# UNIVERSIDAD DE MURCIA

## FACULTAD DE MEDICINA

Opiate Addiction: Neuronal Plasticity on Brain Reward System and Emotional Memory-Related Areas

Adicción a Opiáceos: Plasticidad Neuronal en los Circuitos Neuronales de Recompensa y en Áreas de Memoria Emocional

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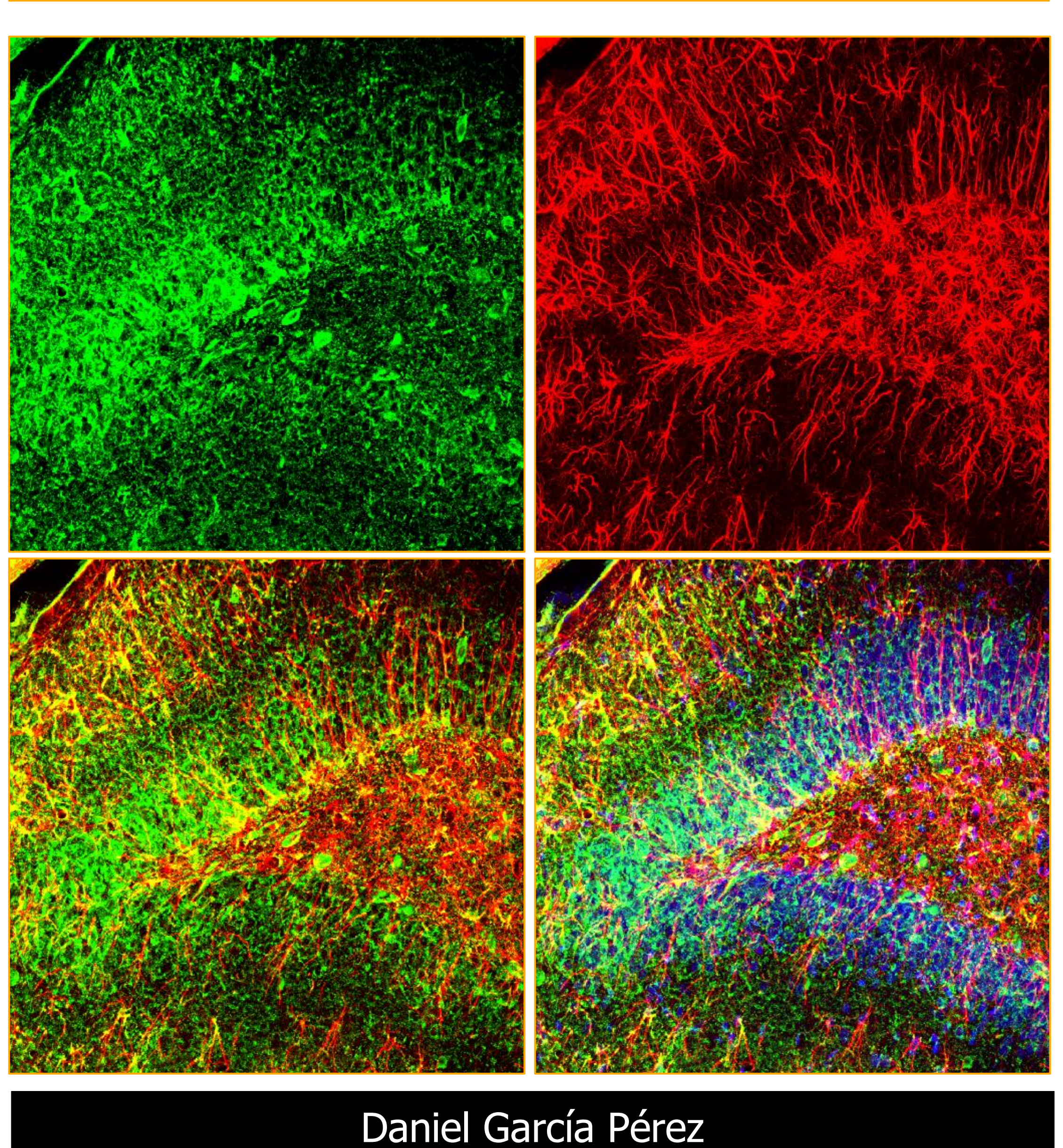
# Opiate addiction: neuronal plasticity on brain reward system and emotional memory-related areas




Daniel García Pérez


Tesis Doctoral (con Mención Europea)

A mis padres, los mejores del mundo

El presente trabajo de investigación ha dado lugar a las siguientes publicaciones:

**García-Pérez D**, Laorden ML, Milanés MV, Núñez C. Glucocorticoids regulation of FosB/ΔFosB expression induced by chronic opiate exposure in the brain stress system. PLoS One. 2012;7(11):e50264. doi: 10.1371/journal.pone.0050264.

**García-Pérez D**, Sáez-Belmonte F, Laorden ML, Núñez C, Milanés MV. Morphine administration modulates expression of Argonaute 2 and dopamine-related transcription factors involved in midbrain dopaminergic neurons function. Br J Pharmacol. 2013;168(8):1889-901. doi: 10.1111/bph.12083.

**García-Pérez D**, López-Bellido R, Hidalgo JM, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. Addict Biol. 2015;20(1):104-19. doi: 10.1111/adb.12083.

**García-Pérez D**, López-Bellido R, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Dysregulation of dopaminergic regulatory mechanisms in the mesolimbic pathway induced by morphine and morphine withdrawal. Brain Struct Funct. 2014;220(4):1901-19. doi:10.1007/ s00429-014-0761-5.

**García-Pérez D**, Núñez C, Laorden ML, Milanés MV. Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal. Addict Biol. 2014. doi: 10.1111/adb.12209.

**García-Pérez D**, Luisa Laorden M, Núñez C, Victoria Milanés M. Glial activation and midkine and pleiotrophin transcription in the ventral tegmental area are modulated by morphine administration. J Neuroimmunol. 2014;274(1-2):244-8. doi: 10.1016/ j.jneuroim.2014.07.017.

**García-Pérez D**, Laorden ML, Milanés MV. Acute morphine, chronic morphine and morphine withdrawal differently affects pleiotrophin, midkine and receptor protein tyrosine phosphatase β/ζ regulation in the ventral tegmental area. Mol Neurobiol (under review).

**García-Pérez D**, Laorden ML, Milanés MV. Regulation of pleiotrophin, midkine, receptor protein tyrosine phosphatase β/ζ and their intracellular signalling cascades in the nucleus accumbens during opiate administration. Int J Neuropsychopharmacol. 2015. doi: 10.1093/ijnp/pyv077.

**García-Pérez D**, Ferenczi S, Kovács KJ, Laorden ML, Milanés MV, Núñez C. Glucocorticoid homeostasis in the dentate gyrus is essential for opiate withdrawal-associated memories. Biol Psychiatry (under review).

**García-Pérez D**, Ferenczi S, Kovács KJ, Laorden ML, Milanés MV, Núñez C. Different contribution of glucocorticoids in the basolateral amygdala to the formation and expression of opiate withdrawal-associated memories. Addict Biol (under review).

El presente trabajo de investigación ha dado lugar a las siguientes comunicaciones a Congresos:

7th Forum of European Neuroscience (FENS) 2010
Amsterdam, The Netherlands, July, 3-7th, 2010
Title: Corticotropin-releasing factor-2 receptor (CRF2R) mediates morphine withdrawal-induced increased noradrenaline turnover in the hypothalamic paraventricular nucleus.

European Opioid Conference (EOC) 2011
Cracow, Poland, April, 13-14th, 2011
Title: Δfosb expression in the brain stress system from adrenalectomized rats during morphine dependence.
Published in: Pharmacological Reports, 2011; 63: 255

XIV Congress of the Spanish Neuroscience Society (SENC) 2011

Salamanca, Spain, September, 28-30th, 2011

Title: Role of glucocorticoids in ΔFosB expression in CRF neurons during morphine dependence.

XXXIII Congress of the Spanish Pharmacology Society (SEF) 2011

Málaga, Spain, October, 3-5th, 2011

Title: Glucocorticoids regulation of ΔFosB expression in TH and dynorphin neurons during morphine dependence.

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8th Forum of European Neuroscience (FENS) 2012

Barcelona, Spain, July, 14-18th, 2012

Title: Tyrosine hydroxylase activity is modified in the dopaminergic reward circuit during morphine dependence and withdrawal.

8th Forum of European Neuroscience (FENS) 2012

Barcelona, Spain, July, 14-18th, 2012

Title: Chronic morphine administration modulates the expression of transcription factors involved in midbrain dopaminergic neurons function.

European Opioid Conference (EOC) 2013

Guildford, United Kingdom, April, 10-12th, 2013

Title: Morphine regulates Argonaute 2 and TH expression but not miR-133b in midbrain dopaminergic neurons (Oral presentation).

European Opioid Conference (EOC) 2013

Guildford, United Kingdom, April, 10-12th, 2013

Title: Orexins receptor type 2 (OX2R) antagonist, TCSOX229, attenuates somatic expression of naloxone-precipitated morphine withdrawal syndrome.

European Opioid Conference (EOC) 2013

Guildford, United Kingdom, April, 10-12th, 2013

Title: μ opioid receptor (mor) regulation by microrna let-7a in the hypothalamus during morphine dependence and withdrawal.

European Opioid Conference (EOC) 2013
Guildford, United Kingdom, April, 10-12th, 2013
Title: Argonaute 2 regulates TH expression during chronic morphine and naloxone-induced morphine withdrawal.

XXV Congreso Nacional Sociedad Española Farmacología (SEF 2013)
San Pedro del Pinatar-Murcia, Spain, September, 16-19th, 2013
Title: Morphine administration modulates expression of dopamine-related transcription factors involved in midbrain dopaminergic neurons function.

XXV Congreso Nacional Sociedad Española Farmacología (SEF 2013)
San Pedro del Pinatar-Murcia, Spain, September, 16-19th, 2013
Title: Assessing students' knowledge and understanding of pharmacology by a re-evaluation with different types of exams.

Reunión Científica Red Trastornos Adictivos 2013 (RTA 2013)
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Title: Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b, in midbrain dopaminergic neurons.

IX Congreso Nacional Estudiantes de Farmacia de Alicante (CEFA 2013)
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Title: Midkine and pleiotrophin expression in the brain reward system is modulated by morphine administration (Oral presentation).

XVI Congress of the Spanish Neuroscience Society (SENC) 2015
Granada, Spain, September, 23-25th, 2015
Title: Glucocorticoids signaling is essential for opiate withdrawal-associated memories.

**Daniel García Pérez** ha sido beneficiario de las siguientes becas y ayudas:

- "Beca para estudios de máster universitario conducente al doctorado" del Programa Séneca 2009. Fundación Séneca - Agencia de Ciencia y Tecnología de la Región de Murcia. 2009/2010.
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- Proyecto SAF/FEDER 2007-62758, Ministerio de Ciencia e Innovación, MICINN. Implicación del CRF extrahipotalámico en la tolerancia/dependencia de morfina.
- Proyecto SAF/FEDER 2009-07178, Ministerio de Ciencia e Innovación, MICINN. Implicación del sistema cerebral del estrés en la dependencia de opiáceos. Mecanismos celulares y moleculares.
- Proyecto SAF/FEDER 2010-17907, Ministerio de Ciencia e Innovación, MICINN. Mecanismos neurobiológicos implicados en la preferencia de plaza a morfina y en la aversión a naloxona. Papel de los receptores de CRF1.
- Proyecto SAF/FEDER 2013-49076-P, Ministerio de Ciencia e Innovación, MICINN. Estrés y memoria: Implicación en los estados aversivos de la adicción y en las recaídas; mecanismos moleculares.
- Proyecto 15405/PI/10, Fundación Séneca - Agencia de Ciencia y Tecnología de la Región de Murcia. Relación entre estrés y adicción a opiáceos: papel de las orexinas y su interacción con los sistemas de CRF y NA.
- Red de Trastornos Adictivos (RETICS RD12/0028/0003), Instituto de Salud Carlos III.
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# RESUMEN

Los opiáceos, como la morfina, son unos potentes analgésicos que se usan para tratar diversas formas de dolor agudo y crónico. Sin embargo son también muy adictivos, lo que limita su uso médico. Además, el uso recreativo de los opiáceos y opioides (morfina, heroína), ha aumentado notablemente en los últimos años. El uso crónico de opiáceos produce cambios neuroadaptativos a nivel cerebral que conllevan efectos indeseables, como la adicción, la cuál es un notable problema médico y sanitario. La creciente evidencia implica a varios mecanismos de regulación génica (incluyendo epigenéticos, moleculares, celulares y a nivel de circuitos neuronales) en los cambios que las drogas de abuso provocan en el cerebro, siendo el conocimiento de éstos una posible estrategia terapéutica para el tratamiento de la adicción.

Recientemente se ha sugerido que la patología adictiva es el resultado de procesos maladaptativos de plasticidad neuronal causados por el consumo de sustancias de abuso, y que conducen a cambios a largo plazo en la expresión génica en áreas concretas del sistema nervioso central (CNS), dando lugar a profundas anormalidades del comportamiento que persisten a lo largo de toda la vida del adicto. Debido a estas características de la adicción, se ha sugerido el importante papel que podrían desempeñar los mecanismos epigenéticos (metilación de DNA, modificación de histonas y RNAs no codificantes) en dicha patología. Así, actualmente ya se sabe que las sustancias adictivas pueden alterar mecanismos epigenéticos que modularían (silenciando o sobre-expresando) la expresión de genes implicados en la neuroplasticidad, perturbando los niveles intracelulares de proteínas y modificando los procesos de señalización dependientes de diferentes neurotransmisores, alterando en último término el procesamiento de la información en diferentes circuitos neuronales: circuitos de recompensa, circuitos del estrés, memoria y control de la función ejecutiva, entre otros. Además, en la última década se ha propuesto la importancia del estrés en la génesis y mantenimiento de los procesos adictivos a sustancias de abuso, así como en las recaídas en el consumo. Por otra parte, cada día es más evidente que las drogas de abuso producen alteraciones en el sistema inmune del CNS. Por ello, se ha propuesto que los astrocitos contribuirían a la plasticidad sináptica que se produce durante el desarrollo de la adicción mediante la síntesis y liberación de sustancias, como las citoquinas. De hecho, los opiáceos inducen cambios en la morfología de las células de la glia y expresión del marcador inmunohistoquímico proteína glial fibrilar ácida (GFAP) de una forma región-específica. Estos datos apuntan la posibilidad de que los astrocitos contribuyan a la plasticidad sináptica durante el desarrollo de la adicción.

En base a estos hallazgos, nos hemos planteado el estudio diferentes circuitos neuronales, neurotransmisores, factores de transcripcion y microRNAs (miRs) que contribuirían a la plasticidad neuronal y memorias aversivas que llevarían al establecimiento del fenotipo adictivo. Esta Tesis aborda: 1) la caracterización de los cambios en factores de transcripción responsables del fenotipo dopaminérgico en la vía mesolímbica (Nurr1 y Pitx3) tras la administración aguda de morfina (intoxicación/binge), durante la dependencia y tras la inducción del síndrome de abstinencia. Además

se ha estudiado la posible relación entre dichos cambios y factores epigenéticos (miR-133b); 2) la contribución de los astrocitos a la plasticidad sináptica durante la adicción a opiáceos, mediante la síntesis y liberación de citoquinas (midkina –MK- y pleiotrofina –PTN-) en los circuitos de recompensa. 3) los aspectos subyacen en las modificaciones transcripcionales, post-transcripcionales y epigenéticas en el sistema cerebral del estrés y en los circuitos responsables de diferentes tipos de memorias, tanto a nivel neuroquimico, como celular y de comportamiento. Hemos centrado el presente estudio en varios neurocircuitos implicados en la adicción a opiáceos: i) el eje hipotálamo-hipófisis-adrenal (HPA) y el sistema extrahipotalámico del estrés, ii) el sistema dopaminérgico de recompensa, y iii) circuitos relacionados con las memorias aversivas.

- El uso crónico de las drogas de abuso altera el sistema cerebral del estrés. La exposición repetida a opiáceos conlleva la acumulación del factor de transcripción ΔFosB, particularmente en áreas cerebrales relacionadas con la recompensa y el estrés. La evidencia sugiere que las hormonas relacionadas con el estrés (como los glucocorticoides –GCs-) podrían producir adaptaciones en el sistema cerebral del estrés que podrían incluir alteraciones a nivel de la expresión génica y de factores de transcripción. El primer objetivo del presente estudio se diseñó después de considerar lo anterior. Examinamos el papel de los GCs en la expresión de ΔFosB en poblaciones neuronales específicas del sistema cerebral del estrés durante la dependencia de morfina. Para ello, la expresión de ΔFosB se cuantificó en ratas control (sham) y adrenalectomizadas (ADX) que desarrollaron dependencia de morfina.

- En los últimos años, diferentes estudios resaltan las modificaciones post-transcripcionales mediadas por los miRs en la adicción y otras patologías neurológicas y enfermedades neurodegenerativas. Algunos de ellos, como el miR-133b, han sido identificados como mediadores de la diferenciación, maduración y función de las neuronas dopaminérgicas del mesencéfalo. miR-133b actuaría mediante la regulación a la baja de factores de transcripción implicados en el desarrollo y el fenotipo de estas neuronas, como Nurr1 y Pitx3, que poseen una función crítica a nivel mesencefálico al regular la transcripción de diferentes genes implicados en el metabolismo de la dopamina (DA). Los cambios epigenéticos, como el silenciamiento génico mediado por miRs/Argonauta 2 (Ago2), también representan complejos mecanismos que regulan la plasticidad neuronal. Entre los miembros de la familia Ago, sólo Ago2 parece tener un papel importante en la ejecución del silenciamiento mediado por los diferentes miRs. Segundo objetivo: Dada la importante implicación de la transmisión dopaminérgica en los desórdenes adictivos, hemos centrado esta parte de nuestro estudio en identificar los marcadores dopaminérgicos que resultan alterados con la administración aguda y crónica de morfina, así como durante el síndrome de abstinencia inducido por naloxona, tanto en el área tegmental ventral (VTA) como el núcleo accumbens (NAc). Para ello, hemos determinado: i) la expresión del miR-133b y Ago2 en el VTA; ii) el contenido de tirosina hidroxilasa (TH) y su

actividad; iii) la actividad dopaminérgica (recambio de DA y activación de TH) en el sistema mesolímbico de ratas tratadas con morfina aguda, ratas dependientes de morfina y durante el síndrome de abstinencia inducido por naloxona. El tercer objetivo fue estudiar: i) posibles cambios en los niveles de mRNA y proteína de Nurr1 y Pitx3, así como la expresión de Ago2 y de TH en regiones específicas del sistema mesolímbico; ii) actividad dopaminérgica en el NAc; iii) co-localización cuantitativa de Nurr1 y Pitx3 en el VTA en las neuronas que expresan TH, iv) cambios en la plasticidad de subpoblaciones neuronales dopaminérgicas del VTA en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia.

- Las neuronas dopaminérgicas localizadas en el mesencéfalo y que proyectan al estriado se caracterizan por la expresión de autoreceptores receptor de dopamina tipo 2 (DRD2) que inhiben la síntesis y liberación de DA. Además, transportador de dopamina (DAT; que media la recaptación de DA), TH (enzima limitante de la síntesis de catecolaminas) y transportador vesicular de monoaminas 2 (VMAT2), han sido identificados como importantes reguladores de la función dopaminérgica. Por otro lado, Nurr1 y Pitx3 son esenciales para la expresión de genes implicados en el metabolismo de la DA, como *Th*, *Dat*, *Vmat2* and *Drd2*. El cuarto objetivo fue: Los efectos del síndrome de abstinencia a morfina sobre la expresión de Nurr1 y Pitx3 nos llevó a investigar la expresión de otros genes y proteínas que están implicadas en la regulación de la función dopaminérgica, algunos de los cuáles son dianas de Nurr1 y Pitx3. Para ello, las ratas recibieron morfina de forma aguda o crónica. Otro grupo fue sometido a un síndrome de abstinencia a morfina. Se estudió: i) la expression de *Dat*-DAT, *Vmat2*-VMAT2, *Drd2*-DRD2 y DRD1 en VTA/NAc; ii) la co-localización de Nurr1 y/o Pitx3 con neuronas que expresan TH en el VTA, así como el porcentaje de neuronas dopaminérgicas que expresan Nurr1 y Pitx3; iii) la posible correlación entre la expresión de Nurr1/Pitx3 y los niveles de los diferentes marcadores dopaminérgicos.

- También se ha propuesto que los astrocitos, mediante la síntesis y liberación de factores de crecimiento/citoquinas, como MK y PTN, jugarían un papel fundamental en los cambios en la plasticidad sináptica del CNS durante el desarrollo de la adicción. Por ello, el quinto objetivo fue: Dado que PTN y MK tienen los mismos efectos que las neurotrofinas y que los astrocitos pueden liberar MK y PTN después de diversos estímulos, nuestro siguiente objetivo fue: i) estudiar posibles cambios en la expresión de PTN, MK, su receptor receptor proteína tirosina fosfatasa β/ζ (RPTPβ/ζ), así como alteraciones en sus vías de señalización intracelular en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia en el VTA y NAc; ii) la posible activación de los astrocitos durante los tratamientos descritos, que podría conllevar la liberación de diferentes factores; iii) identificar las subpoblaciones celulares que producen y secretan PTN y/o MK, así como aquellas que expresan RPTPβ/ζ.

• En ratas dependientes de morfina, las memorias aversivas del síndrome de abstinencia pueden generar un estado motivacional que dé lugar a una búsqueda y consumo compulsivo de la droga. En la actualidad, los mecanismos que subyacen en la generación de las memorias aversivas del síndrome de abstinencia permanecen desconocidos. Es interesante recalcar que entre las regiones cerebrales que se ven afectadas por las drogas de abuso, se encuentran quellas que son sustratos neuronales de la memoria, incluyendo el hipocampo, la amígdala y la corteza prefrontal. Esto coincide con los hallazgos de que algunas de las alteraciones más importantes observadas clínicamente en la adicción (por ejemplo el deseo compulsivo de consumir droga y las recaídas) reflejan anormalidades en los circuitos tradicionales de la memoria, actuando las memorias a largo plazo de las experiencias de consumir la droga como conductores de la patología adictiva. Las hipótesis actuales de los mecanismos moleculares que intervienen en el aprendizaje y la memoria, sugieren que la rápida regulación génica y síntesis de nuevas proteínas conllevan modificaciones sinápticas persistenentes que constituyen un mecanismo para la estabilización de memorias a largo plazo. Egr1 y Arc son genes de expresión inmediata que juegan un papel crucial en la plasticidad y la memoria. Se ha propuesto que Arc tendría un papel fundamental en la consolidación de diferentes tipos de memoria, con algunos trabajos recientes implicando el mRNA y la proteína Arc en los procesos adaptativos que ocurren tras el estrés, así como en la plasticidad maladaptativa que ocurre en la adicción a drogas. Por otra parte, se ha demostrado que los GCs modulan la consolidación de la memoria. Los GCs ejercen sus acciones en diferentes regiones cerebrales, incluyendo el hipocampo, la amígdala y la corteza prefrontal, que tienen alta densidad de receptores de glucocorticoides (GRs) y son regiones importantes para la formación de la memoria. En roedores, el componente afectivo negativo de la dependencia de opiáceos se puede ver reflejado en diferentes tests comportamentales. Entre ellos, el test de aversión condicionada de plaza (CPA) es el más sensible para la medida del estado negativo emocional que produce el síndrome de abstinencia. Con estos antecedentes el sexto objetivo fue: En esta parte del estudio, hipotetizamos que los GCs en diferentes estructuras límbicas (como BLA y el DG en el hipocampo) podrían mediar el componente afectivo negativo del síndrome de abstinencia a morfina, y que por tanto jugarían un papel crucial en las memorias aversivas relacionadas con la abstinencia a opiáceos. Para comprobar nuestra hipótesis utilizamos el paradigma de CPA en animales ADX y que desarrollaron dependencia de morfina. A continuación estudiamos: i) posibles modificaciones epigenéticas (miR-124a y miR-212) y activación o inhibición de diferentes vías intracelulares (pCREB, Egr1 y BDNF) en amígdala basolateral (BLA) y giro dentado (DG) durante la consolidación de memoria y la recuperación de memoria; ii) cambios en la expresión de Arc como marcador de plasticidad neuronal; iii) identificación de las diferentes subpoblaciones neuronales donde se expresaban las proteínas de interés; iv) correlaciones entre los miRs y la regulación de las diferentes proteínas.

Las conclusiones de nuestro trabajo se pueden resumir en:

1. El presente trabajo demuestra que los GCs están críticamente involucrados en la acumulación de FosB/ΔFosB en el sistema cerebral del estrés durante la administración crónica de morfina, lo que resultaría en cambios duraderos en los patrones de expresión génica en las áreas relacionadas con el estrés.

2. Aunque no hallamos cambios significativos en los niveles de miR-133b en el VTA, proponemos el papel de Ago2 en combinación con otros miRs en la regulación de la estabilidad y/o traducción del mRNA de TH en respuesta a la administración crónica de morfina y al síndrome de abstinencia a opiáceos provocado por la administración de naloxona. Además, la dependencia de morfina y el síndrome de abstinencia están asociados con alteraciones en los factores de transcripción involucrados en el mantenimiento de las neuronas dopaminérgicas del sistema mesolímbico de recompensa. Por ello, nuestros resultados sugieren un papel importante de Nurr1 y Pitx3 en los cambios en la expresión génica durante la dependencia y abstinencia a opiáceos, y que la regulación epigenética de TH puede estar asociada, al menos en parte, a los mecanismos moleculares que contribuyen a los cambios en la función dopaminérgica inducida por los opiáceos.

3. La dependencia de morfina y el síndrome de abstinencia a la misma están asociados con alteraciones de la mayoría de los marcadores dopaminérgicos (DAT, VMAT2, DRD2) en la vía mesolímbica de recompensa, que se correlacionan con alteraciones en los factores de transcripción encargados del mantenimiento de las neuronas dopaminérgicas (Nurr1 y Pitx3). La correlación entre los marcadores dopaminérgicos y Nurr1/Pitx3 añaden evidencia a nuestros anteriores resultados, y pueden reflejar el papel de estos factores de transcripción durante la dependencia y abstinencia a morfina.

4. Debido a la activación glial en el VTA durante la administración aguda y crónica de morfina, junto con el incremento de los niveles de MK y PTN, proponemos la importancia de estas citoquinas al mediar, al menos en parte, las adaptaciones tróficas que se observan durante el fenómeno de adicción.

5. Nuestros experimentos demuestran que la expresión de Egr-1 y Arc está asociada con el recuerdo de la memoria del síndrome de abstinencia y sugieren que estos genes contribuirían a la plasticidad y a la reconsolidación que acompaña el proceso de recuerdo. Además, los GCs juegan un papel esencial durante la formación de la memoria aversiva a largo plazo del síndrome de abstinencia, ya que los animales ADX-dependientes muestran reducida aversión de plaza y niveles de Egr-1 y Arc disminuidos en el DG. Además nuestros datos demuestran que los GCs participan en los procesos transcripcionales que se requieren para la consolidación de memoria del síndrome de abstinencia e influencian mecanismos epigenéticos que siguen al recuerdo en el BLA.

# ABSTRACT

Opiate drugs, such as morphine, are a class of powerful analgesics that are used for treating many forms of acute and chronic pain. However, they are also highly addictive, which limits their medical use. The non-medical use of opiates (heroine, morphine) has increased greatly in recent years. Chronic use of opiates causes brain neuroadaptation that lead to undesirable effects, namely opiate addiction that is a significant medical and public health problem. Increasing evidence implicates various mechanisms of gene regulation (including epigenetic, molecular, cellular and circuit level effects) in the changes that drugs of abuse induce in the brain, indicating a potential therapeutic strategy for addiction therapy.

It has been suggested that drug addiction is a result of maladaptive synaptic plasticity mechanisms that are initiated during drug taking. Long-term changes in gene expression in different brain areas would mediate the long-lasting addictive behaviors. Because of these altered behaviors are long-lasting, epigenetic mechanisms have been proposed as mediators of this phenomenon. Thus, it has been described that several drugs of abuse can modulate some epigenetic mechanisms that, in turn, would enhance or repress different genes related with neuroplasticity, intracellular protein levels and/or signaling cascades depending on neurotransmitters action. All these changes would lead to altered functioning of different neuronal circuits: brain reward system, brain stress system, learning and memory or decision-making. In the last decade, it has been suggested a prominent role for stress on drug seeking and taking and the development of drug addiction, as well as on drug reinstatement. On the other hand, there is increasing evidence that drugs of abuse produce alterations in CNS immunology. Thus, it has been proposed that astrocytes contribute to the synaptic plasticity during the development of drug addiction by the synthesis and release of substances, such as cytokines. Thus, opiates induce profound changes in glial cellular morphology and phenotypic receptor/immunohistological marker expression, glial fibrillary acidic protein (GFAP) in a region specific-manner. These data raise the possibility that astrocytes contribute to the synaptic plasticity during the development of drug addiction.

All these data prompted us to study different brain networks, neurotransmitters, transcription factors and microRNAs (miRs) that would contribute to neuronal plasticity and aversive memories during the development of the addicted phenotype. The current Thesis investigates: 1) changes in the transcription factors that characterize the dopaminergic phenotype (Nurr1 and Pitx3) in the mesolimbic circuit after acute and chronic morphine and during morphine withdrawal. Moreover, the possible relationship between such transcription factors and epigenetic mechanisms (miR-133b) was also studied; 2) the role of astrocytes in synaptic plasticity during morphine dependence by means of the synthesis and release of cytokines (midkine –MK- and pleiotrophin –PTN-) in the brain reward system; 3) an altered epigenetic, transcriptional and post-transcriptional regulation of limbic and memory circuits, and the relationship between behavioral and molecular changes. We have focused the present study on several neurocircuits involved in opiate addiction: i) the hypothalamic-pituitary-

adrenal (HPA) and extrahypothalamic stress systems; ii) the dopaminergic reward pathways; and iii) aversive memories-related neuronal circuits.

- Chronic use of drugs of abuse profoundly alters stress-responsive system. Repeated exposure to opiates leads to accumulation of the transcription factor ΔFosB, particularly in brain areas associated with reward and stress. Recent evidence suggests that stress-related hormones (e.g. glucocorticoids –GCs-) may induce adaptations in the brain stress system that are likely to involve alterations in gene expression and transcription factors. The first objective of the present study was designed after considering the above. We examined the role of GCs in regulation of ΔFosB expression in specific populations of the brain stress system during morphine dependence. For that, expression of ΔFosB was measured in control (sham-operated) and adrenalectomized (ADX) rats that were made dependent on morphine.

- In recent years, studies highlight post-transcriptional modifications mediated by miRs in addiction and other neurological disorders and neurodegenerative diseases. Some of them, such as miR-133b, have been proposed to regulate the differentiation, maturation and function of dopaminergic neurons by downregulating transcription factors involved in the development and physiological function of midbrain dopaminergic neurons, such as Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in dopamine (DA) metabolism in the mesolimbic pathway. Epigenetic changes such as miRs/Argonaute 2 (Ago2)-induced gene silencing represent complex molecular signature that regulate cellular plasticity. Among the Ago family members, only Ago2 seems to have an important role in miRs generation and execution of miR-mediated gene silencing. Our second objective was: Given the important implications of DA neurotransmission in addiction disorders, we have focused this part of our study on identifying the DA markers that are altered in association with acute and chronic morphine exposure, as well as with morphine withdrawal in the ventral tegmental area (VTA) and nucleus accumbens [NAc(medial shell)]. For that, we have determined i) the expression of miR-133b and Ago2 in VTA; ii) tyrosine hydroxylase (TH) content and activity; and iii) dopaminergic activity (DA turnover and TH activation) in the mesolimbic system from rats acutely injected with morphine, in morphine-dependent rats and during naloxone-induced morphine withdrawal. Thus, the third objective was to study: i) Nurr1 and Pitx3 mRNAs and proteins changes as well as the expression of Ago2 and TH mRNA and protein levels in specific region of the mesolimbic system; ii) dopaminergic activity in the NAc; iii) quantitative co-localization of Nurr1 and Pitx3 in the VTA TH-positive neurons; and iv) the plasticity changes in VTA DA neurons subpopulations in response to morphine, morphine dependence and morphine withdrawal.

- DA neurons located in the midbrain and projecting to the striatal complex are primarily characterized by expression of dopamine receptor 2 (DRD2) autoreceptor subtype, which inhibits DA synthesis and release. In addition, dopamine transporter (DAT), which mediates the reuptake of

DA, TH (the rate-limiting enzyme for DA synthesis) and vesicular monoamine transporter 2 (VMAT2) have been identified as important regulators of DA function. On the other hand, Nurr1 and Pitx3 are crucial for expression of the set of genes involved in DA metabolism, such as *Th*, *Dat*, *Vmat2* and *Drd2*. Our fourth objective was**:** The long-lasting effects of opiate withdrawal on Nurr1 and Pitx3 expression prompted us to investigate expression of other genes and proteins that are involved in the regulation of DA function, some of which represent putative targets of Nurr1 and Pitx3. For that, rats were exposed to acute and chronic morphine administration as well as to morphine withdrawal and analized: i) the expression of *Dat*-DAT, *Vmat2*-VMAT2, *Drd2*-DRD2 and DRD1 in VTA/NAc(shell), dysfunction of which is causally linked to addiction; ii) the co-localization of Nurr1 and/or Pitx3 with TH-positive neurons in the VTA as well as the percentage of DA neurons expressing Nurr1 and Pitx3; and iii) the possible correlation between Nurr1/Pitx3 expression and DA markers levels in the VTA and/or NAc(shell).

• It has also been proposed that astrocytes, through the synthesis and release of growth factors/cytokines, such MK and PTN, would play a critical role in long-term synaptic plasticity in the CNS during drug addiction. Given these facts, the fifth objective was**:** Given the important implications of DA neurotransmission in addiction disorders and the complexity of opiate-induced neuroadaptive responses in the brain reward dopaminergic system, the present study was focused on: i) identifying whether the expression of PTN, MK, Receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ) and their intracellular signaling pathways [thymoma viral proto-oncogene (Akt) and extracellular-signal regulated kinase (ERK)] are altered in association with acute and chronic morphine exposure as well as with morphine withdrawal in the VTA and NAc; ii) the possible activation of astrocytes, which could lead to the release of astrocyte-related soluble factors; iii) we also aimed to identify those cell subpopulations that produced and secreted PTN and/or MK and those that expressed RPTPβ/ζ in response to morphine administration or morphine withdrawal.

