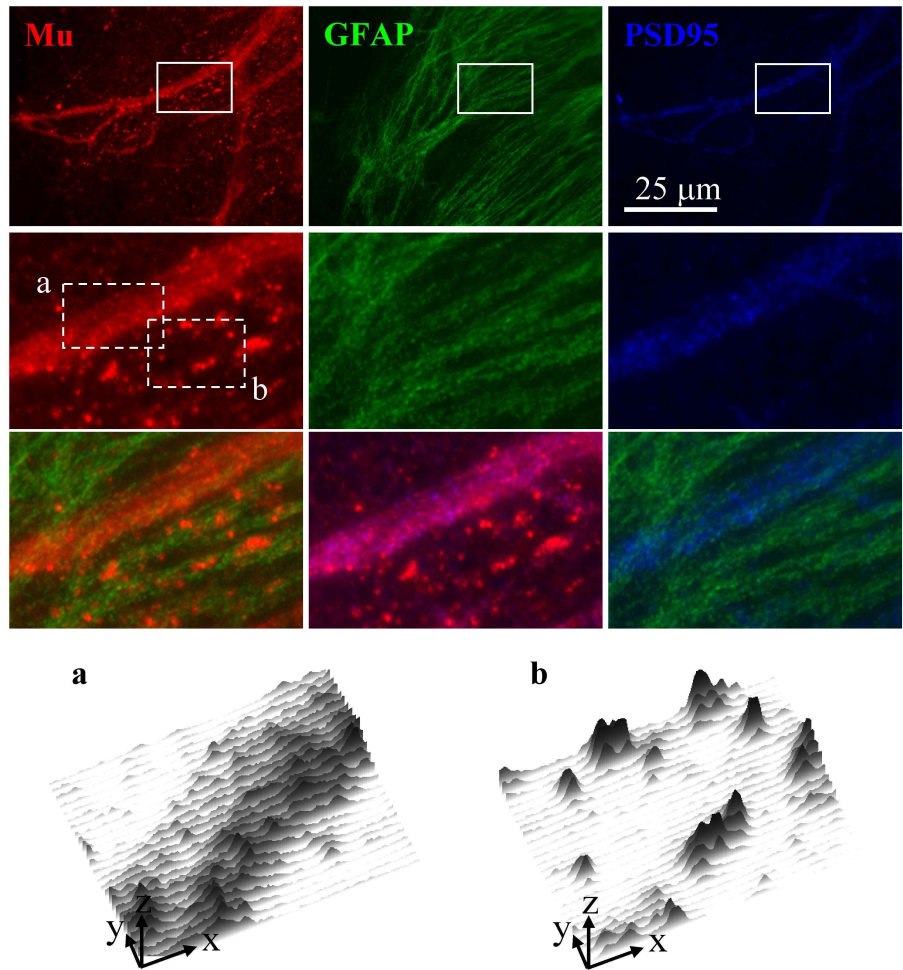


Fig. 3. Expression of Mu-opioid receptors (MOR) in the H 1000 media. Immunocytochemistry, layouts, and labels are same as in Fig. 2. Note significant increases in the MOR signal intensities in both neuron and astrocytes, being more significant in astrocytes.

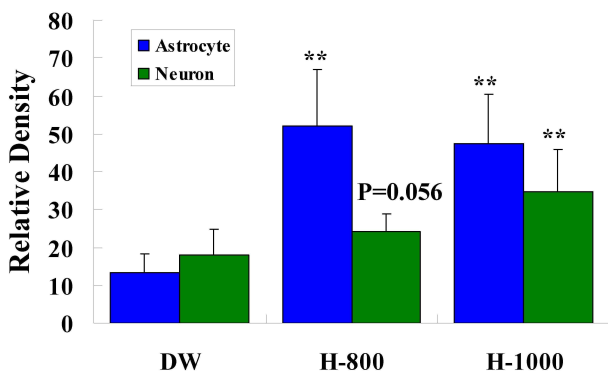


Fig. 4. Relative intensities of Mu-opioid receptors (MOR) IR signals. The IR signal intensities of MOR clusters were measured in 0-255 gray scales. DW=distilled water. Statistic significance was assessed by Mann-Whitney U-test. **, $p < 0.001$.

