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Investigation of Morphine-Induced Dopamine Release in the Nucleus Accumbens by Fiber Photometry

Nucleus Accumbens'te Morfine Bağlı Dopamin Salınımının Fiber Fotometri ile Araştırılması

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ÖZET

Amaç: Morfin, akut ve kronik ağrı tedavisinde sıklıkla kullanılan bir opiyattır. Nucleus accumbens (NAc) ödül yolunun en temel merkezidir. Bağımlılık gelişimi ve ödül yolağının aktif olması bölgedeki dopaminerjik aktivitenin değişimi ile ilişkilendirilmiştir. Ardışık morfin kullanımı ventral tegmental alandaki dopaminerjik nöronların aktivasyonu yoluyla nükleus akumbenste dopamin düzeylerini artırır. Son zamanlarda popüler olmaya başlayan fiber fotometri sistemiyle dopaminerjik sensörlerden hızlı, anlık, tekrarlanabilir ve in vivo dopaminerjik sinyal kaydı yapılmaktadır. Konuyla alakalı çeşitli çalışmalar olmasına rağmen mevcut çalışmalar sınırlıdır. Çalışmanın amacı, yüksek teknolojili fiber optik fotometri sistemi kullanarak morfin bağımlılığı ve yoksunluğu sırasında NAc'deki dopaminerjik sinyalleri dopaminerjik sensör (GRABDA) vasıtasıyla araştırmaktır.

Gereç ve Yöntemler: Erkek Wistar sıçanlar morfin (M) ve morfin+nalokson (M+N) olmak üzere iki gruba ayrılmıştır. Fiber fotometri kaydı için tüm hayvanların sağ NAc bölgesine GRABDA enjekte edilmiştir. GRABDA, yapısında dopamin reseptörü 2'ye (DRD2) dayalı bir tanıma alanı içerir. Bu alana dopamin bağlandığında sensördeki yeşil floresan protein aktive olur. Oluşan floresan değişim, bölgedeki dopaminerjik aktiviteyi ve sinyal iletimini yansıtır. Daha sonra aynı bölgeye bir fiber optik kablo diş çimentosu ile sabitlenmiştir. 15 günlük dinlendirmenin ardından, 5 gün boyunca 10mg/kg morfin intraperitoneal olarak enjekte edilmiştir. Son enjeksiyondan sonra M+N grubuna 3mg/kg nalokson enjekte edilmiştir. Dopaminerjik sinyali ifade eden ortalama ΔF/F (%) değerleri, fiber fotometrik kayıtlar toplandıktan sonra Python ile hesaplanmıştır. ΔF/F (%) değeri, DRD2 reseptörlerine dopamin bağlanma düzeyini, normalize ederek yüzdelik olarak ifade eder. İstatistiksel karşılaştırmalar karma-ANOVA testi ve post-hoc karşılaştırmalar tahmini marjinal ortalamalar kullanılarak yapıldı.

Bulgular: M grubunun ΔF/F (%) değerleri morfin enjeksiyonu ile başlangıca kıyasla artmıştır ($p<0.05$). Nalokson enjeksiyonları sonucunda, M+N grubunun ΔF/F (%) değerleri M grubuna kıyasla önemli ölçüde azalmıştır ($p<0.05$). Morfin bağımlılığı NAc dopaminerjik sinyalini artırır. Nalokson bu artışı baskılar.

Sonuç: NAc'deki dopaminerjik aktivitenin yeni biyosensörlerle fiber fotometri ile saptanması, sıvı kromatografisi gibi eski tekniklerden daha etkili kanıt sağlama potansiyeline sahiptir. Konunun daha iyi anlaşılması için spesifik nöronal ve bölgesel çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Bağımlılık, dopamin, fiber fotometri, GRAB sensörleri, morfin, nalokson, sıçan.

ABSTRACT

Objective: Morphine is an opiate frequently used in the management of acute and chronic pain. Sequential use of morphine increases dopamine levels in the nucleus accumbens (NAc) through activation of dopaminergic neurons in the ventral tegmental area. The aim of the study was to investigate dopaminergic signals in the NAc during morphine dependence and withdrawal using fiber photometry system with dopaminergic sensor (GRABDA).