• In opiate-dependent rats, aversive memories of drug withdrawal can generate a motivational state leading to compulsive drug seeking and taking. However, the mechanisms underlying generation of drug withdrawal memories remain unclear. Interestingly, among the brain regions affected by drugs of abuse are those that are key neural substrates for behavioral memory, including the hippocampus, amygdala and prefrontal cortex. This coincides with the increasing realization that some of the most important features of addiction seen clinically (eg, drug craving and relapse) reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology. Current hypothesis on the molecular mechanisms od learning and memory suggest that rapid regulation of gene programs and synthesis of new proteins leading to persistent synaptic modifications constitute a key mechanism for the stabilization of long-term memory. Early growth response 1 (Egr1) and activity-regulated cytoskeletal-associated protein (Arc) are Immediate-

early gene (IEG) known to play major roles in plasticity and memory. Arc has been proposed to have an important role in consolidation of many forms of memory, with recent works implicating *Arc* mRNA and protein in adaptation to stress as well maladaptive plasticity connected to drug addiction. GCs mediate and modulate memory consolidation. They exert their actions on brain regions, including the hyppocampus, amygdala and prefrontal cortex, that are enriched in glucocorticoid receptor (GR) and are important for long-term memory formation. In rodent, the negative affective component of opiate dependence could be reflected by several behavioral alterations, among which conditioned place aversion (CPA) is the most sensitive measurement for the negative motivational state produced by opiate withdrawal, which is considered as a Pavlovian conditioned paradigm. For these reasons the sixth objective was**:** We hypothesized that GCs in limbic structures (eg., the basolateral amygdala – BLA- and hyppocampal dentate gyrus –DG-) could specifically mediate the negative motivational component of opiate withdrawal and might play a crucial role in the aversive memories of opiate withdrawal. To test this hypothesis, we used the CPA paradigm in control (sham-operated) and ADX animals that were rendered dependent on morphine. Then, we studied: i) epigenetics modifications (miR-124a y miR-212) and intracellular pathways activated or inhibited (phosphorylated cAMP response element binding protein –pCREB-, Egr1 y brain derived neurotrophic factor –BDNF-) in the BLA and the DG during memory consolidation and memory retrieval; ii) changes in Arc expression as a marker of neuronal plasticity; iii) we also aimed to identify those cell subpopulations that produced and secreted the different proteins that we studied; iv) correlation analysis between different miRs and target proteins levels.

The conclusions from present work are:

1. Present work provides evidence that GCs are critically involved in FosB/ΔFosB accumulation in the brain stress system after chronic morphine exposure, which might result in lasting changes of gene expression pattern in stress-related areas.

2. Although no significant changes of miR-133b levels are detected in the VTA, a role for Ago2 and specific miRs is hypothesized in regulating TH mRNA stability and/or translation in response to chronic morphine administration and naloxone-induced morphine withdrawal. Moreover, morphine dependence and withdrawal are associated with consistent alteration of transcription factors involved in the maintenance of dopaminergic neurons in the mesolimbic drug-reward pathway. Thus, our results suggest an important role for Nurr1 and Pitx3 in contributing to the changes in gene expression during opiate dependence and withdrawal and that epigenetic regulation of TH could be associated, at least in part, to the molecular mechanisms contributing to opiate-induced changes in midbrain dopaminergic function.

3. Morphine dependence and withdrawal are associated with consistent alteration of most of the DA markers (DAT, VMAT2, DRD2) in the mesolimbic drug-reward pathway which correlated with alteration of transcription factors involved in the maintenance of dopaminergic neurons (Nurr1 and Pitx3). The correlations between DA markers and Nurr1/Pitx3 add evidence onto previous results, and may reflect engagement of these transcription factors during morphine dependence and withdrawal.

4. Because of the glial activation in the VTA during acute and chronic morphine administration besides the enhancement in MK and PTN, we propose a role for these cytokines in mediating, at least in part, the trophic adaptations that are observed during drug addiction.

5. Our experiments demonstrate that Egr-1 and Arc expression in the DG are associated with morphine-withdrawal memory retrieval and suggest that these genes may contribute to plasticity and reconsolidation accompanying the retrieval process. Besides, GCs play an essential role during the formation of long-term withdrawal-aversive memories, given that ADX-dependent animals exhibit reduced place aversion and decreased Egr-1 and Arc levels in the DG. In addition, our data provide the first evidence that GCs mediate transcriptional events required for morphine-withdrawal memory consolidation and influence epigenetic mechanisms following retrieval in the BLA, supporting the idea that targeting GCs in this amygdalar area may provide important insights into the role of essential signaling cascades mediating aversive drug memories.

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# ABBREVIATIONS

**ACTH:** Adrenocorticotrophin hormone
**ADX:** Adrenalectomized
**Ago:** Argonaute
**Akt:** Thymoma viral proto-oncogene
**AMPA:** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
**Arc:** Activity-regulated cytoskeletal-associated protein
**ATP:** Adenosine triphosphate
**BDNF:** Brain derived neurotrophic factor
**BLA:** Basolateral amygdala
**BNST:** Bed nucleus of the stria terminalis
**CaMKII:** Calcium/calmodulin-dependent protein kinase type II
**cAMP:** Cyclic adenosine monophosphate
**CDK5:** Cyclin-dependent kinase 5
**CeA:** Central nucleus of the amygdala
**CNS:** Cental nervous system
**CORT:** Corticosterone
**CPA:** Conditioned place aversion
**CREB:** cAMP response element binding protein
**CRF:** Corticotropin-releasing factor
**CRFR1:** CRF receptor 1
**DA:** Dopamine
**DAT:** Dopamine transporter
**DG:** Dentate gyrus
**DNA:** Deoxyribonucleic acid
**DOR:** Delta opioid receptor
**DR:** Dopamine receptor
**DRD1:** Dopamine receptor 1
**DRD2:** Dopamine receptor 2
**DSM:** Diagnostic and Statistical Manual of Mental Disorders
**e.g.:** For example
***Egr:*** Early growth response
**ERK:** Extracellular-signal regulated kinase
**GABA:** Gamma-aminobutyric acid
**GABAergic:** GABA releasing
**GCs:** Glucocorticoids

| | |
|---|---|
| **GFAP:** | Glial fibrillary acidic protein |
| **GLT:** | Glutamate transporter |
| **GR:** | Glucocorticoid receptor |
| **HPA:** | Hypothalamic-pituitary-adrenal |
| **HRs:** | High responders |
| **i.c.v.:** | Intra-cerebroventricular |
| **IEG:** | Immediate-early gene |
| **IP3:** | Inositol-1,4,5-trisphosphate |
| **IRS2:** | Insulin receptor substrate-2 |
| **ISuE:** | Intensified Sustained Escalated |
| **kDa:** | Kilodaltons |
| **KO:** | Knock out |
| **KOR:** | Kappa opioid receptor |
| **LC:** | Locus coeruleus |
| **LoC:** | Loss Control |
| **LRs:** | Low responders |
| **LTD:** | Long-term depression |
| **LTP:** | Long-term potentiation |
| **MAPK:** | Mitogen-activated protein kinase |
| **mGluR:** | Metabotropic glutamate receptor |
| **miRs:** | microRNAs |
| **MK:** | Midkine |
| **MOR:** | Mu opioid receptor |
| **MR:** | Mineralocorticoid receptor |
| **MSN:** | Medium spiny neuron |
| **NA:** | Noradrenaline |
| **NAc:** | Nucleus accumbens |
| **NAc(Core)**: | Core of the NAc |
| **NAc(Shell):** | Shell of the NAc |
| **NeuroD:** | Neurogenic differentiation |
| **NFκB:** | Nuclear factor kappa B |
| **NGF:** | Nerve growth factor |
| **NMDA:** | N-Methyl-D-aspartate |
| **nt:** | Nucleotide |
| **NT3:** | Neurotrophin-3 |

**NT4/5:** Neurotrophin-4/5
**NTS:** Nucleus tractus solitarius
**p75NTR:** p75 neurotrophin
**PFC:** Prefrontal cortex
**PI3K:** Phosphatidylinositol 3′-kinase
**PLCγ:** Phospholipase Cγ
**POMC:** Proopiomelanocortin
**PTN:** Pleiotrophin
**PVN:** Paraventricular nucleus
**ReS:** Recreational Sporadic
**RNA:** Ribonucleic acid
**RPTPβ/ζ:** Receptor protein tyrosine phosphatase β/ζ
**SN:** Substantia nigra
**SUDs:** Substance Use Disorders
**TH:** Tyrosine hydroxylase
**TLR4:** Toll-like receptor 4
**TOR:** Target of rapamycin
**TORC2:** TOR complex 2
**TrkB:** Tropomyosin-related kinase B
**UTR:** Untranslated region
**VGluT:** Vesicular glutamate transporter
**VMAT2:** Vesicular monoamine transporter 2
**VP:** Ventral pallidum
**VTA:** Ventral tegmental area

# PRESENTACIÓN

El Grupo de Farmacología Celular y Molecular se ha ocupado, durante años, del estudio de diferentes factores transcripcionales y post-transcripcionales relacionados y/o responsables de la dependencia de opiáceos y del síndrome de abstinencia a los mismos. En la última década se ha propuesto la importancia del estrés en la génesis y mantenimiento de los procesos adictivos a sustancias de abuso, así como en las recaídas en el consumo. Nuestro Grupo se ha interesado por las bases neurobiológicas que relacionan estrés y adicción, y ha profundizado en el estudio de los diferentes factores implicados en dicha conexión: genes de expresión inmediata, proteínas quinasas, así como neurotransmisores y hormonas.

Recientemente se ha sugerido que la patología adictiva es el resultado de procesos maladaptativos de plasticidad neuronal causados por el consumo de sustancias de abuso, y que conducen a cambios a largo plazo en la expresión génica en áreas concretas del sistema nervioso central (CNS), dando lugar a profundas anormalidades del comportamiento que persisten a lo largo de toda la vida del adicto. Debido a estas características de la adicción, se ha sugerido el importante papel que podrían desempeñar los mecanismos epigenéticos (metilación de DNA, modificación de histonas y RNAs no codificantes) en dicha patología. Así, actualmente ya se sabe que las sustancias adictivas pueden alterar mecanismos epigenéticos que modularían (silenciando o sobre-expresando) la expresión de genes implicados en la neuroplasticidad, perturbando los niveles intracelulares de proteínas y modificando los procesos de señalización dependientes de diferentes neurotransmisores, alterando en último término el procesamiento de la información en diferentes circuitos neuronales: circuitos de recompensa, circuitos del estrés, memoria y control de la función ejecutiva, entre otros.

En base a estos hallazgos, nos hemos planteado el estudio de diferentes circuitos neuronales, neurotransmisores, factores de transcripción y microRNAs (miRs) que contribuirían a la plasticidad neuronal y memorias aversivas que llevarían al establecimiento del fenotipo adictivo. Esta Tesis aborda: 1) la caracterización de los cambios en factores de transcripción responsables del fenotipo dopaminérgico en la vía mesolímbica (Nurr1 y Pitx3) tras la administración aguda de morfina (intoxicación/binge), durante la dependencia y tras la inducción del síndrome de abstinencia. Además se ha estudiado la posible relación entre dichos cambios y factores epigenéticos (miR-133b). Para realizar estos estudios se estableció una colaboración con la Dra. Raquel E Rodríguez (INCYL, Salamanca); 2) los aspectos que probablemente subyacen en las modificaciones transcripcionales, post-transcripcionales y epigenéticas en el sistema cerebral del estrés y en los circuitos responsables de diferentes tipos de memorias, tanto a nivel neuroquímico, como celular y de comportamiento. Para ello se han utilizado modelos animales con déficit de glucocorticoides [(GCs), hormona del estrés] y el paradigma de la aversión condicionada de plaza (CPA) como modelo experimental de memorias aversivas. Para llevar a cabo este trabajo se estableció una colaboración con la Dra. Krisztina Kovács (Institute of Experimental Medicine, Budapest, Hungría); 3) la contribución de los astrocitos a la

plasticidad sináptica durante la adicción a opiáceos, mediante la síntesis y liberación de citoquinas (midkina –MK- y pleiotrofina –PTN-) en los circuitos de recompensa.

Este trabajo constituye la presente Tesis Doctoral cuyos resultados han sido parcialmente publicados en siete manuscritos y tres en revisión.

# INTRODUCTION

Cellular and Molecular Pharmacology Group has focused on the research of different transcriptional and post-transcriptional factors influencing opiate dependence and withdrawal. In the last decade, it has been suggested a prominent role for stress on drug seeking and taking and the development of drug addiction, as well as on drug reinstatement. We have focused our interest on the neurobiological basis connecting stress and addiction and the relationship between different factors that mediate this disease: neurotransmitters, hormones, immediate early genes…

It has been suggested that drug addiction is a result of maladaptive synaptic plasticity mechanisms that are initiated during drug taking. Long-term changes in gene expression in different brain areas would mediate the long-lasting addictive behaviors. Because of these altered behaviors are long-lasting, epigenetic mechanisms have been proposed as mediators of this phenomenon. Thus, it has been described that several drugs of abuse can modulate some epigenetic mechanisms that, in turn, would enhance or repress different genes related with neuroplasticity, intracellular protein levels and/or signaling cascades depending on neurotransmitters action. All these changes would lead to altered functioning of different neuronal circuits: brain reward system, brain stress system, learning and memory or decision-making.

All these data prompted us to study different brain networks, neurotransmitters, transcription factors and miRs that would contribute to neuronal plasticity and aversive memories during the development of the addicted phenotype. The current Thesis investigates: 1) changes in the transcription factors that characterize the dopaminergic phenotype (Nurr1 and Pitx3) in the mesolimbic circuit after acute and chronic morphine and during morphine withdrawal. Moreover, the possible relationship between such transcription factors and epigenetic mechanisms (miR-133b) was also studied. For this purpose, we have collaborated with Dr. Raquel Rodriguez (INCYL, Salamanca); 2) an altered epigenetic, transcriptional and post-transcriptional regulation of limbic and memory circuits, and the relationship between behavioral and molecular changes. For that purpose we have used bilaterally adrenalectomized rats and the CPA paradigm, as expression of aversive memories related to morphine withdrawal. We collaborated with Dr. Krisztina Kovács (Institute of Experimental Medicine, Budapest, Hungary) to develop this experiments; 3) the role of astrocytes in synaptic plasticity during morphine dependence by means of the synthesis and release of cytokines (MK and PTN) in the brain reward system.

All these results that compose the current Thesis, have been published in seven research articles and three manuscripts that are under revision currently.

# I. BACKGROUND

## 1. DRUG ADDICTION

Opiates are the most potent compounds known today to control pain, and are also among the most used drugs of abuse (1). Drug addiction is a chronic relapsing disorder characterized by compulsive drug seeking and taking (2,3). *Diagnostic and Statistical Manual of Mental Disorders* (DSM) provides a series of behavioral symptoms (items) that if present in an individual, allows making the diagnosis of specific pathological behaviors such as drug dependence. DSM-IV (4) describes possible behavioral alterations characterizing loss of control that can be grouped into three main categories: 1) difficulty on limiting drug use and seeking; 2) extremely strong motivation to procure the drug; 3) maintaining drug use despite awareness of negative consequences. The DSM-V (5) replaces the two separate DSM-IV categories of abuse and dependence with a single category: Substance Use Disorders (SUDs) along with different severities of SUD.

The compulsive use of drugs despite serious negative consequences defines addiction as a mental illness (6). Research supports the idea that addiction is a true psychiatric pathology and not an iatrogenic disease resulting from a vice. Thus, addiction should not be considered a condition for which the individual is largely responsible and for which he or she should be punished, but as a disease that needs treatment (7).

Recently, Piazza and Deroche-Gamonet (2) proposed a general theory of transition to addiction that is composed of three phases (Figure 1). The first step is a non-pathological phase of drug-taking in which there is Recreational Sporadic (ReS) drug use. The ReS phase starts when the individual learns to take drugs as one among many of his or her recreational activities. Drug use is then sporadic and occupies a small portion of the behavioral repertoire. This can be considered normal behavior and is present in a large proportion of the human population when both legal and illegal drugs are taken into account. The second step, Intensified Sustained Escalated (ISuE) drug use, starts with an increase in frequency, amount, and motivation to take drugs. Drug-taking intensifies and becomes sustained; additionally, drug-related problems appear but are not sufficient to promote spontaneous abstinence. The ISuE phase is the first moderate pathological state, one in which the individual takes too much drug but behavior is still organized and the individual is, generally, well integrated into society. The third and final step signals the beginning of the most serious pathological state. In this case, the individual largely has Loss Control (LoC) of drug intake and becomes fully addicted. In this phase, drug-taking becomes the individual's major goal-directed activity, invading most of the space normally occupied by other components of his or her normal behavioral repertoire. Then, social degradation is inevitable, and relapses to addiction -even after prolonged abstinence- is the rule. When the individual goes from liking drugs to pathologically mourning them when they are not available, the process of transition to addiction is complete. It is important to emphasize that in the

transition from ReS to ISuE, the individual progresses from taking drugs sporadically and moderately to taking them frequently and in large quantity. Thus, drug intake first changes principally quantitatively (from recreational to sustained). In contrast, in the transition from ISuE to LoC, the principal difference is not how much drug is taken but where the individual is able to confine drug-taking (qualitatively changes). In this theory, physical and/or emotional withdrawal is observed as an additional process that promotes excessive drug intake, thus contributing to the transition to addiction (2).

The establishment of an allostatic state has been proposed by Le Moal and Koob as the crucial phenomenon in the addiction process (8,9,10). The idea is that following extended drug use, reward systems adapt to the daily overexposure of the brain to drugs by shifting the homeostatic set point (allostasis) to adapt to this continuous overstimulation. Because of this shift, the drug state progressively becomes the normal state and the nondrug state is now perceived as a pathological, or at least, as an unpleasant state, so drugs progressively shift from being strongly wanted to also strongly needed (8,11,10).

### 1.1. DRUG-CENTERED THEORIES

Drug-centered theories of addiction include all views of addiction for which taking a drug repeatedly is the major cause of addiction. According to these theories, addiction is principally a consequence of drug intake and results from the psychopharmacological changes that follow chronic drug use. These theories focused on specific drug-induced changes, such as tolerance (12), sensitization (13,14), withdrawal and allostasis (10,15,16) or drug-induced cognitive changes in impulsivity (17), decision making (18,19) and conditioning (20). These views of addiction were reinforced by the very significant neurobiological alterations at molecular, cellular (21,22,23,24), synaptic (25,26), and network levels (27,28,29,30) that follow chronic drug intake.

**Figure 1:** Recently, Piazza and Deroche-Gamonet proposed a general theory of transition to addiction that is composed of three consecutive and independent phases: consecutive, because entering one phase is a necessary condition to go to the next one; independent, because entering one phase is not a sufficient condition to progress to the next one.

Reproduced from ref (2): Piazza PV, Deroche-Gamonet V. A multistep general theory of transition to addiction. Psychopharmacology. 2013. 229:387-413.

### 1.2. INDIVIDUAL DIFFERENCES IN THE ETIOLOGY OF ADDICTION

It has been proposed that a "vulnerable" phenotype that predisposes to addiction exists (31). Individual differences in the responses to drugs of abuse have been widely demonstrated in humans (32,33) and in laboratory animals (31,34,35). It was showed that when low unitary doses of drugs are

made available, some rats (high responders, HRs) rapidly escalated their drug intake, reaching and maintaining a high level, whereas others (low responders, LRs) do not (36) (Figure 2). Moreover, self-administration-prone subjects can be identified because of their behavioral reactivity to a stress challenge (36). Thus, animals with a higher locomotor response to novelty (HR) compared with low responder animals (LR), showed the highest sensitivity to drugs (36,31). These observations were confirmed when it was showed that HR rats had a higher preexisting dopaminergic activity (37,38,39) and a higher corticosterone (CORT) secretion in response to stress (40) than did LR rats.

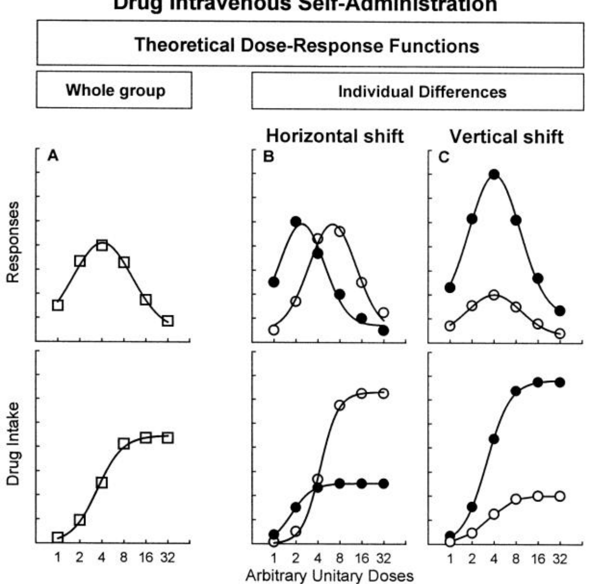



**Figure 2:** According to the model of horizontal shifts, vulnerable subjects are the most sensitive, i.e., the ones leftward shifted, and these individuals would develop self-administration at low unitary doses of the drug (Fig. 2B, top). However, at higher doses, "resistant" subjects, which are the less sensitive and rightward shifted, would provide an equal rate of self-administration (Fig. 2B, top) and even take In vertical shifts (Fig. 2C), vulnerable subjects, which are the upward shifted ones, present a higher rate of responding across doses (Fig. 2C, top) and consume the highest quantities of the drug (Fig. 2C, bottom). Consequently, these vulnerable individuals would have the highest chances to develop drug abuse also in environmental conditions in which both low and high drug doses are available.

Reproduced from ref (35): Piazza PV, Deroche-Gamonent V, Rouge-Pont F, Le Moal M. Vertical shifts in self-administration dose-response functions predict a drug-vulnerable phenotype predisposed to addiction. J Neurosci. 2000. 20:4226-32.

The role of individual vulnerabilities has been also established for the most severe SUDs, which is characterized by loss of control (41). After three months of self-administration, the three addiction-like behaviors according to the DMS-IV [1) high motivation for the drug, measured by a progressive ratio reinforcement schedule; 2) inability to refrain from drug seeking even if the drug was not available, measured by active responses during periods of signaled drug non-availability; 3) drug use despite negative consequences, measured by resistance to foot-shock-induced punishment at cocaine self-administration], appeared in a small percentage of rats, whereas the largest proportion of rats maintained good control over drug intake (41). Importantly, vulnerability to loss of control was completely unrelated to vulnerability to sustained drug use (41,42). Recent publications support the existence of these two independent vulnerable phenotypes by showing that vulnerability to loss of control is associated with a different type of biological modification than the vulnerability to develop sustained escalated drug use (43,44).

Thus, individual-centered theories of addiction support that addiction results from a pathological response to the drug that is generated in a few individuals by a vulnerable biological

phenotype. The main differences among theories in this family principally concern different degrees of etiological relevance given to genetic factors (45,46,47,48), environmental factors (31,49,50), developmental factors (51,52,53), and gene plus environment interactions (54,55,56).

## 2. NEUROCIRCUITRY OF ADDICTION

### 2.1. MESOCORTICOLIMBIC CIRCUIT

All drugs of abuse exert their primary rewarding effects on the mesolimbic dopamine (DA) reward pathway, which consists of DA neurons originating in the ventral tegmental area (VTA) and extending to the striatal complex including the nucleus accumbens (NAc), the prefrontal cortex (PFC), the hippocampus and the amygdala (57). Addictive substances increase, directly or indirectly, firing of dopaminergic neurons in the VTA of the midbrain and a subsequent increase of DA release in the NAc (58,59). The NAc not only receives dopaminergic projections from the VTA, but also glutamatergic projections from a number of neocortical, allocortical, and thalamic areas, and plays a central role in the neurobiological processes of reward, learning, impulsivity, and addiction (60). Opiates bind to the three classical opioid receptors, mu (MOR), delta (DOR), and kappa (KOR), and mediate DA release by reducing the inhibitory gamma-aminobutyric acid (GABA) release on VTA DA neurons (61,62,63). It has also been established that morphine increases DA level through the MOR in the NAc, which may mediate reinforcing effects of morphine (64). Relevant to these observations, endogenous opioid peptides, such as enkephalins or dynorphins, are upregulated in the NAc after exposure to morphine, and modulate DA release in the midbrain (65). Two major distinctions exist between DA release following motivational biological stimuli versus following exposure to an addictive drug. First, the release of DA by addictive drugs is of greater amplitude and duration than the one that can be achieved through physiological mechanisms. The second major difference is that tolerance develops to the release of DA by biological stimuli, whereas addictive drugs release DA every time the drug is taken. In chronic users, increased dosage is required due to tolerance, but with sufficient dose, a DA increase reliably occurs (66,67).

The association between increased DA transmission and learning behaviors to obtain reward has led to an understanding that DA release is a key event to facilitate learning. The release of DA has been proposed to imbue an event with salience, creating an internal sense that this event requires the development of a behavioral response (68). Thus, once a behavior designed to obtain a reward or avoid a negative consequence has been learned, the role of DA changes from promoting new learning to enabling the use of learned information to efficiently execute the adaptive behavioral response (69).

Thus, the increase in DA is backward-shifted and is activated by discriminative stimuli that predict drug availability more than by the drug itself (70). Consequently, conditioned stimuli associated with drug delivery become important discriminative stimuli of drug availability and, under some training conditions, secondary reinforcers. It is noteworthy that exactly the same phenomenon happens with natural reinforcers, showing that these neurobiological mechanisms activated by drugs are part of the normal learning sequence (71).

More than 90% of the cells in the NAc are GABA releasing (GABAergic) medium spiny neurons (MSNs) with a small portion of cholinergic aspiny cells and GABAergic interneurons (72). MSNs in the striatum are typically divided into two groups, based on DA receptors (DR) (Figure 3). To date, five different subtypes of DR have been cloned, and based on their structural and pharmacological properties, a general subdivision into two groups has been made: the D1-like receptors (DRD1)-like, which stimulate intracellular cyclic adenosine monophosphate (cAMP) levels, comprising D1 (73,74) and D5 (75,76), and the D2-like receptors (DRD2)-like, which inhibit intracellular cAMP levels, comprising D2 (77,78), D3 (79), and D4 (80) receptors. In contrast to dorsal striatum, the discrete separation of DRD1-MSNs in the direct pathway and DRD2-MSNs in the indirect pathway does not apply in NAc (72) (Figure 4). DRD2 are located postsynaptically in soma and dendrites to regulate firing rate of neurons and presynaptically in axons to regulate DA synthesis and release (81). However, it has been shown that DRD2 located postsynaptically in GABA projecting neurons of the striatum also regulate DA release (82).

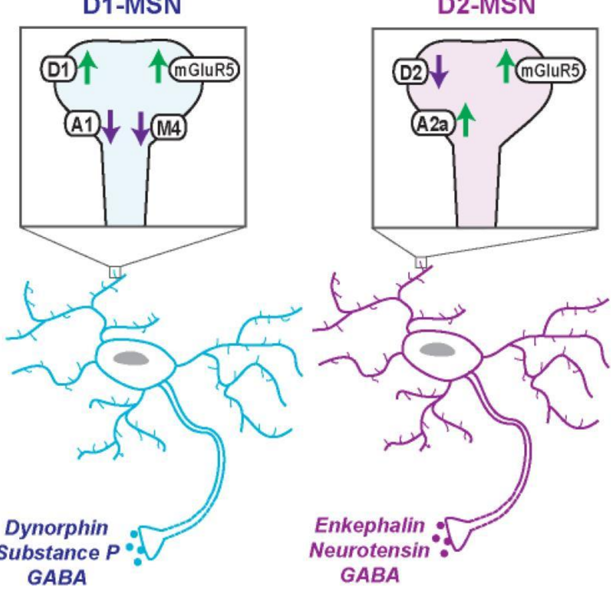



**Figure 3:** DRD1 and DRD2 MSNs in the NAc with co-localized receptors and neuropeptides. DRD1-MSNs also express M4 cholinergic receptors, A1 adenosine receptors, dynorphin, and substance P, whereas DRD2-MSNs co-express A2a adenosine receptors, enkephalin, and neurotensin.

Reproduced from ref (72): Smith RJ, Lobo MK, Spencer S, Kalivas PW. Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways). Curr Opin Neurobiol. 2013. 23:546-52.

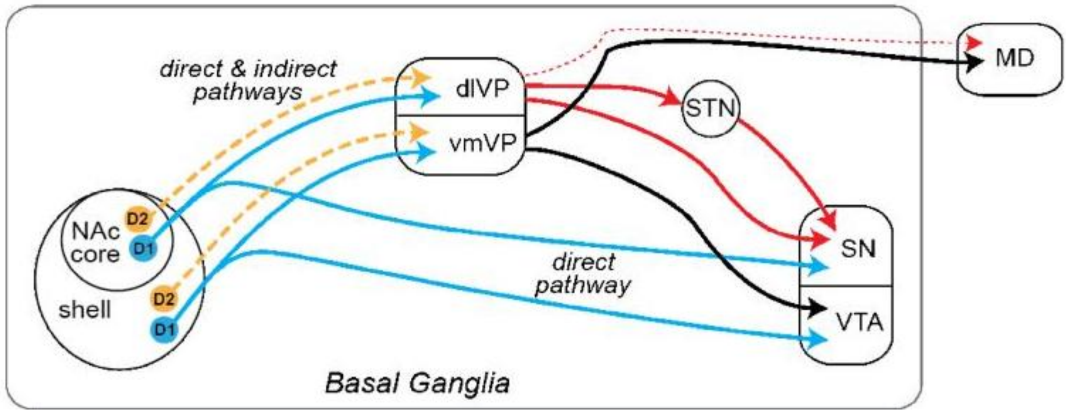



**Figure 4:** Output pathways of NAc DRD1-MSNs and DRD2-MSNs. Both, DRD1-MSNs and DRD2-MSNs in NAc project to ventral pallidum (VP), whereas DRD1-MSNs alone project to ventral mesencephalon output structures.

Reproduced from ref (72): Smith RJ, Lobo MK, Spencer S, Kalivas PW. Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways). Curr Opin Neurobiol. 2013. 23:546-52.

DA has different affinity for DRD1-like and DRD2-like receptors. DRD1 receptor, which is known as the low-affinity DA receptor, is thought to be preferentially activated by the transient, high concentrations of DA mediated by phasic bursts of dopaminergic neurons (83,84). In contrast, it is hypothesized that DRD2-like receptors, which are known to have a high affinity for DA, can detect the lower levels of tonic DA release (85). The effects of DR activation are complex and distinctions exist between activation of DRD1-like versus DRD2-like receptors (81). One signaling pathway of particular interest in neurons is the mitogen-activated protein kinases (MAPK), extracellular-signal regulated kinases (ERK), which are activated by DRD1 and DRD2 (81)( Figure 5,6).

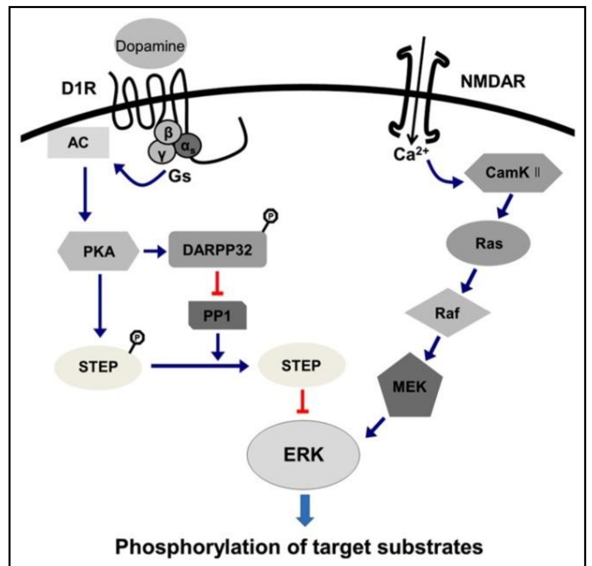



**Figure 5:** DRD1 receptor-mediated ERK activation signaling pathway.

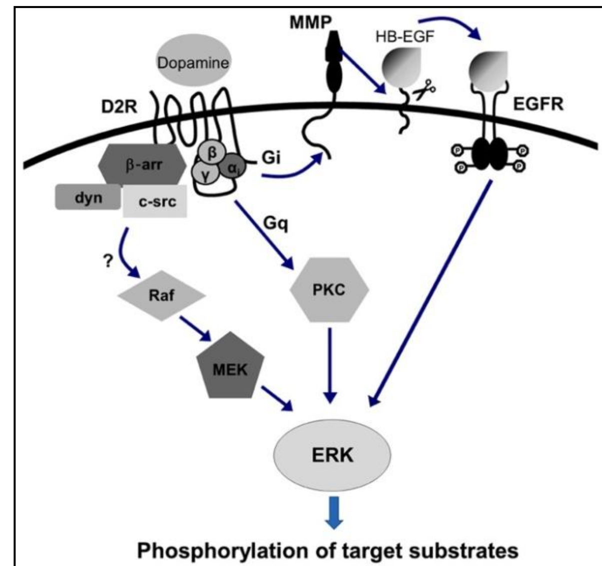



**Figure 6:** DRD2 receptor-mediated ERK activation signaling pathway.

Figure 5 and 6 reproduced from ref (81): Baik JH. Dopamine signaling in reward-related behaviors. 2013. 7:152.

Importantly, DRD1-MSN and DRD2-MSN cell populations are implicated in distinct aspects of NAc function. NAc DRD1-MSNs are critical for acquiring reward-based learning, and blockade of DRD2-MSNs is associated with loss of behavioral flexibility when the task calls for a switch in learning strategies (86). Opposing roles for the cell types in learning is also supported by the finding that selective stimulation of DRD1-MSNs in dorso-medial striatum induces persistent reinforcement, while stimulating DRD2-MSNs causes transient punishment (87). Concerning drug-associated behaviors, DRD1-MSNs in the NAc or dorso-medial striatum are involved in the development of place preference and sensitization, whereas DRD2-MSNs oppose these behaviors (88,89,90,91).

## 2.2. THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

In response to stressful stimuli, secretory neurons of the paraventricular nucleus (PVN) of the hypothalamus discharge corticotropin-releasing factor (CRF) that in turn increases both the secretion of adrenocorticotrophin hormone (ACTH) and the transcription of its precursor, the proopiomelanocortin (POMC) gene, in the anterior pituitary. ACTH stimulates the release of GCs (cortisol in humans and CORT in rodents) and the transcription of genes encoding several steroidogenic enzymes in the adrenal gland. GCs exert diverse effects on target tissues to mobilize energy for the body to deal with the stressor, and also exert a negative feedback through the inhibition of the synthesis and secretion of CRF and POMC (92,93,94,95) (Figure 7). GCs are highly lipophylic and, thus, rapidly enter the brain and bind directly to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (96,97). MRs have a high affinity for the natural steroids CORT and aldosterone and are mostly saturated during basal CORT levels, whereas GRs become occupied by higher levels of CORT (98,99).