Discussion

We carried out immunocytochemistry to investigate whether DSW affects the expression of MOR. These results indicate that DSW promotes expression of MOR in both neurons and astrocytes, more significantly in the latter. Astrocytes express functional mu, delta, and kappa opioid receptors in a region-specific manner [3,14,18]. There is very little on possible mechanisms involved in this study. It would be helpful if any of the individual minerals contained within DSW been assessed for their effects on MOR expression. It would be also very interesting if a single mineral or combination of minerals could be identified which would replicate the observed benefits of DSW.

The East Sea is connected with four other open seas through narrow and shallow straits. Due to relatively isolated oceanography, it is a bowl-shaped 'Ocean Miniature' where water exchange between the East Sea and other connected oceans are very limited. Therefore, it is believed that more than 90% of all East Sea water is DSW. In the previous report we showed that the rat hippocampal neurons grown in the presence of the East Sea DSW are healthier and more dendritic branches are formed than distilled water groups [10]. In this study we investigated whether the health in gross morphology accompanies synaptic 'health' or maturation. Because DSW [25% (v/v)] needs to be added into the culture medium, culture media were made using a powder form [minimal essential medium (MEM; Gibco, #11700-077) containing 10% (v/v) fetal bovine serum (Gibco # 26140-079)]. Our other previous study showed the effects of the DSW on the synaptic maturation of cultured rat hippocampal neurons [9]. Immunocytochemical examination of DIV21 showed that PSD 95, α -isoform of type II Ca^{2+} /calmodulin-dependent protein kinase and synGAP α 1 clusters were strengthened and coupling rates of Synaptic vesicle 2 and NR 2B were significantly increased in neurons grown in the presence of H 800 and H 1000 DSW. Our results indicate that DSW promotes the formation of excitatory post-synaptic signal transduction NMDA- or MAGUK-associated complexes and functional synapses [9].

The acidic saline animal model of pain has been suggested to mimic chronic widespread pain such as fibromyalgia. In this model, repeated intramuscular injections of acidic saline produce a widespread hyperalgesia that persists without evidence of significant peripheral tissue damage or inflammation, and is believed to be centrally maintained [17]. Kim et al. [8] examined the changes of pain-related neurotransmitters in specific brain regions of this model after DSW drinking. Preliminary results showed that compared to controls, acid injected rats demonstrated strong expression of serotonin in red and raphe nucleus. Acid injected rats showed significant reductions of the serotonin expression in red and raphe nucleus after DSW drinking. This study suggests DSW might be helpful for pain and anxiety [8].

In this study we showed that DSW upwelled in the off-shore YangYang promotes expression of MOR in both neurons and astrocytes. Considering increased MOR expression in CNS of rats and significant reductions of the serotonin expression in red and raphe nucleus of acid injected rats [8] after DSW treatment, it is believed that DSW can

be helpful for pain control.

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초록 : 배양된 쥐 해마신경세포에서 μ -아편양 수용체의 발현에 대한 해양심층수의 영향

문일수² · 김성호^{1*}

(¹인제대학교 의과대학 내과학교실, ²동국대학교 의과대학 해부학교실)

해양심층수는 깊이 200 m 정도 혹은 그 이상의 해수를 말한다. 해양심층수는 무기물질이 풍부하여 여러 분야 적용을 위해 많은 관심이 쏠리고 있다. 본 연구에서는 동해 양양 부근에서 취수한 해양심층수에서 배양된 쥐 해마신경세포의 μ -아편양 수용체 발현에대한 영향을 조사하였다. 경도 800 및 1,000 심층수가 25% (v/v) 포함된 minimal essential media에서 해마신경세포를 배양해 대조군(증류수 첨가)과 비교하였다. 경도 800 및 1000심층수 첨가 배양액에서 배양된 신경세포 및 별아교세포에서 μ -아편양 수용체의 면역반응 신호가 매우 증가했다. 흥미롭게도 신경세포보다 별아교세포에서 μ -아편양 수용체의 면역반응 신호 증가가 더 뚜렷했다. 통계 분석시 경도 800 및 1000에서 배양된 별아교세포에서 μ -아편양 수용체 군에 대한 상대적 강도가 약 4배 증가했다. 이런 증가는 통계적으로 매우 유의했다($p < 0.001$). 대조적으로 신경세포에선 이런 증가가 상대적으로 덜 뚜렷하였고, 경도 1000 배양에서만 통계적으로 유의하였다($p < 0.001$). 이 결과들은 심층수가 신경세포 및 별아교세포에서 μ -아편양 수용체의 발현을 항진 시킨다는 것을 나타내었다.