Materials and Methods: Male Wistar rats were divided into two groups as morphine (M) and morphine+naloxone (M+N). GRABDA was injected into the right NAc region. GRABDA contains a recognition domain based on the dopamine receptor 2 (DRD2) in its structure. When dopamine binds to this domain, the green fluorescent protein in the sensor is activated. The resulting fluorescence change reflects dopaminergic activity and signal transmission in the region. Afterwards, a fiber optic cable was fixed to NAc. After 15 days of rest, 10mg/kg morphine was injected intraperitoneally for 5 days. 3mg/kg naloxone was injected into the M+N group after the last injection. Average ΔF/F (%) values expressing dopaminergic signalling were calculated with Python after collecting fiber photometric records. The ΔF/F (%) value expresses the normalised level of dopamine binding to DRD2 receptors as a percentage. Statistical comparisons were made using a mixed-ANOVA test and post-hoc comparisons were made using estimated marginal means.

Results: The ΔF/F (%) values of the M and M+N groups increased with morphine injection compared to baseline period ($p<0.05$). As a result of naloxone injections, the ΔF/F (%) values of the M+N group significantly decreased compared to the M group ($p<0.05$). Morphine addiction increases dopaminergic signalling in the NAc, while naloxone suppresses this effect.

Conclusions: Fiber photometry with a novel biosensors enables more efficient detection of dopaminergic activity compared to older methods such as liquid chromatography. Further studies are needed for a better understanding of specific neuronal and regional processes.

Keywords: Addiction, dopamine, fiber photometry, GRAB sensors, morphine, naloxone, rat.

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INTRODUCTION

Morphine is an opiate widely used in chronic and acute pain management (1). The development of dependence may often limit morphine usage in many clinical cases. The drug addiction can cause social and financial problems worldwide (2, 3). Five types of opioid receptor have been discovered: mμ (MOR), kappa, delta, nociception and zeta (4). It's reported that opiates' habit-forming effects in the ventral tegmental area are mediated by mu and delta opioids receptors (5). Main effects of morphine are mediated through activation of the MOR, as the analgesic, rewarding and withdrawal aversive effects of morphine are abolished in MOR-deficient mice (6, 7). Naloxone is often used to reverse the clinically disabling effects of opioid overdose. Naloxone blocks the effects of opioids on their receptors by acting as a competitive antagonist of MORs. It has been widely used to induce withdrawal symptoms in rodents and to induce a withdrawal syndrome that produces adverse, stress-like states (8).

The nucleus accumbens (NAc) contains a high density of opioid receptors (9, 10). Morphine increases MOR-mediated dopamine release in the NAc (11). MOR agonists induce dopamine release in both core and shell areas of the NAc (12). MOR regulates dopamine release through disinhibition via GABA'ergic interneurons in the ventral tegmental area (VTA) (13). Dopaminergic projection to the NAc from VTA is the primary fundamental signalling circuit in opiate addiction (14). In addition, all drugs that cause addiction are known to increase dopaminergic activity in the NAc (15).

Dopamine is significantly implicated in the integration of reward and addiction mechanisms (16). In the human adult brain, dopaminergic neurons are found in the mesencephalon, diencephalon, and olfactory bulb. These neurones are most abundant in the ventral region of the mesencephalon, VTA and retrobulbar area (17). Dopaminergic neurons in the brain have four main pathways to the target areas. These mesolimbic, nigrostriatal, mesocortical and tuberoinfundibular system pathways play roles in integration of central motor coordination and several limbic functions such as some central mechanisms related to feeding, reward mechanisms, creation of attention and consciousness and some processes of learning (18). The mesolimbic dopaminergic system is vital for the behavioural changes caused by drug abuse (19). Dopamine exerts its diverse physiological effects by binding to five different G protein-coupled receptor subtypes (DRD1-5) (20). DRD1 and DRD5 receptors exert mainly stimulatory effects through activation of adenylate cyclase and elevation of intracellular levels of cyclic AMP (cAMP) (21). DRD2, DRD3 and DRD4 are generally inhibitory. They inhibit adenylate cyclase and reduce cAMP levels (22). Antagonists of the DRD1 and DRD2 receptors inhibit the reinstatement of cocaine seeking in rats (23). Dopamine levels in the NAc are increased after systemic injection of morphine, and pre-treatment with MOR or DRD1 receptor antagonists blocks the morphine-induced effects (24).