The HPA axis is activated following acute administration of many addictive substances, causing increased ACTH and CORT levels in plasma (100). Chronic administration of drugs of abuse in the same animal models results in either a sustained increase in HPA axis function, in the case of cocaine and amphetamine, or a reduced effect of the initial activating effects of the drug, in the case of morphine, nicotine, and alcohol (101,102,103,104). Human studies demonstrate similar perturbations following illicit drug use, with slight differences. As in animal models, acute administration of cocaine (105), alcohol (106) and nicotine (107) increases cortisol levels, whereas acute exposure to opiates decreases cortisol levels (108,109). Activation of the HPA axis is maintained in cocaine addicts (110), whereas following chronic opiate use, HPA responses are reduced over time (111), a more typical response to repeated exposure to a stressor (112,113).

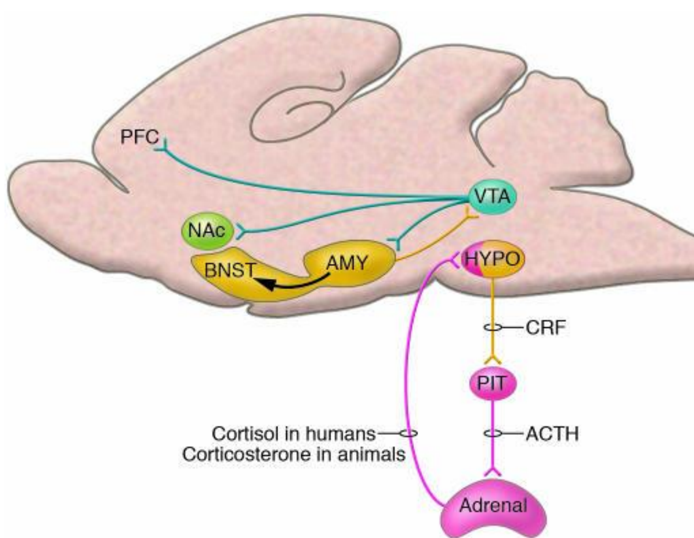



**Figure 7:** The mesolimbic DA reward pathway is depicted in blue. The hypothalamic CRF projections are directed to the pituitary gland (PIT) and stimulate the endocrine output of the HPA axis (red) including the release of ACTH, which acts on the adrenal gland to stimulate the secretion of GCs. Central CRF circuitry (yellow) consists of CRF-containing cell bodies located in the central nucleus of the amygdala, which projects to the BNST. CRF projections from the amygdala also innervate the VTA, thus completing the circuit.

Reproduced from ref (114): Cleck JN, Blendy JA. Making a bad thing worse: adverse effects of stress on drug addiction. 2008. 118:454-461.

## 2.3. THE EXTRAHYPOTHALAMIC CRF CIRCUIT

In addition to its crucial role in the activation of the HPA axis, CRF functions as a neurotransmitter/neuromodulator within the CNS, coordinating extra-hypothalamic aspects of the stress response (115,116). The placement of CRF and its receptors, CRF receptor 1 (CRFR1) and CRFR2, throughout the limbic system and neocortex suggests a critical role for this peptide in affective disorders, including addiction (117). Both CRF receptors are seven transmembrane domain G-protein coupled receptors that function by interacting with $G_s$-protein, thus increasing cAMP levels, although may also couple other G-proteins (118).

The extrahypothalamic CRF circuit, also called the extended amygdala, is an important structure for emotional and affective behavior, and has been delineated in mediating stress-induced relapse. Structures comprising the extended amygdala overlap with those of the reward pathway, including the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and the shell of the NAc [NAc(shell)] (119,120) (Figure 7). Thus, the extended amygdala represents a common anatomical substrate where brain arousal-stress systems with hedonic processing systems are integrated in order to produce the negative emotional states that promote negative reinforcement mechanisms, associated with the development of addiction (60).

CRF from extrahypothalamic sources was demonstrated to play a key role in the behavioral responses to stressors (121). Cocaine, morphine and alcohol tend to increase CRF expression acutely (122,123,124,125,126); however, the direction in which CRF expression is altered chronically and following withdrawal from these drugs depends on both the brain region and the drug studied. For example, following chronic morphine administration increased CRF levels were observed in the

hypothalamus, while decreased levels have been reported in the BNST (124,125). The differences in CRF expression among the different drugs highlight the distinctive pharmacological and molecular mechanisms throughout the CNS that each drug uses in exerting its addictive properties (114).

### 2.4. NORADRENERGIC PATHWAYS

Literature points for a role of noradrenergic transmission within the reward system in drug addiction. α1-adrenergic receptors control a neural pathway responsible for the release of DA in the NAc (127). Additionally, noradrenergic neurons modulate the DA cell firing pattern via excitatory postsynaptic α1-adrenoceptors (128). Moreover, DA release in the PFC is regulated by local noradrenergic nerve terminals, and electrical stimulation of the locus coeruleus (LC) neurons increases both, extracellular DA and noradrenaline (NA) in the PFC (129).

On the other hand, NA also augments CRF release in different nuclei such as PVN, BNST and CeA (60), and this CRF would induce the release of NA by the brainstem noradrenergic areas (feed-forward system). Interestingly, CRF1R expression in the VTA, LC and nucleus tractus solitarius (NTS) catecholaminergic neurons has been observed (130,131). Thus, CRF neurons would activate the NA cell firing rate, and noradrenergic neurons would estimulate CRF release (132,133). Moreover, an augment of noradrenergic activity in the PFC was detected as a consequence of CRF intra-cerebroventricular (i.c.v.) administration (134). Noradrenergic afferents from the NTS to the extended amygdala and PVN mediate not only the development of the motivational effects of opiate withdrawal, but also the stress-induced reinstatement of drug seeking behavior (135,136).

Previous works from our laboratory found an increased enzymatic activity of tyrosine hydroxylase (TH; the rate limiting enzyme in catecholamine synthesis) and TH activation in the NTS during morphine withdrawal (137,138). In addition, α1- and α2-receptor antagonists blocked the increased NA turnover in the PVN, the enhancement in CORT levels and c-Fos expression in CRF cells from the PVN during naloxone-precipitated morphine withdrawal, suggesting that the HPA axis is activated during morphine withdrawal through the modulation of noradrenergic connections from the NTS to the PVN (139,140,141).

## 3. NEUROPLASTICITY UNDERLYING TRANSITION TO ADDICTION

Different types of molecular and cellular adaptations occur in specific brain regions to mediate addiction-associated behavioral abnormalities. These include alterations in gene expression achieved in part via epigenetic mechanisms, plasticity in the neurophysiological functioning of neurons and

synapses, and associated plasticity in neuronal and synaptic morphology mediated in part by altered neurotrophic factor signaling (142).

It has been reported a relationship between neuroplasticity, motivated learning, brain circuitry, and the stages of addiction (Figure 8) (143). Two different categories of neuroplasticity have been proposed: first, relatively transient changes in neuronal function that continue for hours up to weeks of drug abstinence. Transient neuroplasticity corresponds to the necessary changes that are antecedent to develop a new behavior, and is referred to as social use. Secondly, relatively stable changes lasting from weeks to being relatively permanent changes. Thus, repeated drug insults cause enduring changes in the synaptic physiology of brain circuits regulating cognitive and emotional responding to important environmental stimuli, which guides the execution of learned behavior. This is illustrated in Figure 8a as two phases of relapse. The first phase of relapse (regulated relapse) refers to a relatively declarative decision-making process whereby the addict consciously decides to relapse. In the second phase (compulsive relapse) the addict is not making a conscious choice. Figure 8b maps the current understanding of biological reward memory and learning processes onto the stages of addiction. Thus, the acquisition of memories and developing adaptive behavioral responses to important stimuli is referred to as acquisition and corresponds to social drug use. The counterpart to regulated relapse is the retrieval of declarative memories, that is, memories that are verbalized and are used in conscious decision-making. Finally, compulsive relapse can be considered equivalent to habit or procedural memories. The retrieval of procedural memories is not verbalized, and guides the unconscious execution of adaptive motor behaviors. Figure 8b also illustrates how key brain circuits and corresponding neurotransmitters map onto stages of addiction. Thus, learning to become addicted through social drug use critically involves dopaminergic cells in the VTA that release DA. As drug-seeking becomes well-learned, a reliance of the behavior on glutamatergic projections from PFC to the NAc emerges. Thus, regulated relapse strongly depends on the retrieval of drug-associated memories and the integration of these declarative memories through glutamatergic projections from the PFC to the NAc. While glutamate continues to play a dominant role in compulsive relapse in this model, the glutamatergic circuit shifts from more declarative, executive prefrontal circuitry to habit circuitry involving classic cortico-striato-thalamic motor pattern generators, and the procedural memories that drive the unconscious engagement of well-learned behaviors.

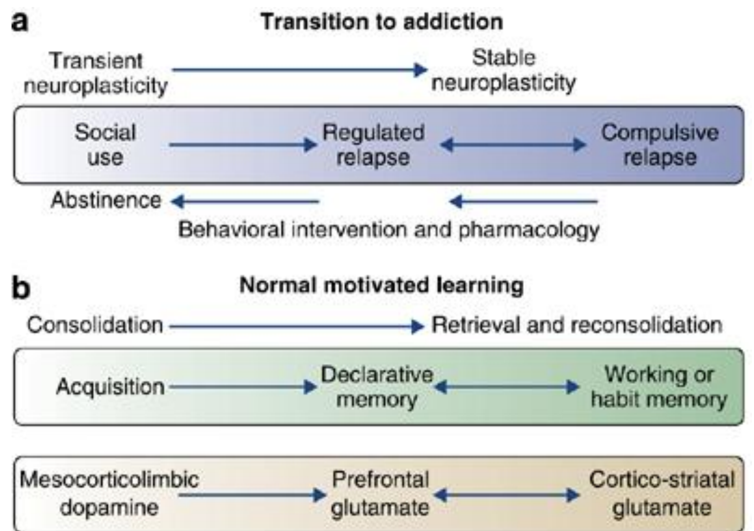



**Figure 8:** Relationship between neuroplasticity, brain circuitry, and the stages of addiction

Reproduced from ref (143): Kalivas PW, O'Brien C. Drug addiction as a pathology of staged neuroplasticity. Neuropsychopharmacology. 2008. 33:166-180.

### 3.1. CELLULAR PLASTICITY AND TRANSCRIPTIONAL MECHANISMS

Opiate-induced plasticity in different regions includes cellular plasticity (homeostatic changes in intracellular signaling cascades). Regulation of gene expression is considered a plausible mechanism of drug addiction, given the stability of behavioral abnormalities that define an addicted state (144). Numerous transcription factors, proteins that bind to regulatory regions of specific genes and thereby control levels of their expression, have been implicated in the addiction process (145). Among several others, a Fos family protein (ΔFosB), cAMP response element binding protein (CREB), Nur transcription factors subfamily and Pitx3 display very different regulation by drugs of abuse within the brain's reward circuitry, and in turn mediates, distinct aspects of the addiction phenotype.

#### 3.1.1. CREB

CREB is a transcription factor implicated in diverse aspects of neural plasticity (146). Both psychostimulants and opiates increase CREB activity, acutely and chronically, in multiple brain regions, including the NAc and dorsal striatum (147,148,149) (Figure 9). In addition, CREB activation occurs in both the dynorphin and enkephalin subtypes of MSN (147). Drug activation of CREB in NAc has been shown to represent a classic negative feedback mechanism, whereby CREB serves to reduce the animal sensitivity to the rewarding effects of drugs (tolerance) and to mediate a negative emotional state during drug withdrawal (dependence)(150,151,152). These effects have been shown recently to drive increased drug self-administration and relapse, presumably through a process of negative reinforcement (153). However, some studies show that CREB is necessary for the rewarding effects of addictive drugs and biological reinforcement (154,155,156), raising the possibility that

whereas acute regulation of CREB is required for motivated behaviors, repeated upregulation of CREB induces tolerance to the reinforcing effects of rewarding stimuli(143). CREB activity has also been directly linked to the functional activity of NAc MSNs, since CREB overexpression increases the electrical excitability of these neurons (157).

Drugs of abuse activate CREB in several brain regions beyond the NAc. In the VTA, chronic administration of cocaine or opiates activates CREB within dopaminergic and non-dopaminergic neurons. This effect seems to promote or attenuate the rewarding responses of drugs of abuse, depending on the subregion of the VTA affected (145). Interestingly, a large body of literature has shown that CREB, acting in the hippocampus and the amygdala, is a key molecule in behavioral memory (158,159,160).

Numerous target genes for CREB have been identified (161,162,149). CREB mediates the induction of dynorphin expression in NAc neurons, which results in activation of KOR on VTA DA neurons and thereby suppresses dopaminergic transmission to the NAc and impairs reward (150). Certain glutamate receptor subunits are also implicated, such as the GluA1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subunit and GluN2B N-Methyl-D-aspartate (NMDA) subunit, as well as $K^+$ and $Na^+$ ion channel subunits, which together would be expected to control NAc cell excitability (157,163). Brain derived neurotrophic factor (BDNF) is another target gene for CREB in the NAc (148). CREB induction has also been shown to contribute to cocaine induction of dendritic spines on NAc MSNs (164).

#### 3.1.2. ΔfosB

The 35-37 kilodaltons (kDa) ΔFosB isoforms accumulate with chronic drug exposure due to their extraordinarily long half-lives (165,166,167,168). The stability of the ΔFosB isoforms provides a novel molecular mechanism by which drug-induced changes in gene expression can persist despite relatively long periods of drug withdrawal (Figure 9). Thus, ΔFosB is proposed to function as a sustained "molecular switch" that helps to initiate and then to maintain an addicted state (169,170).

ΔFosB-overexpressing mice selectively in the dynorphin-containing MSNs (DRD1-type) show augmented locomotor responses to cocaine after acute and chronic administration (171). They also show enhanced sensitivity to the rewarding effects of cocaine and of morphine in place conditioning assays (171,161,172), and work harder for cocaine (173). Additionally, ΔFosB overexpression in NAc exaggerates the development of opiate physical dependence and promotes opiate analgesic tolerance (172). In contrast, ΔFosB-overexpressing mice are normal in several other behavioral domains, including spatial learning as assessed in the Morris water maze (171). On the

other hand, targeting ΔFosB expression to the enkepahlin-containing MSNs in NAc and dorsal striatum (DRD2-type) fail to show most of these behavioral phenotypes (172).

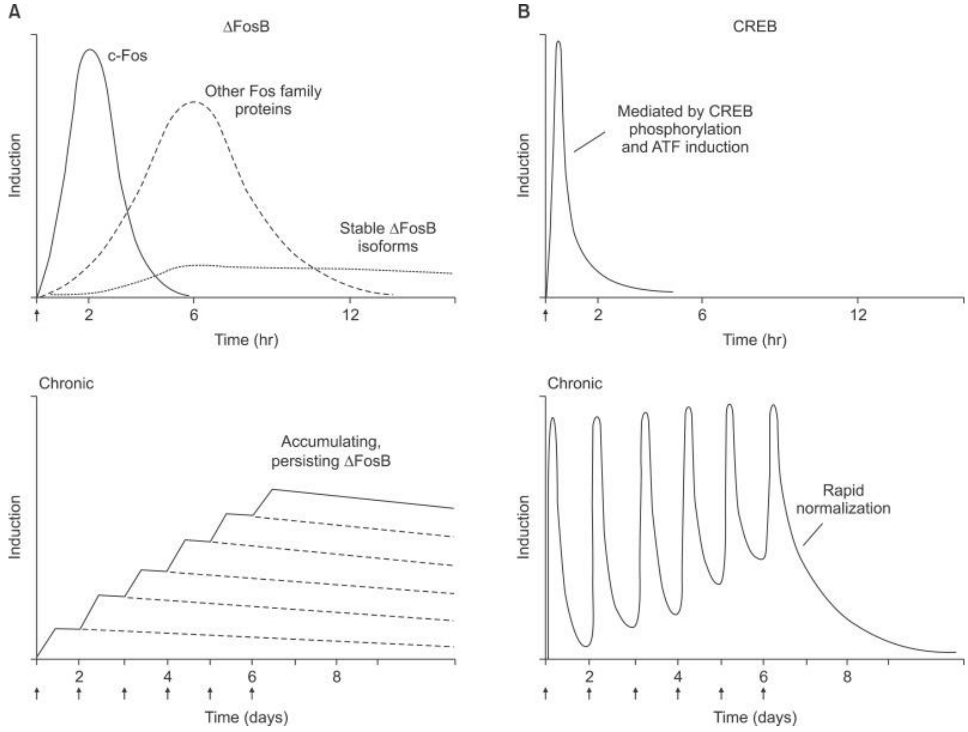



**Figure 9:** A) Fos family transcription factors, which include c-Fos, FosB, Fra1, and Fra2, heterodimerize with Jun family proteins (c-Jun, JunB, or JunD) to form active activator protein-1 (AP1) transcription factors. Fos family proteins are highly unstable and return to basal levels within hours of drug administration. However, after chronic drug administration, ΔFosB accumulates within the same brain regions whereas all Fos family members show tolerance. B) CREB forms homodimers that can bind to genes at cyclic AMP response elements (CREs), but activates transcription after it has been phosphorylated at Ser133. CREB activation is highly transient in response to acute drug administration and reverts to normal levels within a day or two after withdrawal.

Reproduced from ref (145): Nestler, EJ. Transcriptional mechanisms of drug addiction. Clin Psychopharmacol Neurosci. 2012. 10:136-143.

ΔFosB functions primarily as a transcriptional activator, while it serves as a repressor for a smaller subset of genes (161). ΔFosB appears to be induced by drugs of abuse (174). In dynorphin-producing cells in the NAc, ΔFosB represses dynorphin gene expression (172). Since dynorphin is thought to activate KOR on VTA dopaminergic neurons, which inhibits dopaminergic transmission and thereby downregulate reward mechanisms (175,176), ΔFosB repression of dynorphin expression could contribute to the enhancement of reward mechanisms. Another ΔFosB target is cFos: as ΔFosB accumulates represses the *c-Fos* gene, which helps to create the molecular switch: from induction of several short-lived Fos family proteins after acute drug exposure to the predominant accumulation of ΔFosB after chronic drug exposure (167).

### 3.1.3. Nur transcription factors subfamily and Pitx3

Nur transcription factors subfamily (Nur77 -NGFI-B, NR4A1-, Nurr1 -NR4A2- and Nor-1 -NR4A3-) are orphan members of the nuclear receptor superfamily(177,178,179,180), and share their classic structural organization (181)(Figure 10). Although Nur transcription factors have a well-recognized ligand binding domain structure, their transcriptional activity is not regulated by endogenous ligands (Benoit et al., 2004). Since the binding of ligands does not trigger the transcriptional activity of Nur factors, changes of their expression levels and post-transcriptional modifications appear keys to regulate their activity (182). Nur factors show some basal expression in specific nuclei of the rodent brain (183), but also behave as immediate early genes. Thus, their mRNA expression is induced independently of protein synthesis in several cell types by multiple kinds of stimuli (184,185,186,187).

Nur factors are associated to several functions (187), such as inflammatory (188,189) and oncogenic processes (190). However, some of the functions are exclusive for one factor, like the essential role of Nurr1 in the induction and maintenance of midbrain DA neurons. Unlike Nur77 and Nor-1, Nurr1 is expressed under basal physiological conditions in dopaminergic neurons of the substantia nigra (SN) and the VTA (191,192,193,194), and Nurr1 absence results in agenesis of dopaminergic neurons (195,196,197). Dopaminergic markers like TH, the dopamine transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) are not expressed in the ventral mesencephalon of Nurr1 null mice at birth (198,199).

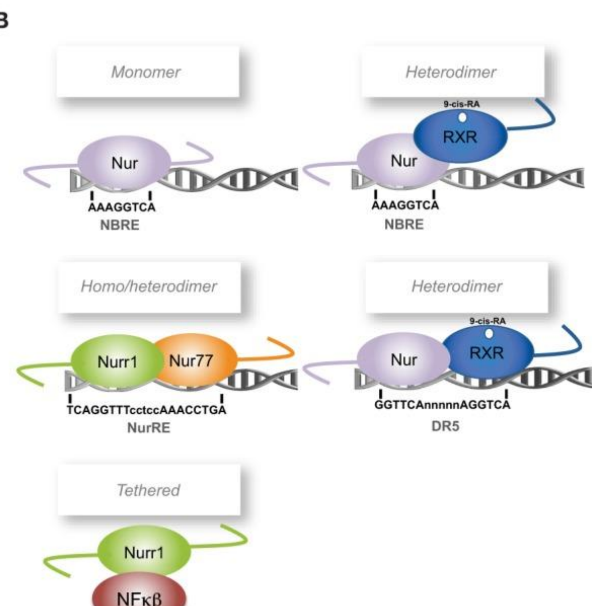



**Figure 10:** A) Nur transcription factors, structural organization: (1) a non-conserved N-terminal region containing the transcriptional activation function-1 (AF-1), (2) a conserved DNA binding domain (DBD) located in the middle of the proteins, and (3) a moderately conserved C-terminal domain, which encloses the ligand-binding domain (LBD) and the ligand-dependent transcriptional activation function 2 (AF-2). B) To exert their function as transcriptional regulators, Nur factors bind to nerve-growth-factor inducible gene B (NGFI-B)-responsive element (NBRE) as monomers, and to Nur-responsive element (NurRE) as homodimers or heterodimers between Nur factors.

Reproduced from ref (182): Campos-Melo D, Galleguillos D, Sánchez N, Gysling K, Andrés ME. Nur transcription factors in stress and addiction. Front Mol Neurosci. 2013. 6:44.

On the other hand, a progressive decline in the expression of the same genes in the SN and VTA is observed when Nurr1 is ablated during adulthood (200,201,202). For instance, aged Nurr1-deficient mice have analogous motor impairment to Parkinson's disease associated with decreased DA levels in the striatum (203). Moreover, the decreased number of TH positive cells in SN observed in aged Nurr1-deficient mice correlates with decreased DA release in the striatum (204). Nurr1-deficient mice also show some symptoms related to schizophrenia, associated with dysfunctions in DA neurotransmission (205,206). Importantly, it has been described that different set of genes are controlled by lower versus higher Nurr1 levels (207).

Evidence suggests that Nurr1 expression is regulated by DA signaling, mainly through DRD2. DRD2 knock out (KO) mice have increased Nurr1 expression in midbrain dopaminergic neurons (208) and the loss of DA in rat striatum generates a rapid increase of Nurr1 expression in DA neurons of the SN (194). Regarding drug administration, studies in cocaine abusers showed a reduction of Nurr1 expression in the SN (209). Similarly, rats chronically treated with cocaine show a down-regulation of the expression of Nurr1 mRNA and protein in the ventral midbrain (210). Additionally, chronic use of heroin in humans decreases Nurr1 mRNA in the VTA (211).

Another critical transcription factor for the development, survival and maintenance of dopaminergic neurons is the homeobox protein Pitx3 (212). At birth, Pitx3-deficient mice have a virtually complete loss of SN dopaminegic neurons, resulting in a 90% reduction in dorsal striatal DA (213,214,215,216). Although normal at birth, the VTA is progressively affected postnatally with a noticeable decline in DA cells (217,216), while no other brain regions are affected (213,214). Interestingly, within the CNS, Pitx3 is only expressed within the SN and VTA DA cells (218).

The gene encoding for Pitx3 activates the transcription of genes directly involved in the differentiation of dopaminergic neurons (219,220). A binding site for Pitx3 has been identified at the promoter region of *Th* gen (221), and a role for the transcription factor Pitx3 in TH regulation has been shown (220). Other genes identified as important regulators of DA function, such as *Dat* and *Vmat2*, are also targets of Pitx3. It has been indicated that robust expression of *Dat* and *Vmat2* genes can be detected when both Nurr1 and Pitx3 coexist (219,221). On the other hand, alterations in Pitx3 transcription and protein levels have been detected after acute and chronic cocaine and methamphetamine administration (Leo et al., 2007; Krasnova et al., 2011).

### 3.1.4. Neurotrophins

Neurotrophic signaling has been shown to modulate neural plasticity and behavior (222). Neurotrophin family members share significant homology (223), and include: nerve growth factor

(NGF) (224), BDNF (225,226), neurotrophin-3 (NT3) (227), and neurotrophin-4/5 (NT4/5) (228). Importantly, the pro- (immature) forms of the neurotrophins mediate signaling cascades distinct from the mature peptides (229). The p75 neurotrophin (p75NTR) receptor binds both the immature and mature forms of all four neurotrophins (230,231,232). BDNF also binds the tropomyosin-related kinase B (TrkB) receptor (233). BDNF receptor activation induces stimulation of different signaling pathways: the phosphatidylinositol 3′-kinase (PI3K), MAPK, phospholipase Cγ (PLCγ) and nuclear factor kappa B (NFκB), which influence a range of cellular functions including neuronal survival, growth, differentiation, and structure (234).

Studies using conditional deletions of BDNF or the TrkB receptor show that they are required for proliferation and maturation of dendritic spines in developing neurons as well as the maintenance and proliferation of spines on neurons throughout the adult brain (235,236,237,238). According to this, accumulating evidence suggests an essential role for this neurotrophic factor signaling in the neuronal remodeling that occurs after chronic drug administration. Stimulants produce a widespread, but transient, induction of BDNF protein in the NAc, PFC, VTA, CeA and the basolateral amygdala (BLA) (148,239,240). In contrast, after chronic morphine treatment, it has been shown that the number of BDNF immunoreactive cells in the VTA is decreased (241). Moreover, several neurotrophin signal cascades have been shown to be regulated within the mesolimbic DA system by opiates and stimulants. Stimulants dramatically increase ERK phosphorylation in numerous brain regions following acute or chronic drug administration (242,243,244,245,246,247). The effects of opiates on ERK signaling are less clear. Recently, it has been reported that ERK phosphorylation is decreased in the NAc (248), PFC (249), and VTA (234) after chronic morphine, an effect that is consistent with decreased neurite branching seen in these regions. In contrast, it has also been reported increased ERK activity in the VTA after chronic morphine (250,251,252).

It is well established the influence of BDNF on various phases of the addiction process. Local infusion of BDNF into the VTA or NAc augments locomotor and rewarding responses to cocaine, while global loss of BDNF exerts the opposite effect (253,254,255). Importantly, BDNF signaling may produce profoundly different effects on neuronal morphology and behavior depending on the brain region examined. For example, increased BDNF in the NAc enhances cocaine-induced behaviors (148,254), whereas in the PFC BDNF suppresses these same behaviors (256). The level of BDNF in the VTA, as well as NAc and amygdala, progressively increases during abstinence (239). This progressive increase has been hypothesized to underlie the progressive increase in drug-seeking that occurs during cocaine withdrawal (257,240). Interestingly, it has been described that stimulating BDNF receptors in the amygdala, NAc, or VTA promotes (254,255,148,258), whereas microinjection of BDNF into the PFC inhibits drug-seeking (256). Moreover, BDNF infusions in the hippocampus

are antidepressant-like, whereas infusions of BDNF in the VTA or NAc produce prodepressant-like effects (259,260,261).

### 3.2. SYNAPTIC PLASTICITY

Opiate-induced plasticity also includes synaptic plasticity, described as persistent changes in glutamatergic and GABAergic synaptic transmission (262,263). Focused on glutamate transmission, decreased levels of basal extrasynaptic glutamate are observed in rodents in the NAc(Core) following chronic exposure to drugs of abuse (264), resulting in a lack of tone on inhibitory presynaptic mGluR2/3 subtype of metabotropic glutamate receptors(mGluR) and a dramatic enhancement of excitatory transmission at PFC-NAc(Core) synapses (264).

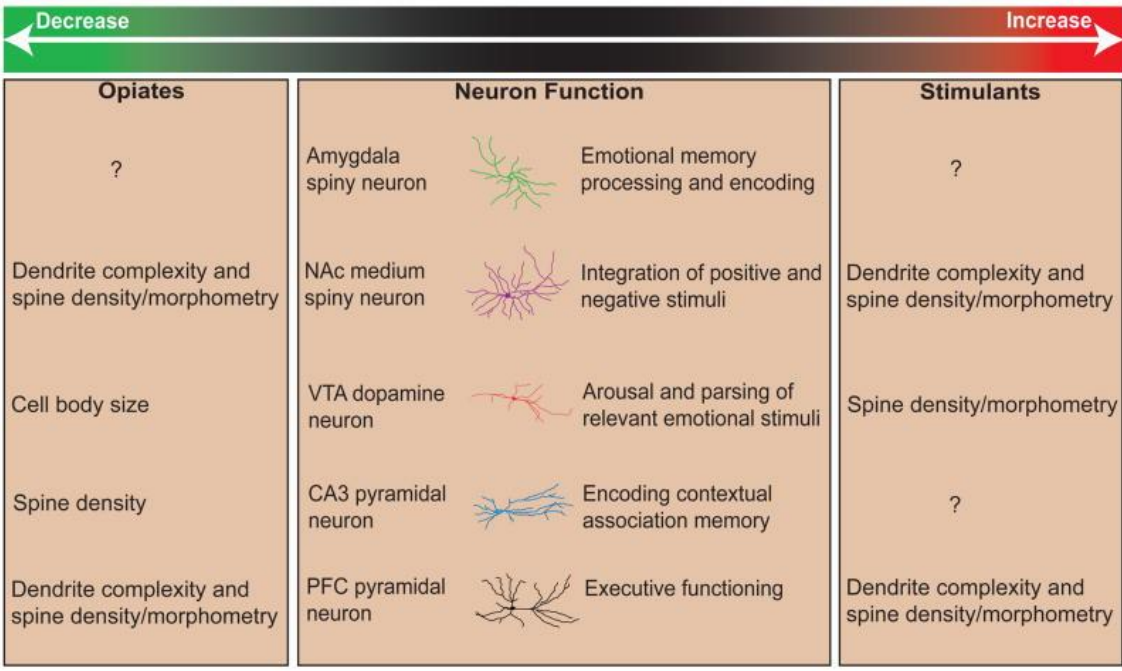
Moreover, there is a general reduction in prefrontal cortical measures of cellular metabolism and blood flow in individuals addicted to a variety of different drugs (265). This hypofrontality has been characterized as a strong indicator of reduced ability to regulate drug-seeking (266). A reduction in frontal cortical neurons has also been reported in drug-free cocaine addicts (267). Interestingly, when exposed to a cue previously associated with drug use that precipitates the desire for drug, there is marked activation in the PFC (265,268,269), which has been positively correlated with the intensity of cue-induced desire for the drug. Moreover, consistent with addiction being characterized in part by reduced response to biological rewards, when cocaine addicts were presented with a sexual stimulus, prefrontal activation was significantly impaired compared to controls (270). Also, addicts show reduced levels of DRD2 receptors in the striatum (271). Since reduced DRD2 receptors indicates blunting of DA transmission, it is not surprising that addicts report reductions in high or pleasure in response to methylphenidate relative to control subjects. In contrast, whereas the methylphenidate induces strong cravings in the addicts, there is no craving in comparison subjects (143).

There is also increasing evidence for the importance of whole cell plasticity, also referred to as homeostatic plasticity (272), which involves changes in the intrinsic excitability of an entire nerve cell in a manner that it is not synapse-specific. Chronic opiates increase the intrinsic excitability of noradrenergic neurons of the LC (273). This increased excitability is mediated via CREB-dependent induction of certain isoforms of adenylyl cyclase (273,274,275,276). This hyperexcitabilty of LC neurons represents a classic mechanism of tolerance and dependence and drives some of the signs and symptoms of opiate withdrawal. Interestingly, CREB mediates a similar form of whole cell plasticity in NAc MSNs, which are also rendered hyperexcitable by chronic exposure to drugs of abuse (157). Another example of whole cell plasticity is the hyperexcitability of VTA dopaminergic neurons that occurs after chronic exposure to opiate drugs (277,278). This adaptation is not mediated by CREB, but achieved instead via regulation of neurotrophic signaling cascades (277).

3.3. STRUCTURAL PLASTICITY

Opiate-induced plasticity also includes a third type of plasticity: structural plasticity (long-lasting changes in neuronal morphology) (279). Changes in synaptic plasticity are associated with morphological changes at synapses. For example, long-term depression (LTD) and the generation of silent synapses are associated with the formation of thin or stubby dendritic spines, whereas long-term potentiation (LTP) is associated with larger, mushroom-shaped spines (280,281). Chronic exposure to stimulant drugs of abuse increases the dendritic spine density of MSNs in the NAc (282,283,279). However, particular circuits display different forms of plasticity (Table 1). Literature suggests very different spine changes occurring at different withdrawal time points and in the NAc(Shell) versus NAc(Core) subregions (264,284,285,286). Although the induction of dendritic spines after repeated treatment with psychostimulants occurs in both DRD1 and DRD2 MSNs, the long-term stability of new spines appears to be greater in DRD1 neurons, supporting the idea that intracellular signaling pathways downstream of DRD1 may mediate longer-term stabilization of spines than in DRD2 neurons (287,288). ΔFosB has been shown to be both necessary and sufficient for the induction of immature spines on DRD1 NAc neurons (289,290,282). Indeed, the persistence of increased dendritic spines in DRD1 MSNs highly correlates with the persistent induction of ΔFosB in DRD1 MSNs and sensitized behavioral response to chronic drug exposure (291,170). Interestingly, cocaine regulation of several genes known to control the reorganization of the actin cytoskeleton, including induction of cyclin-dependent kinase 5 (CDK5), calcium-/calmodulin-dependent protein kinase type II (CaMKII) and NFkB, and repression of the dimethyltransferase G9a, are mediated via ΔFosB (292,289,290,293). In contrast, opiates exert the opposite effect and reduce dendritic spine density of NAc MSNs (283). This phenomenon is surprising, given that CREB and ΔFosB are induced by both stimulants and opiates and are both implicated in stimulant-mediated induction of NAc dendritic spine density.

Other major form of morphological plasticity is the physical reduction in cell soma size of VTA DA neurons induced by chronic opiate administration.(277,294,295) (Table 1), which seems to mediate reward tolerance and is associated with reduced DA release in the NAc. It has been directly linked the opiate-induced VTA neuron shrinkage to reduced activity of downstream BDNF signaling cascades, specifically reduced activity of the insulin receptor substrate-2 (IRS2), thymoma viral proto-oncogene (Akt), and TOR (Target of rapamycin) complex 2 (TORC2) (277,295). The downregulation of BDNF signaling directly modulates the increased excitability that morphine induces in these neurons (277,278). In contrast, stimulants induce BDNF signaling to the NAc, an effect due to increased local synthesis of BDNF as well as increased release from several afferent regions (148).



