Opioids in the hypothalamus control dopamine and acetylcholine levels in the nucleus accumbens

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Abstract

The experimental question is whether hypothalamic opioids, known to stimulate consummatory behavior, control a link to the nucleus accumbens (NAc). It was hypothesized that opioids injected in the hypothalamic paraventricular nucleus (PVN) alter the balance of dopamine (DA) and acetylcholine (ACh) in the NAc in a manner that fosters appetite for food or ethanol. Rats were implanted with two guide shafts, one in the NAc to measure extracellular DA and ACh by microdialysis and the other in the PVN for microinjection of opioid μ- and δ-agonists, an antagonist, or saline vehicle. The compounds tested were morphine, the μ-receptor agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-Enkephalin (DAMGO), the δ-receptor agonist D-Ala-Gly-Phe-Met-NH₂ (DALA), and the opioid antagonist naloxone methiodide (m-naloxone). Morphine in the PVN increased the release of accumbens DA (+41%) and decreased ACh (−35%). Consistent with this, the opioid antagonist m-naloxone decreased DA (−24%) and increased ACh (+19%). In terms of receptor involvement, DAMGO dose-dependently increased DA to up to 209% of baseline. Simultaneously, ACh levels were markedly decreased to 55% of baseline. The agonist DALA produced a smaller but significant, 34% increase in DA, without affecting ACh. In contrast, control injections of saline had no significant effect. These results demonstrate that μ- and δ-opioids in the PVN contribute to the control of accumbens DA and ACh release and suggest that this circuit from the PVN to the NAc may be one of the mechanisms underlying opiate-induced ingestive behavior as well as naltrexone therapy for overeating and alcoholism.

1. Introduction

Hypothalamic peptides are powerful controllers of the urge to eat food and also to consume alcohol (Berthoud and Seeley, 2000; Leibowitz and Hoebel, 2004; Leibowitz, 2007; Schwartz et al., 2000). When injected in the paraventricular nucleus (PVN), opioid peptides are found to initiate eating or prolong a meal once it has started (Leibowitz and Hoebel, 2004; Lenard and Berthoud, 2008; Stanley et al., 1988), and they preferentially stimulate the intake of palatable, fat-rich foods [Leibowitz and...
Hoebe, 2004; Naleid et al., 2007). In rats trained to drink ethanol, PVN opioids also have a stimulatory effect on ethanol intake, which may even usurp their stimulatory effect on food intake (Barson et al., 2008). Conversely, injection of an opioid antagonist in the PVN suppresses feeding (Gosnell et al., 1986; Naleid et al., 2007) and ethanol drinking (Barson et al., 2008). The endogenous opioid peptides, enkephalin and dynorphin, are transcribed within the PVN (Swanson and Sawchenko, 1983), and the eating of a high-fat meal (Chang et al., 2007b) or drinking of ethanol (Chang et al., 2007a) increases the expression of these peptides in this nucleus. The neural mechanisms underlying the stimulatory effect of these opioid peptide systems on consummatory behavior have yet to be defined. The purpose of the present study was to determine whether these PVN opioids influence and possibly function through the neurotransmitter systems of the nucleus accumbens (NAc), a structure involved in reward and motivation.

The functions of the dopamine (DA) system in the NAc appear to be critically involved in motivation and reinforcement (Berridge, 2007; Di Chiara and Bassareo, 2007; Salamone et al., 2007; Schultz, 2002; Wise, 2008). Results from local NAc injections of DA or amphetamine suggest that an abrupt rise in extracellular DA can facilitate seeking and intake of food, ethanol, or other drugs of abuse (Kelley et al., 1989; Pal and Thombre, 1993; Samson et al., 1999). With injection in the NAc shell, DA elicits increases in feeding behavior, and specific DA receptor agonists or antagonists can influence effort in seeking food or food-associated conditioned stimuli (Bald and Kelley, 2007). In addition, the ingestion of food, ethanol, or other drugs can themselves cause the release of accumbens DA (Di Chiara et al., 2004; Hernandez and Hoebe, 1988; Martel and Fantino, 1996; Weiss et al., 1996), and daily binging on a sugar solution repeatedly increases the release of DA in the NAc shell, resulting in signs of opioid dependency (Avena et al., 2008b). These studies provide strong evidence for a positive relationship between DA and appetitive or consummatory behavior.