Although morphine dependence and NAc dopamine activity have been investigated in many studies (25-27), there is still a deficiency in this regard. Especially in recent years, fiber photometry and genetically encoded sensors have revolutionized in vivo studies on this subject. Therefore, our aim in this study is to examine NAc dopaminergic activity in morphine dependence in detail against time with fiber photometry in vivo. In addition, we aimed to investigate the time dependent change of NAc dopaminergic activity in morphine withdrawal modelling with naloxone in vivo.

MATERIALS AND METHODS

Twelve adults male Wistar rats weighing 300-350 g were used in the experiments. The animals were kept in plastic cages (temperature 22 ± 2°C) with food and tap water, and a 12-h light/dark cycle.

Virus Injection and Ferrule Implantation

Animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively) by intraperitoneally and their heads were shaved. Betadine and 70% ethanol were used to sterilize the surgical field. Animals were placed in a stereotaxic device with two ear bars and dental fixation. Terramycin cream was applied to the eyes of the rats to protect them from possible infections and to prevent dry eyes. The stereotaxic coordinates of the NAc region were determined from the rat brain atlas and the projection point on the bone tissue was marked. This pinpoint area was precisely drilled with a dentist's round and made suitable for intracerebral injection. Approximately 0.5 μL units/rat of pAAV-hsyn-GRAB_DA2m (GRABDA2m; Addgene #140553) was injected into the right NAc shell region of rats (bregma 1.70 mm, lateral + 0.80 mm and ventral - 7.0 mm from the brain surface) with a Hamilton syringe. A period of 10 minutes was allowed for the virus to diffuse into the area and the syringe was gently removed from the site after injection. Two small screws were implanted in order to fix the dental cement to the skull. Afterwards, an optical cable (400 μm diameter aperture; FP400URT Multimode, NA 0.50; Thorlabs) was placed in the previously prepared ferrules (SFLC440-10 with 400 μm diameter aperture, 6.4 mm length; Thorlabs) to target the NAc. After the ferrules were placed, the skull was covered with dental acrylic and the surgical procedure was completed. The rats were allowed to beg for two weeks to recover and for the virus to infect the area. Thus, dopaminergic receptors in the NAc region were labelled with green fluorescent protein (GFP). Rats that did not regain their preoperative body weight during this period were not used in subsequent experiments. The rats were randomly divided into two groups as morphine (M) and morphine+naloxone group (M+N). All animals were administered morphine (10 mg/kg) intraperitoneally between 09:00-10:00 every morning for 5 days. On the fifth day, morphine groups were injected with saline 15 minutes after morphine injection. On the fifth day, naloxone (3 mg/kg) was injected into the naloxone groups 15 minutes after morphine injection.

This study was approved by the Necmettin Erbakan University KONUDAM Experimental Medicine Application and Research Center Animal Experiments Local Ethics Committee (Ethics Number: 033-2021). Experimental studies

were conducted in the laboratories of the Experimental Medicine Application and Research Center. Animal rights are protected within the scope of the 'Guide for the Care and Use of Laboratory Animals'.

Fiber Photometry Recording and Analysis

Fiber photometry recordings were performed while the rats were free to move in their cages. The recordings were collected with a fiber photometry system (BFMC6; Doric Lenses) that transmits 405 nm as the control signal and 470 nm as the genetically encoded sensor signal to a fiber optic cable coupled to the fiber cannula.

Thomas Akam's open access GitHub library "Photometry data preprocessing" was used for fiber photometry analysis in our study (28). Briefly, raw signal data of 405nm control and 470nm GRABDA2m signals were acquired. To reduce high-frequency noise, the signals were low-pass filtered using a zero-phase filter with a cut-off frequency of 10Hz. The effects of photobleaching were removed with a double exponential fit and a 0.001 high-pass filter. Motion correction was applied to the signals. For normalization, ΔF/F was calculated as signal changes (ΔF) divided by initial fluorescence (F). The change in ΔF/F (%) was calculated according to the percentage of ΔF/F.

