Brief Communications

Dopamine Acts as a Partial Agonist for α2A Adrenoceptor in Melanin-Concentrating Hormone Neurons

Christian O. Alberto, Robert B. Trask, and Michiru Hirasawa
Division of Biomedical Sciences, Faculty of Medicine, Memorial University, St. John's, Newfoundland and Labrador A1B 3V6, Canada

Melanin-concentrating hormone (MCH) is a hypothalamic neuropeptide that promotes positive energy balance and anxiety. Since dopamine (DA) is also closely implicated in these functions, the present study investigated the effect of DA on MCH neurons. Using whole-cell patch-clamp recordings in rat brain slices, we found that DA hyperpolarizes MCH neurons by activating G-protein-activated inwardly rectifying K^+ (GIRK) channels. Pharmacological study indicated that the effect was mediated by α2A adrenoceptors, not DA receptors. DA-induced outward current was also observed in the presence of tetrodotoxin or the dopamine β-hydroxylase inhibitor fusaric acid, suggesting that DA directly binds to α2A receptors on MCH neurons, rather than acting presynaptically or being transformed into norepinephrine (NE) in the slice preparation. The effects of NE and DA were concentration-dependent with EC_{50} of 5.9 and 23.7 μM, respectively, and a maximal effect of 106.6 and 57.2 pA, respectively, suggesting that DA functions as a partial agonist. Prolonged (5 min) activation of α2A receptors by either DA or NE attenuated the subsequent response to DA or NE, while 5 s applications were not sufficient to induce desensitization. Therefore, a history of α2A receptor activation by DA or NE can have a lasting inhibitory effect on the catecholaminergic transmission to MCH neurons. Our study suggests that α2A receptors expressed by MCH neurons may be one of the pathways by which DA and NE can interact and modulate mood and energy homeostasis, and this cross talk may have functional implications in mood disorders and obesity.

Introduction

Melanin-concentrating hormone (MCH) is an important regulatory peptide implicated in energy balance, reward, and mood. MCH promotes positive energy balance by increasing food intake (Qu et al., 1996; Shimada et al., 1998; Ludwig et al., 2001) and sleep (Verret et al., 2003; Willie et al., 2008), while inhibiting metabolism and locomotion (Shimada et al., 1998; Marsh et al., 2002). MCH also modulates behavioral responses to palatable food and drugs of abuse by its action in the nucleus accumbens (Borowsky et al., 2002; Smith et al., 2005; Chung et al., 2009). Involvement of MCH in emotion and mood disorders has been put forward by studies demonstrating that MCH receptor antagonists exert anxiolytic and antidepressant effects in animal models (Borowsky et al., 2002; Georgescu et al., 2005).

Dopamine (DA) and norepinephrine (NE) are classical neurotransmitters with a significant overlap in the breadth of physiological functions with MCH. Direct interactions between these neurochemical systems are highly likely, since MCH is expressed predominantly within in the lateral hypothalamus (LH), perifornical area (PFA), and zona incerta (Bittencourt et al., 1992), where significant NE and DA projections and DA cell bodies are found (Leibowitz and Brown, 1980; Baldo et al., 2003; Sita et al., 2003). Catecholamine signaling in the LH/PFA suppresses food intake (Leibowitz and Brown, 1980; Adamson et al., 2010), which entails the ascending projections from the brainstem (Leibowitz and Brown, 1980). It is also well established that NE and DA are involved in wakefulness, reward, and mood (Saper et al., 2005; Nestler and Carlezon, Jr., 2006; Itoi and Sugimoto, 2010).

At the cellular level, NE has been shown to directly hyperpolarize MCH neurons through α2 adrenoreceptors (α2Rs) and Ba^{2+}-sensitive G-protein-activated inwardly rectifying K^+ (GIRK) channels (Gao et al., 2003; van den Pol et al., 2004). However, the effect of DA on MCH neurons has yet to be demonstrated. The present study provides evidence showing that DA inhibits MCH neurons by activating α2A adrenoceptors (α2ARs) rather than their cognate receptors. Such cross talk between DA and NE may be important for the regulation of the MCH system and related functions.