In contrast to DA, there is evidence that acetylcholine (ACh) in the NAc may show an inverse relationship to consummatory behavior and opioids that promote consumption. Morphine decreases extracellular ACh while releasing DA (Rada et al., 1991, 1996), and ACh rises during withdrawal from morphine, ethanol, or sugar as DA levels decline (Avena et al., 2008a; Rada et al., 1991, 2004b). Also, ACh rises with ordinary satiety during a meal (Avena et al., 2006; Mark et al., 1992) and in response to an aversively conditioned taste stimulus (Mark et al., 1995). Thus, it has been proposed that ACh levels increase in the NAc as consummatory responses are inhibited and in association with negative mood states (Hoebe et al., 2007).

The orexigenic peptide, galanin, is similar to the opioids in that it stimulates food and ethanol intake when injected in the PVN (Kyrkouli et al., 1990; Leibowitz et al., 1998; Rada et al., 2004a; Schneider et al., 2007). Prior research has shown that PVN injection of galanin activates the mesolimbic system, increasing the release of DA in the NAc shell while reducing levels of ACh (Rada et al., 1998). Interestingly, these effects were seen only in rats that exhibited enhanced feeding in response to galanin injection. Further, the feeding response elicited by galanin is found to be blocked by an opioid antagonist (Dube et al., 1994). This evidence has led us to test the possibility that opioids in the PVN may act in a similar manner to galanin, altering the balance between accumbens DA and ACh, enhancing DA and decreasing ACh release, in a way that favors ingestive behavior. Support for this possibility would implicate PVN opioid receptors in the control of DA-mediated motivation for food or ethanol.

To test this hypothesis, the present experiment used microdialysis in the NAc to measure DA and ACh after PVN injection of opioids. Different opioid agonists were tested, in addition to the opioid antagonist, naloxone methiodide (m-naloxone), which remains close to the injection site for a longer period than naloxone (Schroeder et al., 1991). The doses used in the PVN were the same as those known to stimulate food or ethanol intake when injected in this nucleus (Barson et al., 2008; McLean and Hoebe, 1983; Naleid et al., 2007; Quinn et al., 2003; Stanley et al., 1988). The findings of this study provide strong support for a close relationship between the PVN opioid peptides that promote consummatory behavior and the accumbens neurotransmitters involved in reward-related behavior.

2. Results

2.1. Morphine in the PVN enhances DA release and attenuates ACh

Morphine (12.7 nmol) injected in the PVN significantly increased DA release while it reduced extracellular ACh in the NAc. This opiate increased DA release more than saline.
(n=5/group; F(1,8)=9.00, p<0.05), and this effect shifted over time (F(11,88)=2.92, p<0.01), with extracellular DA levels in morphine-injected animals increasing to 141±13% of baseline at 120 min post-injection (Fig. 1A). In addition, morphine compared to saline affected levels of the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC; F(1,8)=44.98, p<0.01), which increased to 151±8% of baseline at 160 min post-injection (F(11,88)=17.10, p<0.001), and of the DA metabolite homovanillic acid (HVA; F(1,8)=83.91, p<0.001), which increased to 200±15% of baseline at 180 min post-injection (F(11,88)=18.13, p<0.001). Conversely, as seen in Fig. 1B, morphine decreased ACh release more than saline (F(1,8)=10.96, p<0.05), and the levels of ACh reached a nadir of 65±10% of baseline at 80 min post-injection (F(11,88)=7.56, p<0.001). Saline injection in the PVN did not significantly affect DA or ACh release (Figs. 1A and B) or levels of the metabolites.

Post-hoc tests revealed that these effects in the NAc resulting from morphine in the PVN were long lasting. Levels of DOPAC, which were significantly greater than baseline starting at 60 min post-injection (p<0.01), continued to be higher at the conclusion of the test 200 min later (p<0.001). The levels of HVA, which were significantly higher than baseline beginning 80 min post-injection (p<0.01), also continued to be significantly greater at 200 min (p<0.001). Similarly, ACh was significantly lower than baseline beginning at 60 min post-injection (p<0.01), and it remained significantly reduced for the full 3 h recording (p<0.01).

