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Layer selective presynaptic modulation of excitatory inputs to hippocampal CA1 by μ -opioid receptor activation

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Abstract

Chronic and acute activation of μ -opioid receptors (MOR) in hippocampal CA1 disrupts rhythmic activity, alters activity-dependent synaptic plasticity and impairs spatial memory formation. In CA1, MORs act by hyperpolarizing inhibitory interneurons and suppressing inhibitory synaptic transmission. MOR modulation of inhibitory synaptic function translates into an increase in excitatory activity in all layers of CA1. However, the exact anatomical sites for MOR actions are not completely known. Therefore, we used voltage-sensitive dye imaging, whole cell patch clamping, photolysis of CNB-caged GABA, and micro-sectioned slices of rat hippocampus to investigate the effect of MOR activation in CA1. First, we investigated the effect of MOR activation using a MOR agonist DAMGO on the direct activation of GABA receptors by photolysis of CNB-caged GABA in all layers of CA1. MOR activation did not affect hyperpolarizations due to direct GABA receptor activation in any layer of CA1, but MOR activation did suppress GABAergic inhibitory postsynaptic potentials suggesting that MOR activation acts by presynaptically inhibiting interneuron function. We next examined whether MOR activation was equivalently effective in all anatomical layers of CA1. To do this, cuts were made between anatomical layers of CA1 and isolated layers were stimulated electrically (5 pulses at 20 Hz) to produce excitatory postsynaptic potentials (EPSPs). Under these conditions, MOR activation significantly increased EPSP areas in stratum radiatum (SR), stratum pyramidale (SP) and stratum oriens (SO) relative to stratum lacunosum-moleculare (SLM). When compared to the effect of GABA_A and GABA_B receptor antagonists on EPSP areas, the effect of DAMGO was proportionately larger in SR, SP and SO than in SLM. We conclude that MOR activation is more effective at directly modulating activity in SR, SP and SO, and the smaller effect in SLM is likely due to a smaller MOR inhibition of GABA release in SLM.

Keywords

Interneuron; synaptic inhibition; voltage-sensitive dye imaging; caged-GABA; photolysis

Introduction

One of the largest obstacles to successful drug abuse rehabilitation is relapse (Kreek, 2001). Frequently, relapse is triggered by exposure of the recovered addict to objects previously associated with drug use. The formation of these associations requires the declarative memory

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system, and in particular the hippocampus (White, 1996). In models of opiate abuse, an intact hippocampus is required for animals to learn to self-administer μ -opioid receptor (MOR) agonists (Olmstead and Franklin, 1997a), and in some cases animals can be trained to self-administer MOR agonists directly into the hippocampus (Corrigall and Linseman, 1988; Stevens et al., 1991; Self and Stein, 1993; but see Olmstead and Franklin, 1997b). Nonetheless, precisely how the activation of MORs affects hippocampal circuit function and how this translates into alterations in the formation of long term memories is not completely understood.

At the circuit level, MOR activation has been shown to modulate spatial memory and dramatically affect the induction of synaptic plasticity in hippocampal CA1 pyramidal neurons (Mansouri et al., 1997; Pourmotabbed et al., 1998; Mansouri et al., 1999; Wagner et al., 2001; Pu et al., 2002). Interestingly, the manner by which synaptic plasticity was modulated depended on the history of an animal's exposure to chronic morphine or heroin. In addition to effects on synaptic plasticity, MOR activation disrupted synchronous rhythms in hippocampal slices thought to be important for the coding of information and the formation of memories (Whittington et al., 1998; Faulkner et al., 1998; Faulkner et al., 1999). Thus, MOR activation had profound effects on synaptic plasticity and network function in hippocampal CA1.

In CA1, MORs are thought to be localized exclusively to inhibitory interneurons (Bausch et al., 1995; Kalyuzhny and Wessendorf, 1997; Drake and Milner, 1999; Drake and Milner, 2002), and activation of these receptors has been shown to hyperpolarize these cells (Madison and Nicoll, 1988; Wimpey and Chavkin, 1991; Svoboda and Lupica, 1998; Svoboda et al., 1999) and inhibit the release of GABA (Nicoll et al., 1980; Masukawa and Prince, 1982; Swearengen and Chavkin, 1989; Wimpey et al., 1990; Lupica et al., 1992; Cohen et al., 1992; Capogna et al., 1993; Reikling, 1993; Lupica, 1995). However, not all interneurons equally express MORs. Perisomatically projecting parvalbumin-expressing basket cells exhibit a much higher percentage of colocalization with MORs compared to all other subtypes of interneurons (Drake and Milner, 2002; Stumm et al., 2004). The distal dendritic projecting somatostatin-containing interneurons displayed a smaller amount of coexpression with MOR, while calretinin, vasoactive intestinal peptide, and cholecystokinin-containing interneurons have little to no MOR expression (Drake and Milner, 2002; Stumm et al., 2004). Consistent with these anatomical studies, physiological studies have shown that perisomatically projecting basket cells were approximately twice as likely to be hyperpolarized by MOR activation than dendritically projecting interneurons (Svoboda et al., 1999). Together, the anatomical and physiological data suggested that MOR would have a complex effect on excitatory activity in hippocampal CA1, but MOR activation would primarily act by disinhibiting the output of CA1 pyramidal cells with only a small effect in the dendritic layers.

More recent physiological studies have shown that MOR activation significantly affected the dendritic layers of CA1 by increasing the size of excitatory inputs in CA1 and augmenting excitatory activity that propagated between layers of CA1 (McQuiston and Saggau, 2003; McQuiston, 2007). The simplest explanation for these observations was that MORs were found in sufficient concentrations in CA1 dendritic layers to be as effective at modulating excitatory activity in the dendritic layers of CA1 as they were at modulating excitatory activity in the soma of pyramidal cells. However, these studies were done in intact slices in which inhibition at a particular site in a CA1 pyramidal cell may influence a more distant compartment of the same cell through the cable properties of its dendrites (McQuiston, 2007). Thus, an effect of MOR activation on inhibition in a more proximal layer of CA1 could possibly influence a more distal dendritic layer. The present study was undertaken to preclude this possibility of influence from distal neuronal compartments. We first examined a presynaptic or postsynaptic mechanism of action for the effect of MOR activation on GABA receptor function in all layers of hippocampal CA1 through the use of voltage-sensitive dye (VSD) imaging and the photolysis of CNB-caged GABA. We then used VSD imaging to measure excitatory

postsynaptic potentials (EPSP) in micro-dissected rat hippocampal CA1 slices. The slices were dissected to isolate the stratum lacunosum-moleculare (SLM) from the stratum radiatum (SR), and the SR from the stratum pyramidale (SP). We then measured the effects of MOR activation on excitatory activity from isolated layers of CA1 and directly compared the effect of MOR activation between the anatomical layers.

