DOPAMINERGIC MODULATION OF MELANIN-CONCENTRATING HORMONE EXPRESSING NEURONS

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DOPAMINERGIC MODULATION OF MELANIN-CONCENTRATING HORMONE EXPRESSING NEURONS

By

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Abstract

Melanin-concentrating hormone (MCH) is a neuropeptide that acts centrally as an anabolic signal, while also mediating behaviours such as reward, activity level, and anxiety. MCH expressing neurons are limited in their expression being isolated to the lateral hypothalamus (LH) and nearby zona incerta (ZI). Despite this limited area of expression the neurons project widely throughout the brain. The neurotransmitter/neuromodulator dopamine (DA), which is best known for its role in reward and locomotion, is released within the LH area. DA action within the LH area is known to influence MCH related behaviours: for example, an injection of DA into the LH will decrease food intake. Since MCH promotes food intake and DA has anorexic properties in the LH it is hypothesized that DA inhibits the activity of the MCH expressing neurons. However, DA's mechanism of action on MCH neurons is not known. This study was carried out to determine the cellular mechanisms by which DA influences MCH expressing neurons. To determine this MCH neuronal activity was monitored using whole cell patch-clamp recordings from LH/ZI containing rat brain slices. MCH neurons were identified and distinguished from nearby orexin neurons by their electrophysiological characteristics and post-hoc immunohistochemistry. All drugs were bath applied. In order to monitor DA's presynaptic influence γ-aminobutyric acid (GABA) release was recorded through pharmacologically isolated miniature inhibitory postsynaptic currents (mIPSCs). Results showed that DA inhibits the activity of MCH expressing neurons through hyperpolarization and a reduction in action potential firing.

Furthermore, DA induced a dose dependent outward current. This current was the result of G protein-activated inwardly rectifying K^+ (GIRK) channel activation. Surprisingly, initiation of the GIRK current was not dependent on DA receptor activation but rather on the highly expressed adrenergic α_{2A} receptor. Norepinephrine (NE) also produced a reversible, dose dependent outward current in MCH neurons when acting on the adrenergic receptors. The DA and NE induced outward currents were of similar magnitudes, indicating both have similar efficacies when acting on MCH neurons. Monitoring mIPSCs revealed that DA had no consistent effect on their frequency or amplitude, indicating that DA does not influence synaptic GABA transmission. Overall this study demonstrates that DA has a direct postsynaptic inhibitory influence on MCH neurons. This inhibition is accomplished though activation of adrenergic receptors and the initiation of a GIRK current. Furthermore, this study reveals a cross talk between dopaminergic and adrenergic signalling at the cellular level within the LH area.

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List of Abbreviations

AC – Adenylyl cyclase

ACSF – Artificial cerebrospinal fluid

ACTH - Adrenocorticotropic hormone

AMCA - Aminomethylcoumarin acetate

ATP – Adenosine triphosphate

BRL44408 - 2-[(4,5-Dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1- methyl-1H-isoindole maleate

CART - Cocaine-and amphetamine regulated transcript

CNS – Central nervous system

Cy2 – Cyanine dye that fluoresces green

Cy3 - Cyanine dye that fluoresces red

DA – Dopamine

D-AP5 - D-2-amino-5-phosphonopentanoate

D1-like – D1-like dopamine receptors

D2-like – D2-like dopamine receptors

DMSO - Dimethyl sulfoxide

DNQX - 6,7-Dinitroquinoxaline-2,3-dione

GABA - γ-aminobutyric acid

GAD- Glutamic Acid Decarboxylase

GIRK - G Protein-activated Inwardly Rectifying K^+

ICV – Intracerebroventricular

IgG – Immunoglobulin G

I_h – H current

IR-DIC - Infrared differential inference contrast

HPA - Hypothalamic-pituitary adrenal

LH - Lateral hypothalamus

MCH - Melanin-concentrating hormone

MCHR1 – MCH receptor 1

MCHR2 - MCH receptor 2

mEPSC - miniature excitatory postsynaptic current

mIPSC - miniature inhibitory postsynaptic current

NAc - Nucleus accumbens

NE – Norepinephrine

NK3 - Neuokinin receptor

NPY – Neuropeptide Y

Ob-R - Leptin receptor

OXR1 – Orexin receptor 1

OXR2 – Orexin receptor 2

PBS - Phosphate buffered saline

PTX - Pertussis toxin

RT-PCR - Reverse transcriptase polymerase chain reaction

SCH23390 - R-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride

SD - Sprague Dawley

SKF81297 - 6-Chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine hydrobromide

SN – Substantia nigra

TTX - Tetrodotoxin

VGLUT1 - Vesicular glutamate transporter 1

VTA – Ventral tegmental area

ZI - Zona incerta

CHAPTER 1

Introduction

1.1 Overview

Melanin-concentrating hormone (MCH) is a cyclic neuropeptide that centrally participates in the regulation of numerous behaviours including feeding, reward, stress, and locomotor activity. Through animal studies MCH has been shown to function as an anabolic signal in energy homeostasis. It communicates hedonic aspects of feeding, activates the stress axis, and decreases an animal's activity level. Due to these functions an unbalanced regulation of the MCH system may be associated with the development and maintenance of obesity. In order to fully understand the MCH system and to develop pharmaceutical therapies for diseases like obesity, it is crucial that the cellular mechanisms regulating MCH neuronal activity are described.

The neurotransmitter/neuromodulator dopamine (DA) may be one of the critical regulators of the MCH system. This is because similar to MCH, DA influences behaviours such as feeding, response to reward, and locomotion. Also, DA projections are known to intersect MCH's neuronal origins and terminal fields. Thus, some of the physiological functions of DA may be mediated by MCH. However, despite the possibility that DA directly influences MCH neuronal activity, its mechanism of action is not known. My goal is to determine DA's effect on MCH neuronal activity and the mechanisms regulating it.

1.2 History and structure

The MCH neuropeptide was first identified due to its skin lightening role in Teleosts (Baker & Ball, 1975;Westerfield *et al.*, 1980). The primary sequence was first identified in 1983 from isolated chum salmon pituitaries as a 17-amino-acid peptide (Kawauchi et al., 1983), with a dicysteine bridge at positions 5 and 14 forming a ring structure. Subsequent to its discovery in fish, MCH was identified it the mammalian hypothalamus (Vaughan et al., 1989). The mammalian form of MCH is a cyclic 19-amino-acid peptide that is highly homologous to the chum salmon MCH. Identification of the MCH receptor was made collectively (Bachner et al., 1999;Chambers et al., 1999;Lembo et al., 1999;Saito et al., 1999;Shimomura et al., 1999) and denoted MCHR1. MCH binds to MCHR1 with nanomolar affinity and the receptor can couple to G_i, G_o, or G_q proteins to activate multiple intracellular signaling pathways (Hawes et al., 2000). Subsequent to the identification of MCHR1, a second MCH receptor has been identified in humans (An et al., 2001;Hawes et al., 2000;Rodriguez et al., 2001) and denoted MCHR2. MCH2R was found to be a pseudogene in rodent species, but is functional in dogs, ferrets, rhesus monkeys, and humans (Tan et al., 2002). However, due to the lack of available animal models the functional importance of MCHR2 remains largely unknown. The overall homology of MCHR2 to MCHR1 is relatively low for a family of receptors that bind to the same ligand with about a 38% amino-acid similarity (Sailer et al., 2001). This indicates that the two receptors are more divergent in evolution than other GPCR families.

1.3 Expression in the CNS

Central nervous system (CNS) expression of the MCH neuropeptide is limited to the lateral hypothalamus (LH) and the zona incerta (ZI). These neurons project widely throughout the brain and are distributed throughout the CNS in patterns that generally conform to known projection fields of the LH and ZI (Bittencourt et al., 1992). These include the prefrontal cortex, dorsal and ventral striatum, piriform cortex, olfactory tubercle, hippocampal formation, nucleus accumbens (NAc), amygdala, and various nuclei in the hindbrain, such as the nucleus tractus solitarius and the parabrachial nucleus. MCHR1 and MCHR2 expression coincides with MCH neuronal projection fields (An et al., 2001;Hervieu et al., 2000;Hill et al., 2001;Saito et al., 2000).

1.4 Neurochemical properties

Through quantitative immunocytochemical identification and analysis with laser microdissection and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) a multi-transcriptional profiling of the intrinsic characteristics of MCH neurons in the rat LH was established (Harthoorn et al., 2005). MCH neurons were shown to express the neuropeptides MCH, cocaine-and amphetamine regulated transcript (CART), and dynorphin. Furthermore, MCH neurons expressed the glutamate marker vesicular glutamate transporter (VGLUT)-1 and the γ-aminobutyric acid (GABA) markers glutamic acid decarboxylase (GAD)-65 and GAD-67, thus indicating the presence and possible dispersal of both excitatory and inhibitory transmitters.

1.5 Physiological Roles of MCH

1.5.1 Food intake

MCH was first thought to be involved in food intake because it was expressed within the LH. The LH has long been thought of as the "hunger center" of the brain due to classic lesion/stimulation studies (for review see Schwartz et al., 2000). This notion was supported when it was observed that MCH mRNA was increased in leptin deficient, obese, *ob/ob* mice (Zhang et al., 1994). An increase in MCH RNA was also observed after fasting in both lean and *ob/ob* mice (Qu et al., 1996). Furthermore, intracerebroventricular (ICV) injection of MCH tripled food intake in rodents, an effect that lasted for up to six hours (Qu et al., 1996;Rossi et al., 1997). MCH's role as an orexigenic neuropeptide was confirmed through gene knockout and over expression studies. It was discovered that when the MCH gene was knocked out (MCH-/-) mice were hypophagic and lean compared to their wild type littermates (Shimada et al., 1998). Also, their resting energy expenditure was elevated, which accounted in part for their leanness. Furthermore, when the MCH gene was over expressed in mice fed a high fat diet they where 12.6% heaver then the wild-type controls fed the same diet. Also the obese mice were found to be hyperphagic (Ludwig et al., 2001). During in-vitro brain slice experiments the MCH neuropeptide was shown to stimulate orexigenic neurons of the arcuate nucleus, while inhibiting anorexigenic neurons of the ventromedial hypothalamus (Davidowa et al., 2002), thus providing cellular correlates of its role as an orexigenic neuropeptide.