2.2. M-naloxone attenuates DA and enhances ACh release, the opposite of morphine’s effect

M-naloxone (1 nmol) injected in the PVN reduced extracellular DA and increased ACh in the NAc. M-naloxone compared to saline decreased DA release (n=8/group; F(1,14)=5.72, p<0.05), and this effect of m-naloxone injection changed over time (F(8,112)=3.38, p<0.01), with extracellular DA levels decreasing to 76±5% of baseline at 60 min post-injection (Fig. 2A). Although m-naloxone did not significantly affect metabolite levels compared to saline (DOPAC; F(1,14)=1.64, ns; HVA; F(1,14)=1.38, ns), this opiate agonist significantly affected these metabolites over time (DOPAC; F(8,112)=2.86, p<0.05; HVA; F(8,112)=2.59, p<0.05). The metabolite DOPAC decreased to 85±5% of baseline at 120 min post-injection, and HVA decreased to 87±6% after 120 min. In contrast, as seen in Fig. 2B, m-naloxone increased ACh release more than saline (n=5/group; F(1,8)=8.64, p<0.05), with levels increasing to 119±10% above baseline and reaching a maximum at 20 min (F(8,64)=2.80, p<0.05). Saline injected in the PVN did not affect DA or ACh release (Figs. 2A and B) or levels of the metabolites.

2.3. DAMGO, like morphine, enhances DA release and attenuates ACh

Given that morphine acts primarily on μ- and δ-receptors, we next tested DAMGO and DALA in the PVN. Of the two receptor-specific agonists, DAMGO showed the larger effect, with both doses (2 and 4 nmol) increasing DA release and metabolism while decreasing extracellular ACh. Across time, DAMGO significantly affected levels of DA (F(16,136)=5.58, p<0.001), DOPAC (F(16,136)=9.89, p<0.001), HVA (F(16,136)=11.55, p<0.001), and ACh (F(16,128)=3.36, p<0.001). At the higher dose (4 nmol), DAMGO compared to saline stimulated DA release (n=7/group; F(1,12)=16.91, p<0.01), causing it to increase to 209±23% of baseline at 80 min post-injection (Fig. 3A; F(8,96)=8.85, p<0.001). This dose of DAMGO compared to saline also affected DOPAC levels (F(1,12)=18.36, p<0.01), which increased to 220±29% of baseline at 100 min (F(8,96)=15.97, p<0.001), and HVA levels (F(1,12)=27.04, p<0.01), which increased to 235±31% at 100 min (F(8,96)=20.58, p<0.001). Levels of ACh concur-
dently decreased (F(1,12)=10.44, p<0.05), reaching a nadir of 55±6% at the 40 min time point (Fig. 3B; F(8,96)=8.01, p<0.001). At the lower dose (2 nmol), DAMGO had similar effects although they were smaller in magnitude. It increased DA release compared to saline (n=6; F(1,11)=7.30, p<0.05) and raised extracellular DA levels to 135±14% of baseline at 60 min (Fig. 3A; F(8,88)=3.73, p<0.01). This lower dose of DAMGO also affected DOPAC levels (F(1,11)=21.95, p<0.01), which increased to 151±13% at baseline at 80 min (F(8,88)=14.31, p<0.001), and HVA levels (F(1,11)=33.85, p<0.01), which increased to 190±18% also at 80 min (F(8,88)=22.93, p<0.001). As with the higher dose, levels of ACh after 2 nmol of DAMGO were also affected (n=5; F(1,10)=8.46, p<0.05), declining to 69±5% of baseline at 40 min post-injection (Fig. 3B; F(8,80)=6.61, p<0.001). Saline injected in the PVN did not affect DA or ACh release (Figs. 3A and B) or metabolite levels.

Direct comparisons between the two doses of DAMGO revealed a significantly greater DA release at the higher dose (4 nmol) compared to the lower dose (2 nmol) across time (F(8,88)=2.64, p<0.05). This occurred at two time points, 80 and
100 min (p<0.05) (Fig. 3A). The increase in DOPAC levels at the higher dose was also significantly greater than the lower dose across time (F(8, 88)=2.87, p<0.05), occurring at the 100- and 120-min time points (p<0.05). In contrast, no significant differences were observed with the measurements of ACh (Fig. 5B) and HVA.