Brain Slice Imaging

Brains were fixed in 4% paraformaldehyde. Total brains were then cut into 70 um pieces in PBS using a vibratome. The sections placed in the wells were imaged with a Cell Discoverer 7 fluorescence microscope (Carl Zeiss, Germany). Histological verification was performed at the end of the experiments, and sterotaxic treatments that did not reach the shell region of the NAc were not included in the groups.

Statistical analysis

Statistical comparisons were made using a mixed-ANOVA test and post-hoc comparisons were made using estimated marginal means. All data were statistically analyzed with IBM SPSS 22 software and presented as mean ± standard error of the means (mean ± SEM). $p<0.05$ was accepted as significant.

RESULTS

We demonstrated that the injection of the dopamine sensor and the fiber optic cable was in the NAc region by photographing brain sections under an immunofluorescence microscope (Figure 1).

To determine the dynamics of dopaminergic activity, we performed in vivo fiber photometry recording from the rat right NAc shell region infected with pAAV-hsyn-GRAB_DA2m. To better understand the effect of morphine withdrawal, we first obtained a baseline recording (baseline period). Then we continued to record the effects of morphine administration (morphine period). Finally, we injected saline in the M group and naloxone in the M+N group and continued recording (saline or naloxone period). We completed the fiber photometry recording in 3 periods for a total of 25 minutes (Figure 2A). Fiber photometry recording in groups is shown in the heat map graph (Figure 2B).

We recorded baseline fiber photometric values for 5 min to detect dopaminergic activity in the NAc region before morphine injection of the animals. There was no significant difference in baseline period mean ΔF/F (%) between M and M+N groups (Figure 3).

There was no statistically significant difference between the mean ΔF/F (%) values of the M and M+N groups during the morphine period ($p>0.05$, Figure 4).

During the saline or naloxone period, the mean ΔF/F (%) values

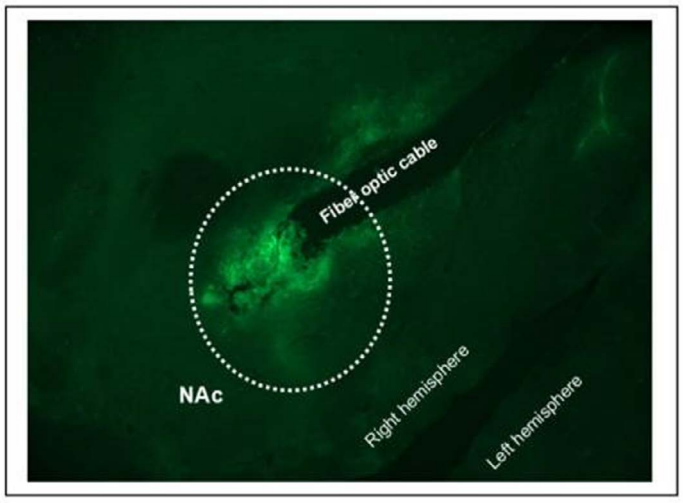

Figure 1. Microscopic image of injected GRABDA viruses and fiber optic ferule in NAc region. Rat brain section were visualized with immunofluorescence microscopy. Green color indicates GFP originating from GRABDA sensors. Abbreviations: NAc, Nucleous accumbens.

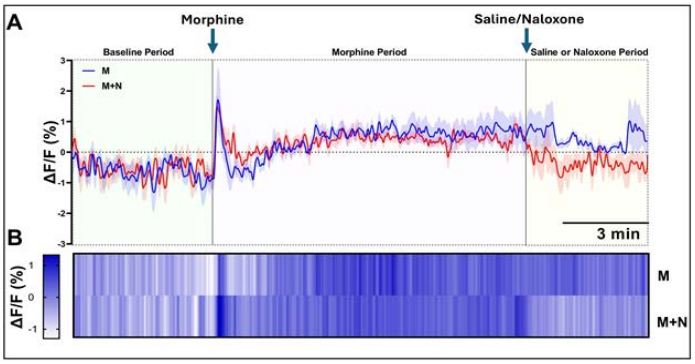

Figure 2. GRABDA sensor-induced ΔF/F (%) change over time in the NAc region according to groups. A, The mean ΔF/F (%) of the M group is shown as mean (blue trace) ± SEM (blue shading); the mean ΔF/F of the M+N group is shown as mean (red trace) ± SEM (red shading). B, The mean ΔF/F (%) of the M and M+N groups is shown as a heatmap plot. Abbreviations: M, morphine group; M+N, morphine+naloxone group.