1.5.2 Reward

It has been suggested that signaling between the LH and NAc plays an important role in communicating the hedonic, or rewarding, aspects of feeding (Saper et al., 2002). Evidence for this view include the fact that the MCHR1 is robustly expressed in the ventral and dorsal striatum, the terminal fields of midbrain dopaminergic neurons which are involved in mediating reward (Saito et al., 2001). Furthermore, injection of MCH directly into the medial NAc shell increases food intake (Georgescu et al., 2005). Research to date suggests that MCH signaling in the NAc is a starvation induced signal that influences the reward pathway to increase the drive to feed (Georgescu *et al.*, 2005;Kelley & Berridge, 2002;Saper *et al.*, 2002).

1.5.3 Other Behaviours

MCH is believed to play a physiological role to suppress excessive activity. In support of this, MCH injected ICV in rats has been shown to inhibit the increase in locomotor activity, grooming, and rearing induced by neuropeptide E-I (peptide produced from the same prohormone as MCH) injection (Sanchez et al., 1997). Also, genetic inactivation of MCHR1 in mice results in increased spontaneous locomotor activity (Marsh et al., 2002).

MCH neuronal activation has also been described to promote sleep following sustained waking (Modirrousta et al., 2005). If MCH does in fact decrease activity level and promote sleep, then direct inhibition of MCH neurons by arousal systems would be a means to prevent the opposing effects of MCH. This is in fact the case with

neurotransmitters that can initiate arousal such as norepinephrine (NE), serotonin, muscarine, and cholinergic agonists, inhibit the activity of MCH neurons (van den Pol et al., 2004;Wollmann et al., 2005) .

MCH is also believed to play a role in the physiological regulation of anxiety and stress. Several lines of evidence show that MCH activates stress responses and induces depressive and anxiety-like behaviours, while the blockade of MCHR1 results in antidepressant and anxiolytic effects in various rodent models. MCH has been shown to activate the hypothalamic-pituitary adrenal (HPA) axis (Kennedy et al., 2003), with ICV injection of MCH increasing circulating adrenocorticotropic hormone (ACTH) up to 10 min post injection. Furthermore, rats given the MCHR1 antagonist, SNAP7941, showed a decrease in anxiety and depression during a forced swim test (Borowsky et al., 2002). Antidepressant effects have also been observed after MCHR1 antagonist injection into the medial shell of the NAc (Georgescu et al., 2005). This suggests that MCHR1 may play a substantial role in mediating anxiety and depressive-like behaviours and may form the basis for future pharmaceutical approaches to the treatment of these diseases.

1.6 Electrophysiological properties

Knowledge of the electrophysiological properties of MCH neurons is crucial for the identification of the cells in in-vitro brain slice preparation while also providing a foundation for the understanding of all MCH governed behaviours. MCH neurons are intermixed with another population of peptidergic neurons, i.e. orexin neurons, which have similar morphological characteristics. Because they cannot be distinguished by their

appearance with differential interference contrast optics, with which we identify neurons suitable for recordings in brain slices, differences in their electrophysiological properties are used. Orexin neurons are also known as an important regulator of food intake (for review see Sakurai T., 2006) and thus a significant research topic, however, this thesis will only focus on MCH neurons. MCH neuronal electrophysiological properties do not correspond to the spontaneously active orexin neurons. In a previous study we found in in-vitro acute brain slice preparation that MCH neurons were relatively hyperpolarized with an average resting membrane potential of -58.8mV, while the orexin neurons were comparatively depolarized at an average of -48.6mV (Alberto et al., 2006). Other electrical properties of MCH neurons provide insight into their lack of spontaneous activity. They do not have an I_h (sag during a hyperpolarizing current pulse) or rebound depolarization (after a hyperpolarizing current pulse) as the orexin neurons do. However, they do show strong spike rate adaptation during depolarization current pulses (Alberto et al., 2006;Eggermann et al., 2003;Gao et al., 2003). Overall compared to spontaneously active orexin neurons the electrophysiological properties of MCH neurons appear to maintain quiescence when synaptic activity is absent.

1.7 Neurotransmitter interactions

MCH neurons receive a variety of inputs from different neurotransmitter systems that regulate their activity. For example, glutamate, ATP and orexin A and orexin B depolarize and increase the activity of MCH neurons (van den Pol et al., 2004). However, neuropeptide Y (NPY), a potent orexigenic hypothalamic neuropeptide, inhibits MCH

neurons (van den Pol et al., 2004). This may be a negative feedback mechanism due to the fact that MCH stimulates NPY neurons (Davidowa et al., 2002). Other neurotransmitters, including norepinephrine, serotonin, muscarine or cholinergic agonists, inhibit MCH neurons (van den Pol et al., 2004;Wollmann et al., 2005).

Numerous cell surface receptors have been identified on MCH neurons including GABA receptors, glutamate receptors, OXR1 and OXR2 orexin receptors, leptin receptors, neuokinin receptors, chemokine receptors, muscarinic receptors, serotoninergic receptors, and α_2-adrenoceptors (Gao et al., 2003;Griffond et al., 1997;Guyon et al., 2005;Hakansson et al., 1999;van den Pol et al., 2004). DA receptors have also been identified in the LH area (Bubser et al., 2005;Fetissov et al., 2002). Overall MCH neurons receive numerous excitatory and inhibitory afferents signals that are processed by the corresponding cell surface receptor. The cellular mechanisms that are initiated by the activated receptors determine the cellular response and consequently the functional behaviour.

1.8 DA expression in CNS and association with MCH neurons

The neurotransmitter/neuromodulator DA is largely known for its involvement in modulating the reward and motor systems. DA is expressed within neurons located in several regions of the mammalian CNS including the substantia nigra (SN), ventral tegmental area (VTA), and the retrorubal area. Dopaminergic neurons are also located in the hypothalamus within the arcuate nucleus and ZI. Some of these areas form the origin for the three main domaminergic pathways, the nigrostriatal, the mesolimbocortical, and

the tuberoinfundibular pathway (for review see Bjorklund & Dunnett, 2007). With respect to MCH neurons the majority of the DA fibers within the LH area arise from the VTA (Leibowitz & Brown, 1980;Yoshida *et al.*, 2006), and, DA has been shown to be released within the LH (Fetissov et al., 2000). Furthermore, DA expressing neurons exist within the ZI (Bjorklund et al., 1975). Therefore, DA is positioned to directly interact with MCH neurons and possibly influence their activity.

DA receptors are expressed widely throughout the brain with high expression levels in the striatum, NAc and the prefrontal cortex (Bouthenet et al., 1991;Dearry et al., 1990). There are five subtypes of DA receptors classified into 2 groups based on sequence homology. DA receptors 1 and 5 are known as D1-like, while DA receptors 2, 3, and 4 are the D2-like. All DA receptors are G protein dependent with D1-like coupled to G_S and activating adenylyl cyclase (AC), while D2-like receptors are coupled mainly to $G_{i/o}$ and inhibit AC (for review see Missale et al., 1998). Activation of either receptor can modulate cellular activity through numerous cellular mechanisms such as the modulation of cellular calcium and potassium channels, and the Na^+-K^+-ATPase pump. Also, the D2-like receptors can inhibit the Na^+/H^+ exchanger and activate arachidonic release (for review see Missale et al., 1998).

1.9 DA influences MCH related behaviours

DA has largely been described based on its involvement with the reward and motor systems, however DA has been shown to modulate MCH related behaviours. For example DA has been shown to play an important role in regards to energy homeostasis.

Transgenic rodent models with altered DA receptors or reuptake have a strong influence on weight gain and food reinforcement. It was shown that when DA transporters are eliminated, therefore increasing DA levels in the extracellular space, there is a reduction in energy intake (Epstein & Leddy, 2006). Furthermore, DA has been shown to have an inhibitory impact on food intake when acting within the LH. DA receptor activation inhibits feeding (Sederholm et al., 2002), while the D2-like receptor antagonist sulpiride blocks the anorexic effect of DA in the LH (Parada et al., 1988). Also, different energy states (fasting/obesity) modulate DA receptor expression and DA release within the LH (Fetissov et al., 2002). Therefore, DA's anorexic properties within the LH may be mediated at least partially though the modulation of MCH neuronal activity.

1.10 Summary and Hypothesis

It is evident that MCH expressing neurons play substantial roles in numerous physiological functions including stimulating food intake and maintaining energy homeostasis. In contrast, behavioural studies have shown DA to have a negative effect on food intake and energy homeostasis when acting within the LH area where MCH neurons exist. Therefore, I hypothesized that DA inhibits the activity of MCH neurons. Using the in-vitro patch-clamp technique I have examined the effect of DA on the electrophysiological activity of MCH neurons and the cellular mechanisms involved.

CHAPTER 2

Materials and Methods

2.1 Animal Model

Male Sprague Dawley (SD) rats (60-90g) were used in this study and were obtained from Memorial University Vivarium (VT1000 Leica Microsystems). The rats were housed with food and water available *ad-libitum* and with a room temperature of 21°C ± 1°C. Also controlled were light/dark cycle (12/12hrs), humidity, and air circulation. All experiments were conducted in accordance with the guidelines set by the Canadian Council on Animal Care and were approved by the Memorial University of Newfoundland Internal Animal Care Committee. Only the number of animals necessary to produce reliable results was used.