2.4. DALA in the PVN enhances DA release but does not affect ACh

DALA (16 nmol) injected in the PVN significantly increased DA release but had little effect on ACh levels. Injection of DALA increased DA release more than saline (n=5/group; F(1, 8)=7.81, p<0.05). This effect changed over time (F(8, 64)=5.08, p<0.001), with extracellular DA levels in DALA-injected animals increasing to 134±10% of baseline at 40 min post-injection (Fig. 4A). In addition, DALA affected DOPAC levels (F(1, 8)=10.70, p<0.05), which increased to 136±11% of baseline 60 min after injection (F(8, 64)=7.09, p<0.001), and also HVA levels (F(1, 8)=13.07, p<0.05), which increased to 144±13% of baseline at 60 min (F(8, 64)=10.23, p<0.001). Unlike morphine and DAMGO, DALA did not significantly affect ACh levels (F(1, 8)=1.26, ns), which only decreased to 90±12% of baseline after 40 min (Fig. 4B; F(8, 64)=1.20, ns). Saline injected in the PVN had no effect on DA or ACh release (Figs. 4A and B) or the metabolites.

2.5. Histology

Histology showed that microinjections were made at the dorsal aspect of the PVN and that the microdialysis probes were located in the medial shell region of the NAc or in the transition zone between the shell and the core (Fig. 5).

3. Discussion

It was hypothesized that PVN injection of opioids would activate the mesolimbic DA system and inhibit the ACh system in a manner similar to galanin (Rada et al., 1998) and consistent with opioid-induced changes in ingestive behavior (see Introduction). Results of the present study provide evidence for a strong influence of PVN opioids on the balance of DA and ACh in the NAc shell and transition zone. When injected in this hypothalamic nucleus, the general opioid agonist morphine, the specific μ-agonist DAMGO, and the specific δ-agonist DALA were found to enhance extracellular levels of DA relative to ACh, while the opioid antagonist naloxone produced the opposite effect.

The action of morphine in the PVN was to enhance the release of DA in the accumbens. The fact that PVN naloxone injection produced the opposite effect confirms the role of opioid receptors in this hypothalamic site and suggests that endogenous opioids exert a tonic effect on DA levels in the

Fig. 3 – Effects of DAMGO (2 and 4 nmol) and saline injected into the PVN. (A) DAMGO 2 nmol (solid triangles) and 4 nmol (solid squares), but not saline (open squares), increased extracellular DA in the NAc, with the higher dose leading to a significantly greater response. (B) DAMGO 2 nmol and DAMGO 4 nmol, but not saline, decreased extracellular ACh in the NAc but did not produce significantly different responses from each other (mean±SEM, **p<0.01, *p<0.05 for main effects of ANOVA).

Fig. 4 – Effects of DALA (16 nmol) or saline injected in the PVN. (A) DALA (solid squares), but not saline (open squares), increased extracellular DA. (B) Neither DALA nor saline caused a significant change in extracellular ACh in the NAc (mean±SEM, *p<0.05 for main effects of ANOVA).
accumbens. While morphine and naloxone can affect all opioid receptor types, they act preferentially at the μ-receptor and somewhat less at the δ-receptor (Gillan et al., 1980). Therefore, tests were undertaken using specific agonists of these receptors. The effects of the μ-receptor agonist DAMGO in the PVN exceeded those of morphine, while the δ-receptor agonist DALA produced the smallest effect. At the higher dose, DAMGO doubled the measurable levels of DA in the NAc compared to baseline, suggesting the possibility of a very strong influence of μ-opioid inputs to the PVN on DA release in the accumbens. The δ-agonist DALA also stimulated extracellular DA, although this effect was considerably smaller and required a higher dose. Whereas it is difficult to draw sharp demarcations between μ- and δ-receptor function given the likelihood of some overlap in agonist action and the coupling of some opioid receptors into heterodimers (Levac et al., 2002; Mansour et al., 1995; Pasternak, 2004), it is interesting that this difference between DAMGO and DALA in the release of accumbens DA corresponds with the greater effects of μ-agonists compared to δ-agonists in the PVN on food intake (Stanley et al., 1988). This evidence provides a link between the neurochemical changes induced by the PVN opioids and the behavioral effects of these opioids.