Decrease — Increase

| Opiates | Neuron Function | | Stimulants |
|---|---|---|---|
| ? | Amygdala spiny neuron | Emotional memory processing and encoding | ? |
| Dendrite complexity and spine density/morphometry | NAc medium spiny neuron | Integration of positive and negative stimuli | Dendrite complexity and spine density/morphometry |
| Cell body size | VTA dopamine neuron | Arousal and parsing of relevant emotional stimuli | Spine density/morphometry |
| Spine density | CA3 pyramidal neuron | Encoding contextual association memory | ? |
| Dendrite complexity and spine density/morphometry | PFC pyramidal neuron | Executive functioning | Dendrite complexity and spine density/morphometry |

Table 1: In several regions of the central nervous system opiates are observed to reduce neuronal size or extensions, while stimulants (e.g., cocaine and amphetamine) exert the opposite types of changes.

Reproduced from ref (234): Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ. Neurotrophic factors and structural plasticity in addiction. Neuropharmacology. 2009. 56 Suppl 1:73-82.

The regulation of the actin cytoskeleton, that can both stabilize or change spine morphology, is a candidate for a process that may underlie changes in spine density (296,297,298). Accordingly, there is an enduring increase in actin cycling after withdrawal from chronic psychostimulant administration (299). In addition to alterations in spine morphology, another consequence of increased actin cycling would be alterations in the trafficking of proteins into the postsynaptic membrane (300). A potentially critical change in postsynaptic receptor trafficking is an enduring increase in membrane insertion of glutamate receptors (301,302,303).

## 4. STRESS AND ADDICTION

Although many factors can contribute to initial and continued drug use, exposure to either psychological or physiological stress at any point in the addiction cycle seems to worsen this disease, augmenting all drug-seeking behaviors, including initial drug taking, drug craving, and relapse (304,305).

### 4.1. STRESS AND DRUG TAKING

Stress affects different stages of drug addiction. Exposure to physiological as well as physical stressors, including social isolation, tail pinch or footshock, can enhance initial amphetamine and cocaine self administration (306,307,308). Furthermore, repeated exposure to forced swim stress can augment the rewarding properties of cocaine (309). Thus, these studies implicate stress in modulating the initial rewarding effects of addictive drugs. Moreover, CORT release via the HPA axis is vital to the acquisition of drug administration. Inhibiting CORT release by adrenalectomy or pharmacologic treatment blocks cocaine self administration in rats (310,311). Furthermore, CORT release following drug administration in rats increases neuronal activity above the critical levels needed for self administration to occur (312). The ability of CORT to modulate drug reward can be mediated by GRs located on neurons throughout the mesolimbic DA reward pathway (313). Adrenalectomized (ADX) animals exhibit a blunted DA response in the NAc following either drug exposure (314) or stress (315). Similarly, GR antagonists decrease extracellular DA levels in the NAc (316). In addition, GR antagonists locally injected into the VTA decrease morphine-induced increases in locomotor activity (316), thus indicating that activation of GRs in the VTA can mediate DA-dependent behavioral outputs. Interestingly, in mice where the *GR* gene was deleted specifically in the CNS, a decrease in motivation to self-administer cocaine was observed (317). A role for CRF has also been proposed in stress-induced drug taking. For example, pharmacological blockade of CRFR1 inhibits cocaine-induced DA release as well as reductions in the rewarding properties of cocaine and locomotor activating effects (318,319).

### 4.2. STRESS AND WITHDRAWAL

Activation of the HPA axis and increased CORT levels occur following acute withdrawal from most drugs of abuse, both in humans and in animal models (100). During long-term withdrawal, the HPA axis displays an augmented response upon exposure to a stressor (320,321). These data suggest that the stress response can be sensitized by drug exposure and subsequent withdrawal. In addition, previous results from our laboratory showed that the physical signs of opiate withdrawal are modulated by GR signaling (322). Moreover, the somatic signs of opiate withdrawal were also modulated by MR signaling (323).

Also alterations in the CRF peptide and mRNA levels throughout the CNS were observed following acute withdrawal from several drugs of abuse, and these alterations vary depending on the brain region as well as the drug administered. Interestingly, increases in CRF mRNA in the PVN correlate with increases in anxiety behaviors during ethanol, cocaine, and morphine withdrawal, and blockade of the CRF system decreases the anxiety observed in this acute withdrawal phase

(324,325,326). These results suggest that the CRF1R subtype may be involved in the behavioral and somatic signs and in ACTH release (partially) during morphine withdrawal (327,326). Also, CRF2R signaling modulated physical signs of opiate withdrawal (328).

### 4.3. STRESS AND REINSTATEMENT OF DRUG SEEKING

Alterations in the responsiveness of the HPA axis to a stressor during long-term withdrawal might play a role in the ability of stressors to reinstate drug seeking long after the drug is removed (304,305). Exposure to an acute stressor can effectively reinstate drug seeking of various drugs (329,330,331,332). Stress facilitates relapse by activating central CRF brain circuits, since animals reinitiate lever pressing following an i.c.v. CRF injection (333).

The significance of the extended amygdala in the addiction cycle is evident primarily in relapse. Inactivation of the CRF projection from the CeA to the BNST blocks stress-induced cocaine reinstatement (334,335), and local injections a nonspecific CRF receptor antagonist into the BNST, but not the amygdala, attenuates footshock-induced reinstatement (335). Interestingly, selective CRFR1 antagonists attenuate footshock-induced reinstatement of cocaine or opiate seeking (329,336) but have no effect on drug-induced reinstatement (333,337). These data demonstrate that stress stimulation of the CRF-containing pathway, originating in the CeA and extending into the BNST, and subsequent activation of CRFR1 localized in the BNST, triggers drug seeking in previously addicted animals.

### 4.4. STRESS AND REWARD PATHWAYS

It is well established that the mesolimbic DA reward pathway participates in the response to stress. Acute exposure to a stressor, such as footshock (338) or tail pinch (315), produces an increase in DA release in the NAc. Moreover, exposure to either drugs of abuse or stress produces similar alterations in the electrophysiology of neurons in the mesolimbic reward pathway in animals. Enhanced excitatory synaptic transmission, as evidenced by an increase in glutamate receptor activation, occurs in VTA dopaminergic neurons following exposure to either stress or different drugs of abuse (339). In addition, both stress and drugs of abuse cause alterations in dendrites (340,341,342). Thus, rats subjected to chronic restraint stress exhibit decreases in dendritic branching in the medial PFC (342). As stated above, alterations in dendritic branching are also observed following exposure to addictive drugs, with an increase occurring following exposure to cocaine and amphetamine, whereas reductions in branching occur following exposure to morphine (340,341). Together, these findings indicate that stress and drugs of abuse act similarly to affect the neurochemistry, electrophysiology and morphology of neurons involved in reward pathways. Also, common mechanisms can be observed

at the molecular level. Chronic stress increases ΔFosB in the NAc as well as in the PFC and BLA (343). CREB is regulated by both acute and chronic drug treatment throughout brain reward areas (344,155) and various stressors, including shock, repeated immobilization or forced swim are associated with increased CREB phosphorylation in several regions of the brain (345), including the NAc (151).

Importantly, CRF has been detected in the VTA (346). In both, cocaine-naive and cocaine-experienced rats, CRF is released into the VTA following an acute footshock, but only in cocaine-experienced animals glutamate and DA are released in the VTA in conjunction with CRF in response to a stressor (347). This release of glutamate and DA is dependent upon CRF, as local injections of CRF antagonists into the VTA attenuated the release of these two neurotransmitters (346,347). In addition, administration of CRFR2 antagonists, but not CRFR1 antagonists, locally into the VTA blocked the ability of footshock to reinstate cocaine seeking in a self-administration paradigm (347). Thus, these studies suggest a role for CRF in modulating DA cell activity, specifically following drug experience.

4.5. STRESS AND THE NORADRENERGIC SYSTEM

The noradrenergic system has been implicated in stress-induced reinstatement of drug seeking. Animal models have demonstrated that an α2-adrenergic receptor agonist (lofexidine) reduced opiate withdrawal symptoms by decreasing noradrenergic outflow in the CNS (348) and attenuated footshock-induced reinstatement of drug seeking (349). Moreover, selectively lesioning noradrenergic projections to forebrain areas effectively blocks stress-induced reinstatement in rats (350). Furthermore, local injections of noradrenergic antagonists into the BNST and CeA inhibited the ability of footshock to reinstate drug seeking (351).

Previous results from our laboratory have proposed a role for CRF and GR signaling in noradrenergic-mediated opiate withdrawal. Thus, we showed that the TH activation and stimulation of noradrenergic pathways innervating the PVN are modulated by GR signaling (322). However, pretreatment with spironolactone (a MR antagonist) resulted in no significant modification of the increased NA turnover or TH activation that occurred during morphine withdrawal (323). Interestingly, increased GCs levels are responsible for induction of TH mRNA expression, phosphorylation, and enzyme activity in the NTS during morphine withdrawal (138). Also, CRF2R signaling modulated not only the physical signs of opiate withdrawal, but also the TH activation and stimulation of noradrenergic pathways innervating the PVN (328).

### 4.6. STRESS AND MOLECULAR MECHANISMS

The mechanisms by which stress affects the addiction process are not well understood. It is important to elucidate the molecular mechanisms underlying the interactions between stress and drug abuse, as an understanding of this may help in the development of novel and more effective therapeutic approaches to block the clinical manifestations of drug addiction. Some knowledge on this area has focused on stress-regulation of different transcription factors.

CREB-deficient mice do not exhibit stress-induced reinstatement of cocaine-conditioned place preference, but do exhibit reinstatement of drug seeking to a priming dose of cocaine (331). This deficit in stress- but not drug-induced reinstatement indicates a specific requirement for CREB in stress-induced behavioral responses to drugs of abuse. BDNF, a putative CREB target gene, was increased following withdrawal from chronic cocaine (239). The increase in BDNF in these brain areas positively correlated with the response of the rats to drug-associated cues and relapse to drug-seeking (239,148).

Several lines of evidence indicate that Nur transcription factors play a prominent role in adaptive responses to stress, regulating the transcription of target genes in the HPA axis: CRF and POMC (352,353) and different enzymes essential for the production of GCs in the adrenal gland (354,355,356). In addition, the expression of Nur transcription factors increases in the limbic nuclei associated to the extra-hypothalamic aspects of the stress response, suggesting that Nur factors could also underlie enduring changes induced by chronic stress (357,358). Interestingly, the increased Nurr1 expression has been suggested as a possible compensatory mechanism that may counteract the observed reduction of DA levels induced by stress (359,360,361). Importantly, Nur factors have been proposed to integrate stress and drug addiction signaling (362).

## 5. EPIGENETIC MECHANISMS

A crucial role for epigenetic mechanisms in driving lasting changes in gene expression has been proposed, which has prompted research aimed at characterizing the influence of epigenetic regulatory events in mediating the lasting effects of drugs of abuse (363). There is robust evidence that repeated exposure to drugs of abuse induces changes within the brain in three major modes of epigenetic regulation: non-coding RNAs, histone modifications such as acetylation (364,365,366) and methylation (290,367) and DNA methylation (368,369).

The complete sequencing of the mammalian genome and its transcriptional products has revealed a large number of expressed RNAs that are not translated into proteins. Such non-coding RNAs have been shown to play crucial regulatory roles in cell function (370,371). Most studied are miRs, ~22 nucleotide (nt) non-coding RNAs that participate in gene regulation. They bind to 3′ untranslated regions (UTRs) of their mRNA targets, inhibiting the transcripts translation and/or destabilizing them (372,373)(Figure 11). However, miR targeting can also occur in coding regions (374,375). Interestingly, deletion of Argonaute (Ago), a protein crucial for the processing of miRs, alters behavioral responses to cocaine, with distinct effects observed for DRD1- versus DRD2-type MSNs (376).

Since the discovery that miRs are important regulators of gene expression, these molecules have been linked to biological and pathological processes such as drug addiction (377,378). Multiple miRs are reported to be up- or down-regulated by drugs of abuse. For instance, cocaine increases levels of miR-181a and decreases miR-124 and miR-let-7d in rat striatum (379,380,376,381,382), and mimicking the direction of each of these changes enhances cocaine reward. miR-212 is induced in rat dorsal striatum after cocaine self-administration, and serves to inhibit cocaine intake (383). This action was attributed to the ability of miR-212 to indirectly lead to the activation of CREB, a transcription factor that antagonizes cocaine reward (384). Moreover, chronic ethanol suppresses the expression of big potassium channels via induction of miR-9 in striatum and hypothalamus (385). Several additional genes implicated in addiction models, such as ΔFosB, DAT and glutamate receptor subunits, have also been related to drug-triggered alterations in specific miRs (380,386). However, there is very little knowledge about the intracellular signaling pathways through which synaptic transmission and neural activity are translated into epigenetic modifications.

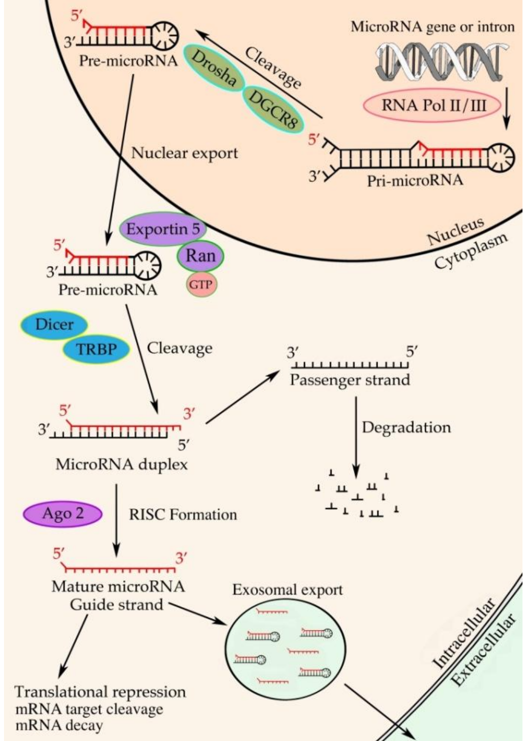



**Figure 11:** miR biogenesis involves transcription of pri-miR by RNA polymerase II/III, cleavage by the Drosha-DGCR8 complex to pre-miR, followed by export to the cytoplasm by Exportin-5. In the cytoplasm, pre-miR is cleaved by the Dicer-TRBP complex to a miR duplex, which is unwound to a guide strand that is bound to Ago2 and incorporated into the RISC, and a passenger strand, which is degraded. Ultimately, miR binding to target mRNAs results in mRNA target cleavage, translational repression, or mRNA decay.

Reproduced from ref (373): Møller HG, Rasmussen AP, Andersen HH, Johnsen KB, Henriksen M, Duroux M. A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. Mol Neurobiol. 2013.47:131-44.

5.1. OPIATES AND miRs

As stated above, miRs have been linked to drug addiction. Thus, literature supports a role for several miRs in mediating different aspects of opiate addiction. miR-let-7 represses MOR expression and works as a mediator moving MOR mRNA to P-bodies, leading to translation repression (377). It has been identified that miR-23b represses MOR translation efficiency (387), whereas fentanyl regulates the expression of miR-190 and one of its targets, neurogenic differentiation 1 (NeuroD) (388,389).

miR-133b, regulates the differentiation, maturation, and function of dopaminergic neurons by downregulating the transcription of its target Pitx3 in the dopaminergic system (390). In zebrafish embryos, morphine decreases miR-133b level and increases the mRNA levels of Pitx3 and Dat. Moreover, naloxone effectively abolished the morphine-induced changes in the expression levels of miR-133b, Pitx3, Th, and Dat (391,392)(Figure 12). In addition, inhibition of ERK 1/2, even when the MOR is activated by morphine, produces the same effect as knockdown of the receptor, i.e., the expression of miR-133b increases, and hence, the expression of Pitx3, TH, and DAT decreases, which reduces the level of dopaminergic neuron differentiation (392).

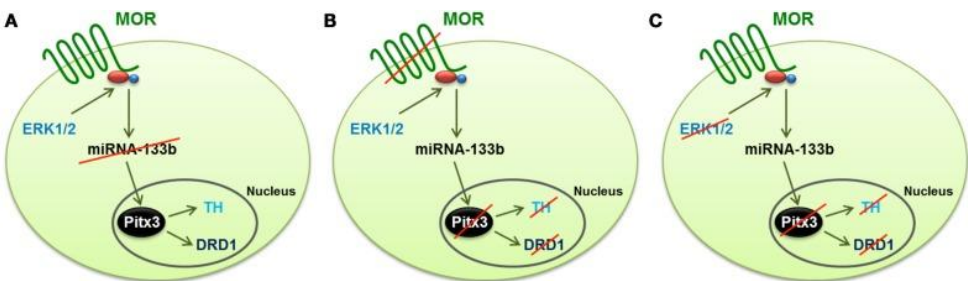



**Figure 12:** Schematic representation of the mechanism by which morphine regulates the differentiation of dopaminergic neurons through the control of the miR-133b.

Reproduced from ref (392): Rodríguez RE. Morphine and microRNA Activity: Is There a Relation with Addiction? Front Genet. 2012. 3:223.

## 6. MEMORY MODULATION

Addiction-related molecular and cellular adaptations involve most of the same brain regions that mediate classic forms of memory. This coincides with the increasing realization that some of the most important features of addiction seen clinically (eg, drug craving and relapse) reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology (143,269,20). Memory modulation systems influence different

neurobiological processes underlying the consolidation of new information, memory extinction, memory recall and working memory. Hormones of the adrenal medulla (adrenaline) and adrenal cortex (cortisol or CORT) are released during and immediately after the kind of emotionally arousing stimulation typically used in training tasks (393,394,395), and there is extensive evidence that these hormones enhance memory consolidation (396,397). Drugs and hormones affecting several other systems also enhance memory consolidation, e.g., CRF (398) or ACTH (399).

Adrenaline effects are most likely mediated by activation of β-adrenoceptors located on vagal afferents that project to the NTS in the brain stem (400). Early studies also reported evidence that adrenaline induces the release of NA in the brain (401). Thus, the NTS appears to be an interface between peripheral adrenergic activation and brain processes regulating memory consolidation. It has also been proposed that opioids and GABA impair memory by decreasing NA release in the brain (402,403,404).

Post-training injections of GCs produce dose- and time-dependent enhancement of memory (405,406,407). The memory-modulating effects of GCs appear to involve the selective activation of the low-affinity GRs (408,409,410). GCs are known to act through intracellular and intranuclear receptors and can affect gene transcription by direct binding of receptor homodimers to DNA (411,412) or via protein-protein interactions with other transcription factors such as Jun or Fos (413). However, GCs may also act more rapidly by interacting with membrane receptors and/or potentiating the efficacy of the NA signal cascade via an interaction with G-protein-mediated actions (414,415,416). Adrenergic activation is essential in enabling GC enhancement of memory consolidation (417), and training-induced emotional arousal is required in enabling GC effects on memory consolidation (405).

## 6.1. MEMORY CONSOLIDATION

In rodents, when an affective component is paired with a particular environment, it triggers the onset of association between affective consequences with the context, and results in memory formation/consolidation. It has long been known that noradrenergic activation of the BLA is crucially involved in strengthening the consolidation of long-term memory (418,419,397) (Figure 13). NA or a β-adrenoceptor agonist infused into the BLA immediately post-training enhances the retention of many different types of emotionally arousing training experiences, including inhibitory avoidance (420,421), contextual fear conditioning (422,423) or water-maze spatial training (403). In contrast, intra-BLA infusions of a β-adrenoceptor antagonist impair the consolidation of memory for these training experiences (403,424). However, it has been recently published that the BLA noradrenergic inervation also modulates long-term memory consolidation of object-in-context recognition training, a

low-arousing behavioral task designed to assess episodic-like memory in rats (425). Evidence that post-training intra-amygdala infusions of a synthetic cAMP analog enhance retention (426) is consistent with the hypothesis that activation of β-adrenoceptors modulates memory via a direct coupling to adenylate cyclase.

There is extensive evidence that the BLA is a critical locus of the synergistic actions of GCs and emotional arousal-induced noradrenergic activation in influencing memory consolidation (Figure 13). Systemic or intra-BLA infusions of GCs modulate memory consolidation (406,427). A β-adrenoceptor antagonist infused post-training into the BLA blocks the memory-enhancing effects of GCs (417). It has been suggested that activation of GRs in the BLA may facilitate memory consolidation by potentiating the NA-induced signaling cascade through an interaction with G-protein-mediated effects(428). *In vivo* microdialysis suggested that GCs also facilitate the training-induced release of NA in the amygdala (429).

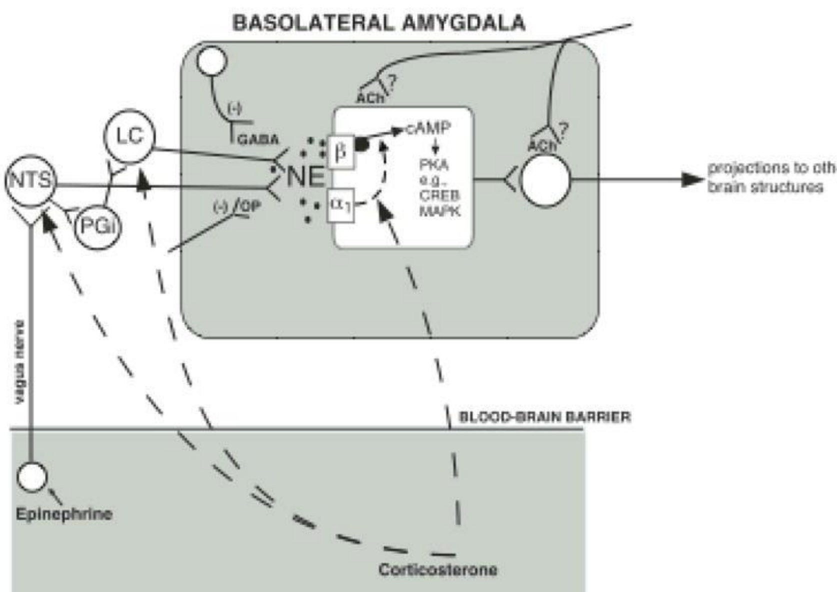



**Figure 13:** Role of the noradrenergic system of the BLA in memory consolidation. NA is released in the BLA and binds to both β-adrenoceptors and $\alpha_1$-adrenoceptors at postsynaptic sites. The β-adrenoceptor is coupled directly to adenylate cyclase to stimulate cAMP formation. The $\alpha_1$-adrenoceptor modulates the response induced by β-adrenoceptor stimulation.

Reproduced from ref (430): Roozendaal B, McGaugh JL. Memory modulation. Behav Neurosci. 2011. 125:797-824.

As was found with adrenaline, GC effects on memory consolidation also appear to involve brain stem nuclei, including the NTS, which send noradrenergic projections to the BLA. A GR antagonist infused into the NTS attenuates the memory-enhancing effects of systemically administered dexamethasone (431). Moreover, the finding that post-training infusions of the GR agonist RU 28362 into the NTS enhance inhibitory avoidance retention and that intra-BLA infusions of a β-adrenoceptor antagonist block the enhancement (431) provides additional evidence that the NTS influence on memory consolidation involves noradrenergic activation of the BLA (432).

It is recognized that spatial information about a given environment is acquired through the hippocampus and then moves to the cortex for long-term storage (433). The amygdala projects directly and indirectly to the hippocampus (434,435). Evidences of BLA-hippocampus interactions in memory consolidation have been proposed since unilateral post-training intra-hippocampal infusions of the specific GR agonist enhance rats retention of inhibitory avoidance training and the enhancement is blocked selectively by ipsilateral infusions of a β-adrenoceptor antagonist into the BLA (430). Lesions of the BLA, BNST or NAc also block the enhancement induced by GR activation in the hippocampus (436). Studies of BLA influences on hippocampal neuroplasticity provide additional important evidence of amygdala-hippocampal interactions. Electrical stimulation of the BLA enhances the induction of LTP in the dentate gyrus (DG) of the hippocampus (437,438). Also, selective lesions of the BLA or infusions of a β-adrenoceptor antagonist into the BLA block the induction of LTP in the DG (439,440). Importantly, the effects of BLA stimulation on DG LTP are influenced by NA and CORT (441,442).

### 6.2. MEMORY RETRIEVAL

Memory retrieval describes when animals are re-exposed to a previously paired environment, and thus, they can prefer or avoid the paired environment due to the association between the context and positive or aversive memories. Stress exposure or CORT administered systemically shortly before testing for memory of different training tasks produces temporary impairment of retention performance (443,444,445), indicating that GCs impair retention by influencing memory retrieval. Since the β-adrenoceptor antagonist propranolol administered systemically 30 minutes before inhibitory avoidance retention testing blocks the memory retrieval impairment induced by concurrent injections of CORT (446), and the stimulation of $\beta_1$-adrenoceptors with systemic injections of the selective agonist xamoterol induces memory retrieval impairment comparable to that seen after CORT administration (447), GC effects on memory retrieval impairment involve activation of noradrenergic mechanisms. Peripheral administration of the opioid antagonist naloxone or DRD2 antagonists also blocks the impairing effect of concurrently administered CORT or dexamethasone on memory retrieval (444,448).

Many studies have reported evidence that the hippocampus is involved in retrieval of spatial and contextual information (449,450). Inactivation of the hippocampus with local infusions of the glutamatergic AMPA/kainate receptor antagonist or the GABAergic agonist muscimol impairs memory retrieval (451,452). Since administering a GR agonist into the hippocampus shortly before retention testing also impairs retrieval of spatial memory (453,447), the GC-induced memory retrieval impairment depends, in part, on GR activation in the hippocampus. Moreover, a β-adrenoceptor antagonist infused into the hippocampus prevents the retrieval-impairing effect of a GR agonist

administered concurrently (447). In contrast, infusions of the protein-synthesis inhibitor anisomycin do not block CORT effects on memory retrieval (454), suggesting that stress and CORT may influence memory retrieval through a protein synthesis-independent mechanism, a finding consistent with the rapid onset of stress and GC effects on memory retrieval.

Memory retrieval of emotionally arousing information also induces the activation of the BLA (455,456). Furthermore, intra-BLA infusions of NA or AMPA receptor antagonist affect retrieval of memory for inhibitory avoidance training (457,458). In contrast, intra-BLA infusions of a GR agonist do not appear to affect memory retrieval (453). However, the BLA interacts with the hippocampus in mediating GC effects on memory retrieval. Lesions of the BLA or infusions of a β-adrenoceptor antagonist into the BLA block the impairing effect of a GR agonist infused into the hippocampus on memory retrieval (447,453) indicanting that, similarly to memory consolidation, BLA regulates memory retrieval via interactions with other brain regions.

### 6.3. EGR1

Among the studies of the functional role of inducible transcriptional regulators in synaptic plasticity and memory processes, many have focused the Early Growth Response (Egr) family (459,460). The Egr family comprises four members: Egr1 (also known as NGFI-A*,* Krox-24*,* Zif268*,* Tis8 *or* ZENK), Egr2 (Krox-20), Egr3 (Pilot) and Egr4 (NGFI-C) (461,462). In addition to their basal expression, the expression of Egr family members can be induced in different brain areas after a variety of neuronal stimulation paradigms, such as the LTP induction or maximal electroconvulsive shocks inducing seizure activity, or in behavioral circumstances after exposure to novel environments of specific learning experiences (461,463,464).

Egr1 is rapidly induced in dentate granule cells following the induction of LTP (465,466) and its expression has been widely used as a marker of neuronal plasticity in a learning context (463,464). Interestingly, Egr1 is involved in the maintenance of LTP (467), in the consolidation of different forms of long-term memory (460,468,467,469) and in the reconsolidation of memory after reactivation during retrieval (470,471). In addition, enhanced memory strength was reported in mice over-expressing Egr1 (472). In parallel, Egr1-deficient mice show inability to form long-term memories in a variety of behavioral tasks, while short-term memory, as well as early-LTP, is intact (467).

Although a wide range of neurotransmitter systems can induce expression of Egr family members (461), the cell-signaling pathways leading to the regulated expression of Egr members in synaptic plasticity and learning have not been precisely defined. In the hippocampus, activity-dependent expression of Egr1 is dependent upon NMDA receptor (465,473,466) and MAPK/ERK

activation (474), both of which are known to play a key role in synaptic plasticity and learning. Conversely, Egr1 can be negatively regulated by the protein phosphatase calcineurin, a negative regulator of neuronal signaling and of memory formation (472). In addition, Egr1 has been shown to be regulated by BDNF/TrkB and GCs can enhance the memory of stressful events driving Egr1 expression (475,476).

### 6.4. ARC AND MEMORY

Changes in dendritic spine morphology are thought to be involved in synaptic plasticity (477), and cytoskeletal actin is the major structural component of the dendritic spine (478). Cytoskeletal actin role in synaptic plasticity is mediated by dynamic transformation between G-actin (monomeric globular actin) and F-actin (polymeric filamentous actin) through polymerization and depolymerization (actin rearrangement) (479,480,481). Literature supports a role for actin rearrangement in LTP maintenance and behavioral adaptations (479,482,483) and demonstrates that changes in the actin cytoskeleton in certain neuronal cell types alter behavioral responses to different drug of abuse (299,484). Importantly, actin rearrangements in the amygdala and the hippocampus are required for the acquisition and consolidation of the aversive memories of morphine withdrawal (485).

The immediate-early gene (IEG) activity-regulated cytoskeletal protein (Arc) is found at recently activated synapses and interacts with the cytoskeletal protein actin (486,487) (Figure 14). For instance, Arc controls LTP consolidation through regulation of local actin polymerization in the DG in vivo (488). Like Egr1, Arc is required for synaptic plasticity and for several forms of long-term memory (489). Arc, however, is a direct effector protein at the synapse. Upon cell activation, *Arc* mRNA traffics to dendrites and accumulates at sites of synaptic activity, where it is locally translated (490,486,487) and plays important roles in the structural modifications of the synapse (491,492). The expression of Arc is regulated as an IEG (493), but also as a late-response gene by a protein synthesis-dependent mechanism (494,495). Moreover, Arc has been identified as a direct target of the Egr1 (496,495). Furthermore, Arc synthesis is required for both the induction and consolidation of LTP elicited by local BDNF infusion, thus identifying Arc as a key molecular effector of BDNF in synaptic plasticity (488).

Noradrenergic stimulation of the BLA, which enhances memory consolidation, also increases dorsal hippocampal levels of Arc (497). Additionally, inactivation of the BLA impairs memory consolidation and decreases Arc protein levels or actin rearrangements in the dorsal hippocampus (497,485)}. The finding that intra-BLA infusions of muscimol attenuate the increase in Arc mRNA induced by contextual fear conditioning provides further evidence that the BLA modulates memory consolidation via regulation of Arc expression in the hippocampus (498).

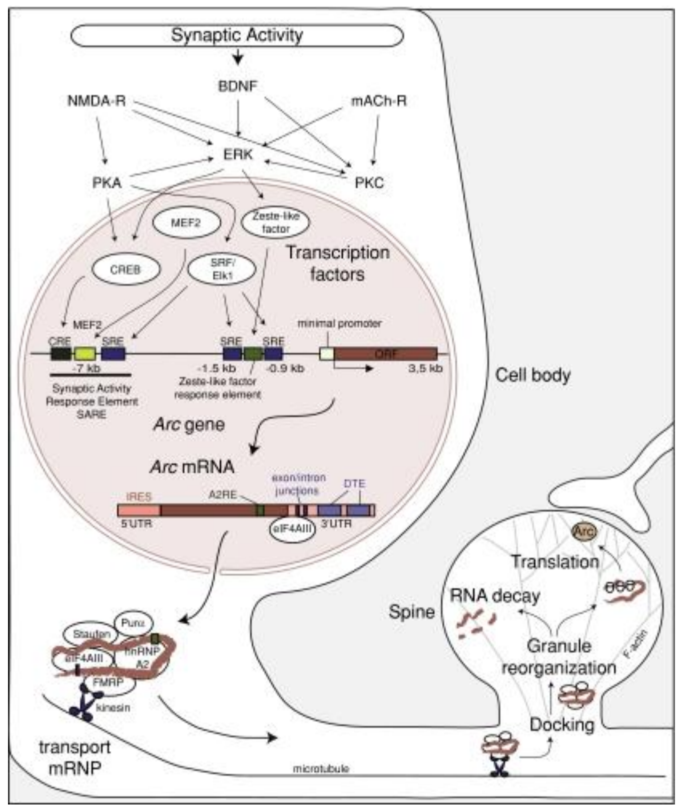



**Figure 14:** *Arc* expression is induced by signaling cascades that regulate transcription factors in response to synaptic activity. The diagram depicts the genomic organization of the synaptic activity-responsive element (*SARE*) and other regulatory elements upstream of the *Arc* open reading frame that serve as binding sites for these transcription factors. Following transcription, *cis*-regulatory elements of the *Arc* mRNA regulate its assembly into transport mRNPs. The dendritic localization and stability of the mRNA in dendrites is a result of active microtubule-based transport and local F-actin-dependent docking. Upon translation, *Arc* RNA is subject to rapid nonsense-mediated RNA decay.

Reproduced from ref (499): Bramham CR, Alme MN, Bittins M, Kuipers SD, Nair RR, Pai B, Panja D, Schubert M, Soule J, Tiron A, Wibrand K. The Arc of synaptic memory. Exp Brain Res. 2010. 200:125-40.

## 6.5. DRUG ADDICTION AND MEMORY

The aversive memories of drug withdrawal play a key role in the induction of a motivational state leading to drug seeking and taking (60). CPA test is one of the most sensitive measurement for the negative motivational state produced by opiate withdrawal in both chronically dependent (500) and acutely dependent animals (501). CPA is a pavlovian conditioning paradigm in which drug withdrawal is paired with a particular environment, which triggers the onset of association between the negative affective consequences of withdrawal with context (aversive memory formation), and then the animals are reexposed to the paired environment in drug-free state, leading to avoidance of the paired environment (aversive memory retrieval). Thus, CPA can be used to explore the neurobiological mechanisms underlying the formation and retrieval of aversive memories of drug withdrawal in drug-dependent animals. Place aversion is the reflection of the aversive memories formed by the negative affective consequences of morphine withdrawal with a particular environment (502). Interestingly, the amygdala and dorsal hippocampus are critically involved in mediating the negative affective component of morphine withdrawal in morphine-dependent rats (485).