2.2 Slice Preparation

The rats were deeply anesthetized with halothane and decapitated. The brain was removed and with the aid of a vibratome 250μm coronal hypothalamic brain slices were generated at 0–2°C in buffer solution composed of the following (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 25 glucose, 30 sucrose, 3 pyruvic acid, 1 ascorbic acid. Slices were then incubated at 33–34°C for 30-45 min and then held at room temperature in artificial cerebrospinal fluid (ACSF) composed of (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, 10 glucose, 1 ascorbic

acid. Both solutions were continuously bubbled with a combination of O_2 (95%) and CO_2 (5%).

2.3 Setup

A hemisected brain slice was placed in the recording chamber of the microscope setup and continuously perfused with 33–34°C ACSF at 1.5-2.0ml/min. Whole-cell patch-clamp recording was performed using a Multiclamp 700B amplifier and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). The pipette tip resistance of the recording electrode was 3–7 MΩ when filled with the internal solution composed of (in mM): 128 K-gluconate, 8 KCl, 2 $MgCl_2$, 0.2 EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP, pH 7.3. A high chloride concentration internal solution composed of (in mM): 132 KCl, 2 $MgCl_2$, 0.2 EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP, pH 7.3, was used to aid in miniature inhibitory postsynaptic current (mIPSC) detection. Biocytin (1-1.5 mg/ml) was also included in the internal solution to label recorded neurons.

2.4 Cell Identification

To aid in the localization of MCH neurons, an infrared differential interference contrast microscope (IR-DIC) (DMLFSA; Leica Microsystems) was used for visual detection. Neurons adjacently lateral and dorsal to the fornix with a diameter of 10–20μm were selected. Once the cells were patched they were injected with a series of hyperpolarizing and depolarizing 300ms step pulses in current clamp mode in order to characterize the electrophysiological properties of the cell.

2.5 Electrophysiological Recordings

2.5.1 Current-clamp mode

Current-clamp mode was used to monitor membrane potential and action potential firing. The current was clamped at 0 pA so the cells could remain at rest. Because MCH neurons are silent at rest in our preparation, 200 pA depolarization current pulses 300msec in duration were given every 20-60 sec to monitor action potential firing. Membrane currents were filtered at 10 kHz, sampled at 5 kHz and stored for off-line analysis.

2.5.2 Voltage-clamp mode

Voltage-clamp mode was used to monitor the direction of current flow. Cells were held at a membrane potential of –70 mV in the presence of tetrodotoxin (TTX) (1μM) to block Na^+-dependent action potentials. During mIPSC experiments, DNQX (10μM) and D-AP5 (10μM) were used to block glutamatergic transmission in addition to TTX. Membrane currents were filtered at 1 kHz, digitized at 5 kHz and stored for off-line analysis. A 20 mV hyperpolarizing pulse lasting for 100 ms was applied every 20–60 s throughout each experiment, and the steady state current and decay rate of the capacitance transient were monitored as measures of input resistance and series/access resistance, respectively. Cells that showed significant change in these parameters were excluded from additional analysis.

2.6 Immunohistochemistry

After the electrophysiological recordings were completed a representative number of cells were saved for immunohistochemical analysis. When the electrophysiological recording was completed the brain slice was immediately placed in 4% paraformaldehyde in 0.1 M PBS overnight at 4°C, then washed and stored in PBS before the addition of primary antibodies. To aid in distinguishing between MCH neurons and the orexin neurons that occupy similar areas slices were incubated with anti-orexin A goat polyclonal IgG (1:3000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-MCH rabbit polyclonal IgG (1:2000 dilution; Phoenix Pharmaceuticals, Belmont, CA) for 3 days at 4°C. Since all orexin neurons contain both the orexin A and the orexin B neuropeptides only anti-orexin A was used. Slices were then washed and treated for 3 h with a combination of Cy3-conjugated donkey anti-goat antibody, Cy2-conjugated donkey anti-rabbit antibody, and streptavidin-conjugated aminomethylcoumarin acetate (AMCA), all at 1:500 dilution at room temperature. Antibodies were diluted with PBS with 0.03% Triton X. Slices were then washed, mounted, and examined under a fluorescence microscope for detection of orexin A (Cy3), MCH (Cy2) immunoreactivity, and biocytin (AMCA).

2.7 Data Analysis

Current and voltage-clamp data was detected and analyzed using Clampfit 9.2 software (Axon Instruments). mIPSC's were further detected and analyzed using minianalysis 6.0 software (Synaptosoft, Decatur, GA). The data are expressed as mean $\pm$

SE. Statistical comparisons were performed by using appropriate tests (i.e., Kolmogorov Smirnov test for testing individual cells, and unpaired or paired Student's *t* tests, or ANOVA for group comparison as appropriate). Dose response curves and EC_{50} calculations were performed using GraphPad Prism software. GraphPad Prism and minianalysis 6.0 were used for statistical analysis. A value of $p < 0.05$ was considered to be significant.

2.8 Chemical Compounds

All drugs were bath perfused at final concentrations as indicated, by diluting aliquots of 1000X stock in the ACSF immediately before use. DA and NE stock solutions included the anti-oxidant ascorbic acid (1 mM) and were light protected during the recordings to minimize oxidation. Dimethyl sulfoxide (DMSO) was used to solubilize appropriate compounds in stock solutions and the final concentration of DMSO was 0.1%.

SKF 81297, quinpirole, SCH 23390, sulpiride, tertiapin-Q, yohimbine, BRL 44408, and imiloxan were purchased from Tocris Bioscience (Ellisville, MO), dopamine, norepinephrine, biocytin, DNQX, and D-AP5 from Sigma Aldrich (St. Louis, MO), and tetrodotoxin from Alomone Labs (Jerusalem, Israel).

CHAPTER 3

Results

3.1 Identification of MCH expressing neurons

MCH expressing neurons were targeted based on their location within the LH and ZI and relatively large size. This was accomplished by using an IR-DIC microscope. However, orexin expressing neurons are also located in similar regions of the LH. Therefore, differentiating between the two types of cells was of extreme importance. Identification of MCH neurons was accomplished through immunohistochemical and electrophysiological means. Neurons were filled with biocytin via patch pipette, and their neurochemical phenotype was confirmed by an immunohistochemical method following recordings (Fig. 1B1, B2). Cells that were found to be MCH immunopositive ($n = 17$) had distinct electrophysiological characteristics that were different from that displayed by orexin neurons (Fig. 1A1, A2). None of the MCH neurons displayed an I_h upon injection of a hyperpolarizing current and no rebound depolarization was reveled at the current offset. The average membrane potential was -57.95 ± 1.54mV and the majority of them did not fire spontaneously (15 of 17, 88.2%). Also, fourteen of the seventeen neurons (82.4%) displayed strong spike rate adaptation (Fig. 1A1, A2). These results are consistent with other previous studies (Alberto et al., 2006;Eggermann et al., 2003;Gao et al., 2003).

A total of 65 MCH neurons from 40 rats were used in this study of which 17 cells were identified as MCH immunopositive and having typical electrophysiological characteristics as described above. Forty-eight cells were identified based on the electrophysiological properties only. Cells that did not meet the criteria of MCH neurons were excluded from the study.

3.2 Effect of DA on MCH neurons (current-clamp)

100 μM DA application caused a reversible hyperpolarization of MCH neurons (Fig. 2A,top). Since one of the identifying characteristics of MCH neurons is their lack of action potential firing at rest, a depolarizing current (200 pA, 300 msec) was given each minute to monitor DA's influence on action potential firing (Fig. 2A, bottom). As shown in Fig. 2B, 10 and 100 μM DA application caused a decrease in action potential firing and increased the latency to the first induced action potential ($n = 2$ respectively). In addition, DA application reduced input resistance during a –200 pA, 300 msec current pulse (Fig. 2B3, $n = 2$ respectively). However, DA application had no significant effect on spike threshold or width. Control values were -32.15 ± 0.03mV and 3.9 ± 0.2 msec respectively, while during DA application values were -35.31 ± 0.26 mV and 3.4 ± 0.2 msec respectively ($n = 4$, $p>0.05$).

3.3 Postsynaptic effect of DA on MCH neurons (voltage-clamp)

It is possible that DA inhibits MCH activity by either acting directly on the MCH neuron or by influencing synaptic inputs. To investigate the possible postsynaptic

mechanism voltage-clamp experiments were carried out in the presence of TTX (1 μM). As shown in Fig. 3A, 100 μM DA application caused a reversible outward current. This current is concentraion dependent (Fig. 3B) at concentrations tested (0.1, 1, 3, 10, and 100 μM). 0.1 μM DA application did not induce a significant outward current (0.19 ± 0.08 pA, $n = 3$, $p>0.05$). The lowest concentration to produce a significant current was 1 μM (8.94 ± 2.32 pA, $n = 4$, $p<0.05$). 3 μM application induced a 17.37 ± 3.93 pA outward current ($n= 5$, $p<0.05$), while the current was also significant with the 10 and 100 μM application (22.99 ± 3.36 pA, $n = 5$, $p<0.05$ and 20.52 ± 2.38 pA, $n=13$, $p<0.05$ respectively). Also, there was no statistical difference ($p>0.05$) for the currents produced from the 3, 10, or 100 μM application. This indicates that a concentration of 3 μM can initiate a maximal current with the DA's induced current having a steep concentration dependence. The EC_{50} of the DA induced outward current was 0.80 μM.

3.4 DA activates GIRK channels

To determine the voltage dependence of the DA induced current the membrane potential was ramped before and during DA application and the resulting current response was measured (Fig. 4A1). In the two experiments carried out the reversal potential for the DA induced current was -91mV, as shown in Fig. 4A2, and -89mV (data not shown). The results are similar to the predicted potassium reversal potential of the solutions used in our experiments (-101mV). The DA induced current also displayed an inward rectification similar to the G Protein-activated Inwardly Rectifying K^+ (GIRK) current. To determine the contribution of GIRK channels to DA's effect, DA was applied

in the presence of the GIRK channel blocker tertiapin-Q (300nM). In the presence of Tertiapin-Q a 100μM DA application failed to initiate an outward current. This was statistically different from DA application alone [(Fig. 4B; 2.91 ± 0.85pA, $n = 3$, $p<0.05$) (Fig. 4B2)]. This suggests that a GIRK mediated current is activated by DA application.