In contrast to DA, the release of ACh in the accumbens was reduced by PVN injection of morphine, while it was increased by naloxone. Of the receptor-specific agonists, DAMGO at both doses also diminished ACh release to a comparable extent to that of morphine. While DALA showed a tendency to decrease ACh, this effect did not reach statistical significance. The fact that morphine and DAMGO, but not DALA, suppressed ACh release suggests that the effects of morphine on the accumbens are driven more by μ-receptors than by δ-receptors. This difference in the inhibition of ACh release may also relate to the greater effects of μ-agonists compared to δ-agonists in the PVN on food intake (Stanley et al., 1988). These findings show that ACh, similar to DA, can be controlled from distant areas of the brain. They also demonstrate that these neurotransmitters can be modulated independently, with changes in DA levels not necessarily associated with opposite changes in ACh as indicated by studies showing an increase ACh as well as DA in relation to satiety (Avena et al., 2006; Mark et al., 1992).

Given that the intake of food and ethanol are stimulated by opioids in the PVN, the findings here suggest that this may occur through a shift in the DA/ACh balance of the NAc, with enhanced activity in the mesolimbic DA system accompanied by a reduction in ACh release. This is consistent with the finding that galanin stimulates DA only in rats that, on separate days, respond with a feeding response after galanin injection (Rada et al., 1998). The increase in accumbens DA may influence ingestion in different ways, for example, by enhancing incentive motivation based on the memory of reward that increases willingness to work (Kelley et al., 2005b; Robinson et al., 2007; Salamone et al., 2007). Since the microdialysis probes in the present experiment were located in both the NAc shell and the transition zone, and recent research indicates that this zone responds in a way intermediate between the NAc shell and core (Hipolito et al., 2008; McKittrick and Abercrombie, 2007; Rebec et al., 1997), the release of DA by PVN opiate stimulation may represent the crossover between motivation and motor function. With regard to the decrease in ACh induced by PVN injection of morphine and DAMGO, this may also enhance feeding by diminishing the aversive effects associated with a rise in ACh (Hoebel et al., 2007). There is pharmacological evidence, however, suggesting that accumbens ACh may have a more complex role in controlling food intake and motor learning (Kelley et al., 2005a,b), involving both nicotinic and muscarinic receptor systems (Hoebel et al., 2007; Pratt and Blackstone, 2009). Although the precise roles of accumbens DA and ACh remain a matter of debate, the marked opioid-induced change in the overall balance of DA and ACh observed in the present study takes on particular significance in light of extensive evidence linking PVN and forebrain opiates to pleasure, palatability and the inhibition of satiety that leads to overconsumption (Kelley et al., 2005a; Leibowitz, 2007).

The neurocircuit through which PVN opioids control the release of DA in the NAc is not known, although there are several logical possibilities. One is a direct connection from the PVN to the accumbens, with an action on DA terminals. The PVN contains numerous outputs, which could play this role, including a corticotrophin-releasing factor (CRF) pathway that projects, in part, to the accumbens (Merchenthaler et al., 1982). A second possibility is a known CRF projection from the PVN to the DA cell bodies in the ventral tegmental area (VTA) (Rodauros et al., 2007), where CRF Type 1 receptors stimulate dopaminergic cell firing (Wanat et al., 2008). Central opioid injection enhances...
CRF gene expression in the PVN (Wang et al., 1996), likely through disinhibition involving the GABA interneurons that surround this nucleus (Meister et al., 1988). There is a third possibility, which involves an opioid-to-opioid projection to neurons in the VTA (Quinn et al., 2003) that, in turn, project to the NAc to release DA (Di Chiara, 2002). Other indirect routes from the PVN that control accumbens DA release may involve PVN projections to hindbrain nuclei, such as the parabrachial nucleus and nucleus tractus solitarius, which project back to the midbrain cholinergic system that excites DA cells in the VTA (Rada et al., 2000; Yeomans, 1995). This VTA release of DA into the accumbens may be greater in the transition zone than in the shell (McKittrick and Abercrombie, 2007).