## 7. GLIA AND ADDICTION

Astrocytes, a subset of glial cells, preserve homeostasis of the extracellular space by buffering potassium and glutamate (503). Astrocytes extend thousands of fine membranous processes, many of which make contact with synapses, to provide metabolic support and maintain the integrity of neuronal communication (503). Astrocyte metabolism operates via the generation of adenosine triphosphate (ATP) from glycolysis (504). Lactate serves as a key component of the neuronal metabolic cycle used to generate the ATP serving the energy demands of synaptic transmission (504). Following an action potential, astrocytes act to terminate signaling by clearing glutamate from the synaptic cleft via the $Na^{+}$-dependent glial glutamate transporter (GLT) family, in particular GLT-1 (505). Once removed from the synapse, glutamate is converted to glutamine, which is then released by astrocytes into the extracellular space, where it can be taken up by neurons (506). Following uptake, the enzyme glutaminase converts glutamine back to glutamate so that it can then be loaded into vesicles by the vesicular glutamate transporter (VGluT), restarting the cycle of glutamate synaptic transmission (506). Unlike neurons, astrocytes are connected to each other via gap junction pore proteins, forming functional ensembles of interconnected cells (507). As a result, $Ca^{2+}$ and other small molecules like inositol-1,4,5-trisphosphate (IP3) can flow between adjacent cells in the reticular astrocyte network. This allows for the propagation of transient increases in internal calcium concentration across connected cells, termed calcium waves. Calcium waves play a role in initiating organized gliotransmission in order to synchronize neuronal firing patterns (508). In fact, astrocytes participate in bidirectional communication with neurons and release chemical transmitters (gliotransmission), including taurine, ATP, D-serine, and glutamate (509). Thus, activation of astrocytes induces the synthesis and release of substances capable of modulating the surrounding cells, like neurotransmitters, neurotrophic factors, cytokines, chemokines and extracellular matrix factors (510,511,512).

Moreover, glial cells are considered the immune competent cells of the CNS as well as crucial components of synaptic plasticity (513,514). There is increasing evidence that drugs of abuse produce alterations in CNS immunology. For example, opiates induce profound changes in glial cellular morphology and phenotypic immunohistological marker expression (GFAP: glial fibrillary acidic protein, a cell surface marker of astrocyte reactivity) in specific brain areas (515). Importantly, it has been proposed that the actions of opiates through glial reactivity are involved in the development of opiate dependence (516,517,518) (Figure 15). In addition, morphine has been shown to promote the expression of pro-inflammatory cytokines and chemokines in the CNS (519,520), where these peptides are synthetized and released mostly by glial cells. Some of these molecules exhibit a profile similar to that of neuromodulators or behave like neurotrophic factors (521,522). Interestingly, morphine-mediated increase of cytokines/chemokines appears to be more pronounced in brain areas that

participate in the neuronal network involved in opiate abuse/reward, including the NAc (523). These data raise the possibility that astrocytes would contribute to the synaptic plasticity during the development of drug addiction.

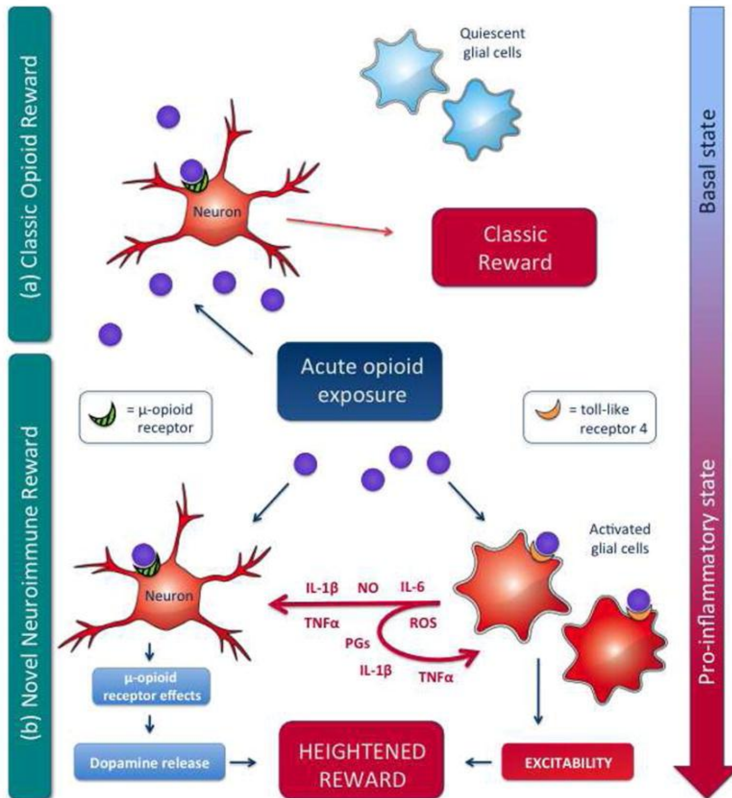



**Figure 15:** a) In the classic view of opioid reward, glia is not accounted for, beyond their homeostatic role. b) Recent research describes non-neuronal actions of opioids and opioid-induced proinflammatory glial activation. The neuronal opioid receptor-dependent and complementary opioid-Toll-like receptor 4 (TLR4) dependent mechanisms produce a heightened opioid reward signal and ensuing presentation of altered behavior.

Reproduced from ref (524): Hutchinson MR, Watkins LR. Why is neuroimmunopharmacology crucial for the future of addiction research? Neuropharmacology. 2014. 76 Pt B:218-27.

## 7.1. ASTROCYTES AND GLUTAMATE HOMEOSTASIS DURING ADDICTION

Glutamate homeostasis is defined as the balance between synaptic and nonsynaptic glutamate, with nonsynaptic glutamate acting to regulate neuronal communication (264). As such, astrocytes tightly regulate synaptic and nonsynaptic glutamate levels through the coordinated release and uptake of glutamate (525,503). Presynaptically, glial-glutamate release can activate Gi/Go-coupled group II mGluR (mGluR2/3) located on cortical glutamatergic neurons synapsing in the NAc (526). Stimulation of glutamate autoreceptors attenuates synaptic release through the activation of presynaptic $K^+$ channels, the inhibition of presynaptic $Ca^{2+}$ channels, and by directly inhibiting vesicular release (526). Glial-derived glutamate can also potentially activate mGluR5, a Gq coupled group I metabotropic glutamate receptor located postsynaptically on GABAergic MSNs in the NAc (527). Activation of these receptors has been linked to synaptic plasticity (528) and the induction of LTP in the NAcCore (529). In addition to mGluRs, glial glutamate release can also activate nonsynaptic ionotropic GluN2B containing NMDA receptors. The mainly synaptic GluN2A containing NMDA receptors have been linked to the induction of LTP, whereas extrasynaptic GluN2B

containing subtypes mediate the induction of LTD (530). Evidence suggests that these receptors play a role in mediating drug-related plasticity, as antagonizing GluN2B-containing NMDA receptors has been shown to inhibit opiate (531), nicotine (532), and alcohol reinstatement (533).

### 7.2. PLEIOTROPHIN AND MIDKINE

MK is a heparin-binding cytokine that promotes growth, survival, differentiation and migration of different target cells (534). MK has an important role in development and repair of the CNS (535). PTN is a secreted, highly conserved cytokine which shares over 50% identity in amino acid sequence with MK, the only other member of the PTN/MK developmentally-regulated gene family (536). The expression of both PTN and MK genes is constitutive and limited to only a few cell types in adults (537). Both genes are up-regulated at sites of injury and repair in inflammatory macrophages, glia, dermal fibroblasts, endothelial cells and other cells (538,539,540), suggesting that PTN and MK signaling may be critical in different steps of differentiation of different cells, both in development and in wound repair.

It has been suggested that MK and PTN could be involved in the addictive cycle to drugs of abuse (Table 2). Thus, PTN mRNA and/or MK mRNA levels are up-regulated after acute amphetamine administration (541) and after injection of delta-9-tetrahydrocannabinol (542) or morphine (543) in brain areas related to addiction, such as the NAc(shell), the PFC and the hypocampus, respectively. Likewise, increased mRNA and protein levels were found in the PFC of alcoholics and smokers (544). Given that these cytokines exert effects that are similar to those of neurotrophins, these findings support the hypothesis that these two cytokines are up-regulated in order to induce neurotrophic or neuroprotective effects during drug consumption (545). MK and PTN bind common receptors, including the protein tyrosine phosphatase β/ζ (RPTPβ/ζ) receptor (546), which is abundantly expressed in the CNS. The interaction of MK or PTN with RPTPβ/ζ establishes a "ligand-dependent inactivation" of RPTPβ/ζ, presumably consequence of RPTPβ/ζ dimerization (547). As a consequence, PTN or MK signaling through RPTPβ/ζ leads to the activation of ERK and PI3K-Akt (548,549), important axes inducing morphological changes and modulating addictive behaviors.

| | Drug addiction | | Neurodegenerative disorders | |
|---|---|---|---|---|
| | **Neurotoxicity** | **Addictive behavior** | **Parkinson's Disease** | **Alzheimer's Disease** |
| *PTN* | ↓ Amphetamine nigrostriatal toxicity ↓ Cocaine neurotoxicity | ↓ Amphetamine relapse ↓ Morphine reward | ↓ nigrostriatal degeneration ↑ functional nigrostriatal recovery | |
| *MK* | ↓ Amphetamine striatal toxicity | ↓ Cocaine relapse | ↓ neurochemical and behavioral dysfunctions in nigrostriatal pathways ↓ olfactory and cognitive dysfunctions of early stages | ↓ amyloid β-peptide plaques cytotoxicity ↓ amyloid β-peptide plaques deposition |

**Table 2:** Proposed roles and effects of PTN and MK in drug addiction, Parkinson's disease and Alzheimer's disease

Reproduced from ref (545): Herradón G, Pérez-García C. Targeting midkine and pleiotrophin signalling pathways in addiction and neurodegenerative disorders: recent progress and perspectives. Br J Pharmacol. 2014. 171:837-48.

# II. OBJETIVOS

Los opiáceos, como la morfina, son unos potentes analgésicos que se usan para tratar diversas formas de dolor agudo y crónico. Sin embargo son también muy adictivos, lo que limita su uso médico. Además, el uso recreativo de los opiáceos y opioides (morfina, heroína), ha aumentado notablemente en los últimos años (550). El uso crónico de opiáceos produce cambios neuroadaptativos a nivel cerebral que conllevan efectos indeseables, como la adicción, la cuál es un notable problema médico y sanitario. La creciente evidencia implica a varios mecanismos de regulación génica (incluyendo epigenéticos, moleculares, celulares y a nivel de circuitos neuronales) en los cambios que las drogas de abuso provocan en el cerebro, siendo el conocimiento de éstos una posible estrategia terapéutica para el tratamiento de la adicción (550,384,551).

Por otra parte, cada día es más evidente que las drogas de abuso producen alteraciones en el sistema inmune del CNS. Por ello, se ha propuesto que los astrocitos contribuirían a la plasticidad sináptica que se produce durante el desarrollo de la adicción mediante la síntesis y liberación de sustancias, como las citoquinas (279,552,545). De hecho, los opiáceos inducen cambios en la morfología de las células de la glia y expresión del marcador inmunohistoquímico GFAP de una forma región-específica(515). Estos datos apuntan la posibilidad de que los astrocitos contribuyan a la plasticidad sináptica durante el desarrollo de la adicción.

Hemos centrado el presente estudio en varios neurocircuitos implicados en la adicción a opiáceos: i) el eje HPA y el sistema extrahipotalámico del estrés, ii) el sistema dopaminérgico de recompensa, y iii) circuitos relacionados con las memorias aversivas.

- El uso crónico de las drogas de abuso altera el sistema cerebral del estrés. La exposición repetida a opiáceos conlleva la acumulación del factor de transcripción ΔFosB, particularmente en áreas cerebrales relacionadas con la recompensa y el estrés. La evidencia sugiere que las hormonas relacionadas con el estrés (como los GCs) podrían producir adaptaciones en el sistema cerebral del estrés que incluirían alteraciones a nivel de la expresión génica y de factores de transcripción.

1. **El primer objetivo** del presente estudio se diseñó después de considerar lo anterior. Examinamos el papel de los GCs en la expresión de ΔFosB en poblaciones neuronales específicas del sistema cerebral del estrés durante la dependencia de morfina. Para ello, la expresión de ΔFosB se cuantificó en ratas control (sham) y ADX que desarrollaron dependencia de morfina.

• En los últimos años, diferentes estudios resaltan las modificaciones post-transcripcionales mediadas por los miRs en la adicción y otras patologías neurológicas y enfermedades neurodegenerativas (553). Algunos de ellos, como el miR-133b, han sido identificados como mediadores de la diferenciación, maduración y función de las neuronas dopaminérgicas del mesencéfalo. miR-133b actuaría mediante la regulación a la baja de factores de transcripción implicados en el desarrollo y el fenotipo de estas neuronas, como Nurr1 y Pitx3, que poseen una función crítica a nivel mesencefálico al regular la transcripción de diferentes genes implicados en el metabolismo de la DA. Los cambios epigenéticos, como el silenciamiento génico mediado por miRs/Ago2, también representan complejos mecanismos que regulan la plasticidad neuronal. Entre los miembros de la familia Ago, sólo Ago2 parece tener un papel importante en la ejecución del silenciamiento mediado por los diferentes miRs (554).

2. **Segundo objetivo**: Dada la importante implicación de la transmisión dopaminérgica en los desórdenes adictivos, hemos centrado esta parte de nuestro estudio en identificar los marcadores dopaminérgicos que resultan alterados con la administración aguda y crónica de morfina, así como durante el síndrome de abstinencia inducido por naloxona, tanto en el VTA como el NAc. Para ello, hemos determinado: i) la expresión del miR-133b y Ago2 en el VTA; ii) el contenido de TH y su actividad; iii) la actividad dopaminérgica (recambio de DA y activación de TH) en el sistema mesolímbico de ratas tratadas con morfina aguda, ratas dependientes de morfina y durante el síndrome de abstinencia inducido por naloxona.

3. El **tercer objetivo** fue estudiar: i) posibles cambios en los niveles de mRNA y proteína de Nurr1 y Pitx3, así como la expresión de Ago2 y de TH en regiones específicas del sistema mesolímbico; ii) actividad dopaminérgica en el NAc; iii) co-localización cuantitativa de Nurr1 y Pitx3 en el VTA en las neuronas que expresan TH, iv) cambios en la plasticidad de subpoblaciones neuronales dopaminérgicas del VTA en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia.

• Las neuronas dopaminérgicas localizadas en el mesencéfalo y que proyectan al estriado se caracterizan por la expresión de autoreceptores DRD2 que inhiben la síntesis y liberación de DA (82). Además, DAT (que media la recaptación de DA), TH (enzima limitante de la síntesis de catecolaminas) y VMAT2, han sido identificados como importantes reguladores de la función dopaminérgica. Por otro lado, Nurr1 y Pitx3 son esenciales para la expresión de genes implicados en el metabolismo de la DA, como *Th*, *Dat*, *Vmat2* and *Drd2* (220,555,221).

4. **Cuarto objetivo**: Los efectos del síndrome de abstinencia a morfina sobre la expresión de Nurr1 y Pitx3 nos llevó a investigar la expresión de otros genes y proteínas que están implicadas en la regulación de la función dopaminérgica, algunos de los cuáles son dianas de Nurr1 y Pitx3. Para ello, las ratas recibieron morfina de forma aguda o crónica. Otro grupo fue sometido a un síndrome de abstinencia a morfina. Se estudió: i) la expression de *Dat*-DAT, *Vmat2*-VMAT2, *Drd2*-DRD2 y DRD1 en VTA/NAc; ii) la co-localización de Nurr1 y/o Pitx3 con neuronas que expresan TH en el VTA, así como el porcentaje de neuronas dopaminérgicas que expresan Nurr1 y Pitx3; iii) la posible correlación entre la expresión de Nurr1/Pitx3 y los niveles de los diferentes marcadores dopaminérgicos.

- También se ha propuesto que los astrocitos, mediante la síntesis y liberación de factores de crecimiento/citoquinas, como MK y PTN, jugarían un papel fundamental en los cambios en la plasticidad sináptica del CNS durante el desarrollo de la adicción (524,545).

5. **Quinto objetivo**: Dado que PTN y MK tienen los mismos efectos que las neurotrofinas y que los astrocitos pueden liberar MK y PTN después de diversos estímulos, nuestro siguiente objetivo fue: i) estudiar posibles cambios en la expresión de PTN, MK, su receptor RPTPβ/ζ, así como alteraciones en sus vías de señalización intracelular en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia en el VTA y NAc; ii) la posible activación de los astrocitos durante los tratamientos descritos, que podría conllevar la liberación de diferentes factores; iii) identificar las subpoblaciones celulares que producen y secretan PTN y/o MK, así como aquellas que expresan RPTPβ/ζ.

# III. AIMS

Opiate drugs, such as morphine, are a class of powerful analgesics that are used for treating many forms of acute and chronic pain. However, they are also highly addictive, which limits their medical use. The non-medical use of opiates (heroine, morphine) has increased greatly in recent years (550). Chronic use of opiates causes brain neuroadaptation that lead to undesirable effects, namely opiate addiction that is a significant medical and public health problem. Increasing evidence implicates various mechanisms of gene regulation (including epigenetic, molecular, cellular and circuit level effects) in the changes that drugs of abuse induce in the brain, indicating a potential therapeutic strategy for addiction therapy (550,384,551).

On the other hand, there is increasing evidence that drugs of abuse produce alterations in CNS immunology. Thus, it has been proposed that astrocytes contribute to the synaptic plasticity during the development of drug addiction by the synthesis and release of substances, such as cytokines (279,552,545). Thus, opiates induce profound changes in glial cellular morphology and phenotypic receptor/immunohistological marker expression, GFAP in a region specific-manner (515). These data raise the possibility that astrocytes contribute to the synaptic plasticity during the development of drug addiction.

We have focused the present study on several neurocircuits involved in opiate addiction: i) the HPA and extrahypothalamic stress systems; ii) the dopaminergic reward pathways; and iii) aversive memories-related neuronal circuits.

- Chronic use of drugs of abuse profoundly alters stress-responsive system. Repeated exposure to opiates leads to accumulation of the transcription factor ΔFosB, particularly in brain areas associated with reward and stress. Recent evidence suggests that stress-related hormones (e.g. GCs) may induce adaptations in the brain stress system that are likely to involve alterations in gene expression and transcription factors.

1. **The first objective** of the present study was designed after considering the above. We examined the role of GCs in regulation of ΔFosB expression in specific populations of the brain stress system during morphine dependence. For that, expression of ΔFosB was measured in control (sham-operated) and ADX rats that were made dependent on morphine.

- In recent years, studies highlight post-transcriptional modifications mediated by miRs in addiction and other neurological disorders and neurodegenerative diseases (553). Some of them, such as miR-133b, have been proposed to regulate the differentiation, maturation and function of

dopaminergic neurons by downregulating transcription factors involved in the development and physiological function of midbrain dopaminergic neurons, such as Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in DA metabolism in the mesolimbic pathway. Epigenetic changes such as miRs/Ago2-induced gene silencing represent complex molecular signature that regulate cellular plasticity. Among the Ago family members, only Ago2 seems to have an important role in miRs generation and execution of miR-mediated gene silencing (554).

2. **Second objective:** Given the important implications of DA neurotransmission in addiction disorders, we have focused this part of our study on identifying the DA markers that are altered in association with acute and chronic morphine exposure, as well as with morphine withdrawal in the VTA and NAc(medial shell). For that, we have determined i) the expression of miR-133b and Ago2 in VTA; ii) TH content and activity; and iii) dopaminergic activity (DA turnover and TH activation) in the mesolimbic system from rats acutely injected with morphine, in morphine-dependent rats and during naloxone-induced morphine withdrawal.

3. **The third objective** was to study: i) Nurr1 and Pitx3 mRNAs and proteins changes as well as the expression of Ago2 and TH mRNA and protein levels in specific region of the mesolimbic system; ii) dopaminergic activity in the NAc; iii) quantitative co-localization of Nurr1 and Pitx3 in the VTA TH-positive neurons; and iv) the plasticity changes in VTA DA neurons subpopulations in response to morphine, morphine dependence and morphine withdrawal.

- DA neurons located in the midbrain and projecting to the striatal complex are primarily characterized by expression of DRD2 autoreceptor subtype, which inhibits DA synthesis and release (82). In addition, DAT, which mediates the reuptake of DA, TH (the rate-limiting enzyme for DA synthesis) and VMAT2 have been identified as important regulators of DA function. On the other hand, Nurr1 and Pitx3 are crucial for expression of the set of genes involved in DA metabolism, such as *Th*, *Dat*, *Vmat2* and *Drd2* (220,555,221).

4. **Fourth Objective:** The long-lasting effects of opiate withdrawal on Nurr1 and Pitx3 expression prompted us to investigate expression of other genes and proteins that are involved in the regulation of DA function, some of which represent putative targets of Nurr1 and Pitx3. For that, rats were exposed to acute and chronic morphine administration as well as to morphine withdrawal and analized: i) the expression of *Dat*-DAT, *Vmat2*-VMAT2, *Drd2*-DRD2 and DRD1 in VTA/NAc(shell), dysfunction of which is causally linked to addiction; ii) the co-localization of Nurr1 and/or Pitx3 with TH-positive neurons in the VTA as well as the percentage of DA neurons expressing

Nurr1 and Pitx3; and iii) the possible correlation between Nurr1/Pitx3 expression and DA markers levels in the VTA and/or NAc(shell).

- It has also been proposed that astrocytes, through the synthesis and release of growth factors/cytokines, such MK and PTN, would play a critical role in long-term synaptic plasticity in the CNS during drug addiction.

5. **Fifth Objective:** Given the important implications of DA neurotransmission in addiction disorders and the complexity of opiate-induced neuroadaptive responses in the brain reward dopaminergic system, the present study was focused on: i) identifying whether the expression of PTN, MK, RPTPβ/ζ and their intracellular signaling pathways (Akt and ERK) are altered in association with acute and chronic morphine exposure as well as with morphine withdrawal in the VTA and NAc; ii) the possible activation of astrocytes, which could lead to the release of astrocyte-related soluble factors; iii) we also aimed to identify those cell subpopulations that produced and secreted PTN and/or MK and those that expressed RPTPβ/ζ in response to morphine administration or morphine withdrawal.

# IV. ARTICLES

**<u>García-Pérez D</u>**, Laorden ML, Milanés MV, Núñez C. Glucocorticoids regulation of FosB/ΔFosB expression induced by chronic opiate exposure in the brain stress system. PLoS One. 2012;7(11):e50264. doi: 10.1371/journal.pone.0050264.

Plos One

**Abstract**

Chronic use of drugs of abuse profoundly alters stress-responsive system. Repeated exposure to morphine leads to accumulation of the transcription factor ΔFosB, particularly in brain areas associated with reward and stress. The persistent effects of ΔFosB on target genes may play an important role in the plasticity induced by drugs of abuse. Recent evidence suggests that stress-related hormones (e.g., glucocorticoids, GC) may induce adaptations in the brain stress system that is likely to involve alteration in gene expression and transcription factors. This study examined the role of GC in regulation of FosB/ΔFosB in both hypothalamic and extrahypothalamic brain stress systems during morphine dependence. For that, expression of FosB/ΔFosB was measured in control (sham-operated) and adrenalectomized (ADX) rats that were made opiate dependent after ten days of morphine treatment. In sham-operated rats, FosB/ΔFosB was induced after chronic morphine administration in all the brain stress areas investigated: nucleus accumbens(shell) (NAc), bed nucleus of the stria terminalis (BNST), central amygdala (CeA), hypothalamic paraventricular nucleus (PVN) and nucleus of the solitary tract noradrenergic cell group (NTS-A(2)). Adrenalectomy attenuated the increased production of FosB/ΔFosB observed after chronic morphine exposure in NAc, CeA, and NTS. Furthermore, ADX decreased expression of FosB/ΔFosB within CRH-positive neurons of the BNST, PVN and CeA. Similar results were obtained in NTS-A(2) TH-positive neurons and NAc pro-dynorphin-positive neurons. These data suggest that neuroadaptation (estimated as accumulation of FosB/ΔFosB) to opiates in brain areas associated with stress is modulated by GC, supporting the evidence of a link between brain stress hormones and addiction.

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0050264

**García-Pérez D**, Sáez-Belmonte F, Laorden ML, Núñez C, Milanés MV. Morphine administration modulates expression of Argonaute 2 and dopamine-related transcription factors involved in midbrain dopaminergic neurons function. Br J Pharmacol. 2013;168(8):1889-901. doi: 10.1111/bph.12083.

British Journal of Pharmacology

**Abstract**

**BACKGROUND AND PURPOSE:**

Alterations in transcription factors that regulate the development and maintenance of dopamine (DA) neurons (such as Nurr1 and Pitx3) play an important role in the pathogenesis of addiction diseases. We have examined the effects of acute and chronic morphine and morphine withdrawal on TH expression and activity as well as expression of Nurr1, Pitx3 and Ago2 in the ventral tegmental area (VTA) and nucleus accumbens (NAc) of the rat.

**EXPERIMENTAL APPROACH:**

Rats were injected acutely with morphine and decapitated 1 or 2 h later. Another set of rats were made dependent on morphine by implantation of two morphine pellets. Precipitated withdrawal was induced by injection of naloxone. Ago2, Pitx3, Nurr1, total TH (tTH), TH phosphorylated at Ser31 and at Ser40, and 3,4-Dihydroxyphenylacetic acid, and DA determination in the VTA and/or NAc were measured using immunoblotting, HPLC and immunofluorescence.

**KEY RESULTS:**

Acute morphine produced a marked increase in TH activity and DA turnover in the NAc, concomitantly with increased Nurr1 and Pitx3 expression in the VTA. In contrast, precipitated morphine withdrawal decreased TH activation, TH expression and did not increase DA turnover in the NAc. These effects paralleled decreases in Ago2 expression, which was accompanied by increased Nurr1 and Pitx3, TH activity and normalized TH protein levels in the VTA.

**CONCLUSIONS AND IMPLICATIONS:**

The combined decrease in Ago2 and increases in Nurr1 and Pitx3 might represent some of the mechanisms that served to protect against accumbal TH regulation observed in morphine withdrawn rats, which may be critical for DA bioavailability to influence behaviour.

http://onlinelibrary.wiley.com/doi/10.1111/bph.12083/abstract;jsessionid=F1A1F51070CEE548FD60818818D67F6A.f01t04

**García-Pérez D**, López-Bellido R, Hidalgo JM, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. Addict Biol. 2015;20(1):104-19. doi: 10.1111/adb.12083.

Addiction Biology

**Abstract**

Epigenetic changes such as microRNAs (miRs)/Ago2-induced gene silencing represent complex molecular signature that regulate cellular plasticity. Recent studies showed involvement of miRs and Ago2 in drug addiction. In this study, we show that changes in gene expression induced by morphine and morphine withdrawal occur with concomitant epigenetic modifications in the mesolimbic dopaminergic (DA) pathway [ventral tegmental area (VTA)/nucleus accumbens (NAc) shell], which is critically involved in drug-induced dependence. We found that acute or chronic morphine administration as well as morphine withdrawal did not modify miR-133b messenger RNA (mRNA) expression in the VTA, whereas Ago2 protein levels were decreased and increased in morphine-dependent rats and after morphine withdrawal, respectively. These changes were paralleled with enhanced and decreased NAc tyrosine hydroxylase (TH) protein (an early DA marker) in morphine-dependent rats and after withdrawal, respectively. We also observed changes in TH mRNA expression in the VTA that could be related to Ago2-induced translational repression of TH mRNA during morphine withdrawal. However, the VTA number of TH-positive neurons suffered no alterations after the different treatment. Acute morphine administration produced a marked increase in TH activity and DA turnover in the NAc (shell). In contrast, precipitated morphine withdrawal decreased TH activation and did not change DA turnover. These findings provide new information into the possible correlation between Ago2/miRs complex regulation and DA neurons plasticity during opiate addiction.

http://onlinelibrary.wiley.com/doi/10.1111/adb.12083/abstract

**<u>García-Pérez D</u>**, López-Bellido R, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Dysregulation of dopaminergic regulatory mechanisms in the mesolimbic pathway induced by morphine and morphine withdrawal. Brain Struct Funct. 2014;220(4):1901-19. doi:10.1007/s00429-014-0761-5.

<u>Brain Structure and Function</u>

**Abstract**

Dopamine (DA) is thought to represent a teaching signal and has been implicated in the induction of addictive behaviours. Previously, it has been proposed that the transcription factors Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in DA metabolism in the mesolimbic pathway, are associated with addiction pathology. The aim of our study was to investigate abnormalities in the mesolimbic pathway associated with morphine dependence and withdrawal. Using quantitative real-time PCR, immunofluorescence, HPLC and Western blotting, here we studied the effects of single morphine administration, morphine dependence and morphine withdrawal on Nurr1 and Pitx3 expression as well as on the DA marker tyrosine hydroxylase (TH) and the turnover of DA in the ventral tegmental area (VTA) and/or nucleus accumbens. We showed that the three experimental conditions caused induction of Nurr1 and Pitx3 in the VTA, which correlated with changes in TH expression during chronic morphine administration. Present data also confirmed the colocalization of Nurr1 and Pitx3 with TH-positive neurons in the posterior VTA. Furthermore, during morphine dependence, Nurr1 was detected in the nucleus compartment of VTA TH-positive neurons, whereas Pitx3 was strongly detected in the nucleus of TH-positive neurons after single morphine administration and during morphine withdrawal. The number of TH neurons, number of Nurr1 or Pitx3-positive cells, and the number of TH neurons expressing Nurr1 or Pitx3 were not modified in the subpopulations of DA neurons. Present data provide novel insight into the potential correlation between Nurr1 and Pitx3 and DA neurons plasticity during opiate addiction in the mesolimbic pathway.

http://link.springer.com/article/10.1007%2Fs00429-014-0761-5

**<u>García-Pérez D</u>**, Núñez C, Laorden ML, Milanés MV. Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal. Addict Biol. 2014. doi: 10.1111/adb.12209.

Addiction Biology

**Abstract**

Dopamine (DA) is thought to represent a teaching signal and has been implicated in the induction of addictive behaviours. Dysfunction of DA homeostasis leading to high or low DA levels is causally linked to addiction. Previously, it has been proposed that the transcription factors Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in DA metabolism in the mesolimbic pathway, are associated with addiction pathology. Using quantitative real-time polymerase chain reaction, immunofluorescence and Western blotting, we studied the effects of single morphine administration, morphine dependence and withdrawal on the DA markers DA transporters (DAT), vesicular monoamine transporters (VMAT2) and DA 2 receptor subtype (DRD2), DA 1 receptor subtype as well as tyrosine hydroxylase (TH) in the ventral tegmental area (VTA) and/or nucleus accumbens (NAc). In addition, Nurr1 and Pitx3 expression was also measured. Present data showed a high degree of colocalization of Nurr1 and Pitx3 with $TH^+$ neurons in the VTA. We found that the increased Nurr1 and/or Pitx3 levels during morphine dependence and in morphine-withdrawn rats were associated to an increase of DAT, VMAT2 and DRD2. Altogether, present data indicate that morphine dependence and withdrawal induced consistent alterations of most of the DA markers, which was correlated with transcription factors involved in the maintenance of DA neurons in drug-reward pathways, suggesting that Nurr1 and Pitx3 regulation might be associated with controlling adaptation to chronic morphine and to morphine withdrawal-induced alterations of DA neurons activity in the mesolimbic pathway.

http://onlinelibrary.wiley.com/doi/10.1111/adb.12209/abstract

**García-Pérez D**, Luisa Laorden M, Núñez C, Victoria Milanés M. Glial activation and midkine and pleiotrophin transcription in the ventral tegmental area are modulated by morphine administration. J Neuroimmunol. 2014;274(1-2):244-8. doi: 10.1016/ j.jneuroim.2014.07.017.

Journal of Neuroimmunology

**Abstract**
Opiates cause persistent restructuring in the mesolimbic reward system. Although a possible role for midkine and pleiotrophin cytokines in the field of synaptic plasticity has been proposed, it has not been assessed whether morphine administration regulates astrogliosis and midkine and pleiotrophin transcription. We observed that single morphine injection and chronic morphine increased glial fibrillary acidic protein expression in the ventral tegmental area (VTA). Interestingly, single morphine injection and chronic morphine increased VTA midkine and pleiotrophin mRNA expression. Given these results, we hypothesize a role for these cytokines in mediating, at least in part, acute neuroprotective effects and chronic neurotrophic adaptations that contribute to drug dependence.

http://www.jni-journal.com/article/S0165-5728(14)00232-X/abstract

# V. CONCLUSIONES

1. El presente trabajo demuestra que los GCs están críticamente involucrados en la acumulación de FosB/ΔFosB en el sistema cerebral del estrés durante la administración crónica de morfina, lo que resultaría en cambios duraderos en los patrones de expresión génica en las áreas relacionadas con el estrés.

2. Aunque no hallamos cambios significativos en los niveles de miR-133b en el VTA, proponemos el papel de Ago2 en combinación con otros miRs en la regulación de la estabilidad y/o traducción del mRNA de TH en respuesta a la administración crónica de morfina y al síndrome de abstinencia a opiáceos provocado por la administración de naloxona. Además, la dependencia de morfina y el síndrome de abstinencia están asociados con alteraciones en los factores de transcripción involucrados en el mantenimiento de las neuronas dopaminérgicas del sistema mesolímbico de recompensa. Por ello, nuestros resultados sugieren un papel importante de Nurr1 y Pitx3 en los cambios en la expresión génica durante la dependencia y abstinencia a opiáceos, y que la regulación epigenética de TH puede estar asociada, al menos en parte, a los mecanismos moleculares que contribuyen a los cambios en la función dopaminérgica inducida por los opiáceos.

3. La dependencia de morfina y el síndrome de abstinencia a la misma están asociados con alteraciones de la mayoría de los marcadores dopaminérgicos (DAT, VMAT2, DRD2) en la vía mesolímbica de recompensa, que se correlacionan con alteraciones en los factores de transcripción encargados del mantenimiento de las neuronas dopaminérgicas (Nurr1 y Pitx3). La correlación entre los marcadores dopaminérgicos y Nurr1/Pitx3 añaden evidencia a nuestros anteriores resultados, y pueden reflejar el papel de estos factores de transcripción durante la dependencia y abstinencia a morfina.

4. Debido a la activación glial en el VTA durante la administración aguda y crónica de morfina, junto con el incremento de los niveles de mRNA de MK y PTN, proponemos la importancia de estas citoquinas al mediar, al menos en parte, las adaptaciones tróficas que se observan durante el fenómeno de adicción.

# VI. CONCLUSIONS

1. Present work provides evidence that GCs are critically involved in FosB/ΔFosB accumulation in the brain stress system after chronic morphine exposure, which might result in lasting changes of gene expression pattern in stress-related areas.