3.5 Receptors mediating DA's response

Next the receptor responsible for the outward current was investigated. There are two classes of DA receptors, namely D1-like (D1/D5) and D2-like receptors (D2/D3/D4). To examine the role of the receptors, the D1-like agonist SKF81297 (10μM) and D2-like agonist quinpirole (10 μM) were simultaneously applied. To our surprise, these agonists failed to mimic the DA induced outward current [(Fig. 5A); -1.55 ± 1.62, $n = 5$, $p>0.05$ (Fig. 5D)]. Furthermore, 100 μM DA induced a significant outward current when applied in the presence of the D1-like antagonist SCH23390 (10 μM) and the D2-like antagonist sulpiride (10 μM) [(Fig. 5B); 22.81 ± 5.13, $n = 3$, $p<0.05$ (Fig. 4D)]. This suggests that DA receptors are not mediating the DA effect. Furthermore, as shown in Fig. 5B, the DA receptor antagonists did not affect the MCH neuron on their own. This suggests that the DA receptor antagonists are not participating in the outward current.

DA has the ability to activate adrenergic receptors (Brown & Caulfield, 1979), and activation of α_2-adreoceptors has been shown to hyperpolarize MCH neurons due to the activation of GIRK channels (van den Pol et al., 2004). Therefore, we hypothesize that DA activates α_2-adrenoceptors on MCH neurons which in turn initiates the outward current. 100 μM DA application in the presence of the α_2-adreoceptor antagonist

yohimbine (5 μM) failed to induce an outward current [(Fig. 5C); -0.53 ± 0.44, $n = 3$, $p>0.05$, (Fig. 5D)]. This suggests dopaminergic activation of α_2-adreoceptors mediates the outward current. α_2-adreoceptors are classified into four subtypes, namely α_{2A}, α_{2B}, α_{2C}, and α_{2D}. Due to the high expression level of the α_{2A} and α_{2B} adrenoceptor subtypes within the LH (Wang et al., 2002;WinzerSerhan et al., 1997), these were investigated. Application of 10-100 μM DA in the presence of α_{2A} antagonist BRL44408 (3 μM) failed to induce an outward current [(100 μM, Fig. 6A); 10-100μM, 2.38 ± 2.11, $n = 5$, $p>0.05$, (Fig. 6C)]. In contrast, application of 10-100 μM DA in the presence of α_{2B} antagonist Imiloxan (3μM) induced an outward current [(100μM, Fig. 6B); 10-100μM, 16.77 ± 7.81, $n = 2$, (Fig. 6C)]. This suggests dopaminergic activation of the α_{2A}-adreoceptor subtype mediates the outward current.

3.6 Comparison of DA vs NE effects on MCH neurons

To determine whether DA was as effective as NE in modulating MCH neurons, the amplitude of DA and NE induced outward currents was compared. We found that NE application initiated an outward current in a dose dependent manner in MCH neurons (Fig. 7A and B). 0.1 μM NE application did not induce a significant outward current (3.10 ± 3.70 pA, $n = 4$, $p>0.05$). The lowest concentration of NE to produce a significant current was 1μM (11.46 ± 4.49 pA, $n = 4$, $p<0.05$). 3μM application induced a 18.98 ± 1.84 pA outward current ($n = 4$, $p<0.05$), while the current was also significant with the 10 and 100μM application of NE(21.20 ± 2.08 pA, $n = 3$, $p<0.05$ and 24.99 ± 2.38 pA, $n=5$, $p<0.05$ respectively). The EC_{50} of the NE induced outward current was 1.23 μM. As

shown in Fig. 7B the dose response curves of DA and NE are very similar with no significant differences between groups at concentrations tested ($p>0.05$), indicating that their effects are of similar magnitudes. In a preliminary experiment an occlusion test was carried out in an attempt to confirm if DA and NE were acting on the same receptor. As shown in Fig. 7C addition of 100 μM NE in the presence of 100 μM DA does not induce any further outward current, supporting the idea that the same receptor is activated. However, the possibility remains that DA and NE are both activating GIRK channels but through the activation of different subtypes of adrenoceptors receptors.

3.7 Synaptic effect of DA on MCH neurons (voltage-clamp)

To determine possible synaptic effects of DA on MCH neurons, mIPSCs were monitored in the presence of TTX (1 μM). Application of 0.1, 1, 3, 10, and 100 μM DA had no consistent effect on mIPSC frequency (Fig. 8A1), with the change in mIPSC frequency fluctuating independent of concentration. Some cells showed a significant increase in mIPSC frequency during DA application. For example 100 μM DA application can increase the frequency of mIPSCs (Fig. 8A2, $p<0.05$), while some cells were unchanged by 100 μM DA application (Fig. 8A3, $p>0.05$) However some cells had significantly decreased frequencies with DA application. For example, 100 μM DA application decreased the frequency of mIPSCs in some cells (Fig. 8A4, $p<0.05$). Also, no consistent effect on mIPSC amplitude was found with DA application (Fig. 8B). This suggests that DA induced hyperpolarization is not mediated by altered GABA release or GABA receptor activity

CHAPTER 4

Discussion

4.1 Distinguishing MCH and Orexin neurons

Animal studies have shown that MCH functions as an anabolic signal in energy homeostasis, communicates hedonic aspects of feeding, activates the stress axis, and decreases an animal's activity level. In order for the MCH neuropeptide to be fully understood, the factors influencing its neuronal activity must be identified. However, the orexigenic neuropeptide orexin is also expressed in similar regions of the hypothalamus as the MCH neurons. Although MCH and orexin are both synthesized by neurons of the LH and maintain many overlapping projections, the two peptides are not co-localized in a single neuron (Broberger et al., 1998;Elias et al., 1998;Peyron et al., 1998). Therefore, distinguishing between the two neuronal types is of extreme importance. This study began with the confirmation of the electrophysiological characteristics associated with MCH and orexin expressing neurons as described in earlier studies (Eggermann et al., 2003;Gao et al., 2003). As shown in Fig. 1 our results confirmed the electrophysiological characteristics of both MCH and orexin neurons (Alberto et al. 2006). MCH neurons have a lower resting membrane potential compared to orexin neurons, do not fire spontaneously, show spike-rate adaptation, do not have an I_h, and do not display rebound depolarization. However, the orexin neuronal properties are different to that of MCH neurons because they have a higher resting membrane

potential, fire spontaneously, display no spike-rate adaptation, posses an I_h, and do display rebound depolarization. Therefore these distinguishing electrophysiological characteristics can be used as a reliable tool in the identification of the MCH and orexin neuronal groups.

4.2 DA hyperpolarizes MCH neurons

Mesolimbic dopaminergic projections are positioned ideally for direct interaction with MCH neurons (Duva *et al.*, 2005;Yoshida *et al.*, 2006;Leibowitz & Brown, 1980). Behavioural studies have also suggested that this interaction may directly modulate the activity of MCH neurons (Parada et al., 1988;Yang et al., 1997). Also, DA expressing neurons are located within the ZI, which in turn could directly influence MCH neurons (Cheung et al., 1998). The present study demonstrates that DA causes a reversible hyperpolarization of MCH neurons. As observed in Fig. 2, DA hyperpolarizes the membrane potential of MCH neurons and reduces the action potential frequency. This reduction in action potential frequency coincides with the DA-induced increase in latency to the first action potential and to the decrease in input resistance. However, the spike threshold and width are not affected. Therefore, the DA induced hyperpolarization may prevent MCH related behaviours, such as food intake, from being initiated.

4.3 DA induces a GIRK mediated current

DA may hyperpolarize MCH neurons through direct postsynaptic mechanisms, presynaptic mechanisms, or a combination of the two. As observed in Fig 3, during the

voltage-clamp protocol, when the MCH neuron is isolated from its synaptic inputs through the use of TTX, DA induces a direct dose-dependent reversible outward current. This indicates that DA may induce its hyperpolarization at least in part through direct postsynaptic mechanisms. As shown in Fig. 4, the current-voltage relationship of the current has an inward rectification with a reversal potential approximately equal to the K^+ equilibrium potential. Furthermore, the GIRK channel blocker tertiapin-Q abolished this current. Thus we concluded that DA activates a GIRK current in MCH neurons.

G protein-dependent stimulation of potassium channels has been described for numerous neurotransmitters in a variety of brain regions. Stimulation of GIRK currents in the CNS has been shown to be pertussis toxin (PTX) sensitive (Saugstad et al., 1996), and hence the involvement of a G_i protein. Major neurotransmitters within the CNS can activate a hyperpolarizing GIRK current, which include: GABA by $GABA_B$ receptors in the hippocampus (Andrade et al., 1986; Otis et al., 1993), norepinephrine (NE) by α_2-adrenoceptors in the locus coeruleus (Velimirovic et al., 1995), 5-HT in the locus coeruleus and cerebellum (Andrade et al., 1986: Andrade and Nicoll 1987), and by DA in the subtantia nigra and in the pituitary lactotrophs (Kim et al., 1995). Activation of a GIRK current by NE has also been shown to induce the hyperpolarization of MCH neurons (van den Pol et al., 2004). Therefore, DA may be activating the GIRK current in MCH neurons through the activation of DA receptors, or through an indirect adrenergic mechanism.

4.4 DA induced outward current is not DA receptor mediated

It has been shown that addition of the D2-like receptor agonist quinpirole to brain slices hyperpolarizes substantia nigra neurons via the activation of a GIRK current during whole-cell patch clamp experiments (Kim et al., 1995). The GIRK current was blocked using the D2-like receptor antagonist sulpiride suggesting the direct activation of the D2-like receptor initiated the activation of GIRK channels, which led to the hyperpolarization. In contrast, as shown in Fig. 5, simultaneous application of D1 and D2 receptor agonists failed to mimic the DA induced outward current in MCH neurons. To ensure the D1 agonist was not counteracting a D2 initiated current, the D2-like agonist quinpirole was applied alone in some cells. Activation of D2 receptors alone also failed to initiate an outward current (data not shown). Furthermore addition of DA receptor antagonist failed to block the DA induced current. This suggests that DA is not initiating the GIRK current through a DA receptor based mechanism.