In contrast to these projection systems controlling DA, the release of accumbal ACh is considered to be from terminals of intrinsic interneurons (Hoover et al., 1978; Meredith et al., 1989). It has been proposed that these ACh interneurons are controlled by a path that travels from the hypothalamus to the NAc (Zhou et al., 2003). The evidence suggests that the opioids may affect the ACh interneurons in the NAc through different mechanisms. They can have direct effects in the NAc, where µ-receptors exist on ACh dendrites and terminals (Svingos et al., 2001) and µ- and δ-agonists are found to inhibit accumbal ACh release (Heijna et al., 1990, 1992). They can also have indirect effects via the VTA and release of DA, which can then, via D2 receptors, inhibit accumbens release of ACh (Zhou et al., 2003). Although DA via D1 receptors may enhance ACh (Zhou et al., 2003), the present results suggest that PVN opioids, by projecting directly to the NAc or indirectly via the VTA, act primarily to reduce ACh.

The findings of the present study, showing PVN opioids to affect the release of accumbens DA and ACh, have implications for the treatment of the overconsumption of food or alcohol. Systemic delivery of naltrexone, a long-acting opioid antagonist, has been used with occasional success in the treatment of binge-eating disorder and bulimia (Alger et al., 1991; Marrazzi et al., 1995). It has also achieved recognition in the treatment of alcoholism (Anton et al., 2008; O’Brien et al., 1996). In light of the effect of m-naloxone in the present experiment, part of the effectiveness of naltrexone therapy may be due, in part, to a PVN opioid-induced decrease in the DA relative to ACh that normally rises in association with the consumption of palatable foods and alcohol.

4. Experimental procedures

4.1. Subjects and surgery

Male Sprague–Dawley rats (200–250 g) were obtained from Taconic Farms (Germantown, NY, USA) and housed individually on a reversed 12:12-h light/dark cycle with lights off at 6:00 am. The subjects had access to LabDiet rodent chow (St. Louis, MO) and water on an ad libitum basis except during the microdialysis tests. All procedures were approved by the Princeton University Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines on the ethical use of animals. Efforts were made to minimize the use and suffering of animals.

All rats underwent surgery to implant guide cannulas for microinjections and microdialysis. They were anesthetized with 10 mg/kg xylazine and 80 mg/kg ketamine (i.p.), supplemented with ketamine as needed. Guide shafts (21-gauge stainless steel, 10 mm in length) were implanted unilaterally and perpendicularly in the PVN (B = −1.8, L 0.4, V 3.8) and NAc (B 1.2, L 0.8, V 4.2), with reference to bregma, the midsagittal sinus, and the level skull surface. Placement was alternated so that half of the subjects in each group had guide shafts in the right hemisphere and half in the left hemisphere. Between procedures, stainless steel stylets were left in the guide shafts to prevent occlusion. Microdialysis probes for the NAc were inserted at least 1 week after surgery and protruded 5 mm from the guide shaft to reach the ipsilateral, posterior medial NAc shell. Injectors for the PVN protruded 4.5 mm from the guide shaft to reach the intended site.

4.2. Microinjection procedures and drugs

A modified version of Parada et al’s (1993) microinjection procedure was used for PVN injections. This technique allows injections of very small volumes without disturbing the animal. The injectors were made of fused silica tubing (37 µm i.d. × 160 µm o.d.) and reached a ventral coordinate 8.3 mm below the skull surface. Drug doses were selected based on results from prior studies that showed their effects, when injected in the PVN, on food or ethanol intake (Barson et al., 2008; McLean and Hoebel, 1983; Naleid et al., 2007; Quinn et al., 2003). Injected drugs were morphine sulfate (12.7 nmol), m-naloxone (1 nmol), and [D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin (DAMGO; 4 nmol and 2 nmol) (Sigma-Aldrich Co., St. Louis, MO) and d-Ala-Gly-Phe-Met-NH2 (DALA; 16 nmol) (American Peptide Co., Inc., Sunnyvale, CA). Drugs were dissolved in preservative-free 0.9% sodium chloride solution (Hospira Inc., Lake Forest, IL) and prepared fresh immediately prior to microinjection. Injections of drug or saline vehicle were given approximately 5 h into the dark cycle, after baseline levels of DA and ACh stabilized. A 10-µl syringe was used to deliver 0.5 µl in 30 s, and the injector was left in place an additional 30 s to allow diffusion. In total, fifty rats were used, with each drug tested in 5–8 animals. Forty-six rats received a single session of microdialysis following microinjection. Four animals were used for two consecutive days of microdialysis. In these cases, the drugs injected each day were different (e.g., DALA on Day 1 and m-naloxone on Day 2) and the order of presentation between days was randomized.