Experimental Procedures

Preparation of hippocampal slices and staining with voltage-sensitive dye

In adherence with an approved Virginia Commonwealth University IACUC protocol, male Sprague Dawley rats (42 to 60 days old) were deeply anaesthetized with ketamine and xylazine, and transcardially perfused with ice cold artificial cerebral spinal fluid consisting of (in mM): Sucrose 230, KCl 2.5, CaCl 1, MgSO₄ 4, NaHPO₄ 1, NaHCO₃ 25, glucose 10. The brain was removed, hemi-sectioned, and horizontal slices containing the mid temporal hippocampus were cut at 350 μ m on a vibratome 3000 (Ted Pella Inc., Redding, CA). Sections were incubated at 32°C for 30 min and afterward permitted to return to room temperature (~22°C). The incubation chamber solution consisted of (in mM): NaCl 125, KCl 3.0, CaCl 2, MgSO₄ 6, NaHPO₄ 1, NaHCO₃ 25, glucose 10. Slices were stained for 30 to 60 min with the VSD NK3630 (0.02 to 0.05 mg/ml) prior to experimentation (Jin et al., 2002).

Voltage-sensitive dye imaging and electrophysiology

After staining with NK3630, razor cuts were made to isolate the anatomical layers in hippocampal CA1. If slices were used for photolysis of CNB-caged GABA experiments, no cuts were made to the slices. Slices were then placed in a glass bottom recording chamber where they were perfused submerged with saline (32 to 34°C) consisting of (in mM): NaCl 125, KCl 3.0, CaCl 1.2, MgSO₄ 1.2, NaHPO₄ 1, NaHCO₃ 25, glucose 25 bubbled with 95% O₂/5% CO₂. The recording chamber was mounted on a fixed stage under an Olympus BX51WI microscope equipped with differential interference contrast (DIC) optics. The image of the slice was collected using transmitted near infrared light (> 775 nm) and a 20x (0.95 NA) water immersion objective. The image was captured (Foresight I-50 frame grabber) with a Dage-MTI NC-70 Newvicon tube or a Dage-MTI IR-1000 CCD camera with contrast enhancement.

For voltage-sensitive dye absorbance measurements, slices were illuminated with a tungsten-halogen 100 W lamp passed through a bandpass filter (705 $\pm$ 30 nm, Chroma Technology, Rockingham, VT). The transmitted light was collected with a Wutech H-469IV photodiode array that is part of the Redshirtimaging integrated Neuroplex II imaging system. The data were acquired, displayed and analyzed with Neuroplex software.

To evoke synaptic activity in the isolated layers of hippocampal CA1, bipolar tungsten stimulating electrodes (approx. 100 k Ω , FHC) were placed in either SO/SP, SR or SLM of CA1 to stimulate afferents in CA1. To evoke inhibitory synaptic potentials in intact slices, bipolar stimulating electrodes were placed at the border of SR and SLM and recordings were made in the presence of the glutamate antagonists DNQX (30 μ M) and D-APV (50 μ M). Electrical current was delivered (100 μ s, 10 to 100 μ A) by a stimulation isolation unit (NL800, Digitimer, Hertfordshire, England) to produce EPSPs or IPSPs. A train of 5 pulses at 20 Hz was routinely used to evoke synaptic activity. Isolated inhibitory postsynaptic potentials were rarely observed in microdissected slices. The signal from one photodiode was spatially averaged with its immediate surrounding photodiodes (consisting of nine photodiodes in total) to represent the electrical response of an anatomical layer of CA1 (Figs. 1A, 2A, 3A, 4A). The averaged VSD signals were used to measure the MOR induced changes in EPSP or IPSP area, and these changes in EPSP or IPSP area were normalized to the area of the controls, MOR activation/Control. This provided a fractional change in EPSP or IPSP area that was

quantitatively compared between different stimuli in the train of electrical pulses, different anatomical layers and different slices. The area of EPSPs was calculated from the sum of signal sample point amplitudes for the first 50 ms following the stimulation of each EPSP. The area of the IPSP was measured from the sum of signal sample point amplitudes taken from the end of the 5 stimuli to the point where the hyperpolarizing signal of the control VSD response returned to baseline. The amplitude of each sample point was calculated relative to the baseline signal preceding any stimulation.

In the photolysis experiments, VSD signals were simultaneously compared to electrophysiological measurements. Whole cell patch clamp recordings from the somata of CA1 pyramidal cells were performed to measure the membrane currents produced by the photolysis of CNB-caged GABA and the electrically-evoked inhibitory postsynaptic responses. Whole cell patch clamp recordings were performed using patch pipettes (4 to 5 M Ω) pulled from borosilicate glass (8250 1.65/1.0 mm) on a Narishige PP830 pipette puller (East Meadow, New York) filled with (in mM): KGluconate 130, NaCl 8, MgATP 2, NaGTP 0.3, HEPES 10, BAPTAK₄ 0.1. Membrane current responses were recorded with a Model 2400 patch clamp amplifier, and further amplified and filtered with a Brownlee 440 amplifier. Membrane potential was voltage-clamped at -55 mV and whole cell capacitance and series resistance were compensated 70 – 80%. The slow inhibitory currents were sampled at a frequency of 1.6 kHz and low pass filtered at 800 Hz.