Materials and Methods

All experiments were performed following the Canadian Council on Animal Care guidelines and as approved by the Memorial University Institutional Animal Care Committee. Sprague Dawley rats (60–70 g, 3- to 4-weeks-old) were obtained from the breeding colony at Memorial University. Males were used for all experiments except one, which used females (see Results, below).

Under deep halothane anesthesia, brains were removed and 250-μm-thick coronal hypothalamic slices were generated in ice-cold buffer solution [containing 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, and 1 ascorbic acid] or artificial CSF (ACSF) [containing the following

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Correspondence should be addressed to Dr. Michiru Hirasawa, Division of Biomedical Sciences, Faculty of Medicine, Memorial University, 300 Prince Philip Drive, St. John's, Newfoundland and Labrador A1B 3V6, Canada, E-mail: michiru@mun.ca.

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(in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 18 $NaHCO_3$, 10 glucose, 1 ascorbic acid]. Slices were incubated at 33–34°C for 30–45 min in ACSF and then at room temperature until the recording. Solutions were continuously bubbled with O_2 (95%)/CO_2 (5%).

A hemisected slice was perfused in a recording chamber at 1.5–2 ml/min with ACSF at 33–35°C. Whole-cell patch-clamp recording was performed using a Multiclamp 700B amplifier and pClamp 9.2 (Molecular Devices). Recording electrodes were 3–9 MΩ (majority, 3–6 MΩ) when filled with an internal recording solution containing the following (in mM): 123 K-gluconate, 2 $MgCl_2$, 8 KCl, 0.2 EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP, pH 7.3, and 290–295 mOsmol. In some experiments, K-gluconate was replaced with equimolar KCl. Using an infrared-differential interference contrast microscope (DM LFSA; Leica Microsystems), neurons in the dorsal PFA, LH, or zona incerta with a diameter of 10–20 μm were selected. Series resistance was 10–30 MΩ (average, 18.7 ± 5.5 MΩ). To characterize the electrophysiological features and excitability of recorded cells, a series of hyperpolarizing and depolarizing 300 ms step pulses were injected in current-clamp mode (−200 to +100 pA in 100 pA increments). Resting input resistance was calculated from the steady-state voltage response to −100 pA current pulse. MCH neurons were characterized by spike adaptation upon positive current injection and the absence of spontaneous firing, I_h, and rebound depolarization upon negative current offset (Alberto et al., 2006) (Fig. 1*B*); only cells that displayed all of these electrophysiological characteristics were used for analysis. Upon recording, a subset of cells was labeled by including biocytin (1–1.5 mg/ml) in the internal solution. Fifty-six cells displaying these typical characteristics were successfully labeled and 46 of these (82.1%) were determined to be MCH-immunopositive, suggesting that MCH neurons are identifiable by electrophysiological characteristics. Ten remaining cells were neither MCH- nor orexin-immunopositive, some of which could be accounted for by technical issues associated with staining of thick sections.

Voltage-clamp experiments used a holding potential of −70 mV, except in a subset of cells where voltage ramps (−140 to −20 mV in 0.6 s) were applied. Membrane currents were filtered at 1 kHz, digitized at 5 and 1 kHz, and stored for off-line analysis. Hyperpolarizing pulses (20 mV, 100 ms) were applied every 20–60 s to monitor input and series/access resistance.

Data are expressed as mean ± SEM. Student's *t* test (unpaired and paired) and one-way ANOVA with Dunnett's posttest were used for statistical comparisons. $p < 0.05$ was considered significant. Regression lines, EC_{50}, maximal drug response, and half-recovery time were calculated using GraphPad Prism 4.