2. Although no significant changes of miR-133b levels are detected in the VTA, a role for Ago2 and specific miRs is hypothesized in regulating TH mRNA stability and/or translation in response to chronic morphine administration and naloxone-induced morphine withdrawal. Moreover, morphine dependence and withdrawal are associated with consistent alteration of transcription factors involved in the maintenance of dopaminergic neurons in the mesolimbic drug-reward pathway. Thus, our results suggest an important role for Nurr1 and Pitx3 in contributing to the changes in gene expression during opiate dependence and withdrawal and that epigenetic regulation of TH could be associated, at least in part, to the molecular mechanisms contributing to opiate-induced changes in midbrain dopaminergic function.

3. Morphine dependence and withdrawal are associated with consistent alteration of most of the DA markers (DAT, VMAT2, DRD2) in the mesolimbic drug-reward pathway which correlated with alteration of transcription factors involved in the maintenance of dopaminergic neurons (Nurr1 and Pitx3). The correlations between DA markers and Nurr1/Pitx3 add evidence onto previous results, and may reflect engagement of these transcription factors during morphine dependence and withdrawal.

4. Because of the glial activation in the VTA during acute and chronic morphine administration besides the enhancement in MK and PTN mRNAs, we propose a role for these cytokines in mediating, at least in part, the trophic adaptations that are observed during drug addiction.

# VII. APPENDIX

## 1. OBJETIVOS SUPLEMENTARIOS

- En ratas dependientes de morfina, las memorias aversivas del síndrome de abstinencia pueden generar un estado motivacional que dé lugar a una búsqueda y consumo compulsivo de la droga (9). En la actualidad, los mecanismos que subyacen en la generación de las memorias aversivas del síndrome de abstinencia permanecen desconocidos. Es interesante recalcar que entre las regiones cerebrales que se ven afectadas por las drogas de abuso, se encuentran quellas que son sustratos neuronales de la memoria, incluyendo el hipocampo, la amígdala y la corteza prefrontal. Esto coincide con los hallazgos de que algunas de las alteraciones más importantes observadas clínicamente en la adicción (por ejemplo el deseo compulsivo de consumir droga y las recaídas) reflejan anormalidades en los circuitos tradicionales de la memoria, actuando las memorias a largo plazo de las experiencias de consumir la droga como conductores de la patología adictiva (20,142). Las hipótesis actuales de los mecanismos moleculares que intervienen en el aprendizaje y la memoria, sugieren que la rápida regulación génica y síntesis de nuevas proteínas conllevan modificaciones sinápticas persistenentes que constituyen un mecanismo para la estabilización de memorias a largo plazo (556). Egr1 y Arc son genes de expresión inmediata que juegan un papel crucial en la plasticidad y la memoria. Se ha propuesto que Arc tendría un papel fundamental en la consolidación de diferentes tipos de memoria, con algunos trabajos recientes implicando el mRNA y la proteína Arc en los procesos adaptativos que ocurren tras el estrés, así como en la plasticidad maladaptativa que ocurre en la adicción a drogas (499). Por otra parte, se ha demostrado que los GCs modulan la consolidación de la memoria (396). Los GCs ejercen sus acciones en diferentes regiones cerebrales, incluyendo el hipocampo, la amígdala y la corteza prefrontal, que tienen alta densidad de GRs y son regiones importantes para la formación de la memoria.

En roedores, el componente afectivo negativo de la dependencia de opiáceos se puede ver reflejado en diferentes tests de comportamiento. Entre ellos, el test de CPA es el más sensible para la medida del estado negativo emocional que produce el síndrome de abstinencia (557).

1. **Sexto objetivo**: En esta parte del estudio, hipotetizamos que los GCs en diferentes estructuras límbicas (como BLA y el DG en el hipocampo) podrían mediar el componente afectivo negativo del síndrome de abstinencia a morfina, y que por tanto jugarían un papel crucial en las memorias aversivas relacionadas con la abstinencia a opiáceos. Para comprobar nuestra hipótesis utilizamos el paradigma de CPA en animales ADX y que desarrollaron dependencia de morfina. A continuación estudiamos: i) posibles modificaciones epigenéticas (miR-124a y miR-212) y activación o inhibición de diferentes vías intracelulares (pCREB, Egr1 y BDNF) en BLA y DG durante la consolidación de memoria y la recuperación de memoria; ii) cambios en la expresión de Arc como marcador de plasticidad neuronal; iii) identificación de las diferentes subpoblaciones neuronales donde

se expresaban las proteínas de interés; iv) correlaciones entre los miRs y la regulación de las diferentes proteínas.

## 2. SUPPLEMENTAL AIMS

• In opiate-dependent rats, aversive memories of drug withdrawal can generate a motivational state leading to compulsive drug seeking and taking (9). However, the mechanisms underlying generation of drug withdrawal memories remain unclear. Interestingly, among the brain regions affected by drugs of abuse are those that are key neural substrates for behavioral memory, including the hippocampus, amygdala and prefrontal cortex. This coincides with the increasing realization that some of the most important features of addiction seen clinically (eg, drug craving and relapse) reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology (20,142). Current hypothesis on the molecular mechanisms od learning and memory suggest that rapid regulation of gene programs and synthesis of new proteins leading to persistent synaptic modifications constitute a key mechanism for the stabilization of long-term memory (556). Egr1 and Arc are IEG known to play major roles in plasticity and memory. Arc has been proposed to have an important role in consolidation of many forms of memory, with recent works implicating *Arc* mRNA and protein in adaptation to stress as well maladaptive plasticity connected to drug addiction (499). GCs mediate and modulate memory consolidation (396). They exert their actions on brain regions, including the hyppocampus, amygdala and prefrontal cortex, that are enriched in GR and are important for long-term memory formation.

In rodent, the negative affective component of opiate dependence could be reflected by several behavioral alterations, among which CPA is the most sensitive measurement for the negative motivational state produced by opiate withdrawal, which is considered as a Pavlovian conditioned paradigm (557).

1. **Sixth Objective:** We hypothesized that GCs in limbic structures (eg., the BLA and hyppocampal DG) could specifically mediate the negative motivational component of opiate withdrawal and might play a crucial role in the aversive memories of opiate withdrawal. To test this hypothesis, we used the CPA paradigm in control (sham-operated) and ADX animals that were rendered dependent on morphine. Then, we studied: i) epigenetics modifications (miR-124a y miR-212) and intracellular pathways activated or inhibited (pCREB, Egr1 y BDNF) in the BLA and the DG during memory consolidation and memory retrieval; ii) changes in Arc expression as a marker of neuronal plasticity; iii) we also aimed to identify those cell subpopulations that produced and secreted the different proteins that we studied; iv) correlation analysis between different miRs and target proteins levels.

Molecular Neurobiology

# Acute morphine, chronic morphine and morphine withdrawal differently affects pleiotrophin, midkine and receptor protein tyrosine phosphatase β/ζ regulation in the ventral tegmental area

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| Abstract: | Pleiotrophin (PTN) and midkine (MK) are secreted growth factors and cytokines, proposed to be significant neuromodulators with multiple neuronal functions. PTN and MK are generally related with cell proliferation, growth, and differentiation by acting through different receptors. PTN or MK, signaling through receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), lead to the activation of extracellular signal-regulated kinases (ERKs) and thymoma viral proto-oncogene (Akt), which induce morphological changes and modulate addictive behaviors. Besides, there is increasing evidence that during the development of drug addiction, astrocytes contribute to the synaptic plasticity by synthesizing and releasing substances such as cytokines. In the present work we studied the effect of acute morphine, chronic morphine and morphine withdrawal on PTN, MK, and RPTPβ/ζ expression and on their signaling pathways in the ventral tegmental area (VTA). Present results indicated that PTN, MK and RPTPβ/ζ levels increased after acute morphine injection, returned to basal levels during chronic opioid treatment and were up-regulated again during morphine withdrawal. We also observed an activation of astrocytes after acute morphine injection and during opiate dependence and withdrawal. In addition, immunofluorescence analysis revealed that PTN, but not MK, was overexpressed in astrocytes and that dopaminergic neurons expressed RPTPβ/ζ. Interestingly, p-ERK 1/2 levels during chronic morphine and morphine withdrawal correlated RPTPβ/ζ expression. All these observations suggest that the neuroprotective and behavioral adaptations that occur during opiate addiction could be, at least partly, mediated by these cytokines. |

# Acute morphine, chronic morphine and morphine withdrawal differently affects pleiotrophin, midkine and receptor protein tyrosine phosphatase β/ζ regulation in the ventral tegmental area

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**Short title:** Morphine regulates pleiotrophin and midkine**.**

**Key words:** Opiate dependence; reward pathway; pleiotrophin; midkine; glial fibrillary acidic protein.

**Abbreviations**: Pleiotrophin (PTN); midkine (MK); receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ); ventral tegmental area (VTA); glial fibrillary acidic protein (GFAP); GFAP-immunoreactivity (GFAP-IR); dopamine (DA).

## Abstract

Pleiotrophin (PTN) and midkine (MK) are secreted growth factors and cytokines, proposed to be significant neuromodulators with multiple neuronal functions. PTN and MK are generally related with cell proliferation, growth, and differentiation by acting through different receptors. PTN or MK, signaling through receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), lead to the activation of extracellular signal-regulated kinases (ERKs) and thymoma viral proto-oncogene (Akt), which induce morphological changes and modulate addictive behaviors. Besides, there is increasing evidence that during the development of drug addiction, astrocytes contribute to the synaptic plasticity by synthesizing and releasing substances such as cytokines. In the present work we studied the effect of acute morphine, chronic morphine and morphine withdrawal on PTN, MK, and RPTPβ/ζ expression and on their signaling pathways in the ventral tegmental area (VTA). Present results indicated that PTN, MK and RPTPβ/ζ levels increased after acute morphine injection, returned to basal levels during chronic opioid treatment and were up-regulated again during morphine withdrawal. We also observed an activation of astrocytes after acute morphine injection and during opiate dependence and withdrawal. In addition, immunofluorescence analysis revealed that PTN, but not MK, was overexpressed in astrocytes and that dopaminergic neurons expressed RPTPβ/ζ. Interestingly, p-ERK 1/2 levels during chronic morphine and morphine withdrawal correlated RPTPβ/ζ expression. All these observations suggest that the neuroprotective and behavioral adaptations that occur during opiate addiction could be, at least partly, mediated by these cytokines.

## Introduction

Astrocytes perform numerous functions: they provide structural support for nerve cells, modulate the environment around neurons, regulate the production of synapses, maintain the blood--brain barrier and release a range of neuronal growth factors [1, 2]. Since glia play a variety of roles in the central nervous system (CNS), a stimulus that affects their morphology and function has widespread consequences including changes in neurotransmission, metabolism of neurotransmitters, synaptic plasticity and propagation of action potentials [2]. Morphine administration is established as a stimulus that affects glial activity. For example, morphine administration induces region-specific upregulation of glial fibrillary acidic protein (GFAP), a protein constituent found specifically in astrocytes, in specific brain areas [3]. Interestingly, evidence has demonstrated that tolerance and dependence are associated with opioid-induced increases in glial cell activity, resulting in neuroadaptations that may directly contribute to the reinforcing effects of these agonists [4].

Activation of astrocytes induces the synthesis and release of substances capable of modulating the surrounding cells (including neurons) like neurotransmitters, neurotrophic factors, cytokines, chemokines and extracellular matrix factors [5-7]. Morphine not only increases glial activation, but also the consequent production of immune factors (chemokines and cytokines) [8]. In addition, some of these molecules exhibit a profile similar to that of neuromodulators or behave like neurotrophic factors [9, 10]. These data raise the possibility that astrocytes contribute to the synaptic plasticity during the development of drug addiction. Moreover, cytokines, in addition to dopamine (DA) and neurotrophins, have been proposed as key mediators of addiction-related neuronal plasticity [11].

Pleiotrophin (PTN) is a secreted cell signaling cytokine that acts as growth factor and as a neuromodulator with multiple neuronal functions [12]. PTN shares high homology with

another peptide, denominated Midkine (MK) [13]. PTN and MK have been involved in neurodegenerative disorders and in response to chronic drug consumption. PTN is upregulated in cortex and caudate-putamen after injection of a cannabinol [14], and in NAc after acute administration of amphetamine [15]. Likewise, increased mRNA and protein levels were found in the prefrontral cortex of alcoholics and smokers [16]. In addition, PTN is also highly upregulated in substantia nigra of patients with Parkinson disease [17] and treatment with L-Dopa increases PTN levels in striatum [18]. Given that these cytokines exert effects that are similar to those of neurotrophins, these findings support the hypothesis that these two cytokines are up-regulated in order to induce neurotrophic or neuroprotective effects during drug consumption [19]. Different membrane receptors could function as a multi-molecular complex coordinated to transduce the PTN-MK signal into the cell by different signaling pathways [12]. So, PTN and MK bind common receptors, including receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ) [20], which is abundantly expressed in the CNS. The interaction of MK or PTN with RPTPβ/ζ establishes a "ligand-dependent inactivation" of RPTPβ/ζ, presumably consequence of RPTPβ/ζ dimerization [21]. As a consequence, PTN or MK signaling through RPTPβ/ζ leads to activation of ERK and phosphatidylinositol 3-kinase (PI3K)-Akt [22, 23], important axes inducing morphological changes and modulating addictive behaviors.

To date, despite the fact that morphine causes a full addiction syndrome and persistent restructuring in the mesolimbic reward system, the role of PTN and MK in the context of morphine administration has only been assessed in morphine-induced antinociceptive effects [24, 25]. The action of many addictive substances converges on the mesolimbic dopaminergic reward pathway, inducing increased firing of dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain [26, 27]. Given the important implications of DA neurotransmission in addiction disorders and the complexity of opiate-induced neuroadaptive

responses in the brain reward dopaminergic system, the present study was focused on identifying whether the expression of PTN, MK, RPTPβ/ζ and their intracellular signaling pathways (Akt and ERK) are altered in association with acute and chronic morphine exposure as well as with morphine withdrawal in the VTA. Then, we assessed the possible activation of astrocytes, which could lead to the release of astrocyte-related soluble factors. Finally, we also aimed to identify those cell subpopulations that produced and secreted PTN and/or MK and those that expressed RPTPβ/ζ in response to morphine administration or morphine withdrawal.

## Materials and Methods

*Subjects*

Male Wistar rats (n = 65, Harlan, Barcelona, Spain) initially weighting 220–240 g were housed (2-3/cage) on arrival in a room with controlled temperature (22 ± 2°C) and humidity (50 ± 10%), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light-dark cycle (lights on: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the local Committees for animal research (REGA ES300305440012).

*Drug treatment and experimental procedure*

Following habituation, rats were implanted subcutaneously (s.c.) with placebo pellets (lactose) for six days. Another set of rats were made dependent on morphine by implantation (s.c.) of two 75 mg morphine pellets under light ether anaesthesia. This procedure has been shown to produce consistent plasma morphine concentrations beginning a few h after the implantation of the pellets and a full withdrawal syndrome after acute injection of opiate antagonists [28]. On day 7, rats were injected intraperitoneally (i.p.) with either morphine HCl (20 mg/kg; in a volume of 1 ml/kg body weight), naloxone (1 mg/kg; 1 ml/kg body weight) or an equivalent volume of 0.9% saline and sacrificed 2 h later. There were five experimental groups: chronic placebo + acute saline, chronic placebo + acute morphine, chronic morphine + acute saline, chronic placebo + acute naloxone, and chronic morphine + acute naloxone. The weight gain of the rats was checked during chronic treatment to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment

induces a decrease in body weight gain due to lower caloric intake [29]. In addition, the animals were observed for opioid withdrawal behaviors for 30 min before and after naloxone injection.

*Electrophoresis and Western blotting*

Animals were killed by rapid decapitation. The brains were removed, placed (with its ventral surface facing up) on a plaque over crushed ice, and tissue samples of the VTA were dissected out as described in [30]. Brain regions were placed in individual wells, frozen immediately on dry ice and stored at -80°C until assaying. Samples were placed in homogenization buffer, homogenized and sonicated for 30 s prior to centrifugation at 10 000 g for 10 min at 4°C. Samples containing equal quantities of total proteins (20–40 mg, depending on the protein of interest) were separated by 6%, 10% or 12% SDS-PAGE (depending on the molecular weight of the protein of interest) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS containing 0.15 % Tween-20 (TBS-T), 1% BSA for 90 minutes at room temperature (RT), and incubated overnight at 4ºC with the primary antibody diluted in 1% BSA in TBS-T. The following primary antibodies were used: goat polyclonal anti-PTN (1:1000; AF-252-PB, R&D Systems, Minneapolis, MN, USA); rabbit polyclonal anti-MK (1:500; sc-20715, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-RPTPβ/ζ (1:750; 610180, BD Transduction Laboratories, Erembodegm, Belgium); mouse monoclonal anti-phospho-ERK 1/2 (p-ERK 1/2; 1:1000; sc-7383; Santa Cruz Biotechnology); mouse monoclonal anti-ERK 1/2 (1:1000; sc-135900; Santa Cruz Biotechnology); rabbit monoclonal anti-phospho-Akt (p-Akt; 1:2000; #4060, Cell Signaling Technology Inc., Danvers, MA, USA); rabbit polyclonal anti-Akt (1:1000; #9272, Cell Signaling Technology Inc.). Blots were washed and incubated for 90 min at RT in TBS-T with 1% BSA with appropriate horseradish peroxidase (HRP)

conjugated secondary antibodies: anti-rabbit (1:5000; sc-2004, Santa Cruz Biotechnology) anti-mouse (1:5000; sc-2005, Santa Cruz Biotechnology) or anti-goat (1:5000; sc-2350, Santa Cruz Biotechnology). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). We used GAPDH or α-Tubulin as our loading control. Before reprobing, blots were stripped by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37°C. Blots were subsequently reblocked and probed with rabbit polyclonal anti-GAPDH (1:5000; #2118, Cell Signaling Technology Inc.) or rabbit polyclonal anti- α-Tubulin (1:2500; #2144, Cell Signaling Technology Inc.). For Akt or ERK experiments, the same membrane was processed in the following order: incubation with anti-p-Akt or anti-p-ERK 1/2 antibody, stripping, incubation with anti-Akt or anti-ERK antibody, stripping and incubation with anti-GAPDH antibody. The ratios of PTN/GAPDH, MK/GAPDH, RPTPβ/ζ/α-Tubulin, p-Akt/t-Akt, t-Akt/GAPDH, p-ERK 1/t-ERK 1, t-ERK 1/GAPDH, p-ERK 1/GAPDH, p-ERK 2/t-ERK 2, t-ERK 2/GAPDH, p-ERK 2/GAPDH were plotted and analyzed. Protein levels were corrected for individual levels.

*Brain Perfusion and Sectioning*

Another set of rats was deeply anaesthetised with an overdose of pentobarbital (100 mg/kg i.p.) and perfused transcardially with saline following by fixative containing paraformaldehyde (4% paraformaldehyde in 0.1 M borate buffer, pH 9.5). After removal of the perfused brains, they were post fixed in the same fixative for 3 h and stored at 4ºC in PBS containing 30% sucrose until coronal sections (30-µm thickness) were cut rostrocaudally on a freezing microtome (Leica, Nussloch, Germany). The atlas of Paxinos and Watson (2007)

[31] was used to identify the VTA. The sections were cryoprotected and stored at -20ºC until use.

*GFAP Immunohistochemistry*

Sections of the VTA were used for immunohistochemistry to detect astrocytes. Brain sections were rinsed in PBS and an antigen retrieval procedure was applied by treating sections with citrate buffer (10 mM citric acid in 0.05% Tween 20, pH 6.0) at 60°C for 20 min. Endogenous peroxidase activity was inhibited with 0.3% $H_2O_2$ and non-specific binding sites were blocked in 3% normal goat serum (Sigma, USA)/0.3% Triton-X-100 in PBS for 1 h at RT. The sections were incubated for 72 h (4ºC, constant shaking) with primary antibody: mouse monoclonal anti-GFAP (1:400; sc-33673, Santa Cruz Biotechnology). The sections were then incubated for 2 h at RT in biotinylated secondary antibody: anti-mouse (1:500; BA-2000, Vector Laboratories, Burlingame, CA, USA). Tissue was processed by the avidin-biotin immunoperoxidase method (1:250; Vectastain ABC Elite Kit, Vector Laboratories) and immunopositive cells were visualized by addition of the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, USA) and 0.005% $H_2O_2$. Sections were mounted on chrome-alum gelatin-coated slides and dehydrated in graded ethanol series and xylene before being coverslipped.

*Quantification GFAP-positive cells*

Neuroanatomical sites were identified using the Paxinos and Watson (1997) atlas. Photomicrographs were captured by means of Leica microscope (DM 4000B; Leica) connected to a video camera (DFC290, Leica). GFAP-positive cell nuclei were counted using a computer-assisted image analysis system (QWIN, Leica). Positive cells were counted at 20X magnification. A square field (325 µm) was superimposed upon captured image to use as

reference area. The number of astrocytes was counted bilaterally in four to five sections from each animal, and averaged to obtain a single value for each rat. The whole histological quantification was performed blindly. Total counts for different brain regions are expressed as mean ± SEM.

*GFAP Densitometric Analysis*

The same conventional light microscopy described above was used for optical density (OD) study of the nuclei and processes. Photomicrographs were captured at 10X magnification (image size, 936 µm x 702 µm). To avoid observer bias, all sections were quantified by a blinded investigator. The intensity of the different brain areas was evaluated through densitometric analysis using ImageJ 1.43 software (NIH ImageJ, Bethesda, MD, USA). The region of interest was outlined in each image, and a mean optical density value was obtained. Background was determined from unstained areas in the tissue section. The corrected densitometric signal was calculated by subtracting the background from each densitometric determination. Four to five sections from each animal were evaluated. A mean value for different regions of each animal was then obtained. The area outlined in each image was also calculated to assure that there were no differences between the regions of interest analyzed in different groups.

*Immunofluorescence*

Triple immunofluorescence study was carried out to detect GFAP, PTN and MK. Sections were treated with citrate buffer (60°C for 20 min). Non-specific Fc binding sites were blocked with 2% normal horse serum/0.3% Triton-X-100 in PBS for 1 h at RT, and the sections were incubated for 72 h (4ºC, constant shaking) with primary antibodies: mouse monoclonal anti-GFAP (1:400; sc-33673, Santa Cruz Biotechnology), goat polyclonal anti-

PTN (1:400; AF-252-PB, R&D Systems) and rabbit polyclonal anti-MK (1:250; sc-20715, Santa Cruz Biotechnology). Secondary antibodies were applied sequentially for 4 h: Alexa Fluor 488 anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 594 anti-goat IgG (1:1000; A-11058, Invitrogen) and Alexa Fluor 405 anti-mouse IgG (1:1000; A-31553, Invitrogen). After washing, the sections were mounted in ProLong® Gold antifade reagent (Invitrogen).

Double-fluorescent labeling was processed as previously explained. Mesencephalic sections were incubated (72 h, 4ºC) with mouse monoclonal anti-RPTPβ/ζ (1:50; 610180, BD Transduction Laboratories) and goat polyclonal anti-tyrosine hydroxylase (TH; 1:4000; ab101853, Abcam, Cambridge, UK) to identify midbrain dopaminergic neurons. Appropriate secondary antibodies were used: Alexa Fluor 488 anti-goat IgG (1:1000; A-11055, Invitrogen) and Alexa Fluor 594 anti-mouse IgG (1:1000; A-21203, Invitrogen). Sections were incubated in DAPI (1:100 000) for 1 min, and mounted in ProLong® Gold antifade reagent (Invitrogen).

*Confocal analysis*

The brain sections were examined using a Leica DMIRE2 confocal microscope and Leica Confocal Software (Leica Microsystems). Images were captured from low magnification to high magnification (20X to 63X oil objective). Confocal images were obtained using 405-nm excitation for Alexa Fluor 405 or DAPI, 488-nm excitation for Alexa Fluor 488 and 543-nm excitation for Alexa Fluor 594. Emitted light was detected in the range of 450 nm for DAPI, 515-530 nm for Alexa Fluor 488 and 605 nm for Alexa Fluor 594. Every channel was captured separately to avoid spectral crosstalking. Series of optical sections were performed determining an upper and lower threshold using the Z/Y position for Spatial Image Series setting. The optical series covered 20 µm of thickness through the tissue. Images were

deconvolved using Huygens Essential 3.6 by Scientifica Volume Imaging (SVI, Hilversum, The Netherlands).

*Materials*

Morphine HCl and morphine base were supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Naloxone HCl was purchased from Sigma-Aldrich (Sigma Chemical Co, St Louis, MO, USA). Morphine HCl and naloxone HCl doses are expressed as the weight of the salt. Protease inhibitors were purchased from Boehringer Mannheim, (Mannheim, Germany); phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany). Morphine HCl and naloxone were prepared fresh each day by reconstitution in sterile saline (0.9% NaCl; ERN Laboratories, Barcelona, Spain).

*Statistical Analysis*

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a post hoc Newman–Keuls test. Correlations between changes in protein expression were assessed using Pearson correlation. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

## Results

*PTN and MK were up-regulated in the VTA after morphine administration and during precipitated morphine withdrawal*

PTN and MK have been found to be up-regulated in different brain areas after amphetamine, alcohol or delta-9-tetrahydrocannabinol administration (see Introduction); however, their possible alteration after morphine administration or during morphine withdrawal in the mesolimbic pathway has not been determined. This experiment addressed two questions regarding PTN and MK expression. The first was whether acute or chronic morphine treatment was associated with changes in PTN and/or MK in VTA. ANOVA showed significant effect after acute morphine for PTN ($F(2,20) = 8.656$; $p = 0.0023$) and MK ($F(2,19) = 6.949$; $p = 0.0062$) in the VTA. As shown in Fig. 1A,C, *post hoc* comparisons showed that acute morphine administration significantly elevated PTN ($p < 0.01$) and MK ($p < 0.05$) expression in the VTA. This elevation was not seen during chronic morphine administration ($p < 0.01$) compared with acute morphine injection. Two-way ANOVA for PTN expression showed a significant effect of chronic pretreatment ($F(1,21) = 12.65$; $p = 0.0019$), acute naloxone injection ($F(1,21) = 6.73$; $p = 0.0170$) and an interaction between pretreatment and acute treatment ($F(1,21) = 4.92$; $p = 0.0376$). *Post hoc* test revealed that PTN levels in the VTA were increased after naloxone precipitated morphine withdrawal ($p < 0.01$), as shown in Fig. 1B. Two-way ANOVA for MK showed a significant effect of acute naloxone injection ($F(1,23) = 5.885$; $p = 0.0235$) and an interaction between pretreatment and acute treatment ($F(1,23) = 4.516$; $p = 0.0445$). *Post hoc* test revealed that MK levels in the VTA were increased after naloxone precipitated morphine withdrawal ($p < 0.05$), as shown in Fig. 1D.

As our first goal was to establish whether acute morphine administration, chronic morphine or morphine withdrawal affect PTN and or MK VTN expression, it was important

to know if VTA express RPTPβ/ζ, one of the main receptors for PTN/MK, as well as whether morphine, morphine dependence and/or morphine withdrawal alter its expression. So, we next used semiquantitative Western blot to examine whether morphine and or morphine withdrawal affected RPTPβ/ζ expression protein levels. ANOVA showed significant effect after acute morphine ($F(2,18) = 14.6456$; $p = 0.0002$). As shown in Fig. 1E, *post hoc* comparisons showed that acute morphine administration significantly elevated RPTPβ/ζ ($p < 0.001$) expression. However, there was a decrease in its expression during morphine dependence compared with the control group receiving placebo plus saline ($p < 0.05$) and with acute morphine-treated rats ($p < 0.001$). Two-way ANOVA for RPTPβ/ζ expression showed an interaction between pretreatment and acute treatment ($F(1,23) = 4.75$; $p = 0.03976$). *Post hoc* test revealed that RPTPβ/ζ levels in the VTA were decreased in morphine-dependent rats ($p < 0.05$), as compared with the control group (Fig. 1F). No changes were observed in morphine-withdrawn rats.

We next compared the expression of PTN and MK with the induction of RPTPβ/ζ protein levels by Pearson correlation. There were not significant correlations in the different experimental groups between MK expression and RPTPβ/ζ protein levels in the VTA (data not shown). In contrast, we observed that during morphine dependence and withdrawal, the expression of PTN was significantly positively correlated with RPTPβ/ζ levels (Fig. 2).

It is important to notice that robust behavioral signs of the morphine withdrawal syndrome were seen in all of chronically morphine-treated animals after administration of naloxone, indicating the development of dependence.

*Astrocytes were activated by morphine and morphine withdrawal in the VTA*

Astrocytes undergo a process of proliferation, morphological changes, and enhancement of GFAP expression, termed the activation of astrocytes or astrogliosis [32]. In

this work we assessed the activation of astrocytes in the VTA by both, measuring the number of astrocytes (as the number of GFAP-positive cells) in the different groups and studying the levels of GFAP-immunoreactivity (GFAP-IR) by densitometric analysis. ANOVA showed significant effect of morphine administration for GFAP-positive cells ($F(2,12) = 5.909$; $p = 0.0202$) and GFAP-IR ($F(2,14) = 8.632$; $p = 0.0048$) in the VTA. *Post hoc* test revealed that acute morphine elevated the number of GFAP-positive cells and GFAP-IR ($p < 0.05$; $p < 0.01$, respectively) (Fig. 3G,I). In addition, chronic morphine administration also increased GFAP-IR ($p < 0.05$). Two-way ANOVA for GFAP revealed main effects for chronic pretreatment ($F(1,14) = 22.16$; $p = 0.0003$), and significant interaction between acute and chronic treatment ($F(1,14) = 10.530$; $p = 0.0059$). We found that precipitated morphine withdrawal significantly increased the number of GFAP-positive cells in the VTA compared with its control group ($p < 0.001$) and with chronic morphine-treated rats receiving saline ($p < 0.01$; Fig. 3H). Two-way ANOVA for GFAP-IR in the VTA revealed main effect for chronic pretreatment ($F(1,16) = 17.08$; $p = 0.0008$). *Post hoc* test showed that both chronic morphine and morphine withdrawal elevated ($p < 0.05$) GFAP-IR (Fig. 3J).

*PTN but not MK was overexpressed in astrocytes during acute morphine administration and morphine withdrawal in the VTA*

Activation of astrocytes produces the accumulation of intermediate-filament GFAP, consequently they can be recognized by their stellate morphology. Triple immnunofluorescence study revealed that acute morphine (Fig. 4A-A'''') or morphine withdrawal (Fig. 4B-B'''') mediated the activation of astrocytes that expressed high levels of PTN protein, but not MK protein in the VTA. Cells that produced MK are supposed to be

neurons, due to their size and morphology and the fact that MK and RPTPβ/ζ colocalized (data not shown).

*RPTPβ/ζ was expressed in midbrain dopaminergic neurons*

RPTPβ/ζ is a transmembrane tyrosine phosphatase, the extracellular domain of which carries chondroitin sulphate chains. The anti-RPTPβ/ζ antibody used recognizes only the intracellular portion of the RPTPβ/ζ to avoid non-specific labeling of other proteoglycans and specially of phosphacan, a secreted peptide which has the same molecular structure as the extracellular portion of the RPTPβ/ζ.

In order to determine where this receptor is located, we immunolabeled RPTPβ/ζ in combination with specific cell markers on the mesencephalon of morphine-treated rats. At low magnification, we observed that following morphine injection there was a region-specific increase of RPTPβ/ζ protein in the VTA, but weak or absent staining was detected in the interpeduncular nucleus (IP) or red nucleus (RN) (Fig. 5A-A'''). At high magnification, RPTPβ/ζ immunoreactivity was widely distributed throughout the VTA, and double-labeling with anti-TH (the rate-limiting enzyme of DA synthesis) showed that RPTPβ/ζ is located on cell bodies and proximal processes of almost all dopaminergic neurons in the VTA (Fig. 5B-B'''). There were some RPTPβ/$\zeta^+$/$TH^-$ cells, that might represent GABAergic interneurons in the VTA.

*ERK pathway but not Akt was activated by morphine and by precipitated morphine withdrawal in the VTA*

In each experiment, the specific signal of p-Akt or p-ERK proteins was normalized to the corresponding Akt or ERK signals, respectively, and then to the level of GAPDH measured in the same preparation. Previously, it has been described that PTN or MK

signaling through RPTPβ/ζ leads to activation of ERK and Akt pathways [22, 23]. ANOVA for p-Akt showed significant effect after acute or chronic morphine administration ($F(2,18) = 5.265$; $p = 0.0175$) failed to detect significant changes in t-Akt expression in the VTA ($F(2,18) = 2.845$; $p$ = ns) (Fig. 6A,C). *Post hoc* test revealed that p-Akt levels in the VTA were decreased in morphine-dependent rats ($p < 0.05$). Two-way ANOVA revealed that morphine withdrawal had a significant effect on p-Akt ($F(1,21) = 5.190$; $p = 0.0332$). *Post hoc* test revealed that p-Akt levels in the VTA were significantly ($p < 0.05$) elevated in morphine-withdrawn rats compared with the morphine dependent group receiving saline instead naloxone (Fig. 6B). Two-way ANOVA revealed that morphine pre-treatment ($F(1,21)$ = 1.83; $p = 0.0539$], acute naloxone injection ($F(1,21) = 0.04$; $p = 0.8352$) or the interaction between pre-treatment and acute treatment ($F(1,21) = 0.13$; $p = 0.7172$) had no significant effects on t-Akt (Fig. 6D).