Photolysis with voltage-sensitive dye imaging

For photolysis of CNB-caged GABA by ultraviolet (UV) light, UV light pulses were generated with a high power (10 ms pulse, 130 mW at source, wavelength peak 365 nm) UV light emitting diode device (UVILED, Rapp Optoelectronic, Hamburg, Germany). UV light was collected with a 2 mm liquid light guide and coupled into the epifluorescent light path of the Olympus BX51WI microscope via a flash cube (FC70) and dichromatic beamsplitter (DCLP420, Rapp Optoelectronic, Hamburg, Germany). The light was directed into the back aperture of the 20x objective by another dichromatic beamsplitter (400DCXR, Chroma Technology, Rockingham, VT) placed in a filter cube of the fluorescent filter turret of the Olympus BX51WI. A 675 nm long pass filter was included in the filter cube to reduce the amount of unwanted light generated by the UVILED from reaching the photodiode array. Nevertheless, light generated by the UVILED was detected by the photodiode array. This UVILED signal overwhelmed the VSD signal generated by the photolysis of CNB-caged GABA. In order to recover the GABA generated VSD signal, the photodiode array signals induced by UVILED light in the absence of CNB-caged GABA was subtracted from the signals produced in the presence of CNB-caged GABA.

Statistics and data analysis

The data were analyzed using Neuroplex II software and custom written software in visual basic for applications. Statistics were performed using GraphPad InStat (GraphPad Software, San Diego, CA). For paired measurements, statistical significances ($p < 0.05$) were determined by paired t-tests and repeated measures ANOVA, and for unpaired measurements, one-way ANOVA was used. In cases when the distribution of data did not pass the Komolgorov-Smirnov test for normality, a non parametric Wilcoxon signed rank test was used. Post hoc tests were performed for determining differences between individual groups. A test for linear trend was used when comparing the changes in EPSP area between successive events in a train of stimuli. Otherwise, a Student-Newman-Keuls test was used when comparing between different isolated anatomical layers. All reported values are the mean $\pm$ the standard error of the mean (SEM).

Chemicals

All chemicals were purchased from VWR unless otherwise indicated. NK3630 was obtained from Leslie M. Loew (University of Connecticut, Farmington, CT) or Hayashibara Co. (Japan). Bicuculline methchloride (BIC), (2*S*)-3-[[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP 55845), [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin (DAMGO), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), D-2-amino-5-phosphonovalerate (D-APV), and 6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX) were all purchased from Tocris Bioscience (Ellisville, Missouri). γ -aminobutyric acid, α -carboxy-2-nitrobenzyl ester, trifluoroacetic acid salt (CNB-caged GABA) was purchased from Invitrogen (Carlsbad, CA).

Results

Previous studies have suggested that MOR activation can influence excitatory activity in all layers of hippocampal CA1 (McQuiston and Saggau, 2003; McQuiston, 2007). However, the relative effectiveness of MOR activation in the discrete layers of CA1 is not fully understood. Therefore, we performed the following experiments in an effort to determine the effect of MOR activation on excitatory activity in each anatomical layer of CA1 and to determine the synaptic location of MOR activation. First, we examined the synaptic location of the response to MOR activation by examining the effect on responses to exogenous application of GABA. To do this we used VSD imaging to measure the response to the rapid activation of GABA receptors in all layers of CA1 through the photolysis of CNB-caged GABA. VSD imaging permitted the simultaneous measurement of the effect of MOR activation on postsynaptic GABA receptor function in all layers of hippocampal CA1 and the effect of MOR activation on presynaptic GABA release. This allowed us to examine the pre versus postsynaptic effects of MOR activation on GABAergic synaptic function in all layers of CA1. We then measured the effect of MOR activation on EPSPs in isolated layers of hippocampal CA1 by making razor cuts between adjacent layers. A train of 5 electrical stimuli was delivered at 20 Hz to evoke EPSPs. The train of pulses was used to ensure that interneurons of both low and high probability of release were synaptically liberating GABA (Reyes et al., 1998; Gupta et al., 2000). VSD imaging of EPSPs has the advantage of being more sensitive than electrical field EPSP measurements (Jin et al., 2002). To quantify the effect of MOR activation on EPSPs, the change produced by bath application of DAMGO (1 μ M, for five to ten minutes) on EPSP areas was measured and normalized to control conditions (DAMGO/Control). Similarly, the effect of complete GABA receptor blockade by bath application (five to ten minutes) of the GABA_A receptor antagonist BIC (50 μ M) and GABA_B receptor antagonist CGP 55845 (10 μ M) on EPSP areas was normalized to control values ((GABA receptor antagonists)/Control). Furthermore, because DAMGO acts exclusively by suppressing inhibitory interneuron function, the effect of DAMGO was compared to the effect of GABA receptor antagonists by expressing the effect of DAMGO as a percentage of the effect observed in the presence of GABA receptor antagonists (DAMGO/GABA receptor antagonists $\times$ 100%). This permitted the effect of DAMGO as well as the effect of DAMGO relative to GABA receptor antagonists to be compared across isolated anatomical layers.

MOR activation does not inhibit GABA receptor function in any layer of CA1

Electrophysiological studies have shown that MOR modulation does not affect the response of CA1 pyramidal cells to the application of GABA directly onto their apical dendrites (Nicoll et al., 1980), and the amplitude of miniature IPSPs in CA1 pyramidal cells are also unaffected by MOR agonists (Cohen et al., 1992; Capogna et al., 1993; Reikling, 1993; Lupica, 1995; Capogna et al., 1996). Together with anatomical data showing that MOR receptors are mostly found on inhibitory interneurons (Drake and Milner, 1999; Drake and Milner, 2002), these data suggest that MOR mediate their effect in CA1 by presynaptically inhibiting the release of

GABA without affecting GABA receptor function. However, the physiological studies did not examine the activation of GABA receptors on the distal apical or basal dendrites of CA1 pyramidal neurons. Therefore, to investigate the effect of MOR activation on GABA receptors throughout the entire extent of hippocampal CA1, we used the novel technique of combining flash photolysis of CNB-caged GABA with VSD imaging to measure the effect of MOR activation on the responses to uncaged GABA.