Evidence for DA receptor expression within the LH area is conflicting. One study on Zucker rats concluded that both D1 and D2 receptor mRNAs were present within the LH (Fetissov et al., 2002). PCR preparation for this study used a brain tissue chunk containing the LH. With this type of tissue preparation it is possible that surrounding areas outside of the LH were incorporated into the analysis. Therefore, a false positive may have been obtained with the DA receptors actually expressed outside the LH. A more recent study on SD rats, that used the spatially specific *in-situ* hybridization method, showed that D2 mRNA was scarce within the LH and D1 mRNA was not present (Bubser et al., 2005). However, these conflicting results may be due to the rat

strains used. The present study was undertaken with SD rats, therefore the low level on D2 expression and absence of D1 may explain why the D1-and D2-like agonists and antagonists had no effect on MCH neuronal activity. This suggests that the GIRK current is initiated through a DA receptor independent mechanism.

4.5 DA induced outward current is α_{2A}-adrenoceptor mediated

The possibility of insufficient DA receptor expression suggests that DA may be acting within the LH through the activation of adrenergic receptors. Evidence supporting this includes the fact that α_2–adrenoceptors are heavily expressed within the LH (Wang et al., 2002;WinzerSerhan et al., 1997). As previously shown by van den Pol *et al.*, (2004), NE has the ability to induce a hyperpolarizing GIRK current through the activation of the PTX sensitive α_2-adrenoceptors in MCH neurons. DA and NE are both catacholaminergic neurotransmitters and DA is known to show cross reactivity to adrenergic receptors (Brown & Caulfield, 1979). Therefore, the possibility exists that DA is initiating the outward current through the activation of the adrenergic system. As observed in Fig. 5, blocking the α_2-adrenoceptors prevents the initiation of the DA induced outward current. The α_{2A} and α_{2B} adrenoceptor subtypes are the most predominantly expressed α_2-adrenoceptors in the LH area (Wang et al., 2002;WinzerSerhan et al., 1997). Further investigation into the receptor subtype revealed that the α_{2A} receptor activation was initiating the outward current.

NE itself shares similarities with DA's influence on MCH related behaviours, such as inhibiting food intake (Wellman et al. 1993). On the other hand earlier studies

demonstrated that NE release within the hypothalamus was increased during feeding (Hoebel et al., 1989), and that NE dose-dependently increased food intake by chemical stimulation of the LH (Shiraishi, 1991). Wellman et al. (1993) demonstrated that NE effect on food intake was dependent on which adrenergic receptor was activated within the paraventricular nucleus of the hypothalamus. When α_2- receptors are activated it increased food intake, however α_1 – activation decreased food intake. Therefore, a possible explanation of NE's opposing influence on ingestive behaviour may be due to the type of adrenergic receptor that is activated and its location in the hypothalamus. All adrenergic receptors are G-protein coupled with the α_1-receptors coupled to G_q, while the α_2- are coupled to the G_i, and the adrenergic ß-receptors are G_s coupled, for review see Michelotti et al., (2000). Therefore, the possibility exists that activation of the inhibitory G_i protein within the orexigenic MCH neuronal population by the α_2-adrenoceptors would inhibit their activity and thus decrease food intake. On the other hand activation of the stimulatory G_s protein and even the G_q proteins within the LH by the α_1- and ß-receptors may stimulate the MCH and/or orexin neurons and thus increase food intake. Evidence supporting this hypothesis is the fact that α_1-, α_2, and ß-adrenoceptors are all expressed within the LH (Wang et al., 2002;WinzerSerhan et al., 1997; Day et al., 1997; Castillo-Melendez et al., 2000), while NE action within the hypothalamus produces conflicting effects on food intake.

4.6 DA mimics NE's effect on MCH neurons

Activation of adrenoceptors within the CNS has been shown to reduce food intake (Wellman et al., 1993). This evidence taken with the fact that NE activation of α_2-adrenoceptors directly hyperpolarizes MCH neurons, suggests that NE may interact with MCH neurons to inhibit food intake. Also MCH is known to activate the stress responses and induces depressive- and anxiety-like behaviours, while the blockade of MCH receptors results in antidepressant and anxiolytic effects in various models (Kennedy et al., 2003;Borowsky et al., 2002). With this in mind, preventing the release of the MCH neuropeptide into the CNS through adrenergic inhibition of MCH neurons may result in altered HPA axis function. This is indeed the fact with altered HPA axis activation resulting from an increase in central adrenergic signaling (Morilak et al., 2005). Therefore adrenergic activation of α_2-adrenoceptors on MCH neurons may result in the direct regulation of food intake and the stress response. Since NE and DA both inhibit MCH neuronal activity through the activation of α_2-adrenoceptors, which may result in altered MCH related behaviours, the questions arise whether DA's effects on MCH neurons through the adrenergic receptor are of a similar magnitude to the effect of NE itself, and secondly whether the same α_2-adrenoceptors are being activated.

As observed in Fig. 7, NE produced a dose dependent reversible outward current that is in agreement with previous research (van den Pol et al., 2004). The amplitude of the outward current induced by NE and DA is similar at the same concentrations. Even though our data suggest that DA shows cross reactivity with adrenergic receptors this

observation was surprising due to the fact that DA has an affinity for α_2-adrenoceptors 10-28-fold lower than that of NE (Cornil and Ball, 2008). Cornil and Ball (2008) demonstrated this lower affinity in whole rat brain homogenates, therefore the possibility remains that anatomical specificity may determine the magnitude of DA and NE cross reactivity and that DA may have a higher affinity for α_2-adrenoceptors within the LH in order to compensate for the low DA receptor expression.

To examine whether the outward current was initiated through the same receptors, an occlusion experiment was conducted. As shown in Fig. 7, the addition of NE in the presence of DA did not alter the outward current suggesting that in fact the same α_2-adrenoceptors mediated the outward current. Therefore it can be concluded that DA is regulating the same adrenergic system in MCH neurons as NE itself, and with similar efficacies.

4.7 DA's hyperpolarization of MCH neurons is not synaptically mediated.

Since MCH neuronal activity is modulated by GABA and glutamate transmission (van den Pol et al., 2004), the possibility remains that dopaminergic modulation of glutamate and/or GABA synapses may contribute to its inhibitory effect on MCH neuronal activity. To aid in determining the synaptic influence of a neurotransmitter, mIPSC's and miniature excitatory postsynaptic currents (mEPSC's) can be utilized as an electrophysiological tool. Miniature events inform the researcher on the spontaneous release of transmitters that are action potential independent. Therefore, how a compound influences the release machinery of the presynaptic terminals and the conductivity of the

postsynaptic channels can be studied. Previous research within our laboratory focused on DA's influence on mEPSC's and mIPSC's in orexin neurons within the LH. These studies found that DA decreases glutamate release and increases GABA release onto orexin neurons (Alberto *et al.*, 2006; Trask *et al.*, 2005). In contrast, we have found in the present study that DA has no consistent effect on mIPSC frequency or amplitude in MCH neurons (Fig. 8), regardless of the concentration used. DA influenced the frequency and amplitude of mIPSC's by increasing them in some cells, decreasing in some, and having no effect in others. Previous research within the laboratory also revealed that DA had no effect on mEPSC frequency or amplitude in MCH neurons. Taken together, our results suggest that synaptic modulation of glutamate and GABA receptors is not a mechanism by which DA inhibits MCH neurons.

4.8 Conclusion

From this study it can be concluded that DA hyperpolarizes MCH neurons through the activation of a GIRK current by the direct postsynaptic activation of α_{2A}-adrenoceptors. In general the inhibition of MCH neurons by DA may help explain DA's influence on MCH related behaviours, such as DA's anorexigenic properties when acting within the LH. More specifically this is the first study to demonstrate a cross talk between DA and adrenergic signaling at the cellular level within the LH area. If the DA receptor expression is low in the LH, as Bubser *et al.*, (2005) suggested, then this cross talk may provide an explanation on how the DA input to the LH can influence MCH related behaviours. This study suggests that the abundant postsynaptic expression of α_{2A}-

adrenoceptors within the LH may serve as the mediator necessary for DA effects. This mechanism may also help explain why DA and NE have similar effects on MCH related behaviours.