4.3. Microdialysis procedures

Microdialysis probes were constructed of silica glass tubing (37 µm inner diameter, Polymicro Technologies Inc., Phoenix, AZ, USA) inside a 26-gauge stainless-steel tube with a microdialysis tip of cellulose tubing sealed at the end with epoxy (Spectrum Medical Co., Los Angeles, CA, USA, 6000 molecular weight, 0.2 mm outer diameter × 2 mm long) (Hernandez et al., 1986). Probes were inserted and fixed in place with acrylic cement 14–16 h before each experiment to allow neurotransmitter recovery to stabilize. Probes were perfused with buffered Ringer’s solution (142 mM NaCl,
3.9 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂, 1.35 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, pH 7.35) at a flow rate of 0.5 μl/min overnight and at 2.0 μl/min 2 h before starting, and throughout the experiment. Neostigmine (0.3 μM) was added to the perfusion fluid to improve basal recovery of ACh by hindering its enzymatic degradation (Rada et al., 2007). Starting 4 h into the dark cycle and continuing throughout the experiment, samples were collected every 20 min and then each was split, half for DA analysis (20 μl) and half for ACh (20 μl). Food and water were removed immediately before the start of sample collection, when the animals presumably were satiated, since it is known that feeding and drinking can increase accumbens extracellular levels of DA and ACh (Hernandez and Hoebel, 1988; Mark et al., 1992).

4.4. Microdialysis assays for DA, DOPAC, HVA and ACh

All samples were analyzed immediately after collection. DA and its metabolites DOPAC and HVA were analyzed by reverse phase, high performance liquid chromatography with electrochemical detection (HPLC-EC). Samples were injected into a 20-μl sample loop leading to a 10-cm column with 3.2-mm bore and 3 μm C18 packing (Brownlee Co. Model 6213, San Jose, CA, USA). The mobile phase was comprised of 60 mM sodium phosphate, 100 μM EDTA, 1.24 mM heptanosulfonic acid, and 5% vol/vol methanol (pH 3.6). DA, DOPAC, and HVA were measured coulometrically (ESA Co. Model 5100A, Chelmsford, MA, USA) with the conditioning potential at +500 mV and working cell potential set at ~400 mV. The mean basal concentration of DA in the dialysate was 0.41 ± 0.13 nM.

ACh was also measured by reverse phase HPLC-EC using a 20-μl sample loop with a 10-cm C18 analytical column (Chrompack Inc., Palo Alto, CA, USA). ACh was converted to betaine and hydrogen peroxide with an immobilized-enzyme reactor containing acetylcholinesterase and choline oxidase (Sigma Chemicals, St. Louis, MO) and column (Chrompack Inc.). The mobile phase was 200 mM potassium phosphate at pH 8.0. An amperometric detector (EG&G Princeton Applied Research, Lawranceville, NJ, USA) oxidized the hydrogen peroxide on a platinum electrode (BAS, West Lafayette, IN, USA) set at 500 mV with respect to a Ag-AgCl reference electrode (EG&G Princeton Applied Research). The mean basal concentration of ACh in the dialysate was 26.00 ± 7.50 nM.

4.5. Histology

At the end of the experiment, histology was performed to verify microinjector and microdialysis probe placements. Rats were sacrificed by rapid decapitation; brains were kept in formalin for a minimum of 1 week, then sliced in 40-μm sections on a freezing microtome and slide-mounted for microscopic verification. Two animals had probes more than 0.5 mm from the target regions according to the atlas of Paxinos and Watson (1997) and therefore discarded from the analysis.

4.6. Data analysis

Microdialysis data were normalized to percent of the baseline samples (two samples for morphine injection, three samples for all others), and the data were analyzed first by two- or three-way repeated measures ANOVA, with injection type as the between-subject factor and time as the within-subject factor. This was followed by Newman-Keuls post hoc comparisons with one-way repeated measures ANOVA when justified. Where appropriate, differences between doses at specific time points were analyzed by independent-samples t-tests.

Acknowledgments

This research was supported by USPHS Grants AA12882 (B.G.H. and S.F.L.) and DA21518 (S.F.L.) and funds from the E.H. Lane Foundation (B.G.H.).

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