When a 10 ms UV flash in the presence of 1 mM CNB-caged GABA was delivered to the entire CA1 region of a hippocampal slice, hyperpolarizing responses were measured simultaneously in all layers of CA1 using VSD imaging (Fig. 1A). Nine photodiodes from each layer of CA1 were spatially averaged to represent the GABAergic response in each layer (Figs. 1A and 1B). In each layer of CA1, the hyperpolarizing response (Fig. 1B, black) was unaffected by the MOR agonist DAMGO (1 μ M) (Fig. 1B, blue); however, the GABA receptor antagonists BIC (50 μ M) and CGP 55845 (10 μ M) completely inhibited all hyperpolarizing responses in all layers of hippocampal CA1 (Fig. 1B, green traces). In this example a small depolarizing VSD signal remained in GABA receptor blockade. A small depolarizing VSD signal was not consistently observed across slices (it was observed in two other slices). The mechanism or artifact creating the remaining signal was not further investigated. The VSD measurements were confirmed by simultaneous whole cell patch clamp recordings (Fig. 1A, blue arrow), which showed that DAMGO (1 μ M) (Fig. 1B bottom, blue trace) did not affect the outward current produced by the photolysis of CNB-caged GABA (Fig. 1B bottom, black trace). However, the application of GABA receptor antagonists (BIC 50 μ M, CGP 55845 10 μ M) completely suppressed photolysis produced outward current (Fig. 1B bottom, green trace).

Although DAMGO was unable to affect GABA receptor function, DAMGO (1 μ M) did suppress electrically-evoked IPSPs (Fig. 1A, yellow arrows) in each layer of hippocampal CA1 (Fig. 1C) when recorded from the same slice, same photodiodes and during the same application of DAMGO as the data taken for the photolysis experiments. Furthermore, DAMGO did suppress the electrically-evoked inhibitory postsynaptic current measured with the whole cell patch pipette. This was consistently observed and summarized in Fig. 1D. Activation of MOR by 1 μ M DAMGO did not significantly affect the area of hyperpolarizations observed in all layers of CA1, but DAMGO did significantly suppress the amplitude of electrically-evoked IPSPs ($p < 0.05$, $n = 6$, Wilcoxon signed rank test). Taken together, these data indicate that MOR activation was unable to suppress postsynaptic GABA receptor function in any layer of hippocampal CA1.

MOR activation increases the EPSP in isolated SLM

Anatomical studies have demonstrated that interneurons projecting to SLM express MORs (Drake and Milner, 1999; Drake and Milner, 2002). Although there appears to be significant density of MORs in SLM, MOR density was highest in SP (Atweh and Kuhar, 1977; Herkenham and Pert, 1980; Crain et al., 1986; McLean et al., 1987; Mansour et al., 1987; Mansour et al., 1994; Arvidsson et al., 1995; Mansour et al., 1995; Ding et al., 1996). Furthermore, a physiological study reported that interneurons projecting to the apical dendrites of CA1 were less than half as likely to be hyperpolarized by MOR activation as perisomatically projecting interneurons (Svoboda et al., 1999). Other physiological studies have found that there is MOR modulation of excitatory inputs in SLM (McQuiston and Saggau, 2003), but the influence of the MOR modulation from other layers in CA1 through the cable properties of pyramidal cell dendrites could not be discounted. In order to precisely determine the influence of MOR activity directly on excitatory inputs and dendrites in SLM, SLM was isolated from the rest of CA1 by a razor cut between SLM and SR (Fig. 2A), and EPSPs were elicited by electrical stimulation in SLM.

Stimulation (5 pulses at 20 Hz) in isolated SLM of a hippocampal CA1 slice produced EPSPs that propagated throughout SLM but did not propagate past the razor cut separating SLM from SR (Fig. 2A). VSD signals from nine adjacent photodiodes overlying SLM (Fig. 2A, red traces) were spatially averaged for quantification (Fig. 2B). The MOR agonist DAMGO (1 μ M) increased the amplitude of VSD signals (Fig. 2B, blue trace) when compared to control VSD signals (Fig. 2B, black trace), and the effect of DAMGO was reversed by the MOR antagonist CTOP (Fig. 2B, orange trace). However, the increase in VSD signals produced by DAMGO was much smaller than the effect produced by GABA_A (BIC, 50 μ M) and GABA_B receptor antagonists (CGP 55845, 10 μ M) (Fig. 2B, green trace). Thus, MOR activation had a direct effect in excitatory activity in SLM, but it was smaller than the effect produced by GABA receptor blockade.

In order to quantify the effects of DAMGO and GABA receptor blockade on EPSPs, the areas underlying the VSD signals of each stimulus were measured for comparison. The area under each EPSP was normalized to the control VSD signals and plotted as a fractional change in EPSP area (Drug/control) (Fig. 2C). DAMGO significantly increased EPSP areas for the first, third, fourth and fifth stimuli (Fig. 2C, blue line) (paired t-test, $p < 0.01$ stimuli 1 and 5, $p < 0.05$ stimuli 2, 3 and 4, $n = 7$). Although the effect of DAMGO was significantly different between most EPSPs in the train (repeated measures ANOVA, $p = 0.01$, $n = 7$), the only statistical difference occurred between stimuli one and two (Student-Newman-Keuls post hoc test, $p < 0.05$). Interestingly, the effect of DAMGO was much smaller than the effect of blocking all GABA receptors (Fig. 2C, green line) (paired t-test, $p < 0.001$, all stimuli, $n = 7$). Unlike the effects of DAMGO, the effect of GABA receptor antagonists was significantly different between EPSPs in a train (repeated measures ANOVA, $p < 0.0025$, $n = 7$) such that GABA receptor antagonists increased EPSP areas to a greater extent on EPSPs occurring later in a train (post hoc test for linear trend, $p < 0.0001$). In order to directly compare the effects of DAMGO and GABA receptor blockade, the effect of DAMGO was expressed as a percentage of the effect of GABA receptor blockade (Fig. 2D). The effect of DAMGO ranged from 10 to 25 % of the effect of GABA receptor antagonists. However, the effect of DAMGO was relatively greater on the first EPSP compared to the last four EPSPs (repeated measures ANOVA, $p = 0.0003$, Student-Newman-Keuls post hoc test, $p < 0.01$ stimulation 1 vs. all others). Thus, the activation of MOR in SLM had a small effect on EPSPs in the distal dendrites of CA1 pyramidal cells, and this effect was smaller than that observed with GABA receptor antagonists.