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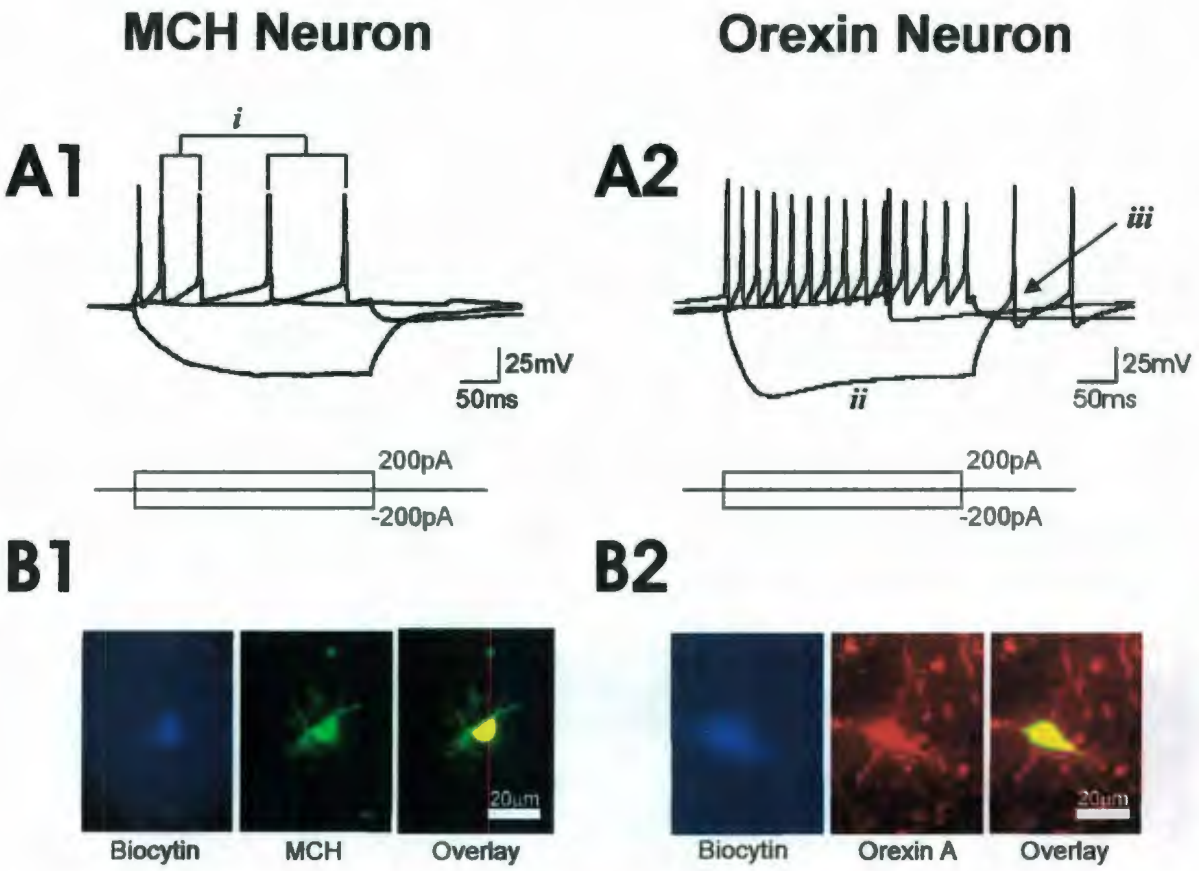

Figure 1. Identification of MCH and orexin neurons. **A1**, Electrophysiological characteristics of an MCH neuron. In a typical MCH neuron, hyperpolarization does not induce an H current and there is no rebound depolarization at the current offset. There are no spontaneous action potential firing at rest and depolarization induces a strong spike rate adaptation(*i*). **A2**, Electrophysiological characteristics of an Orexin neuron. In a typical orexin neuron, hyperpolarization induces an H current(*ii*) and there is a rebound depolarization at the current offset(*iii*). There are spontaneous action potential firing at rest and depolarization does not induce spike rate adaptation. **B1**, Immunohistological identification of a recorded MCH neuron. Left, an example of a cell filled with biocytin during recording. Middle, MCH immunoreactivity is shown in green. Right, overlay showing the biocytin labeled cell is MCH-immunopositive. **B2**, Immunohistological identification of a recorded orexin neuron. Left, an example of a cell filled with biocytin during recording. Middle, orexin A neuropeptide immunoreactivity is shown in red. Right, overlay showing the biocytin labeled cell is orexin A-immunopositive.

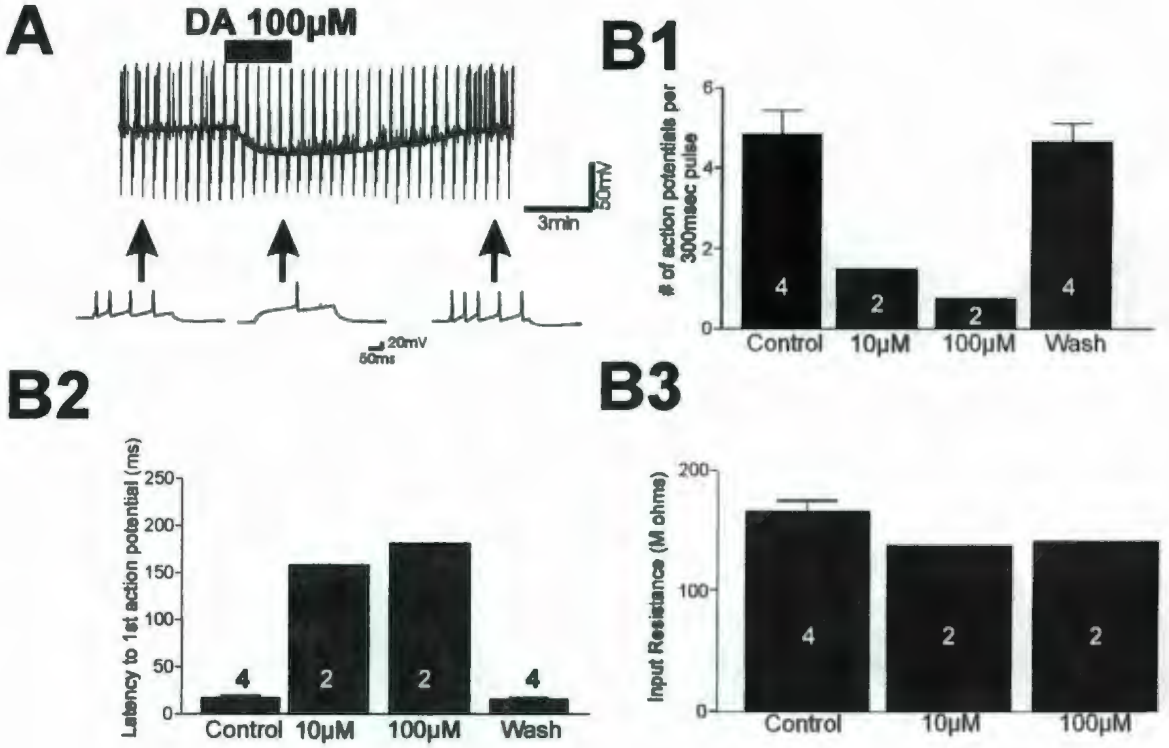

Figure 2. DA hyperpolarizes and diminishes firing of MCH neurons. **A**, Top, Typical recording showing the effect of 100µM DA. The bar above the trace indicates application of DA. Vertical lines are responses to current injections (-200~200pA). Bottom, Representative recording during 200pA injections in expanded time scale taken at the time points indicated by each arrow. Note the reduction in action potential firing and the increase in action potential latency during DA application. **B1**, Effect of 10 and 100µM DA on action potential firing during a 200pA, 300ms current step.. **B2**, Effect of 10 and100µM DA on the latency to the first action potential during a 200pA, 300ms current step. **B3**, Effect of 10 and100µM DA on input resistance during a 200pA, 300ms current step. Error bars indicate SE. Numbers above and within bars represents the number of samples used..

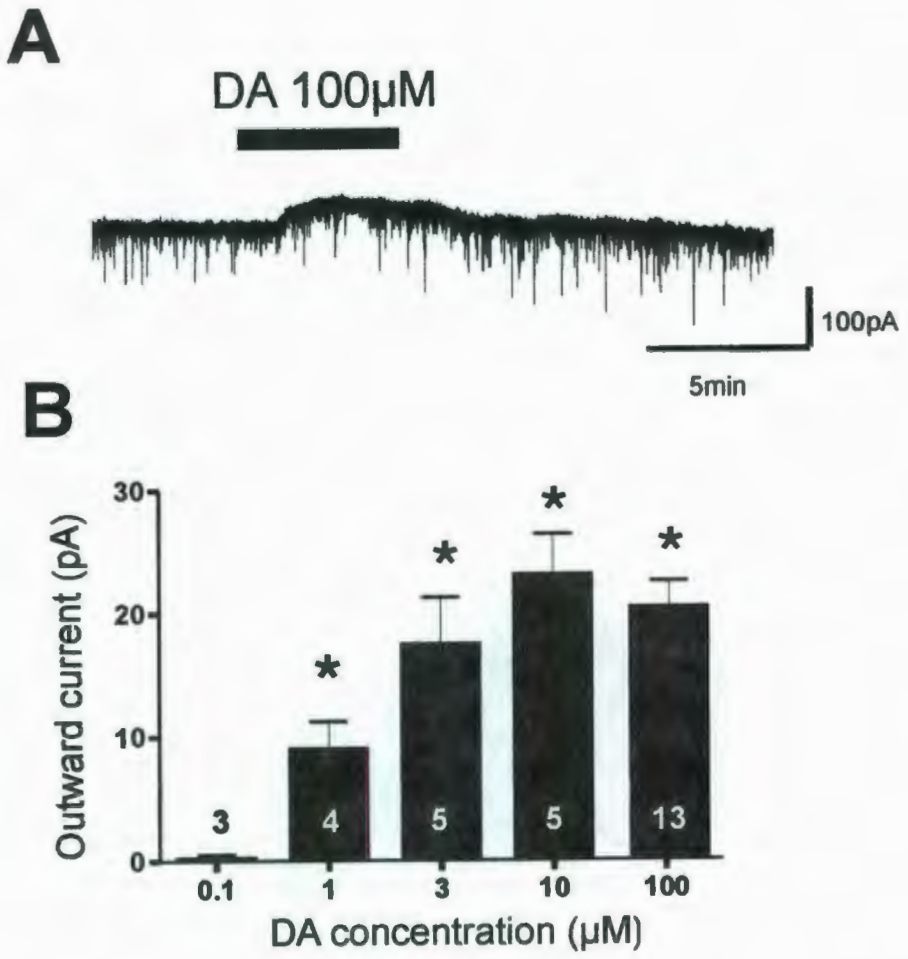

Figure 3. DA induces a concentration dependent outward current in MCH neurons. **A,** Typical recording showing the effect of 100μM DA. **B,** Summary of DA effect at different concentrations. *$p<0.05$. Error bars indicate SE. Numbers above and within bars represents the number of samples used.