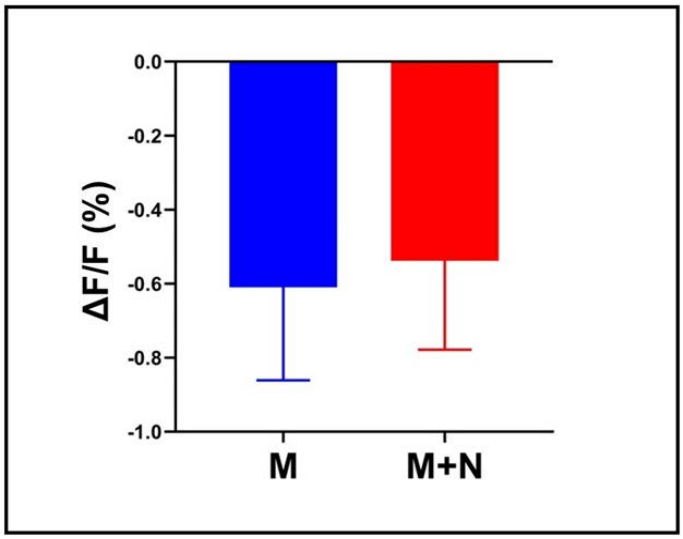

Figure 3. Comparison of NAc ΔF/F (%) baseline period data between groups.
Bar graph comparison of the mean ΔF/F (%) of the M and M+N groups during the baseline period. Data are expressed as mean ± SEM. Abbreviations: M, morphine group; M+N, morphine+naloxone group.

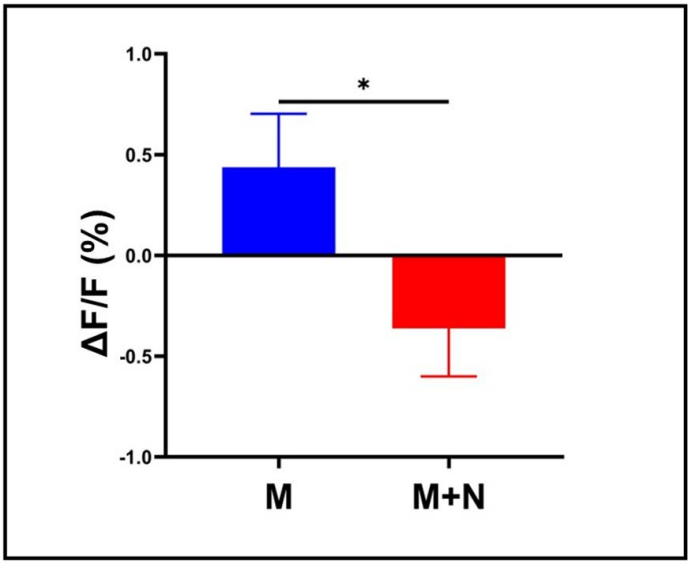

Figure 5. Comparison of mean ΔF/F (%) values after saline or naloxone injection between groups.
Average ΔF/F (%) comparison of the M and M+N groups during the saline or naloxone period. Data are expressed as mean ± SEM. *p<0.05 indicate significant difference between groups. Abbreviations: M, Morphine group; M+N, Morphine+Naloxone group.