All drugs were bath perfused or locally applied by ValveBank perfusion system (Automate Scientific) (for concentrations, see Results, below) by diluting aliquots of 1000× stock in the ACSF before use. Local applications were through a four-channel perfusion pencil with a tip diameter of 250 μm located close to the target neuron (~100 μm from the soma). DA stock included ascorbic acid (1 mM) and DA and NE solutions were light-protected throughout the procedures to minimize oxidation. Tertiapin-Q, LE300, sulpiride, SKF81297, quinpirole, yohimbin, and BLR44408 were purchased from Tocris Bioscience. DA, NE, biocytin, picrotoxin, DNQX, D-AP5, and fusaric acid were from Sigma-Aldrich (St. Louis, MO); and tetrodotoxin was from Alomone Labs.

After recordings, some brain slices underwent immunohistochemical verification as described previously (Alberto et al., 2006). Briefly, slices

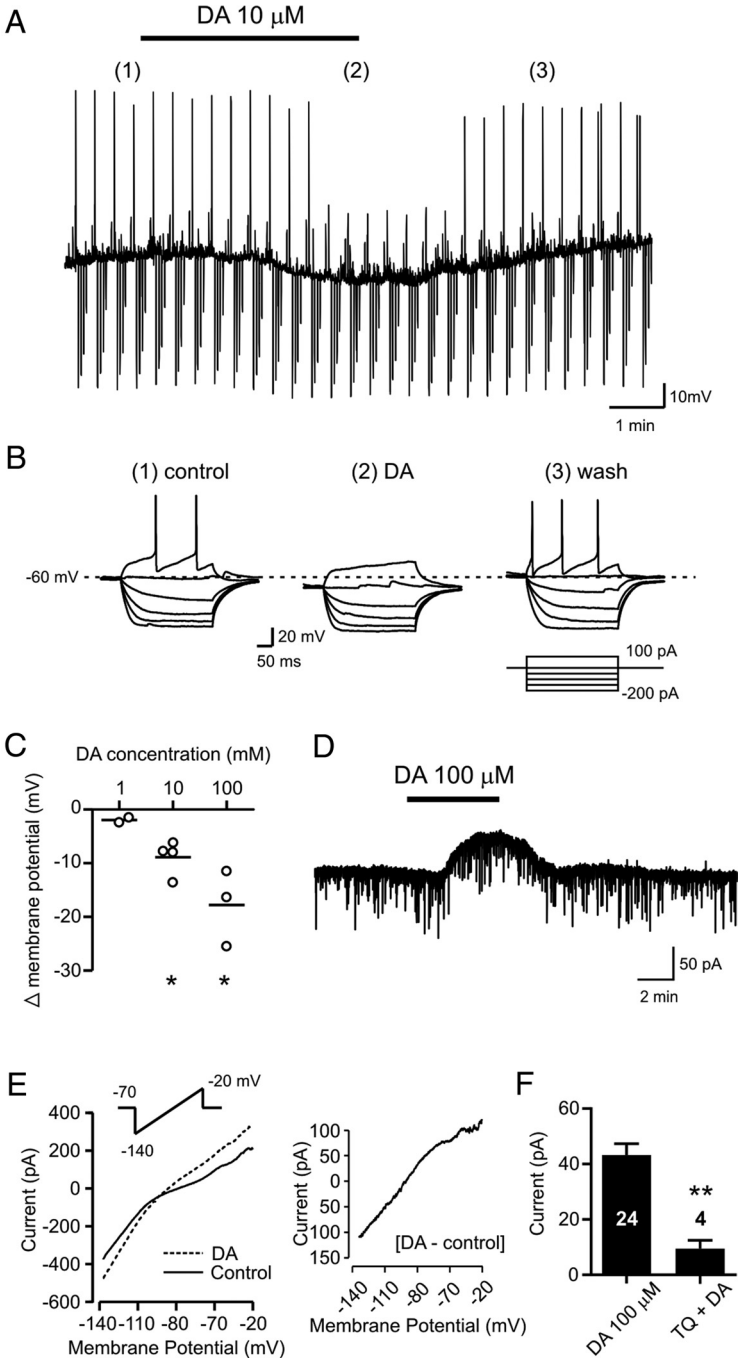

Figure 1. Dopamine inhibits MCH neurons via GIRK channel activation. ***A***, Representative experiment showing a DA-induced reversible hyperpolarization. Vertical lines denote responses to negative and positive current injections. ***B***, Same experiment as in ***A***, except traces from the time points indicated in ***A*** are expanded to show the detailed characteristics of MCH neurons, such as a lack of I_h, rebound depolarization, and spontaneous firing. DA hyperpolarizes MCH cell and abolishes action potentials. ***C***, Changes in resting membrane potential induced by DA. Each dot represents a cell and horizontal lines indicate the average value. $*p < 0.05$, paired *t* test versus baseline. ***D***, Representative voltage-clamp trace showing a DA-induced current. ***E***, Left, Current–voltage relationship in the presence and absence of DA. Inset, Voltage protocol used. Right, Subtracted trace showing the DA-induced current. ***F***, Tertiapin-Q (TQ) attenuates DA current. $**p < 0.01$, unpaired *t* test. Numbers denote the numbers of cells tested.