MOR activation increases the EPSP in isolated SR

As in SLM, anatomical studies have demonstrated that interneurons projecting to SR express MORs (Drake and Milner, 1999; Drake and Milner, 2002). However, a physiological study reported that less than half of interneurons projecting to the proximal apical dendrites in SR were hyperpolarized by MOR activation (Svoboda et al., 1999). Yet other physiological studies have shown that MOR activation can significantly increase the amplitude of excitatory inputs in SR (McQuiston and Saggau, 2003; McQuiston, 2007). To examine the effect of MOR activity on excitatory inputs in SR, SR was isolated from SP by a razor cut between SP and SR (Fig. 3A), and EPSPs were elicited by electrical stimulation in SR.

Stimulation of excitatory afferents in SR produced EPSPs that propagated throughout SR but did not propagate past the cut separating SR from SP (Fig. 3A). VSD signals from nine adjacent photodiodes above SR were spatially averaged to quantify the effect of DAMGO and GABA receptor antagonists (Fig. 3A, red traces). In this particular example, DAMGO increased the amplitude of EPSPs in SR (Fig. 2B, blue trace). The effect of DAMGO was reversed by CTOP (Fig. 3B, orange trace). GABA receptor antagonists had a larger effect on EPSPs in SR relative

to DAMGO (Fig. 3B, green trace). Therefore, both DAMGO and GABA receptor blockade increased EPSPs in isolated SR of CA1.

Normalizing the change produced by DAMGO to control EPSPs showed that DAMGO increased the EPSP area for all 5 stimuli in the train (Fig. 3C, blue traces) (paired t-test, $p < 0.001$ for all stimuli in the train, $n = 15$). Furthermore, there was a linear trend to larger effects of DAMGO for later stimuli in the train (repeated measures ANOVA post hoc test for linear trend $p < 0.0001$, $n = 15$). However, the increases in EPSP area produced by DAMGO were smaller than the increases produced by GABA receptor antagonists (paired t-test, $p < 0.001$, for all stimuli in the train). Similar to the effect of DAMGO, the effect of GABA receptor antagonists showed a linear trend to larger increases in EPSP area for later EPSPs in the train (repeated measures ANOVA post hoc test for linear trend $p < 0.0001$, $n = 15$). The effect of DAMGO varied between 40 and 60% compared to the effect of GABA receptor blockers (Fig. 3D). Furthermore, the effect of DAMGO relative to GABA receptor antagonists decreased with each successive EPSP in the train (repeated measures ANOVA, post hoc test for linear trend $p < 0.0001$, $n = 15$). In summary, DAMGO increased EPSPs in isolated SR, was more effective with successive EPSPs in the train but was not as effective as complete inhibition of GABA receptor function. Furthermore, the effectiveness of DAMGO relative to complete GABA receptor blockade decreased with successive EPSPs in a train.

MOR activation increases the EPSP in isolated SO

MORs have been found to be concentrated in the SP of CA1 (Atweh and Kuhar, 1977; Herkenham and Pert, 1980; Crain et al., 1986; McLean et al., 1987; Mansour et al., 1987; Mansour et al., 1994; Arvidsson et al., 1995; Mansour et al., 1995; Ding et al., 1996). Furthermore, interneurons innervating the somata of CA1 pyramidal cells were consistently hyperpolarized by MOR activation (Svoboda et al., 1999). Therefore, we conducted experiments to examine the effect of MOR activation on activity in SP and SO.

We did this by making a razor cut between SP and SR to isolate SP and SO from the apical dendritic layers of CA1. A bipolar stimulating electrode was placed in SO near SP to evoke EPSPs in SO and SP (Fig. 4A). VSD signals from six adjacent photodiodes in SO and SP were spatially averaged (Fig. 4A, red traces). The bath application of DAMGO increased EPSP amplitudes (Fig. 4B, blue trace). The effect of DAMGO was reversed by CTOP (Fig. 4B, orange trace). However, the increase in EPSPs produced by GABA receptor antagonists was larger than that produced by DAMGO (Fig. 4B, green trace). Therefore, as in SR, DAMGO increased the size of EPSPs in isolated SP and SO, but GABA receptor antagonists were more effective than DAMGO.

Normalizing the change produced by DAMGO relative to control EPSPs showed that DAMGO increased the EPSP area for all 5 stimuli in the train (paired t-test, $p < 0.01$ for all stimuli in the train, $n = 7$). The magnitude of the change in EPSP area varied between different stimuli in the train (repeated measures ANOVA, $p < 0.01$, $n = 7$). Post hoc analysis (Student-Newman-Keuls) showed that stimulus two was significantly larger than stimulus one ($p < 0.001$), four ($p < 0.001$) and five ($p < 0.001$) and that stimulus three was significantly larger than stimulus one ($p < 0.01$), four ($p < 0.05$) and five ($p < 0.01$). However, GABA receptor antagonists had a larger effect on EPSP areas than DAMGO (paired t-test, $p < 0.05$ for stimuli 1, 2, 4, 5, and $p < 0.01$ for stimulus 3, $n = 7$). There was a linear trend to larger effects of GABA receptor antagonists on stimuli that occurred later in the train (repeated measures ANOVA, $p < 0.0001$, post hoc test for linear trend $p < 0.013$, $n = 7$). Student-Newman-Keuls post hoc analysis showed that the effect of GABA receptor antagonists on the first stimulus of the train was significantly different than successive stimuli in the train ($p < 0.01$ for stimuli 2, 3, and 4, $p < 0.05$ for stimulus 5, $n = 7$). Thus, as in SR, DAMGO increased all EPSPs in the train in isolated SO and

SP; however, the effect of DAMGO was smaller than the effect produced by GABA receptor antagonists.

When comparing the effect of DAMGO to inhibition of GABA receptors, DAMGO was proportionately more effective on initial stimuli than later ones (repeated measures ANOVA, $p = 0.0077$, post hoc analysis for linear trend $p = 0.0009$, $n = 7$). Student-Newman-Keuls post hoc analysis showed that stimulus one was significantly different from stimuli three, four and five ($p < 0.05$, $n = 7$). Thus, relative to the effect of GABA receptor antagonists, DAMGO was more effective on initial EPSPs in a train of stimuli in SO and SP.