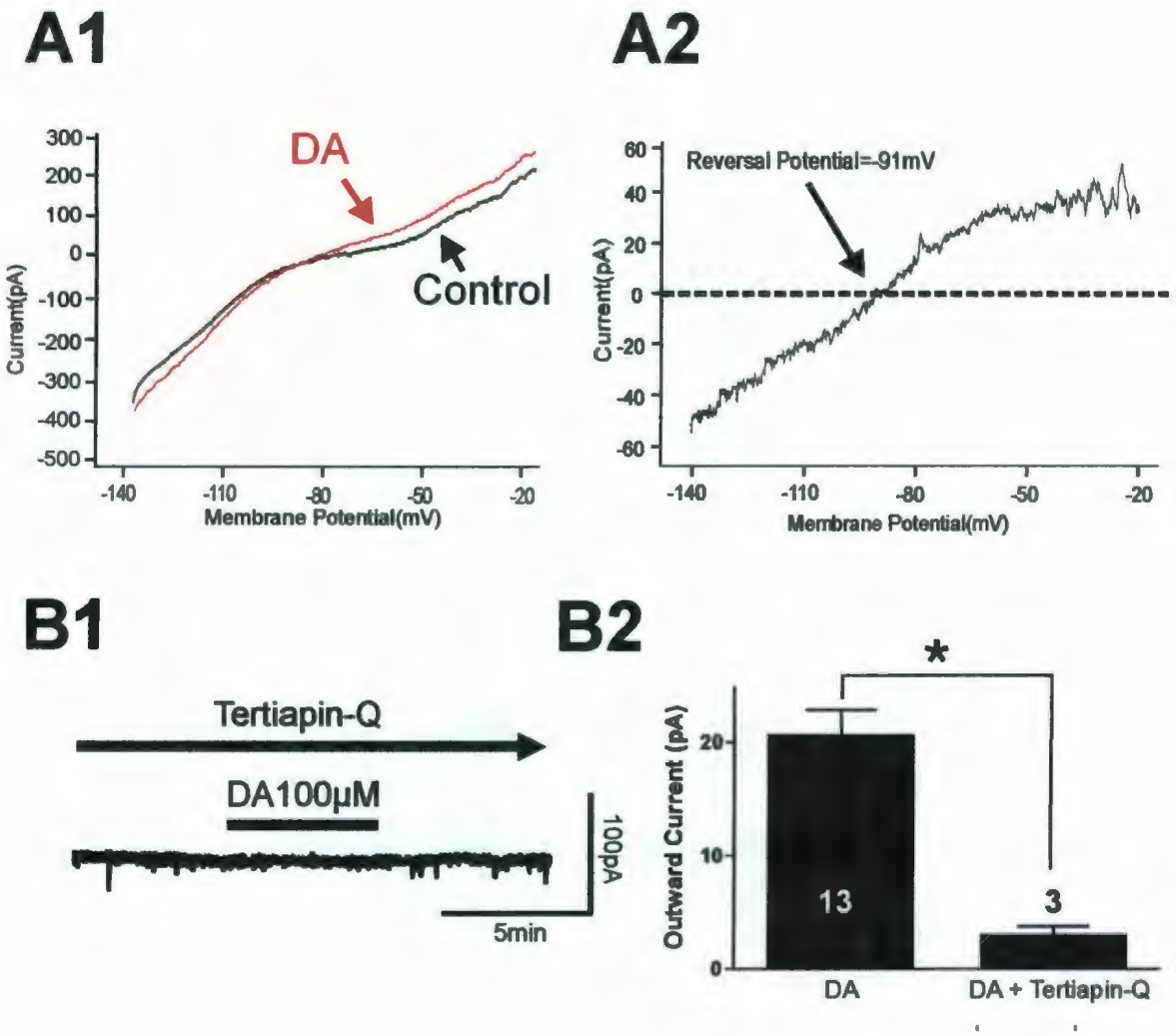

Figure 4. DA-induced outward current due to the activation of GIRK channels in MCH neurons. **A1,** Representative traces showing the effect of DA application on I-V relationship during a 600ms voltage ramp. **A2,**Representative trace showing the total DA induced current with a reversal potential of -91pA (arrow). **B1**, Typical recording showing the effect of 100μM DA in the presence of Tertiapin-Q (300nM). **B2,** Summary of the effect of 100μM DA in the presence of Tertiapin-Q (300nM). *$p<0.05$. Error bars indicate SE. Numbers above and within bars represents the number of samples used.

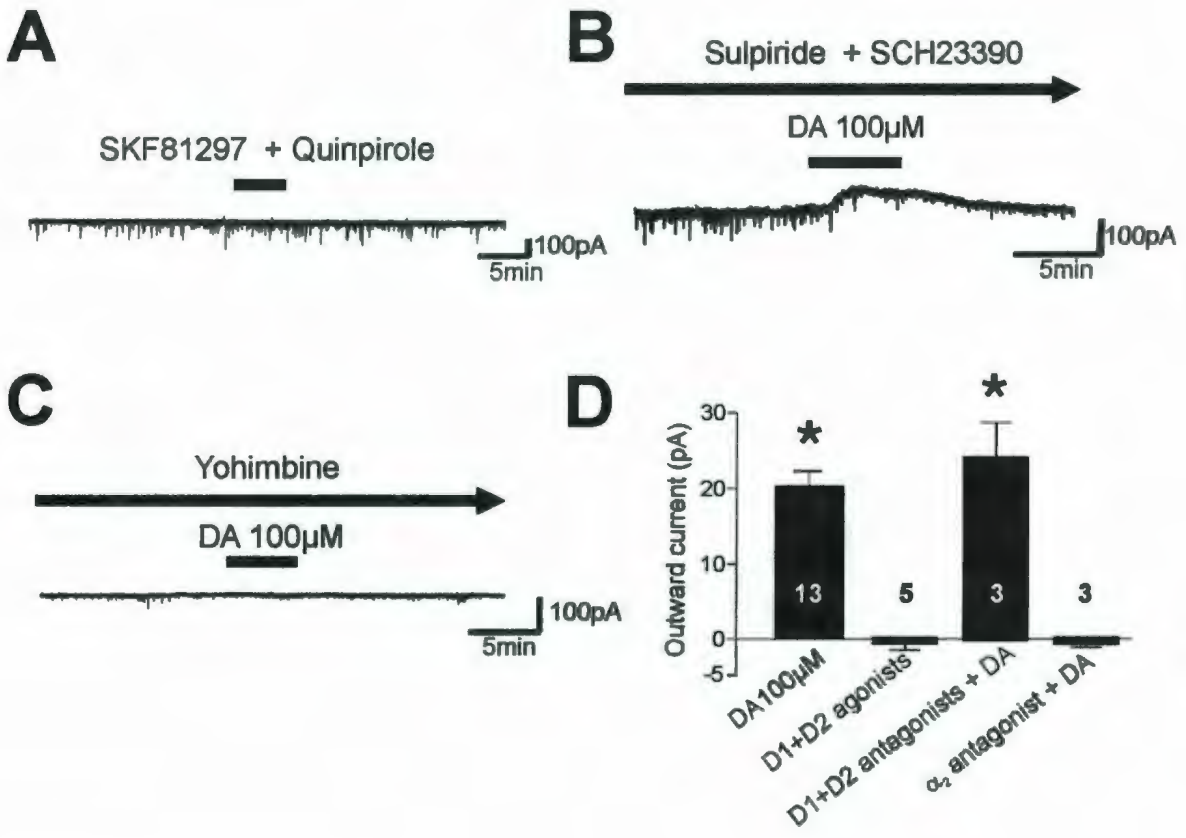

Figure 5. DA-induced outward current is initiated through the activation of alpha$_2$-adrenoceptors and not DA receptors in MCH neurons. **A,** Typical recording showing the effect of SKF81297 (10μM) and Quinpirole (10μM). **B,** Typical recording showing the effect of 100μM DA in the presence of SCH23390 (10μM) and Sulpiride (10μM). **C,** Typical recording showing the effect of 100μM DA in the presence of Yohimbine (5μM). **D,** Summary of the effect of DA, SKF81297 plus Quinpirole, DA in the presence of SCH23390 and Sulpiride, DA in the presence of Yohimbine on MCH neurons. Concentration of DA used was 10-100μM. *$p<0.05$. Error bars indicate SE. Numbers above and within bars represents the number of samples used..