were fixed in 4% paraformaldehyde or 10% formalin overnight, then incubated with anti-MCH rabbit IgG (1:2000; Phoenix Pharmaceuticals) and anti-orexin A goat IgG (1:2000; Santa Cruz Biotechnology) for 3 d, followed by appropriate secondary antibodies and streptavidin-conjugated amino-methyl-coumarin-acetate (1:500). Slices were examined under a fluorescence microscope for MCH, orexin A immunoreactivity, and biocytin.

Results

To test the effect of DA on the excitability of MCH neurons, DA (1–100 μM) was bath-applied while recording the membrane potential in current-clamp mode. In response to DA, MCH neurons were reversibly hyperpolarized in a concentration-dependent manner (Fig. 1*A–C*) with a concomitant decrease in input resistance by 9.8 and 37.2% at 10 and 100 μM, respectively, (10 μM: $n = 4$, $p < 0.05$; 100 μM: $n = 3$, $p < 0.05$, paired *t* test) from a baseline value of 431.0 ± 39.7 MΩ. In voltage clamp, DA (100 μM) induced an outward current that was readily reversible upon wash (Fig. 1*D*). This effect was observed in 100% of cells tested regardless of the localization within the LH, PFA, and zona incerta. Similar DA currents were observed ($p > 0.05$, ANOVA) with high KCl-containing pipette solution ($n = 11$), in the presence of a Na^+ channel blocker tetrodotoxin (1 μM), glutamatergic receptor antagonists DNQX (10 μM) and D-AP5 (10 μM) ($n = 12$), and $GABA_A$ receptor channel blocker picrotoxin (50 μM; $n = 4$), suggesting that the inhibitory effect of DA on MCH neurons is direct and independent of Cl^-. DA also induced an outward current in MCH neurons from female rats (49.4 ± 6.7 pA, $n = 11$), which was not significantly different from those from males (43.4 ± 3.8 pA, $n = 24$, $p > 0.05$, unpaired *t* test). The remainder of the study was performed on brain slices obtained from male rats.

When a voltage ramp was applied to determine the effect of DA over a wide range of membrane potentials, we found that the DA-induced current reversed at −97.1 ± 3.9 mV with some inward rectification, indicative of a K^+ current involvement ($n = 7$) (Fig. 1*E*). Since DA receptors are G-protein coupled, we speculated that GIRK channels were the effector channels. Thus, we tested the effect of tertiapin-Q (300 nM), a selective GIRK channel blocker, which significantly attenuated the DA-induced outward current (Fig. 1*F*).