Therefore, the activation of MORs increased EPSP areas in SO and SP. The effect of MOR was most effective on the second and third stimuli in the train, but the effect of MOR activation was smaller than that of GABA receptor antagonists. Furthermore, when compared to complete inhibition of GABA receptor function, the effect of DAMGO was more effective on initial EPSPs, as observed in the other layers of CA1.

GABA receptor antagonists occlude all effects of MOR activation

We have previously shown that the effects of MOR activation in hippocampal CA1 were prevented when DAMGO was applied in the presence of GABA receptor blockers (McQuiston and Saggau, 2003; McQuiston, 2007). This suggested that the mechanisms through which MOR produced its effect was via the GABAergic system. Therefore, we tested whether the same observation could be made with VSD imaging in the micro-dissected slices.

Regardless of which layer was isolated, all 5 VSD EPSPs in the 20 Hz train had increased amplitudes in SO, SR and SLM following the application of BIC (50 μM) and CGP 55845 (10 μM) (Fig. 5A). However, subsequent application of DAMGO (1 μM) in the presence of these GABA receptor antagonists (BIC and CGP 55845) did not affect the VSD EPSP signals in any isolated layer of CA1. These observations were consistently observed across slices. In all isolated layers of CA1, the GABA receptor antagonists increased the area of all 5 EPSP in the train when compared to control EPSP areas (Fig. 5B, Wilcoxon signed rank test, $p < 0.05$). However, DAMGO in the presence of GABA receptor antagonists did not significantly increase EPSP areas for any EPSP in the train or any isolated anatomical layer of hippocampal CA1 (Fig. 5C). Therefore, in each layer of hippocampal CA1, augmentation of EPSPs by the activation of MORs acted through the modulation of inhibitory interneuron function.

Activation of MORs is least effective in SLM

Both anatomical and physiological studies have suggested that MORs are found in different densities in different layers of CA1 (Atweh and Kuhar, 1977; Herkenham and Pert, 1980; Crain et al., 1986; McLean et al., 1987; Mansour et al., 1987; Mansour et al., 1994; Arvidsson et al., 1995; Mansour et al., 1995; Ding et al., 1996). Consequently, MORs may play varying functional roles across the different layers of hippocampal CA1 (Svoboda et al., 1999). Therefore, we compared the relative effects of MOR activation on EPSPs in isolated layers of hippocampal CA1.

When comparing the effect of DAMGO on EPSP areas across isolated layers of CA1, the effect of DAMGO in SLM was consistently smaller than the effects observed in SR or SO (Fig. 6A). Although there were no statistical differences in the effect of DAMGO between different layers on the first EPSP of the train, DAMGO was more effective on EPSPs in SR and SO compared to SLM on stimuli two through five (One-way ANOVA, $p < 0.05$, Student-Newman-Keuls post hoc test, $n = 7$ in SLM, $n = 15$ in SR, and $n = 8$ in SO). In the second stimulation, the effect of DAMGO on EPSP area in SO was significantly larger than SR (Student-Newman-Keuls

post hoc test, $p < 0.05$, $n = 7$ in SLM, $n = 15$ in SR, and $n = 8$ in SO). Therefore, the activation of MORs had a larger effect on EPSPs in isolated layers of SR and SO compared to SLM.

Because MORs in hippocampal CA1 act by suppressing the function of inhibitory interneurons, the observed smaller effect of DAMGO in SLM may be due to a smaller amount of GABA release following stimulation in SLM compared to what occurs following stimulation in SR and SO. In order to control for this possible difference between the layers, the effect of DAMGO was normalized to the effect of complete GABA receptor inhibition by expressing the effect of DAMGO as a percentage of the effect of GABA receptor antagonists (Fig. 6B). Compared to complete GABA receptor blockade, the effect of DAMGO was significantly smaller in SLM relative to effects observed in SR and SO for all conditions except between SO and SLM in stimulation 5 (One-way ANOVA $p < 0.01$ stimuli 1 to 4, $p < 0.05$ stimulus 5, Student-Newman-Keuls post hoc test magenta asterisks compare bar to SLM, $n = 7$ in SLM, $n = 15$ in SR, and $n = 8$ in SO). Therefore, the activation of MOR was less effective at suppressing inhibition in SLM compared to SR and SO.

Discussion

This study has shown that MOR activation has direct effects in all layers of hippocampal CA1. Furthermore, the effect of MOR activation was presynaptic on inhibitory interneurons because the VSD response to direct application of GABA in all layers of CA1 was not affected by MOR activation, whereas IPSPs were suppressed by DAMGO. However, not all layers were equally affected. MOR activation had a smaller effect on EPSPs in SLM compared to all other layers. Furthermore, when comparing the effect of MOR to the effect of complete GABA receptor blockade, the relative effect of DAMGO in SLM was significantly smaller than the other layers of CA1. Thus, because MORs in hippocampal CA1 act solely by hyperpolarizing interneurons and inhibiting the release of GABA (Lupica and Dunwiddie, 1991; McQuiston and Saggau, 2003; McQuiston, 2007), the smaller effect in SLM was likely due to a reduced ability of MOR activation to inhibit the release of GABA from interneuron terminals in SLM. Therefore, these data show that MOR activation can inhibit the release of GABA in all layers of CA1 and that MOR activation is less efficacious in SLM compared to other layers of CA1.

The most likely explanation for our findings is that MOR activation is less effective at inhibiting the release of GABA from interneuron terminals in SLM compared to other layers of CA1. MORs act exclusively by suppressing interneuron function either by hyperpolarizing somata or inhibiting the release from terminals (Lupica and Dunwiddie, 1991; McQuiston and Saggau, 2003; McQuiston, 2007). Because most of the interneurons that project to the SLM have somata located in SO and SR (Freund and Buzsaki, 1996; Vida et al., 1998; Drake and Milner, 2002), anatomical isolation would make it unlikely for MOR modulation of interneuron somata to influence effects in SLM. However, a somatic influence of neurogliaform interneurons residing in SLM cannot be discounted (Khazipov et al., 1995; Price et al., 2005). Therefore, the effects we observed in SLM were most likely due to MOR inhibition of GABA release from terminals that were directly activated by the stimulating electrode in SLM. These data are consistent with anatomical studies showing that a smaller proportion of interneurons innervating the SLM express MORs relative to interneurons innervating SP (Drake and Milner, 2002). Similarly, interneurons in SO that innervated SLM were less frequently hyperpolarized by MOR activation than interneurons that innervated SP (Svoboda et al., 1999). Therefore, although SLM is affected by MOR activation (McQuiston and Saggau, 2003; McQuiston, 2007), the present VSD imaging studies are in agreement with previous anatomical and physiological studies suggesting that MOR has a smaller direct effect in SLM.