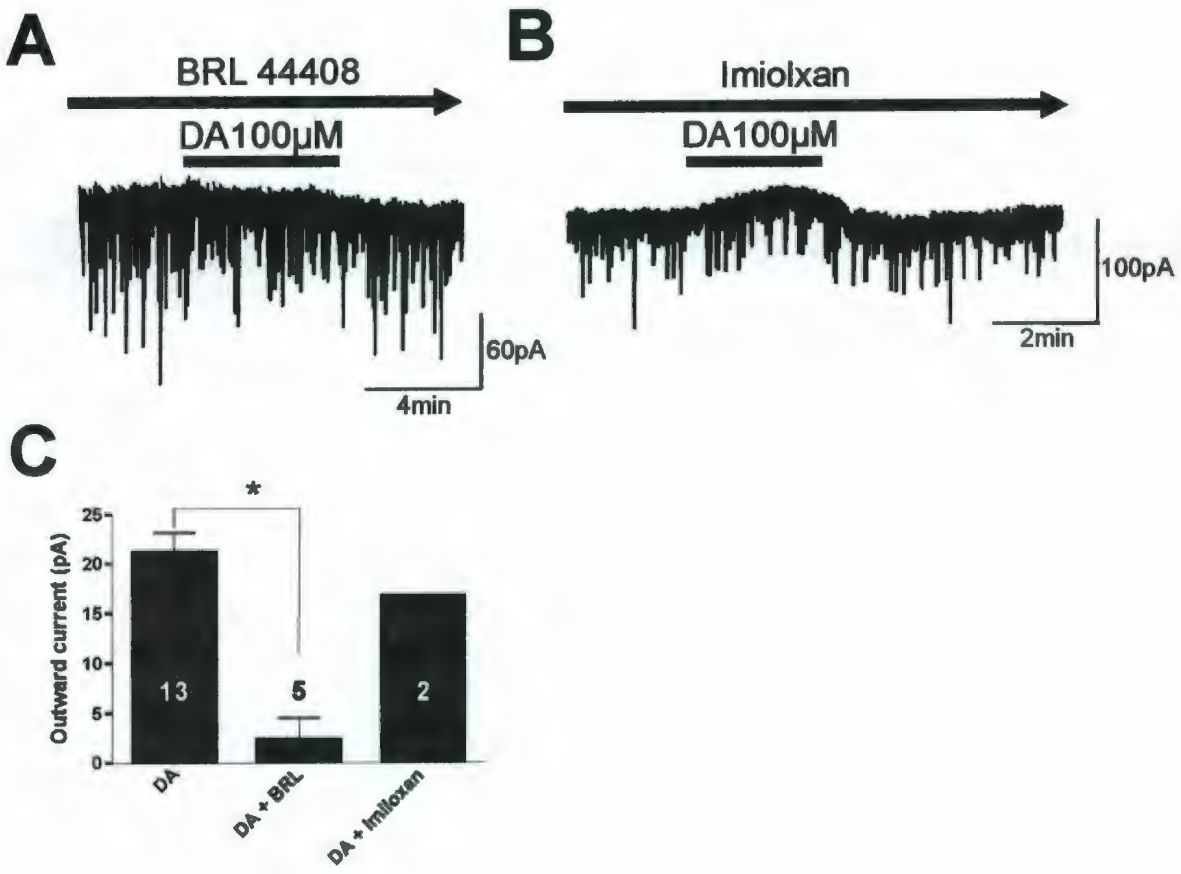

Figure 6. DA-induced outward current is due to the activation of α_{2A}-adrenoceptors in MCH neurons. **A**, Typical recording showing the effect of 100μM DA in the presence of BRL44408 (3μM). **B**, Typical recording showing the effect of 100μM DA in the presence of Imiloxan (3μM). **C**, Summary of the effect on DA, DA in the presence of BRL 44408, DA in the presence of Imiloxan on MCH neurons. The concentration of DA used was 10-100μM. *$p<0.05$. Error bars indicate SE. Numbers above and within bars represents the number of samples used.

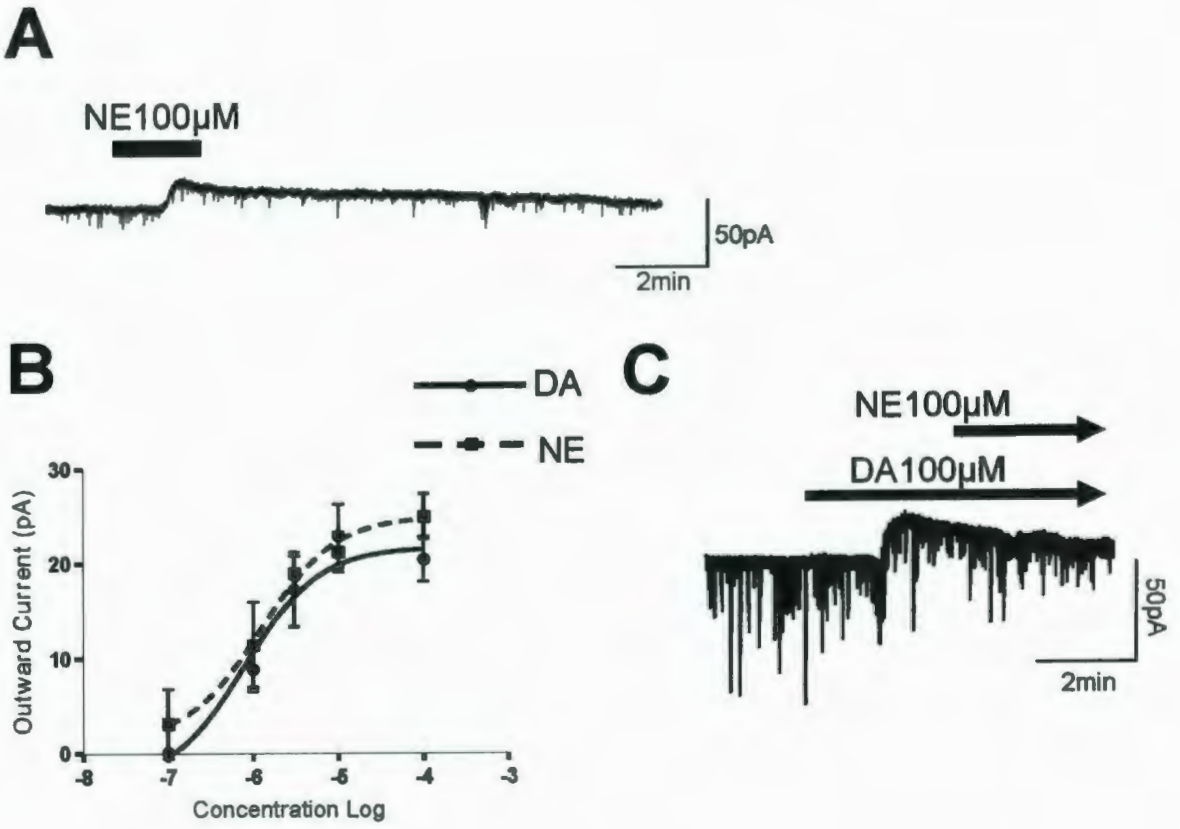

Figure 7. DA and NE-induced outward currents are similar in MCH neurons. **A**, Typical recording showing the effect of 100μM NE. **B**, Concentration dependence of DA and NE-induced outward currents.Number of samples used: 0.1μM (DA=3 NE=4), 1μM (DA=4 NE=4), 3μM (DA=5 NE=4), 10μM (DA=5, NE=3),100μM (DA=13, NE=5). **C**, Preliminary recording showing the effect of 100μM NE in the presence of 100μM DA. Error bars indicate SE.

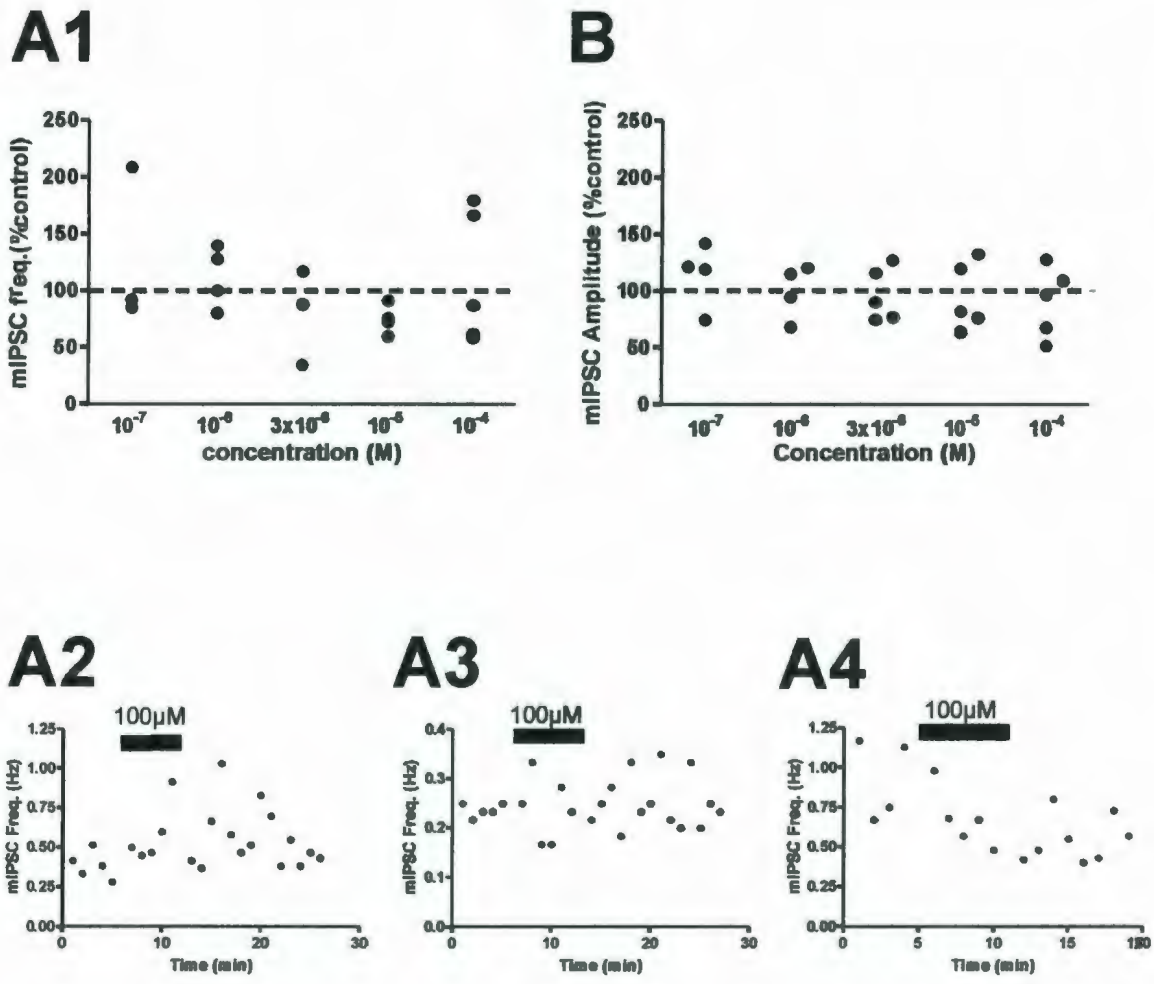

Figure 8. DA has no consistent effect on mIPSC frequency or amplitude. **A1,** Summary of the effect of various concentrations of DA on mIPSC frequency (each filled circle represents a single cell). **A2, A3, A4**, Representative time effect plots of 100µM DA effect on mIPSC frequency. **B**, Summary of the effect of various concentrations of DA on mIPSC Amplitude (each filled circle represents a single cell).