We then sought to identify the receptor subtype that mediated the DA current. As shown in Figure 2*A*, neither the D1-like receptor antagonist LE300 [10 nM (Han and Whelan, 2009)] nor D2-like receptor antagonist sulpiride [10 μM (Alberto et al., 2006)] individually or in combination inhibited the DA effect. Moreover, a mixture of D1- and D2-like receptor agonists SKF81297 (10 μM) and quinpirole (50 μM), respectively, failed to induce an outward current. Because DA is known to bind α2Rs in the brain (Cornil and Ball, 2008) and activation of α2Rs has been shown to hyperpolarize MCH neurons (van den Pol et al., 2004), we turned our attention to α2Rs. Yohimbin (5 μM), the α2R antagonist, almost completely blocked the DA-induced current, suggesting that DA does indeed activate these receptors in MCH neurons. There are three α2R subtypes (α2A, 2B, and 2C) and α2AR have been identified in MCH neurons (Modirrousta et al., 2005). Thus, we tested the α2AR-specific antagonist BLR44405 (3 μM) and found that it largely inhibits the effect of DA (Fig. 2*A*). These receptors also mediate the effect of NE (Fig. 2*B*).

While it seems reasonable to conclude that DA was binding to α2ARs, another possible explanation for the apparent cross talk is conversion of DA to NE within the slice, and subsequent binding of NE to its cognate receptors. Thus, we used fusaric acid to specifically block dopamine β-hydroxylase, the enzyme responsible for synthesizing NE from DA (Hidaka, 1971). It was found that fusaric acid (100 μM) pretreatment for 10 min had no effect on DA-induced outward current (Fig. 2*A*), suggesting that DA itself binds adrenoceptors to exert its effect. Concentration–response curves revealed that NE and DA had an EC_{50} of 5.9 and 23.7 μM, respectively, and calculated maximal effect of 106.6 and 57.2 pA, respectively (Fig. 2*C*). Together, our data suggest that DA activates GIRK channels in MCH neurons by directly interacting with α2ARs as a partial agonist.

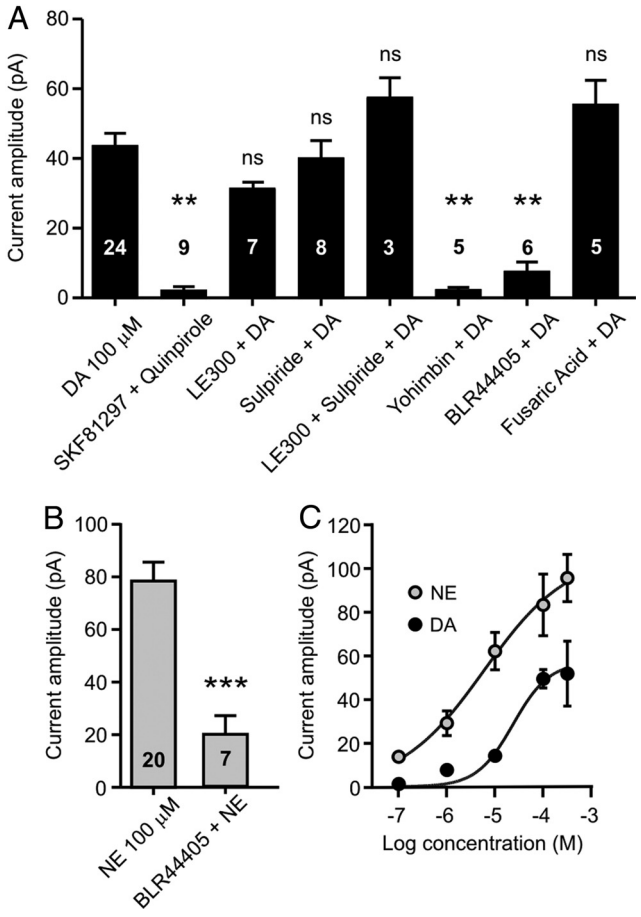

Figure 2. Dopamine acts as a partial agonist of α2A receptors. ***A***, Summary of DA-induced currents in the presence of DA or α2 antagonist. DA effect is blocked by α2 (yohimbin) and α2A antagonist (BLR44405), but not by DA antagonists (LE300 and/or sulpiride). Ineffectiveness of D1- and D2-like agonists is also shown (SKF81297 + quinpirole). DA effect persists in the dopamine-β-hydroxylase inhibitor fusaric acid. $^{**}p < 0.01$ versus DA 100 μM, ANOVA with Dunnett's posttest. ns, Not significant. ***B***, NE response is also inhibited by BLR44408. $^{***}p < 0.0005$, unpaired *t* test. ***C***, Concentration–response curve for DA ($n = 3–8$) and NE ($n = 5–6$), demonstrating that DA is a partial agonist. Numbers in bar graphs indicate cell numbers included.