Because t-ERK showed changes after the different treatments, p-ERKs 1/2 were quantified by Western blot using both t-ERK and GAPDH as loading controls (Fig. 7). ANOVA showed significant effect after acute or chronic morphine for p-ERK 1/t-ERK 1 ($F(2,19) = 11.65$; $p = 0.0007$) and for p-ERK 2/t-ERK 2 ($F(2,19) = 15.26$; $p = 0.0002$). As shown in Fig. 7A,G, *post hoc* comparisons showed that both acute and chronic morphine administration significantly elevated p-ERK 1/ t-ERK 1 ($p = 0.001$; $p = 0.01$) and p-ERK 2/ t-ERK 2 ($p = 0.001$; $p = 0.001$) expression in the VTA. Two-way ANOVA for p-ERK 1/ t-ERK 1 and p-ERK 2/ t-ERK 2 expression showed significant effect for morphine pre-treatment (p-ERK 1: $F(1,24) = 21.15$; $p = 0.0001$; p-ERK 2: $F(1,24) = 41$; $p < 0.0001$) and acute naloxone injection (p-ERK 1: $F(1,24) = 4.62$; $p = 0.0419$; p-ERK 2: $F(1,24) = 8.85$; $p$ = 0.0066). *Post hoc* comparisons showed that morphine withdrawal significantly ($p < 0.001$) elevated p-ERK 1 and p-ERK 2 levels compared with their respective controls and with morphine-dependent rats ($p < 0.05$) (Fig. 7B,H). In addition, p-ERK 2 levels were also

increased ($p < 0.01$) in morphine-dependent rats compared with its control (Fig. 7H). Similar results were seen by using the p-ERK 1/GAPDH and p-ERK 2/GAPDH. Thus, ANOVA showed significant effect after acute morphine for p-ERK 1/GAPDH ($F(2,19) = 17.87$; $p < 0.0001$) and after acute and chronic morphine for p-ERK 2/ GAPDH ($F(2,19) = 14.91$; $p = 0.0002$). As shown in Fig. 7E,K, *post hoc* comparisons showed that acute morphine administration injection significantly elevated p-ERK 1/GAPDH and p-ERK 2/GAPDH ($p < 0.001$) as well as and p-ERK 2/ GAPDH after chronic morphine treatment ($p < 0.01$) expression in the VTA (Fig. 7K). Two-way ANOVA showed significant effect of chronic pretreatment for p-ERK 1/GAPDH ($F(1,24) = 12.41$; $p = 0.0017$) and p-ERK 2/GAPDH ($F(1,24) = 21.31$; $p = 0.0001$) and acute naloxone injection (p-ERK 1/GAPDH: $F(1,24) = 6.14$; $p = 0.0207$; p-ERK 2/GAPDH: $F(1,24) = 6.81$; $p = 0.0154$). As shown in Fig. 7, there was an increase ($p < 0.01$) of p-ERK 1 and p-ERK 2 after naloxone administration to morphine-dependent rats. p-ERK 2 was also elevated during morphine dependence (Fig. 7L).

We next used semiquantitative Western blot to examine the effect of acute morphine administration, chronic morphine and morphine withdrawal on t-ERK 1 (ratio t-ERK 1/GAPDH) and t-ERK 2 (ratio t-ERK 2/GAPDH) protein levels (Fig. 7C-J). ANOVA showed significant effects after acute morphine for t-ERK 1 ($F(2,19) = 6.160$; $p = 0.0097$) and for t-ERK 2 ($F(2,19) = 4.459$; $p = 0.0277$). As shown in Fig. 7C,I, *post hoc* comparisons showed an increase of t-ERK 1 ($p < 0.01$) and t-ERK 2 ($p < 0.05$) after acute morphine administration. This effect was suppressed in chronic morphine-treated rats. Two-way ANOVA for VTA t-ERK1 and t-ERK2 levels failed to detect any significant effects of chronic morphine pretreatment or morphine withdrawal (Fig. 7D,J).

*Relationship between PTN, MK and/or* RPTPβ/ζ *and p-ERK or t-ERK protein levels*

We first compared the expression of PTN, MK and/or RPTPβ/ζ with the induction of p-ERK or t-ERK protein levels in control rats that were injected an acute dose of morphine. There were significant negative correlations between PTN expression and t-ERK 1 and t-ERK 2 protein levels in the VTA (Fig. 8A,B). On the other hand, MK or RPTPβ/ζ expression in acute morphine-injected rats was not correlated with changes on p-ERK or t-ERK levels.

Interestingly, PTN expression during morphine dependence was also negatively correlated with t-ERK 1 (Fig. 8G), and during morphine withdrawal we detected that the increased PTN expression was associated with a decrease in t-ERK 2 levels in the VTA (Fig. 8N).

When we studied MK expression, we found that this cytokine was negatively correlated with p-ERK 1 and p-ERK 2 levels only in morphine withdrawn rats (8O,P), while we observed not significant correlation for MK in other experimental groups.

During morphine dependence and withdrawal, expression of RPTPβ/ζ tended to be positively correlated with p-ERK. There was a significant positive correlation between RPTPβ/ζ and both, p-ERK 1 and p-ERK 2 protein levels during chronic morphine administration (Fig. 8K,L). As depicted in Fig. 8Q,R, a similar trend for positive correlation between RPTPβ/ζ and p-ERK 1/ p-ERK 2 was found after naloxone-induced morphine withdrawal.

## Discussion

The data presented in this study show that different paradigms of morphine treatment promote a diverse profile of activation of cytokines in the brain. While we observed that a single dose of morphine and morphine withdrawal increased the protein levels of PTN and MK in the VTA, chronic morphine administration had the opposite effect, since protein levels returned to basal levels or even decreased. Our results are supported by previous findings showing that acute morphine or withdrawal and not chronic morphine promote the expression of pro-inflammatory cytokines [33]. The present study showed that, PTN up-regulation following morphine administration or during morphine withdrawal is restricted to astrocytes. In agreement with this data, PTN mRNA levels were up-regulated in reactive astrocytes in a model of cryo-injured mouse brain [34]. Besides, in primary cultures of mesencephalon, GFAP-positive astrocytes express PTN mRNA and protein [35] and recently, it has been described that PTN is over-expressed only in astrocytes following an adenoviral vector injection [36]. In contrast, we observed that MK is produced and secreted by non-astrocytic cells following a morphine challenge. Hippocampal pyramidal neurons in adult mouse brain expressed MK, but kainic acid injection induced cell death of pyramidal neurons and enhanced expression of MK by astrocytes [37]. A possible explanation is that, depending on the nature of the insult or the damage/cell death it may produce, MK can be over-expressed by neurons or astrocytes. Moreover, there is evidence that nearly every cell of the CNS, including neurons, is capable of contributing and modifying the central immune signaling [38], for example, by secreting cytokines. For instance, morphine stimulates chemokine CCL2 production by neurons [39].

We also found that the expression of PTN- and MK- target receptor (RPTPβ/ζ) was regulated in the same way that these cytokines by morphine administration. Mainly, PTN can

bind and signal via RPTPζ [40, 41]. Thus, we detected that PTN enhanced expression after chronic morphine and morphine withdrawal would lead to adaptative responses, such as the increase in RPTPβ/ζ levels, which might try to compensate the ligand-dependent inactivation. Although the role and the functions of RPTPs are barely characterized, it has been suggested that each of these enzymes has different and specific functions in neurons [42]. Regarding the RPTPβ/ζ signal, we found a staining pattern in neurons. Our results are in line with previous studies where this protein was found to be located in neurons but not in astrocytes [42, 43]. Moreover, TH-positive neurons in primary cultures of mesencephalon have been described to express RPTPβ/ζ [35]. Although it has been reported that adult control rats do not express RPTPβ/ζ in the VTA [44], we detected (by WB and IF) that systemic morphine injection induced RPTPβ/ζ in VTA-dopaminergic neurons in a region-specific manner. Thus, it suggests that RPTPβ/ζ is tightly up-regulated in the VTA after determinate challenges. This expression pattern supports our hypothesis of an interaction between glial and neuronal function during morphine administration and withdrawal.

PTN and MK have been involved in the regulation of the survival and function of dopaminergic neurons [45, 46]. In addition, many reports attribute neuroprotective roles against drug-induced neurotoxicity to these two cytokines. For instance, amphetamine-induced loss of dopaminergic fibers in the striatum was enhanced in PTN knockout (PTN-/-) mice and even caused dopaminergic cell loss in the substantia nigra (SN) of PTN-/- mice [47]. It is important to note that moderate glial activation is thought to be neuroprotective, while exacerbated activation can be deleterious for the brain [48-50]. Accordingly, amphetamine-induced astrocytosis in the nigrostriatal pathway was increased in PTN-/- mice and in MK-/- mice compared to wild type mice [47, 51, 52]. As exposed above, morphine withdrawal may promote cytokines and other inflammatory responses that have the potential of exacerbating neuronal damage [33]. The increase in pro-inflammatory cytokines may explain why

withdrawal causes caspase-3 dependent apoptosis [53]. So, the enhanced expression of PTN and MK that we observed during acute morphine injection and morphine withdrawal can be regarded as a physiological response to prevent the possible neurotoxic effects of morphine, an excessive astrogliosis or pro-inflammatory cytokines release. Moreover, it has been demonstrated that PTN increases the expression of nuclear receptor related-1 (Nurr1; a transcription factor which is critical for the generation, survival and maintenance of DA neurons) in neural stem cells to promote production of dopaminergic neurons [45]. Previously, we have observed an increase of Nurr1 after acute morphine administration and withdrawal [30], which parallels that of PTN. So, given present results, it is tempting to speculate that neuroprotective effects of PTN during acute morphine injection and morphine withdrawal may be, in part, mediated through the induction of Nurr1.

Astrocytes can display both, hypertrophy and proliferation upon treatment with drugs of abuse. Chronic morphine exposure, achieved by surgically implanted morphine pellets and previously verified to produce opioid tolerance and dependence [54], increased GFAP immunoreactivity in the VTA [3]. This effect was not observed when morphine tolerance and dependence was blocked by concomitant administration of the mu-opioid antagonist, naltrexone [3]. Accordingly, in the present study acute and chronic morphine caused a robust activation of astrocytes, as evidenced by an increase in the level of GFAP-IR in the VTA, while morphine withdrawal maintained the increase GFAP-IR levels. Regarding to proliferation, repeated methamphetamine administration induces proliferation of astrocytes in rodent striatum and SN [47, 51]. Moreover, morphine and opioid signaling have been shown to promote proliferation of astroglia in the postnatal brain [55]. We observed a rapid astrocyte proliferation in the VTA, the brain area where the rewarding properties of morphine are believed to be firstly mediated (via binding to μ opioid receptors located on inhibitory neurons) [56]. A question that arises is whether glial activation (GFAP-IR) observed during

chronic morphine is maintained throughout a longer time or, on the contrary, it slowly decreases. Data supporting this second hypothesis is the evidence that, after two months, methamphetamine induced a long-lasting astrocytic activation and behavioral sensitization, while morphine produced a reversible activation of astrocytes and a reversibility of behavioral sensitization [57].

It has been proposed that PTN/MK signaling may function through a multi-receptor complex, combining different receptors and most probably other adaptor proteins; then, PTN/MK action over previously mentioned receptors could in turn signal through different signal pathways [12]. It has been documented that the interaction of RPTPβ/ζ with PTN or MK blocks the intrinsic tyrosine phosphatase activity of RPTPβ/ζ [19], leading to an increase in the phosphorylation levels in RPTPβ/ζ target proteins. Interestingly, these target proteins include the PI3K-Akt and Ras-ERK signaling, which have been demonstrated to play a crucial role in opiate addiction (for review see [58]).

PTN neuroprotective effects constitute a relevant role, suggesting that PTN signaling pathways are involved in neurodegenerative disorders, as well as in response to injuries and chronic drug consumption [12]. PTN protective effects against amphetamine-induced toxicity in PC12 cells (which express RPTPβ/ζ and synthesize and release DA) were mediated by the ERK 1/2 signaling pathway [51]. The same neuroprotective action could be conferred to RPTPβ/ζ during chronic morphine administration and morphine withdrawal given the positive correlation between RPTPβ/ζ and p-ERK 1/2. Cytokines have also been implicated in withdrawal-related behavior. For example, it was showed that corticotrophin releasing factor (CRF) and cytokines work together to worsen ethanol withdrawal phenotypes [59]. Although the role of PTN and MK in conditioned place aversion (CPA) remains uncharacterized, future studies should address this issue, since robust increased levels of PTN and MK were also

observed during morphine withdrawal. Other studies have shown the important role of ERK signaling in memory formation [60] and the acquisition of CPA [61]. Interestingly, pERK 1/2 levels in the VTA during morphine withdrawal (acquisition of CPA) seem to be controlled through RPTPβ/ζ signaling. In contrast, we assessed that during morphine withdrawal, MK is negatively related to p-ERK 1/2. In agreement, literature shows that although both cytokines could present overlapping or similar functions, they are also clearly involved in different roles [12].

On the other hand, a decrease of Akt signaling was detected in the VTA during chronic morphine. This down-regulation is considered critical to mediate the effects of chronic morphine on soma size and electrical excitability [62, 63]. PTN and MK are also known to be neurotrophic factors for DA neurons [35]. Initial up-regulation of PTN promoted regeneration of axons, but subsequent decline in the expression of PTN has been proposed to be responsible for failure to regenerate denervated nerves [64], highlighting the importance of sustained levels of these growth factors to accomplish their trophic function. Nevertheless, we did not find any correlation between PTN, MK or RPTPβ/ζ and p-Akt levels, so it is reasonable to think that these cytokines do not participate in the decreased dendrite complexity and spine density and cell body size that chronic opiates produce in the mesolimbic system [58].

In summary, given that PTN, MK and RPTPβ/ζ levels increase after acute morphine injection, return to basal levels during chronic opioid treatment and are up-regulated again during morphine withdrawal, we hypothesize that signaling through these cytokines plays a role in mediating, at least in part, neuroprotective and behavioral adaptations that are observed during opiate addiction.

## Conflict of interest statement

The authors declare that they have no conflict of interest.

## Acknowledgments

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## Authors Contribution

DG-P designed and performed the research, analyzed the data and wrote the paper. MLL revised the manuscript. MVM conducted the astrocyte quantification and contributed to editing the paper.

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## Figure Legends

**Fig. 1.** PTN, MK and RPTPβ/ζ protein expression are altered by acute and chronic morphine administration and during morphine withdrawal in the VTA. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of PTN (A, B), MK (C, D) and RPTPβ/ζ (E, F) protein in VTA isolated from rats receiving the above treatments. Each bar corresponds to mean ± SEM. Values are expressed as % of controls. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. pla+sal; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. pla+mor; $^{\#\#}p < 0.01$ vs. pla+nx; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. mor+sal

**Fig. 2.** Correlation between PTN and RPTPβ/ζ. The percent increase in PTN levels was positively correlated with RPTPβ/ζ protein during morphine dependence and morphine withdrawal. No significant correlation was found between PTN expression and RPTPβ/ζ levels after acute morphine injection. $^{\#}p < 0.05$: PTN levels vs. RPTPβ/ζ levels.

**Fig. 3.** GFAP expression is enhanced by acute and chronic morphine administration and maintained during morphine withdrawal in the VTA, while astrocyte proliferation only occurs in acute morphine-injected and morphine-withdrawn rats. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. The analyzed region within the VTA is schematically illustrated in A (modificated from Paxinos and Watson, 2007). A rectangle indicates the size of the photomicrographs. (B-F): Representative photomicrographs showing immunohistochemical detection of $GFAP^{+}$ nuclei and fibers in midbrain coronal sections (scale bar: 200 µm). (B'-F'): high magnifications from each photomicrograph (scale bar: 100 µm). (G, H): Quantitative analysis of astrocytes in the VTA. (I, J): Mean optical

density measurement of GFAP-immunoreactivity in the VTA from rats receiving the treatments mentioned above. (K, L): Reference area used in the densitometric analysis did not differ between groups. SN: Substantia nigra. Each bar corresponds to mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. pla+sal; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. pla+nx; $^{\&\&}p < 0.01$ vs. mor+sal

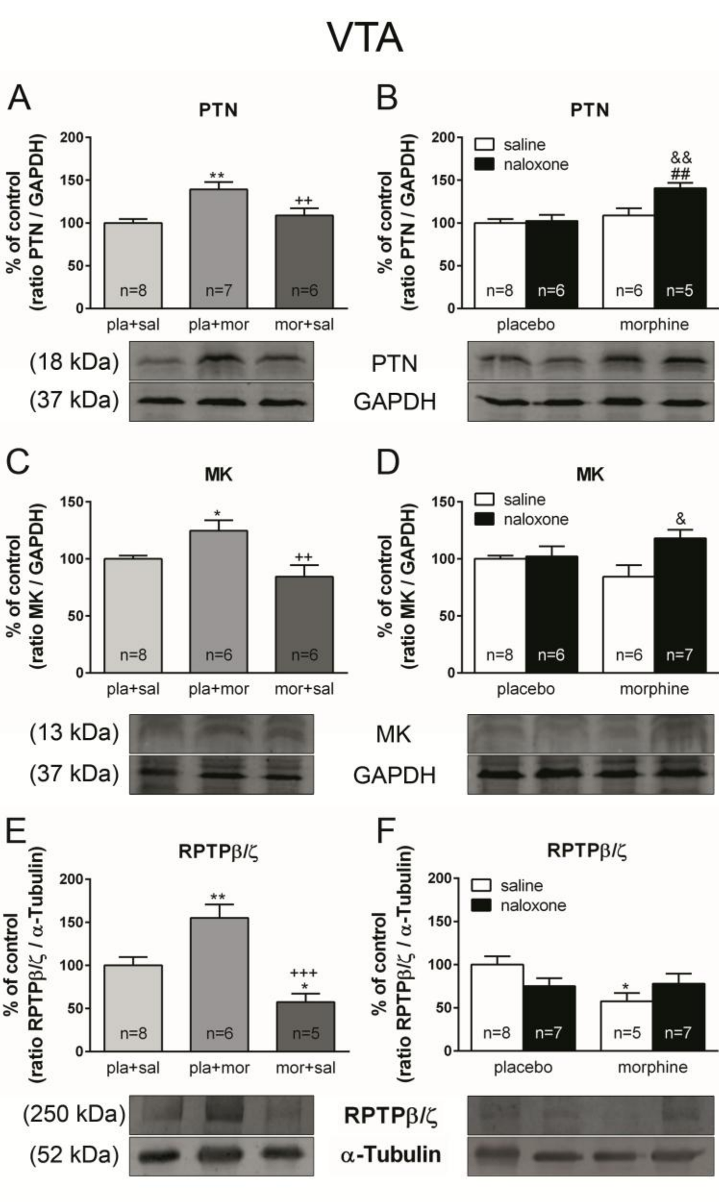
**Fig. 4.** PTN but not MK is overexpressed in astrocytes during acute morphine administration and morphine withdrawal in the VTA. (A-B): Stack of confocal images from the midbrain areas immuno-stained for GFAP (blue), PTN (red) and MK (green) in rats treated with acute morphine or morphine-dependent rats injected with naloxone. Scale bar: A-B, 50 µm

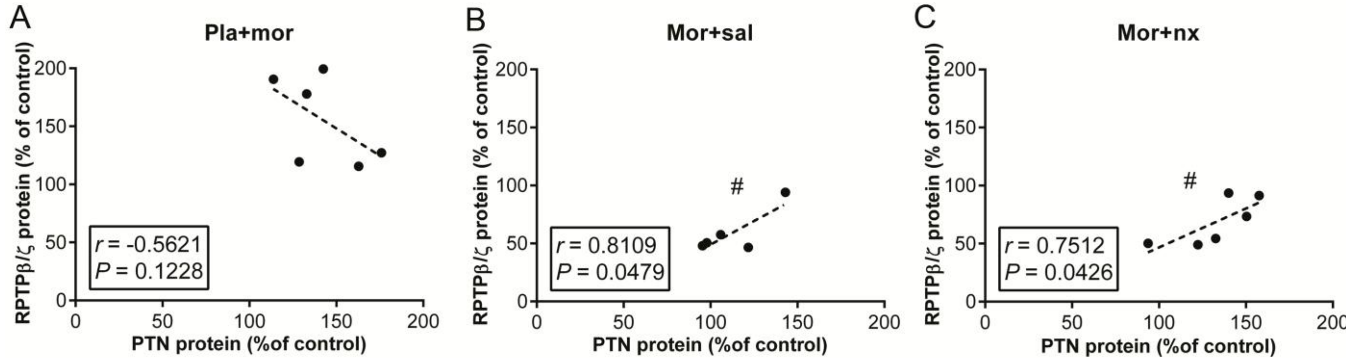
**Fig. 5.** RPTPβ/ζ is expressed in midbrain dopaminergic neurons. (A): RPTPβ/ζ (red) protein in the VTA is region-specific since strong staining in the VTA contrasts with weak or absent staining in the interpeduncular nucleus (IP) or red nucleus (RN). (B): Double-labeling with anti-TH (green) showed that RPTPβ/ζ is located on cell bodies and proximal processes of almost all dopaminergic neurons in the VTA and some non-dopaminergic cells. DAPI (blue) was used as a counterstaining in both nuclei. Scale bar: A, 200 µm; B, 50 µm

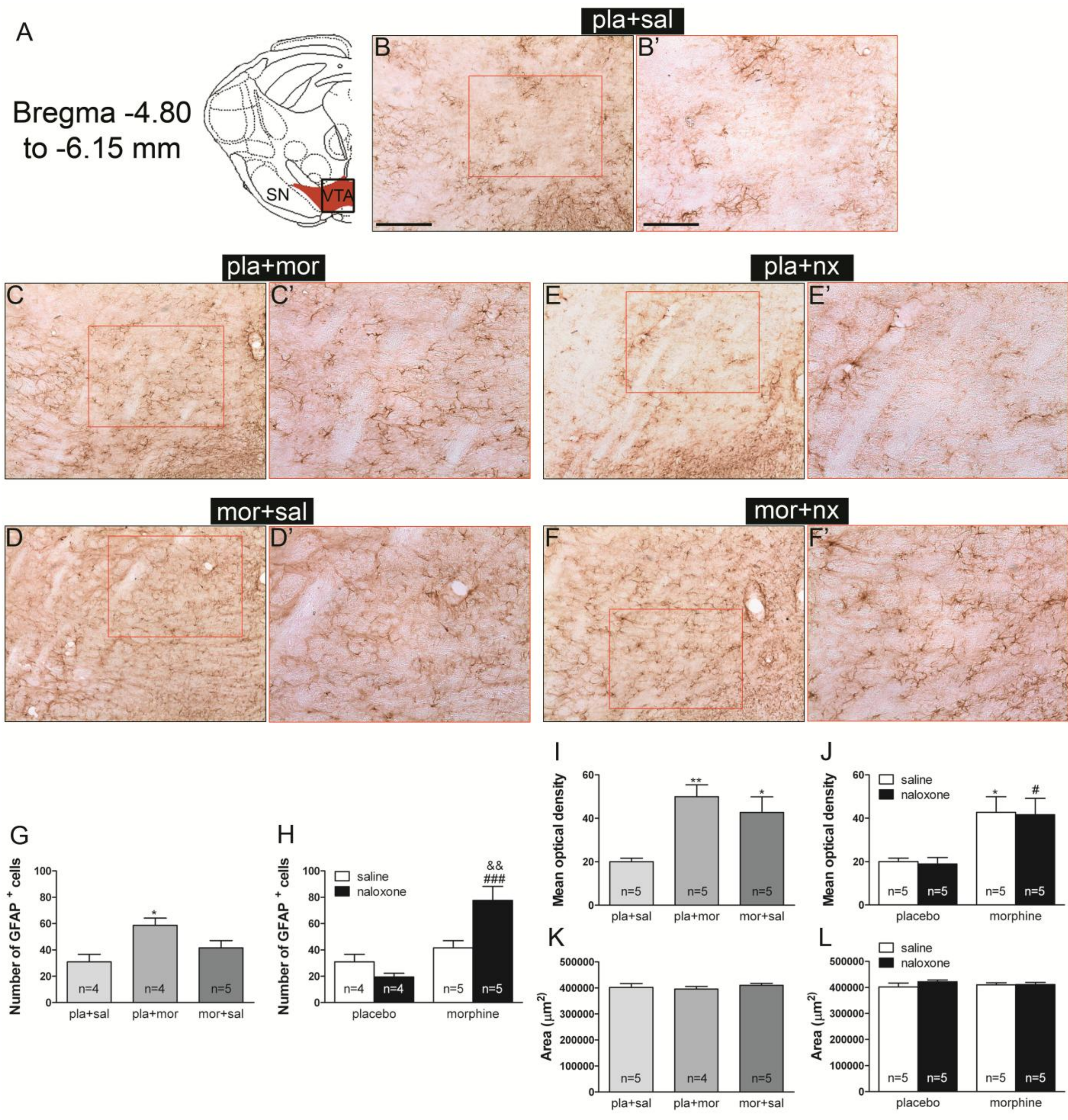
**Fig. 6.** p-Akt levels but not t-Akt levels are diminished during chronic morphine administration in the VTA. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of p-Akt / t-Akt ratio (A, B) and t-Akt levels (C, D) in VTA isolated from rats receiving the above treatments. Each bar corresponds to mean ± SEM. Values are expressed as % of controls. $^{*}p < 0.05$ vs. pla+sal; $^{+}p < 0.05$ vs. pla+mor; $^{\&}p < 0.05$ vs. mor+sal

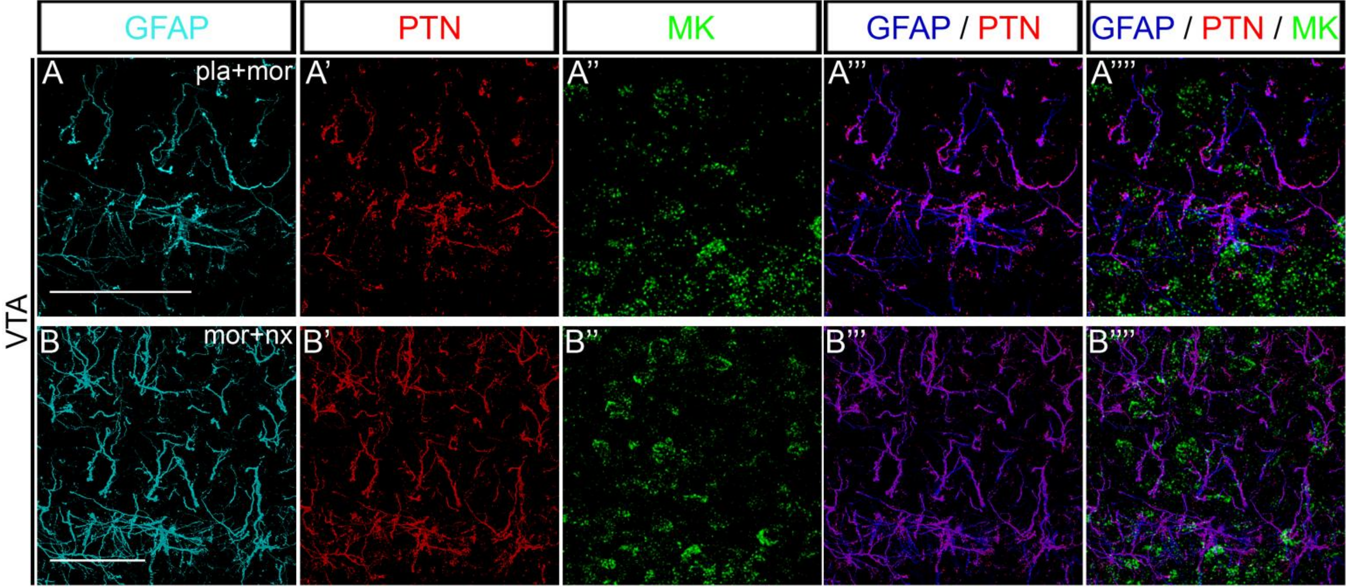
**Fig. 7.** p-ERK / t-ERK ratio, t-ERK levels and p-ERK absolute levels are altered by acute and chronic morphine administration and during morphine withdrawal in the VTA. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of p-ERK 1 / t-ERK 1 ratio (A, B), t-ERK 1 levels (C, D), p-ERK 1 absolute levels (E, F), p-ERK 2 / t-ERK 2 ratio (G, H), t-ERK 2 levels (I, J), p-ERK 2 absolute levels (K, L) in VTA isolated from rats receiving the above treatments. Each bar corresponds to mean ± SEM. Values are expressed as % of controls. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pla+sal; $^{+}p < 0.05$, $^{++}p < 0.01$ vs. pla+mor; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. pla+nx; $^{\&}p < 0.05$ vs. mor+sal

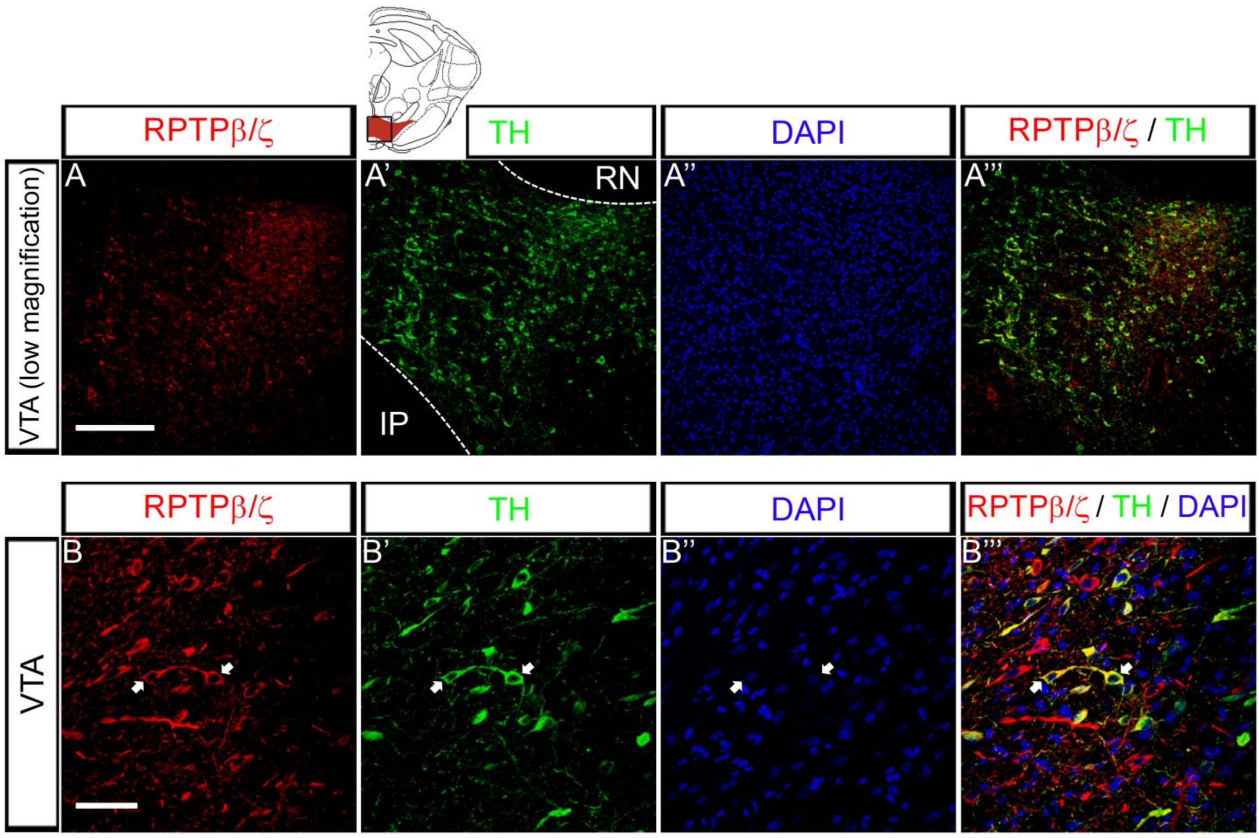
**Fig 8.** Correlation between PTN, MK and/or RPTPβ/ζ and p-ERK or t-ERK protein levels in different experimental groups. PTN tended to be negative correlated with t-ERK 1 and t-ERK 2 expression, while MK was negatively correlated with p-ERK 1 and p-ERK 2 levels only in morphine withdrawn rats. On the other hand, during morphine dependence and withdrawal, expression of RPTPβ/ζ tended to be positively correlated with p-ERK. $^{\#}p < 0.05$: PTN, MK and/or RPTPβ/ζ vs p-ERK 1-2 / t-ERK 1-2; $^{\&}p < 0.05$: PTN, MK and/or RPTPβ/ζ vs p-ERK 1-2 / GAPDH; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$: PTN, MK and/or RPTPβ/ζ vs t-ERK 1-2 / GAPDH.