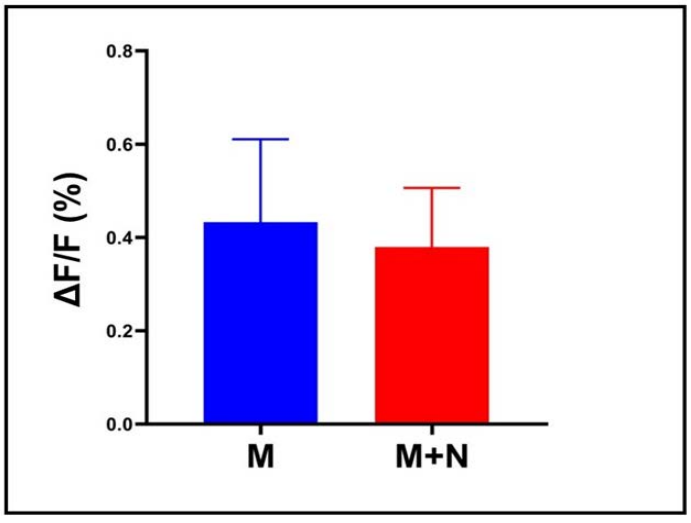

Figure 4. Comparison of mean ΔF/F (%) values of morphine period between groups.
Average ΔF/F (%) comparison of the M and M+N groups during the morphine period. Data are expressed as mean ± SEM. Abbreviations: M, morphine group; M+N, morphine+naloxone group.

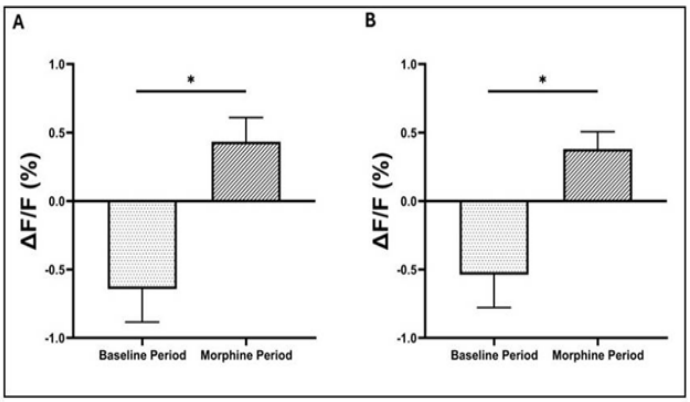

Figure 6. Comparison of mean ΔF/F (%) values of baseline and morphine periods in the M and M+N group.
Comparison of mean ΔF/F (%) values in the baseline and morphine periods of the M (A) and M+N (B) groups. Data are expressed as mean ± SEM. *p<0.05 indicate significant difference between groups. Abbreviations: M, morphine group; M+N, Morphine+Naloxone group.

of the naloxone-injected M+N group were significantly lower than those of the saline-injected M group ($p<0.05$, Figure 5).

When we compared the changes in dopaminergic activity in both M and M+N groups during basal and morphine periods, it was observed that the mean ΔF/F (%) value during morphine period was higher than basal period ($p<0.05$, Figure 6A, B).

DISCUSSION

Morphine injected systemically or into the VTA has been shown to increase the firing rate of dopamine neurons in anaesthetised animals (29, 30). This finding are supported by ex vivo studies showing that MOR agonists directly activate dopamine neurons in the VTA (31). MOR activation causes increased dopamine release by suppressing GABA release in the VTA (32, 33). Increased dopamine in the NAc can potentiate reward responses by activating DRD1 receptors, while its binding to DRD2 receptors can modulate the activity of medium spiny neurons (MSNs) (34). MSNs, which make up 95% of the NAc, can express DRD1 or DRD2 dopamine receptors (35, 36). Furthermore, by expressing MOR, these cells can be directly modulated by opioids such as morphine (37). Morphine injection has been shown to differentially alter excitatory glutamatergic activity in the NAc, D1R-MSNs and D2R-MSNs (38). In addition, it has been reported that dendritic spines of NAc DRD1-MSNs and DRD2-MSNs show distinct plasticity with chronic cocaine exposure (39). NAc dopamine activity during morphine withdrawal has also been assessed in several studies. The effects of morphine withdrawal in the NAc were found to be a consequence of alterations in dopamine neuron firing in the VTA (40). Opioid withdrawal also upregulates c-Fos activity in GABAergic VTA neurones (41). The near-complete disappearance of dopamine release in the NAc during withdrawal has been associated with the cessation of sustained stimulation of high-affinity, inhibitory DRD2 (42). The findings suggest that dopaminergic receptors, particularly DRD2, are actively involved in morphine dependence and withdrawal.