In contrast to observations in SLM, MOR activation produced similar effects on EPSPs in SR and in SO and SP. This may seem contradictory to previous physiological findings that reported

only 50% of SO interneurons projecting to SR were hyperpolarized by MOR activation whereas 91% of the perisomatic projecting interneurons were hyperpolarized by MOR activation (Svoboda et al., 1999). From these previous studies, it was proposed that MOR activation would have larger effects in SP than in SR, although this was not observed in our experiments. However, Svoboda and colleagues (1999) measured MOR effects on the somata of SO interneurons whereas the MOR effects in our experiments were most likely occurring at the interneuron terminals. Furthermore, the effects measured by Svoboda and colleagues (1999) could not be measured in our preparation because the razor cut disconnected interneuronal somata of SO from their axon terminals in SR. Unfortunately, there has been no anatomical data describing frequency of colocalization of MORs with calbindin interneurons that often project to pyramidal cell dendrites in SR (Freund and Buzsaki, 1996). Therefore, it is unknown what proportion of interneuron terminals in SR express MORs. It is possible that a large fraction of interneuron terminals in SR express MORs. Alternatively, it may be that a smaller but sufficient number of interneuron terminals in SR express MORs, resulting in increases in EPSPs in SR that were equivalent to those in SP. This could be because an individual dendritically targeting interneuron can have profound effects on excitatory dendritic events (Miles et al., 1996; Larkum et al., 1999), and therefore MOR expression in a smaller proportion of interneurons that synapse on pyramidal cell dendrites may be sufficient to produce similar sized effects as those occurring in SP. Nevertheless, as seen in our study, MOR activation in SR can produce similar changes in EPSPs compared to SP. Therefore, although MOR activation can augment EPSPs by a similar amount in SR, SO and SP, it remains unclear if MORs are expressed in the same proportion of interneurons projecting to SR relative to their expression in perisomatic interneurons in SP.

A significant finding of this study is that excitatory inputs from CA3 pyramidal neurons in SR were much more affected by MOR activation than inputs into SLM from the entorhinal cortex and nucleus reuniens of the thalamus. However, in addition to exciting the distal dendrites of CA1 pyramidal neurons, excitatory drive into SLM produces significant disinhibitory inhibition of SR and SP (Dvorak-Carbone and Schuman, 1999; McQuiston and Saggau, 2003; Ang et al., 2005; McQuiston, 2007). This SLM-driven inhibition in SR and SP is inhibited by MOR activation (McQuiston and Saggau, 2003; McQuiston, 2007). Accordingly, MOR modulation does have an impact on excitatory inputs in SLM in a circuitous way through its indirect effect on inhibition in the other layers. Therefore, the outcome of MOR modulation will likely result in changes in excitatory inputs from CA3 and/or action potential activity in SP. Consistent with these findings, MOR activation has been shown to modulate synaptic plasticity in SR (Wagner et al., 2001) and disrupt behaviorally rhythmic activity in SP (Whittington et al., 1998). Interestingly, the impact of MOR activity on synaptic plasticity is altered by chronic exposure to the MOR agonists morphine or heroin (Mansouri et al., 1997; Pourmotabbed et al., 1998; Mansouri et al., 1999; Pu et al., 2002). Therefore, it will be interesting to determine which, if any, of the MOR-sensitive interneuron networks that modulate excitatory activity in SR and SP are altered by chronic MOR activation and how this affects activity-dependent changes in synaptic efficacy in hippocampal CA1.

In conclusion, MOR activation in hippocampal CA1 had a significantly larger impact on excitatory activity in SR, SO and SP relative to SLM. These data suggest that MORs will have significant influences on inputs from CA3 pyramidal cells in SR and SO and will also facilitate firing of action potentials in pyramidal cell bodies. In contrast, MOR activation had a small effect on excitatory activity in SLM. These data point to modulation of inputs from CA3 and the output of CA1 pyramidal neurons as sites where chronic morphine may change network function leading to alterations in activity-dependent synaptic plasticity, as has been shown in previous studies (Mansouri et al., 1997; Pourmotabbed et al., 1998; Mansouri et al., 1999; Pu et al., 2002).

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Abbreviations

BIC, bicuculline
 CGP 55845, (2S)-3-[[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl]
 (phenylmethyl)phosphinic acid
 CA1, cornu ammon 1
 CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂
 DAMGO, [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin
 EPSP, excitatory postsynaptic potential
 GABA, γ -aminobutyric acid
 IPSP, inhibitory postsynaptic potential
 MOR, μ -Opioid Receptor
 SLM, stratum lacunosum-moleculare
 SO, stratum oriens
 SP, stratum pyramidale
 SR, stratum radiatum
 VSD, voltage-sensitive dye

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Fig. 1.

μ -Opioid receptor activation has no effect on GABA receptor function in any layer of hippocampal CA1. **A.** VSD signals in response to uncaging of CNB caged-GABA are superimposed on image of hippocampal CA1 slice. Yellow arrow points to tungsten bipolar stimulating electrodes. Blue arrow points to a whole cell patch clamp recording from a pyramidal neuron at the edge of stratum pyramidale (SP). SLM – stratum lacunosum moleculare, SR – stratum radiatum, SO – stratum oriens. Red traces (SO), light blue traces (SP), orange traces (SR) and dark blue traces (SLM) were spatially averaged for quantification and displayed in B and C. **B.** Spatially averaged VSD signals (from A) following the uncaging of CNB caged-GABA (10 ms pulse, 365 nm peak). VSD signals were recorded in the presence of 1 μM DAMGO (blue), GABA receptor antagonists BIC (50 μM) and CGP 55845 (10 μM) (green), or control saline (black). Arrows point to UV flash. Scale: vertical 0.0002 ΔF/F (VSD signals), 50 pA (whole cell current); horizontal 500 ms. **C.** Spatially averaged VSD signals following five electrical stimuli at twenty hertz in the presence of DNQX (30 μM) and APV (50 μM). VSD signals were recorded in the presence of 1 μM DAMGO (blue), GABA receptor antagonists BIC (50 μM) and CGP 55845 (10 μM) (green), or control saline (black). Scale: same as B. **D.** Histogram of effect of DAMGO on the area of the VSD signals produced by uncaging CNB caged-GABA (black) or electrically-evoked IPSPs (green) relative to the area of the signals produced in control saline ((DAMGO/Control)). Asterisks * p<0.05 (Wilcoxon signed rank test).