Agonist-dependent desensitization has been suggested as a general characteristic of GIRK currents (Leaney et al., 2004). Therefore, we tested the hypothesis that DA and NE cross-desensitize each other through α2AR-mediated GIRK current activation. DA or NE (both 100 μM, which induces a robust response) was applied for 3–5 min, and was tested again after a 5 or 10 min wash. When NE was first applied, the subsequent DA current was significantly smaller than that seen when DA was applied for the first time onto the slice (Fig. 3*A*,*E*). A prior DA challenge also attenuated the subsequent DA effect (Fig. 3*B*,*E*). In contrast, NE still induced a current of considerable size following a 5 min DA and 5 min wash, but it was ~50% of the current

induced by NE applied for the first time (Fig. 3*C*,*F*). When NE was applied twice (5 min each, 5 min interval), the second NE current was also significantly desensitized (Fig. 3*D*,*F*). To determine how long it takes for the DA effect to recover from desensitization, DA was applied twice for 5 min each with various intervals, and the second response was normalized to the first one to measure the degree of desensitization. As shown in Figure 3*G*, DA current recovered exponentially with time, with a half-recovery time of 11.9 min ($r^2 = 0.63$).

In contrast to long-term bath applications (3–5 min), brief local applications (5 s) did not induce significant desensitization. When DA was tested following a 5 s NE and 5 min wash, DA still induced a current equivalent to that induced by DA applied for the first time (Fig. 3*H*). Similarly, NE current was not desensitized by prior application of DA for 5 s (Fig. 3*I*). The peak amplitude of DA or NE current was consistent regardless of the mode of application (bath or local), suggesting that the initial peak current was not desensitized even when the compounds were being bath applied (Fig. 3*H*,*I*). This result suggests that significant receptor desensitization occurs only when there is prolonged ligand activity.