Immediate and highly sensitive detection of the dopaminergic VTA-NAc circuit, which forms the basis of drug addiction processes, using the newly developed fiber photometry technique has the potential to be a new research area for studies in this field. Our GRABDA fiber photometry measurements allow us to directly detect changes in dopamine binding to DRD2-MSNs during morphine dependence. Consequently, we are able to examine in greater detail the effects of morphine on the dopaminergic system and the relevance of DRD2 signalling in the NAc to addiction in vivo.

For the past 40 years, researchers have been using animal models to develop behavioural models of addiction and to try to understand the reward mechanisms (43). In these studies, the challenge is to measure dopamine in densely and sparsely innervated regions and to correlate dopamine levels in vivo with behavioural outcomes of interest. Reliable and accurate determination of dopamine levels in vitro or in vivo experiments is often closely related to efficient analytical techniques. In vivo measurements of dopamine concentration have been addressed using classical analytical chemistry techniques such as high-performance liquid chromatography with electrochemical detector system combined with micro dialysis. However, there are limitations that can make long-term measurements with sufficient spatio-temporal resolution and specificity relatively difficult.

Recently, scientists have developed genetically encoded dopamine sensors that overcome some of these technical hurdles. GRABDA sensors take part of the DRD2 receptor (the third intracellular loop, ICL3) and attach a fluorescent protein (cpEGFP) to it. When dopamine binds to the sensory receptor, a structural change takes place. This change affects cpEGFP and produces a fluorescent signal. This process allows the concentration of dopamine to be measured in real time (44, 45). GRABDA biosensors are now superior to chromatographic assays in many ways due to their fast data transfer, their ability to provide instantaneous measurements, the almost unlimited reproducibility of measurements and there in vivo usability (44). Whilst cerebrospinal fluid samples obtained by micro dialysis are typically reflective of a time frame ranging from minutes to hours (46), GRABDA biosensors signals facilitate real-time monitoring of neurochemical changes at the millisecond level. This fluorescence signal in GRABDA biosensors is recorded in vivo by a fiber photometry system (47).

In the present study, it was initially determined that the administration of morphine resulted in a significant augmentation in dopaminergic activity within the nucleus accumbens. This high dopamine level persisted for 20 minutes after morphine injection. A further salient finding was that naloxone significantly reduced morphine-induced dopamine levels. These results suggest that the combined use of GRABDA and a fiber photometry system is a highly effective method for monitoring dopaminergic signalling, especially the association of DRD2 receptors with addiction. The analysis of dopaminergic activity in the central nervous system was performed sensitively and efficiently in this study using a fiber photometer system and GRABDA biosensors. The findings of this study may prove to be of significant value in guiding future research endeavours focused on dopaminergic activation or inhibition in the NAc, particularly in the context of exploring novel therapeutic interventions for morphine addiction.

The analysis of dopamine by chromatography does not allow to follow a temporally continuous process. It can only determine the instantaneous concentration, which reflects the concentration of the sample obtained from the collection of a microdialysis sample of at least 10 minutes. It therefore does not reflect instantaneous changes in dopamine levels in brain tissue. Fiber photometry, on the other hand, offers the unique advantage of monitoring instantaneous changes in dopamine levels over a long period of time, with recordings taken at least 10 times per second. Thus, compared to chromatographic analysis, fiber photometry provides much clearer and more valuable findings in determining the effects of candidate molecules on dopamine levels. In this respect, it can be argued that research on the combination of biosensors and fiber photometry is much more effective in in vivo translational

research related to neuropsychopathologies.

CONCLUSION

This study demonstrates that morphine dependence significantly enhances dopaminergic activity in the nucleus accumbens, as observed through fiber photometry. Naloxone administration effectively suppresses this increase, highlighting its potential role in modulating dopaminergic signalling during withdrawal. The use of a novel biosensor for real-time detection provides a more precise and reliable method compared to traditional techniques, offering valuable insights for understanding addiction mechanisms and developing therapeutic strategies.

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