Fig. 2.

μ -Opioid receptor activation produced a small increase in EPSPs in isolated SLM of hippocampal CA1. **A.** VSD signals superimposed on image of hippocampal CA1 slice. Yellow arrow points to tungsten bipolar stimulating electrode. Blue arrow points to razor cut between SR and SLM. SLM – stratum lacunosum moleculare, SR – stratum radiatum, SO – stratum oriens. Red traces were spatially averaged for quantification and displayed in **B.** **B.** Spatially averaged VSD signals (**A** – red traces) following five electrical stimuli at twenty hertz. VSD signals were recorded in the presence of 1 μ M DAMGO (blue), GABA receptor antagonists BIC (50 μ M) and CGP 55845 (10 μ M) (green), 1 μ M CTOP (orange), or control saline (black). Scale: vertical 0.0005 $\Delta I/I$; horizontal 100 ms. **C.** Line plot of fractional increase in EPSP area (Drug/Control) for each stimulus of the 20 Hz train. Blue – fractional increase produced by DAMGO (1 μ M). Green – fractional increase produced by GABA receptor blockade (BIC, CGP 55845). Blue asterisk – significant difference produced by DAMGO compared to control. Green asterisk – significant difference between effects produced by GABA receptor blockade and DAMGO. **D.** Plot of effect of DAMGO on EPSP area for all five stimuli in the train relative to the effect produced by GABA receptor blockade ((DAMGO/GABA antagonists) $\times$ 100%). Asterisks * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3.

μ -Opioid receptor activation increased EPSPs in SR isolated from cell body layer in hippocampal CA1. **A.** VSD signals superimposed on image of hippocampal CA1 slice. Yellow arrow points to tungsten bipolar stimulating electrode. Blue arrow points to razor cut between SR and SP. Red traces were spatially averaged for quantification and displayed in **B.**

Spatially averaged VSD signals following five electrical stimuli at twenty hertz. VSD signals were recorded in the presence of 1 μ M DAMGO (blue), GABA receptor antagonists BIC (50 μ M) and CGP 55845 (10 μ M) (green), 1 μ M CTOP (orange), or control saline (black). Scale: vertical 0.0008 $\Delta I/I$; horizontal 50 ms. **C.** Line plot of fractional increase in EPSP area (Drug/Control) for each stimulus of the 20 Hz train. Blue – fractional increase produced by DAMGO (1 μ M). Green – fractional increase produced by GABA receptor blockade (BIC, CGP 55845). Blue asterisk – significant difference produced by DAMGO compared to control. Green asterisk – significant difference between effects produced by GABA receptor blockade and DAMGO. **D.** Plot of effect of DAMGO on EPSP area for each stimulus in the train relative to the effect produced by GABA receptor blockade ((DAMGO/GABA antagonists) $\times$ 100%). Asterisks * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4. μ -Opioid receptor activation increased EPSPs in SO and SP isolated from apical dendritic layers in hippocampal CA1. **A.** VSD signals superimpose on image of hippocampal CA1 slice. Yellow arrow points to tungsten bipolar stimulating electrode. Blue arrow points to razor cut between SR and SP. Red traces were spatially averaged for quantification and displayed in **B.** **B.** Spatially averaged VSD signals following five electrical stimuli at twenty hertz. Scale: vertical 0.0002 $\Delta I/I$; horizontal 50 ms. **C.** Line plot of fractional increase in EPSP area (Drug/Control) for each stimulus of the 20 Hz train. Blue – fractional increase produced by DAMGO (1 μ M). Green – fractional increase produced by GABA receptor blockade (BIC, CGP 55845). Blue asterisk – significant difference produced by DAMGO compared to control. Green asterisk – significant difference between effects produced by GABA receptor blockade and DAMGO. **D.** Plot of effect of DAMGO on EPSP area for each stimulus in the train relative to the effect produced by GABA receptor blockade ((DAMGO/GABA antagonists) $\times$ 100%). Asterisks * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5.

μ -Opioid receptor activation produced no effect on EPSPs in any isolated anatomical layer of hippocampal CA1 in the presence of GABA receptor antagonists. **A.** Spatially averaged VSD signals following five electrical stimuli at twenty hertz. VSD signals were recorded in the presence of GABA receptor antagonists BIC (50 μ M) and CGP 55845 (10 μ M) (green), 1 μ M DAMGO and GABA receptor antagonists (blue), or control saline (black). Scale: vertical 0.0005 $\Delta I/I$ (SO and SR) or 0.0002 $\Delta I/I$ (SLM); horizontal 100 ms. **B.** Plot of effect of BIC and CGP 55845 on EPSP area in each anatomically isolated layer for all five stimuli in the train relative to the EPSP area in control saline ((GABA antagonists/Control) $\times$ 100%). **C.** Plot of effect of DAMGO on EPSP area in each anatomically isolated layer for all five

stimuli in the train in the presence of GABA receptor antagonists ((DAMGO/GABA receptor antagonists) $\times$ 100%).

Fig. 6.

μ -Opioid receptor activation was least effective on EPSPs in SLM. **A.** Histogram comparing the fractional increase in EPSP areas produced by DAMGO (1 μ M) in the different isolated anatomical layers SO (black), SR (green) and SLM (magenta). **B.** Histogram comparing the increase in EPSP areas produced by DAMGO relative to GABA receptor blockade ((DAMGO/GABA antagonists) $\times$ 100%) in the different isolated anatomical layers SO (black), SR (green) and SLM (magenta). Magenta asterisk signify statistical difference compared to SLM. Green asterisk signify statistical difference compared to SR. Asterisks * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.