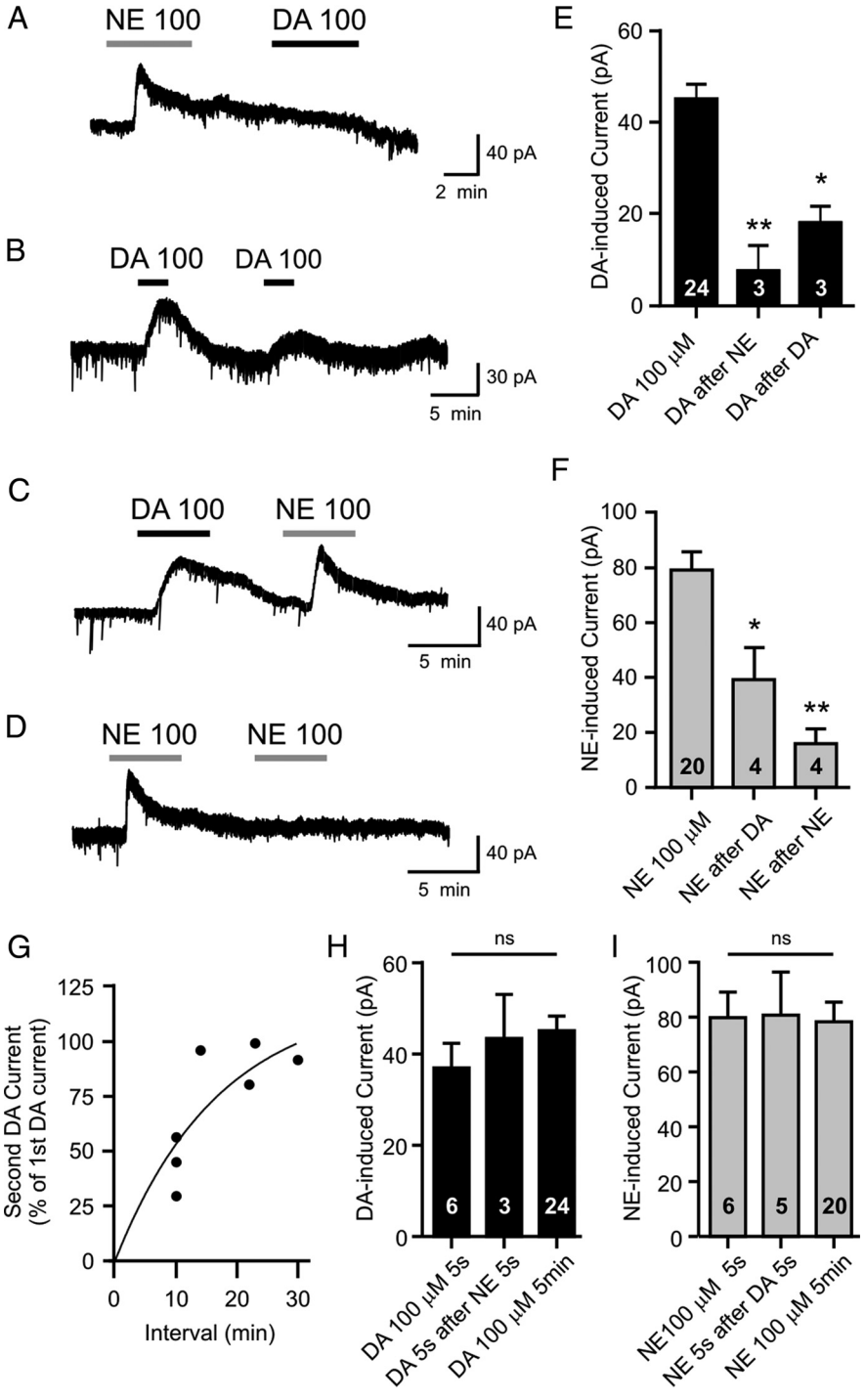

Figure 3. Cross-desensitization of α2A receptors by DA and NE. ***A–D***, Representative traces from MCH neurons showing desensitizing response to repeated applications of NE and/or DA. ***E***, ***F***, Summary of DA (***E***) or NE (***F***) currents following a previous DA or NE application. ***G***, Recovery rate of DA currents from desensitization induced by DA. Second peak current amplitude is normalized to the first one. ***H***, ***I***, α2A receptors do not desensitize by short agonist applications. A 5 s NE application does not desensitize the subsequent DA response (***H***), and vice versa (***I***). For both DA and NE, peak responses to 5 s or 5 min application are comparable. Numbers in bar graphs denote the numbers of cells tested. For comparison, DA 100 μM and NE 100 μM data from Figures 1 and 2 are shown. $*p < 0.05$, $**p < 0.01$. ns, Not significant, ANOVA with Dunnett's posttest.

Discussion

The present study provides evidence that DA directly inhibits MCH neurons by activating α2ARs, but not DA receptors. NE also acts on α2AR subtype to modulate MCH neurons. However, a lack of effect by fusaric acid, the dopamine β-hydroxylase inhibitor, suggests that activation of adrenergic receptors by DA was not due to conversion of DA to NE in the slice preparation, but rather DA itself binding to the receptor. Apparent lack of DA receptor involvement is in agreement with a previous report showing no expression of D1, D2, D3, D4, or D5 receptor mRNA in LH/PFA dorsal to the fornix (Bubser et al., 2005), where MCH neurons are mainly distributed (Bittencourt et al., 1992). DA has been shown to be a functional ligand for α2Rs in several neuronal types (Aguayo and Grossie, 1994; Zhang et al., 1999; Cornil et al., 2002) and displaces α2 ligand virtually everywhere in the brain (Cornil and Ball, 2008). Nonetheless, to the best of our knowledge, this is the first demonstration that DA binds and acts as a partial agonist to endogenously expressed α2A subtype in intact neurons. It is likely that DA binds at least partially within the same catecholamine binding pocket of α2AR as NE, since the radiolabeled α2 antagonist is displaced from these receptors by both DA and NE in competition binding assays (Zhang et al., 1999). According to a study that tested the ability of various ligands to activate α2ARs by measuring the agonist-induced stimulation of [^{35}S]GTPγS binding in isolated cell membranes, NE is much more efficient at activating α2AR than DA (Nyrönen et al., 2001), which may explain the difference in the maximal effects induced by DA and NE in the present study. DA and NE not only share receptors but also the effector channels, namely GIRK

channels in MCH neurons (van den Pol et al., 2004; current study). This is in contrast to what has been shown in normal rat kidney cell line transfected with mouse α2ARs, in which DA and NE induce opposite effects on cAMP accumulation (Zhang et al., 1999).

Our study suggests that EC_{50} of DA is approximately four times higher than that of NE. This may reflect a difference in affinity, as DA has three to four times lower affinity to human and mouse α2ARs than NE in expression systems (Zhang et al., 1999; Nyrönen et al., 2001; Cornil and Ball, 2008). However, our calculated EC_{50}s may be overestimated, because the present study used brain slices in which a combination of diffusion and reuptake can slow down the penetration of bath-applied DA and NE (Nicholson, 1995). Local synaptic and extrasynaptic concentration of endogenous DA in various brain regions has been estimated to be within nanomolar (Ross, 1991; Kawagoe et al., 1992; Garris et al., 1994) to millimolar range when the synaptic geometry is taken into account (Garris et al., 1994). Moreover, using ventral tegmental area slice preparations, DA concentration at postsynaptic DA receptors has been estimated to be >10 μM (Ford et al., 2009). Overall, our result showing that DA significantly hyperpolarizes MCH neurons at 10 μM suggest that the cross talk of DA and α2AR is physiologically relevant for the regulation of the MCH system.

Our study also demonstrated that, because NE and DA activate the same receptors and effector channels, functional cross-desensitization occurs after long applications of either ligand. This indicates that the history of either DA or NE activity could affect the potency of subsequent NE or DA action, in particular after prolonged release onto MCH neurons.

NE and DA are released in the LH in response to nutrient intake (Myers and McCaleb, 1980; Meguid et al., 1995) and injection of NE or DA into the same area reduces feeding (Margules, 1970; Leibowitz and Rossakis, 1978). While the exact subtype of receptors responsible for the satiety effect of NE has not been identified, it is likely that α2ARs expressed by MCH neurons are involved. DA may also induce satiety through the same pathway, although the anorexic effect of DA in the PFA/LH can be reversed by a D2-like receptor antagonist (Leibowitz and Rossakis, 1978). Therefore, other mechanisms may also be important, for example, DA- or NE-mediated inhibition of orexin neurons (Li and van den Pol, 2005; Alberto et al., 2006), another orexigenic cell type localized in the PFA/LH (Sakurai et al., 1998).

α2ARs have been suggested to have anxiolytic and antidepressant properties, as α2AR knock-out mice display increased anxiety- and depression-like behaviors (Schramm et al., 2001; Lähdesmäki et al., 2002). Although α2ARs are widely expressed and have a wide spectrum of functions, the phenotype seen in these animals may at least partially be explained by a lack of suppression on the MCH system. This idea stems from the anxiolytic and antidepressant effects demonstrated by MCH receptor antagonists (Borowsky et al., 2002; Georgescu et al., 2005). Decreasing MCH release from these neurons by activating α2ARs can be expected to have an effect similar to that of MCH receptor antagonists. Furthermore, MCH has been shown to regulate the DA function in the mesolimbic pathway (Smith et al., 2005), which suggests, together with our findings, that DA is capable of regulating its own activity/sensitivity indirectly by regulating MCH neurons.

In summary, our study demonstrates a unique cross talk of DA and NE at MCH neurons. DA binds directly to α2ARs and activates GIRK channels as a partial agonist. Since MCH neurons are implicated in energy balance, reward, and anxiety, targeting these receptors for mood disorders and obesity may be a viable treatment option.

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