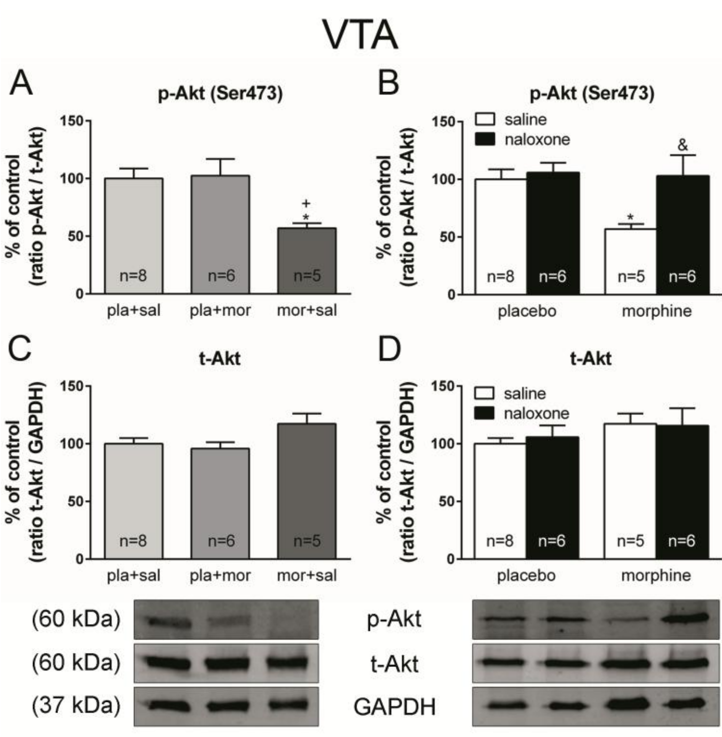

VTA
A
PTN
% of control (ratio PTN / GAPDH)
n=8
n=7
n=6
pla+sal
pla+mor
mor+sal
(18 kDa)
(37 kDa)
B
PTN
saline
naloxone
% of control (ratio PTN / GAPDH)
placebo
morphine
PTN
GAPDH
C
MK
% of control (ratio MK / GAPDH)
(13 kDa)
(37 kDa)
D
MK
MK
GAPDH
E
RPTPβ/ζ
% of control (ratio RPTPβ/ζ / α-Tubulin)
(250 kDa)
(52 kDa)
F
RPTPβ/ζ
RPTPβ/ζ
α-Tubulin

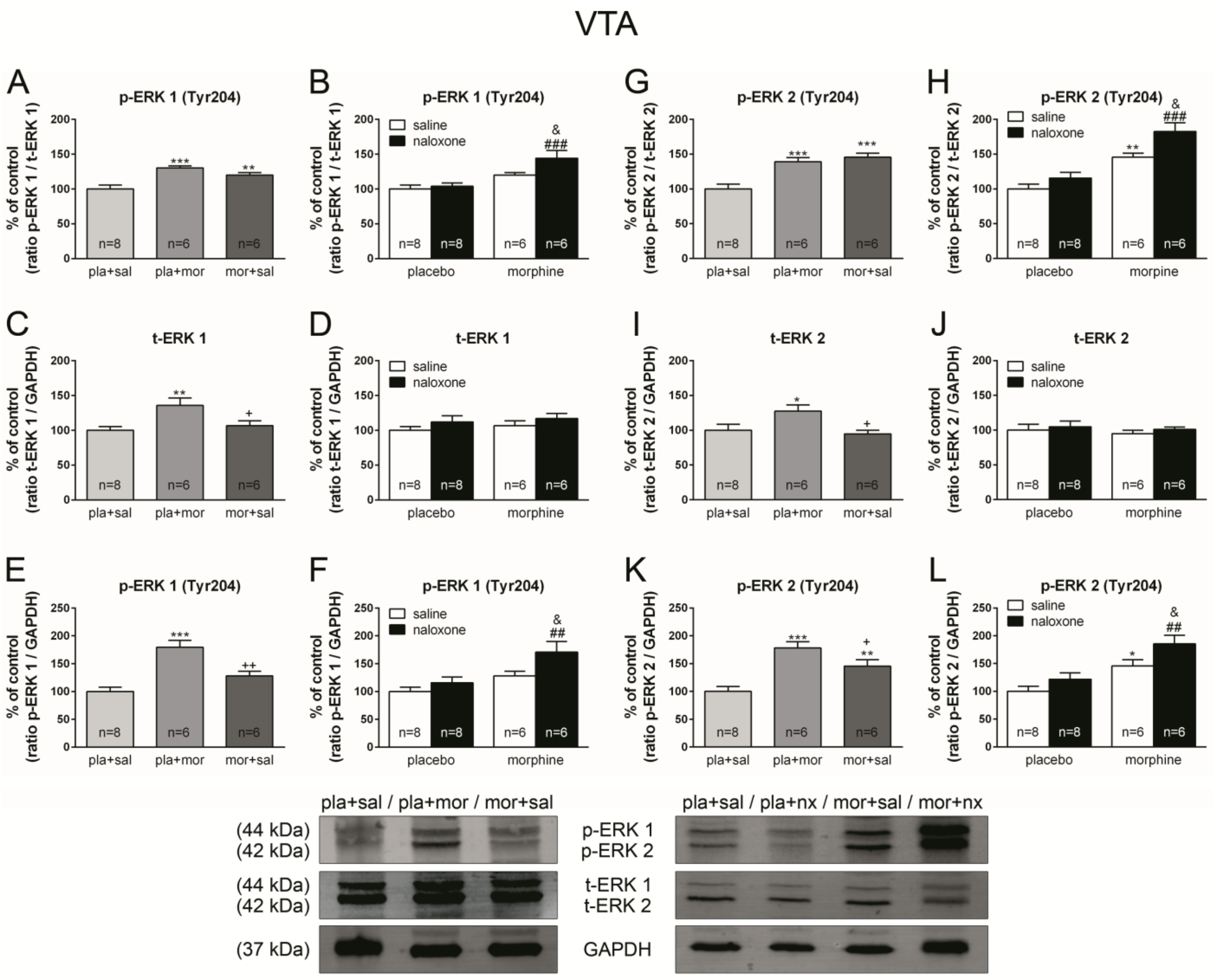

A
Pla+mor
RPTPβ/ζ protein (% of control)
PTN protein (%of control)
r = -0.5621
P = 0.1228
B
Mor+sal
#
r = 0.8109
P = 0.0479
C
Mor+nx
#
r = 0.7512
P = 0.0426

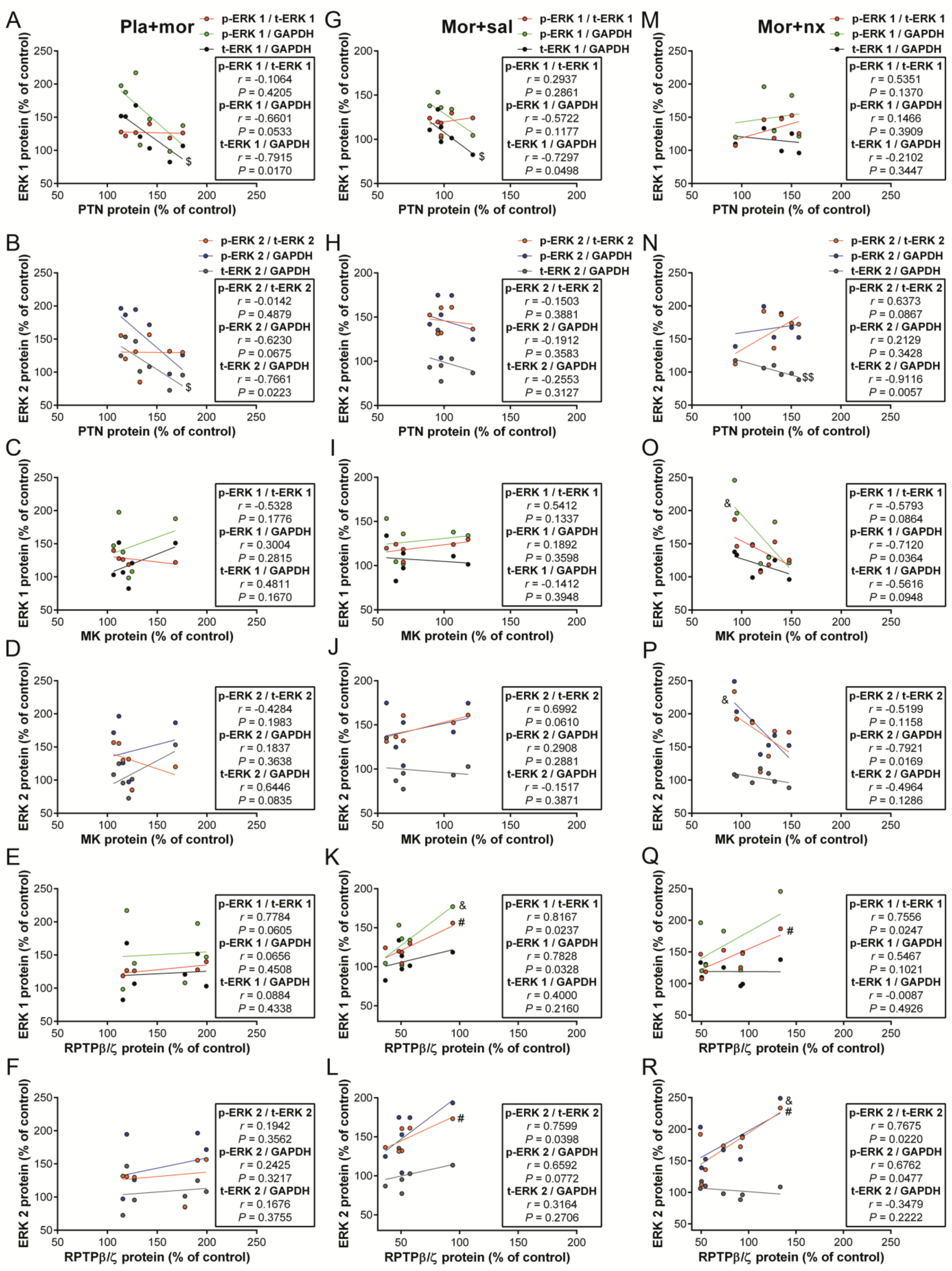

A
Bregma -4.80 to -6.15 mm
SN
VTA
pla+sal
B
B'
pla+mor
C
C'
pla+nx
E
E'
mor+sal
D
D'
mor+nx
F
F'
G
Number of GFAP + cells
n=4
n=4
n=5
pla+sal
pla+mor
mor+sal
H
saline
naloxone
&&
###
placebo
morphine
I
Mean optical density
J
K
Area (μm2)
L


GFAP
PTN
MK
GFAP / PTN
GFAP / PTN / MK
A
pla+mor
A'
A''
A'''
A''''
VTA
B
mor+nx
B'
B''
B'''
B''''


RPTPβ/ζ
TH
DAPI
RPTPβ/ζ / TH
VTA (low magnification)
A
A'
RN
A''
A'''
IP
RPTPβ/ζ
TH
DAPI
RPTPβ/ζ / TH / DAPI
VTA
B
B'
B''
B'''


VTA
A
p-Akt (Ser473)
% of control (ratio p-Akt / t-Akt)
150
100
50
0
n=8
n=6
n=5
pla+sal
pla+mor
mor+sal
B
p-Akt (Ser473)
saline
naloxone
% of control (ratio p-Akt / t-Akt)
placebo
morphine
C
t-Akt
% of control (ratio t-Akt / GAPDH)
D
t-Akt
(60 kDa)
(60 kDa)
(37 kDa)
p-Akt
t-Akt
GAPDH


VTA
A
p-ERK 1 (Tyr204)
% of control (ratio p-ERK 1 / t-ERK 1)
pla+sal
pla+mor
mor+sal
B
p-ERK 1 (Tyr204)
saline
naloxone
placebo
morphine
G
p-ERK 2 (Tyr204)
% of control (ratio p-ERK 2 / t-ERK 2)
H
p-ERK 2 (Tyr204)
morpine
C
t-ERK 1
% of control (ratio t-ERK 1 / GAPDH)
D
t-ERK 1
I
t-ERK 2
% of control (ratio t-ERK 2 / GAPDH)
J
t-ERK 2
E
p-ERK 1 (Tyr204)
% of control (ratio p-ERK 1 / GAPDH)
F
p-ERK 1 (Tyr204)
K
p-ERK 2 (Tyr204)
% of control (ratio p-ERK 2 / GAPDH)
L
p-ERK 2 (Tyr204)
pla+sal / pla+mor / mor+sal
pla+sal / pla+nx / mor+sal / mor+nx
(44 kDa)
(42 kDa)
p-ERK 1
p-ERK 2
t-ERK 1
t-ERK 2
(37 kDa)
GAPDH


A
Pla+mor
p-ERK 1 / t-ERK 1
p-ERK 1 / GAPDH
t-ERK 1 / GAPDH
ERK 1 protein (% of control)
PTN protein (% of control)
p-ERK 1 / t-ERK 1
r = -0.1064
P = 0.4205
p-ERK 1 / GAPDH
r = -0.6601
P = 0.0533
t-ERK 1 / GAPDH
r = -0.7915
P = 0.0170
$
B
p-ERK 2 / t-ERK 2
p-ERK 2 / GAPDH
t-ERK 2 / GAPDH
ERK 2 protein (% of control)
PTN protein (% of control)
p-ERK 2 / t-ERK 2
r = -0.0142
P = 0.4879
p-ERK 2 / GAPDH
r = -0.6230
P = 0.0675
t-ERK 2 / GAPDH
r = -0.7661
P = 0.0223
$
C
ERK 1 protein (% of control)
MK protein (% of control)
p-ERK 1 / t-ERK 1
r = -0.5328
P = 0.1776
p-ERK 1 / GAPDH
r = 0.3004
P = 0.2815
t-ERK 1 / GAPDH
r = 0.4811
P = 0.1670
D
ERK 2 protein (% of control)
MK protein (% of control)
p-ERK 2 / t-ERK 2
r = -0.4284
P = 0.1983
p-ERK 2 / GAPDH
r = 0.1837
P = 0.3638
t-ERK 2 / GAPDH
r = 0.6446
P = 0.0835
E
ERK 1 protein (% of control)
RPTPβ/ζ protein (% of control)
p-ERK 1 / t-ERK 1
r = 0.7784
P = 0.0605
p-ERK 1 / GAPDH
r = 0.0656
P = 0.4508
t-ERK 1 / GAPDH
r = 0.0884
P = 0.4338
F
ERK 2 protein (% of control)
RPTPβ/ζ protein (% of control)
p-ERK 2 / t-ERK 2
r = 0.1942
P = 0.3562
p-ERK 2 / GAPDH
r = 0.2425
P = 0.3217
t-ERK 2 / GAPDH
r = 0.1676
P = 0.3755
G
Mor+sal
p-ERK 1 / t-ERK 1
p-ERK 1 / GAPDH
t-ERK 1 / GAPDH
ERK 1 protein (% of control)
PTN protein (% of control)
p-ERK 1 / t-ERK 1
r = 0.2937
P = 0.2861
p-ERK 1 / GAPDH
r = -0.5722
P = 0.1177
t-ERK 1 / GAPDH
r = -0.7297
P = 0.0498
$
H
p-ERK 2 / t-ERK 2
p-ERK 2 / GAPDH
t-ERK 2 / GAPDH
ERK 2 protein (% of control)
PTN protein (% of control)
p-ERK 2 / t-ERK 2
r = -0.1503
P = 0.3881
p-ERK 2 / GAPDH
r = -0.1912
P = 0.3583
t-ERK 2 / GAPDH
r = -0.2553
P = 0.3127
I
ERK 1 protein (% of control)
MK protein (% of control)
p-ERK 1 / t-ERK 1
r = 0.5412
P = 0.1337
p-ERK 1 / GAPDH
r = 0.1892
P = 0.3598
t-ERK 1 / GAPDH
r = -0.1412
P = 0.3948
J
ERK 2 protein (% of control)
MK protein (% of control)
p-ERK 2 / t-ERK 2
r = 0.6992
P = 0.0610
p-ERK 2 / GAPDH
r = 0.2908
P = 0.2881
t-ERK 2 / GAPDH
r = -0.1517
P = 0.3871
K
ERK 1 protein (% of control)
RPTPβ/ζ protein (% of control)
&
#
p-ERK 1 / t-ERK 1
r = 0.8167
P = 0.0237
p-ERK 1 / GAPDH
r = 0.7828
P = 0.0328
t-ERK 1 / GAPDH
r = 0.4000
P = 0.2160
L
ERK 2 protein (% of control)
RPTPβ/ζ protein (% of control)
#
p-ERK 2 / t-ERK 2
r = 0.7599
P = 0.0398
p-ERK 2 / GAPDH
r = 0.6592
P = 0.0772
t-ERK 2 / GAPDH
r = 0.3164
P = 0.2706
M
Mor+nx
p-ERK 1 / t-ERK 1
p-ERK 1 / GAPDH
t-ERK 1 / GAPDH
ERK 1 protein (% of control)
PTN protein (% of control)
p-ERK 1 / t-ERK 1
r = 0.5351
P = 0.1370
p-ERK 1 / GAPDH
r = 0.1466
P = 0.3909
t-ERK 1 / GAPDH
r = -0.2102
P = 0.3447
N
p-ERK 2 / t-ERK 2
p-ERK 2 / GAPDH
t-ERK 2 / GAPDH
ERK 2 protein (% of control)
PTN protein (% of control)
$$
p-ERK 2 / t-ERK 2
r = 0.6373
P = 0.0867
p-ERK 2 / GAPDH
r = 0.2129
P = 0.3428
t-ERK 2 / GAPDH
r = -0.9116
P = 0.0057
O
ERK 1 protein (% of control)
MK protein (% of control)
&
p-ERK 1 / t-ERK 1
r = -0.5793
P = 0.0864
p-ERK 1 / GAPDH
r = -0.7120
P = 0.0364
t-ERK 1 / GAPDH
r = -0.5616
P = 0.0948
P
ERK 2 protein (% of control)
MK protein (% of control)
&
p-ERK 2 / t-ERK 2
r = -0.5199
P = 0.1158
p-ERK 2 / GAPDH
r = -0.7921
P = 0.0169
t-ERK 2 / GAPDH
r = -0.4964
P = 0.1286
Q
ERK 1 protein (% of control)
RPTPβ/ζ protein (% of control)
#
p-ERK 1 / t-ERK 1
r = 0.7556
P = 0.0247
p-ERK 1 / GAPDH
r = 0.5467
P = 0.1021
t-ERK 1 / GAPDH
r = -0.0087
P = 0.4926
R
ERK 2 protein (% of control)
RPTPβ/ζ protein (% of control)
&
#
p-ERK 2 / t-ERK 2
r = 0.7675
P = 0.0220
p-ERK 2 / GAPDH
r = 0.6762
P = 0.0477
t-ERK 2 / GAPDH
r = -0.3479
P = 0.2222

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**Abstract**

**BACKGROUND:**

Most classes of addictive substances alter the function and structural plasticity of the brain reward circuitry. Midkine (MK) and pleiotrophin (PTN) are growth/differentiation cytokines which, similarly to neurotrophins, play an important role in repair, neurite outgrowth, and cell differentiation. PTN or MK signaling through receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), leads to the activation of extracellular signal-regulated kinases and thymoma viral proto-oncogene. This activation induces morphological changes and modulates addictive behaviors. Besides, there is increasing evidence that during the development of drug addiction, astrocytes contribute to the synaptic plasticity by synthesizing and releasing substances such as cytokines.

**METHODS:**

In the present work we studied the effect of acute morphine administration, chronic morphine administration, and morphine withdrawal on PTN, MK, and RPTPβ/ζ expression and on their signaling pathways in the nucleus accumbens.

**RESULTS:**

Present results indicated that PTN, MK, and RPTPβ/ζ levels increased after acute morphine injection, returned to basal levels during chronic opioid treatment, and were up-regulated again during morphine withdrawal. We also observed an activation of astrocytes after acute morphine injection and during opiate dependence and withdrawal. In addition, immunofluorescence analysis revealed that PTN, but not MK, was overexpressed in astrocytes and that dopaminoceptive neurons expressed RPTPβ/ζ.

**CONCLUSIONS:**

All these observations suggest that the neurotrophic and behavioral adaptations that occur during opiate addiction could be, at least partly, mediated by cytokines.

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Abstract: BACKGROUND: Drug-withdrawal associated aversive memories might trigger relapse to drug-seeking behavior. However, changes in structural and synaptic plasticity, as well as epigenetic mechanisms, which may be critical for long-term aversive memory, have yet to be elucidated.
METHODS: We used conditioned place aversion (CPA) paradigm to uncover the role of glucocorticoids (GCs) on plasticity-related processes that occur within the dentate gyrus (DG) during opiate-withdrawal conditioning (memory formation-consolidation), and after reactivation by re-exposure to the conditioned environment (memory retrieval).
RESULTS: Rats subjected to conditioned morphine-withdrawal robustly expressed CPA, while adrenalectomy impaired naloxone-induced CPA. However, increased locomotor activity was observed in all the animals receiving morphine. Accordingly, activity-regulated cytoskeletal-associated protein (Arc) expression was induced in sham- and ADX-dependent animals during the conditioning phase in the DG. In contrast, memory retrieval elicited increased Arc and early growth response 1 (Egr-1) expression in sham+morphine rats, but not in ADX+morphine animals. Importantly, Arc seemed to regulate CPA score and was selectively expressed in the granular zone of the DG in dopaminoceptive, glutamatergic and GABAergic neurons. We further found that brain derived neurotrophic factor was regulated in the opposite way during the test phase. Our results also suggest a role for epigenetic regulation on the expression of GC receptors and Arc during memory retrieval.
CONCLUSIONS: Our data provide the first evidence that GC homeostasis is important for the expression of long-term morphine-withdrawal memories. Moreover, our results support the idea that targeting Arc and Egr-1 in the DG may provide important insights into the role of these signaling cascades in CPA reconsolidation.

# Glucocorticoid homeostasis in the dentate gyrus is essential for opiate withdrawal-associated memories

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**Key words:** Conditioned place aversion; adrenalectomy; memory consolidation and retrieval; addiction; tanscription factors; miRNAs

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**ABSTRACT**

BACKGROUND: Drug-withdrawal associated aversive memories might trigger relapse to drug-seeking behavior. However, changes in structural and synaptic plasticity, as well as epigenetic mechanisms, which may be critical for long-term aversive memory, have yet to be elucidated.

METHODS: We used conditioned place aversion (CPA) paradigm to uncover the role of glucocorticoids (GCs) on plasticity-related processes that occur within the dentate gyrus (DG) during opiate-withdrawal conditioning (memory formation-consolidation), and after reactivation by re-exposure to the conditioned environment (memory retrieval).

RESULTS: Rats subjected to conditioned morphine-withdrawal robustly expressed CPA, while adrenalectomy impaired naloxone-induced CPA. However, increased locomotor activity was observed in all the animals receiving morphine. Accordingly, activity-regulated cytoskeletal-associated protein (Arc) expression was induced in sham- and ADX-dependent animals during the conditioning phase in the DG. In contrast, memory retrieval elicited increased Arc and early growth response 1 (Egr-1) expression in sham+morphine rats, but not in ADX+morphine animals. Importantly, Arc seemed to regulate CPA score and was selectively expressed in the granular zone of the DG in dopaminoceptive, glutamatergic and GABAergic neurons. We further found that brain derived neurotrophic factor was regulated in the opposite way during the test phase. Our results also suggest a role for epigenetic regulation on the expression of GC receptors and Arc during memory retrieval.

CONCLUSIONS: Our data provide the first evidence that GC homeostasis is important for the expression of long-term morphine-withdrawal memories. Moreover, our results support the idea that targeting Arc and Egr-1 in the DG may provide important insights into the role of these signaling cascades in CPA reconsolidation.

**INTRODUCTION**

Drug addiction is a chronic, relapsing disorder in part due to the strong associations formed between drugs and the stimuli associated with drug use through Pavlovian conditioning (1). These stimuli become strong drivers of continuous use and relapse after abstinence (2). One of the factors thought to be responsible for this vulnerability is conditioned withdrawal, which consists of somatic and affective withdrawal signs elicited in the presence of cues that were previously paired with drug withdrawal (3). Reactivation of these withdrawal memories has been suggested to trigger the relapse of drug seeking behavior in abstinent opiate addicts (4,5). Although much has been learned about the neural basis of reinforcement during drug administration, the neural circuitry that underlies conditioned-cued relapse is only now being clarified.

It has been proposed that the reinforcing properties of drugs of abuse are due, in part, to their ability to enhance memory consolidation. In addition, several molecular and cellular adaptations involved in addiction are also implicated in models of learning and memory. Therefore, recent efforts to develop effective treatments for addiction have focused on manipulations of learning and memory processes involved in encoding cue-drug or cue-drug withdrawal associations (4). Conditioned-place aversion (CPA) paradigm is a highly sensitive animal model for measurement of the negative affective component of drug withdrawal as well as to investigate the neural substrates underlying the aversive memory associated with drug withdrawal (6,7). In rodents, the negative affective component of opiate dependence could be reflected by CPA, in which opiate withdrawal is paired with a particular environment, which triggers the association between negative affective consequences of withdrawal with context (aversive memory formation). When animals are re-exposed to the paired environment in a drug-free state, they avoid the paired environment due to the association between the context and aversive memories of drug withdrawal (aversive memory retrieval). Among the limbic structures that are likely to mediate these component of drug addiction, the basolateral amygdala (BLA) and the hippocampus represent critical neural substrates (8,9). It is well established that the hippocampus is crucially important for memory encoding/consolidation but also for episodic memory retrieval (10). In

this context, much experimental evidence has demonstrated that the granular zone (GZ) of the hippocampal dentate gyrus (DG) has a critical role in learning and memory formation (11,12).

On the other hand, stress hormones such as glucocorticoids (GCs) and noradrenaline mediate and modulate memory consolidation (13,14). GCs exert this action directly on brain regions, including the hippocampus, amygdala and prefrontal cortex, which are enriched in glucocorticoid receptors (GRs) and are important in long-term memory formation (15).

Most of the existing knowledge focuses on the molecular and cellular adaptations that occur in the brain reward regions (VTA and NAc) in response to drugs exposure and withdrawal, with much less information available about other key limbic brain regions also crucial for drug addiction and the molecular mechanisms that are critical to transform opiate withdrawal into a long-term aversive memory.

CPA develops through associative learning and requires synaptic plasticity (16), which is implicated in adapted responses in brain, including memory formation and drug addiction and withdrawal (17). Therefore, we have used CPA to study the plasticity-related processes that occur within the DG during conditioning of the aversive properties of opiate withdrawal (memory formation-consolidation; MC), and after allowing their reactivation by re-exposure to the conditioned environment (memory retrieval; MR). For that, first we have assessed activity-regulated cytoskeletal *(arc)* mRNA expression and effector protein [a plasticity marker; (18)], as well as other transcription factors and receptors involved in memory formation: GR, phosphorylated cAMP response element binding protein (pCREB), brain derived neurotrophic factor (BDNF) and early growth response 1 (Egr-1), among others. Second, we hypothesized that GCs might play a crucial role in the aversive memories of drug withdrawal. To test this hypothesis we have used adrenalectomized (ADX) rats and examining the same neurobiological parameters and determining the role of GC in the induction of CPA.

## METHODS AND MATERIALS

Details are provided in the Supplementary Information (SI).

**Animals**

All experimental procedures were performed in accordance with the European Community Council Directive (2010/63/EU), and local Committees for animal research (REGA ES300305440012). Male Wistar rats (Harlan, Barcelona, Spain) were used.

**Adrenalectomy**

Rats were bilaterally ADX as described previously (19) and implanted subcutaneously (s.c.) with slow-release corticosterone pellets at surgery that provide stable corticosterone levels corresponding to circadian nadir. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation.

**Induction of Opiate Dependence**

Morphine dependence was induced by s.c. implantation (lower back) of two slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base) under light ether anaesthesia. Placebo-pelleted rats received lactose pellets also implanted subcutaneously under anaesthesia.

**Conditioned Place Aversion Protocol**

**Pre-testing Phase**

In the pre-testing phase (day 0), animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. For each rat, one room was randomly chosen to be paired with naloxone and the other to vehicle. Rats were ADX on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

**Conditioning Phase**

Rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. In addition, opiate withdrawal behaviors on sham or ADX animals receiving naloxone on days 8 and 10 were measured for 15 min after the conditioning phase. A cohort of animals, named conditioning memory rats (CM), were decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

**Testing Phase**

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). The difference ($\Delta D = D - D0$) between the time spent in the naloxone-paired compartment after conditioning ($D$) minus the time spent in the same compartment before conditioning (preconditioning test $D0$) reflects the change of preference induced by opiate withdrawal. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

**Tissue processing**

Brains were sliced on a cryostat and kept at -20°C until each region of interest comes into the cutting plane. For DG study, three consecutive 500-µm coronal slides were made corresponding to approximately -2.9 to -4.4 mm from bregma, according to the atlas of (20). Tissues of interest were dissected using a punching device with a 1-mm internal diameter. A second set of animals from each treatment group was used for immunofluorescence staining.

**RNA extraction and quantitative real-time PCR (qPCR)**

qPCR was performed as previously described (21).

List of the genes: gapdh, arc, egr-1, bdnf

List of the microRNA (miRNA) assays: U6 snRNA (cat.: 001973, 4427975), miR-212 (cat.: 002551, 4427975), miR-124a (cat.: 001182, 4427975)

The gene expression was analyzed by ABI Step One 2.1 program. The amplicon was tested by Melt Curve Analysis on ABI Step OnePlus Instrument. Experiments were normalized to gapdh (glyceraldehyde-3-phosphate dehydrogenase) and U6 snRNA expressions.

gapdh for ACAGCCGCATCTTCTTGTGC

gapdh rev GCCTCACCCCATTTGATGTT

arc for CCCCCAGCAGTGATTCATAC

arc rev CAGACATGGCCGGAAAGACT

egr-1 for CACCTGACCACAGAGTCCTTTT

egr-1 rev ACCAGCGCCTTCTCGTTATT

bdnf for AAACGTCCACGGACAAGGCA

bdnf rev TTCTGGTCCTCATCCAGCAGC

U6 snRNA cat:001973

mmu-miR-212 cat: 002551

mmu-miR-124a cat: 001182

**Western Blotting**

Western Blotting protocol (22) and a list of the primary antibodies used in this study can be found at SI. We used GAPDH as our loading control.

**Immunofluorescence study and confocal microscopical analysis**

The tissue for immunofluorescence was processed as previously described (22) (see details in the SI). Equipment and settings are detailed in the SI. Final confocal images are illustrated as they appear throughout the stack of sections as a simple layer or as a transparency of all layers merged together.

**Data Analysis**

Data are presented as mean ± SEM. Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test. Student's *t*-test was used when there were two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant (GraphPad Prism 6: GraphPad Software Inc., San Diego, CA, USA).

**RESULTS**

GCs are released during and immediately after emotionally arousing stimulation used in training tasks, and there is extensive evidence that these hormones enhance memory consolidation (23,24). The animals used in this experiment did not exhibit natural preference for neither the dots nor stripes chambers before the conditioning sessions (Fig. 1B-D,F-I). During the test, morphine dependent rats spent less time in the naloxone-paired compartment than controls (Fig. 1E). The negative score of these animals correlated with reduced number of entries in the naloxone-paired compartment (Fig. 1M). Remarkably, adrenalectomy diminished naloxone-induced CPA in opiate-dependent animals (Fig. 1E). Additionally, in the test phase, the total entries to any chamber were higher in animals receiving opiates (Fig. 1J-L).

ADX animals gained significantly less weight after surgery than sham-operated rats (Fig. 2A). After pellets implantation, sham- and ADX-operated animals receiving morphine showed lower body weight gain than their respective controls (Fig. 2B,C). In addition, ADX reduced the body weight gain from days 4 to 7 in control rats compared with sham+placebo rats (Fig. 2B). In agreement with other studies (25), naloxone administration to sham- or ADX-operated rats dependent on morphine evoked an increase of the body weight loss (Fig. 2D), as well as the appearance of a number of behavioral symptoms characteristic of opiate withdrawal (Fig. 2E-G). It is noteworthy that, on day 10, the ADX+morphine group exhibited an enhancement of the withdrawal score when compared with both sham+morphine and ADX+morphine (day 8) groups (Fig. 2G).

Regarding the activity of the hypothalamic-pituitary-adrenal (HPA) axis after memory formation and retrieval processes, our results revealed that naloxone administration to morphine dependent rats induced an increase in GCs plasma levels, which was not observed in ADX animals after conditioning sessions on day 10 due to adrenalectomy (Fig. 2H). The process of memory retrieval after naloxone-induced CPA did not alter the blood levels of adrenocorticotroph hormone (ACTH) and corticosterone, although ADX rats displayed increased ACTH concentration because of the lack of GCs inhibition on HPA axis (Fig. 2I,J).

To determine which molecular pathways are coupled to the learning-dependent GCs activation required for long-term memory, we examined the effect adrenalectomy on numerous molecular changes that were previously established to underlie long-term synaptic plasticity and memory formation. Our results show that memory consolidation process did not modify neither miR-124 levels nor miR-212 expression in the DG from sham+morphine rats (Fig. 3A,B), but induced a significant increase in Arc mRNA and protein levels (Fig. 3G,J). No other changes were seen in the rest of the parameters studied in sham-operated morphine-dependent animals compared with placebo-pelleted rats. Nonetheless, in ADX animals we observed a diminution of miR-212 in DG from both control and morphine-dependent rats (Fig. 3B), which paralleled an increase of Arc mRNA and protein in morphine-dependent ADX animals (Fig. 3G,J). In addition, Egr-1 protein levels in ADX animals treated with morphine were significantly higher than those in ADX+placebo and sham+morphine groups.

After CPA memory retrieval, the levels of both miR-124a and miR-212 decreased in ADX animals (Fig. 4A,B). These data were accompanied by increasing GR protein levels in ADX animals (Fig. 4K). Both miRNAs were negatively correlated with the levels of GR in the sham+morphine group (Fig. 4F,G). Besides, we observed an enhancement of Arc mRNA and protein levels in sham morphine-dependent animals that did not appear in animals with lack of GCs (Fig. 4E,O), as well as a positive correlation between miR-124a and Arc protein levels (Fig. 4H). Then, we measured the expression of other factors in the DG after the test phase. We observed that both Egr-1 mRNA and protein levels tended to augment in sham+morphine-treated rats, while in ADX animals dependent on morphine there was a significant decrease of Egr-1 mRNA and protein expression (Fig. 4C,M). CREB phosphorylation also augmented after CPA in morphine-treated and in ADX rats (Fig. 4L). In addition, BDNF protein levels were increased in ADX morphine-dependent animals (Fig. 4N). As shown in Fig. 4J, the regulation of BDNF mRNA is opposite to Egr-1 and Arc mRNAs. Interestingly, when we evaluated the correlation between the score and the proteins studied, we found a negative correlation with Arc only in sham+morphine rats (Fig. 4P-U).

As seen in Fig. 5A,C, D1 and D2 receptors were localized in the GZ of DG, although D1 was also found in other regions of the DG. Distinct staining pattern for D1 and D2 was also demonstrated in rat cortex (Fig. S1A). GLS2 and GAD, markers of glutamatergic and GABA-ergic neurons, respectively, were colocalized in the DG, mainly in the GZ (Fig. 5D,E). Besides, D1-positive or GLS2-positive signals were colocalized with GFAP (astrocytic marker) only in the molecular zone, but not in the GZ of the DG (Fig. 5F,G).

According to what has been set out above, we confirmed the co-localization of GR with pCREB, Egr-1, and BDNF in the GZ of the DG after the CPA in sham- and ADX-operated morphine-dependent rats. Representative images are shown in Figure 6. Additionally, positive correlations were obtained between GR and pCREB protein levels in the sham-morphine group (Fig. 6I), as well as between GR and Egr-1 protein levels in ADX-operated rats dependent on morphine (Fig. 6L). In contrast, sham+placebo and ADX+placebo rats displayed negative correlation between GR and Egr-1 (Fig S2C,D). In addition, GR and BDNF protein levels were negatively correlated in the ADX+morphine group (Fig. 6N).

As seen in Fig. 7 and supplementary Fig. S3A, the immunofluorescence study indicated that neurons exhibiting GR staining in the DG after naloxone-induced CPA displayed low Arc signal. Besides, there was a negative correlation between GR and Arc only in sham morphine-dependent rats (Fig. 7H). Arc was found in GABAergic, glutamatergic and dopaminoceptive neurons (Fig 7A-C). Moreover, all neurons that expressed Arc were pCREB- or Egr-1-positive in the GZ of the DG after the test phase (Fig. 8A-D; Fig S3B-C). While all groups exhibited positive correlation between Arc and pCREB (Fig. 8I-J; Fig. S4A-B), Egr-1 was positively correlated with Arc only in sham+placebo rats (Fig. 8K-L; Fig. S4C-D). The majority of Arc-positive neurons in sham+morphine rats did not express BDNF (Fig. 8E). On the other hand, the few $Arc^+$ neurons in ADX+morphine animals were also $BDNF^+$ (Fig. 8F). However, there was no correlation between Arc and BDNF expression neither in sham+morphine rats nor in ADX+morphine rats (Fig. 8M-N). In addition, there was no colocalization of Arc and pTrkB in the DG of morphine-dependent animals (Fig. 8G,H).

**DISCUSSION**

Relapse to drug-taking following prolonged abstinence constitutes an important problem for long-term drug-addiction treatment. Factors contributing to relapse are exposure to conditional drug cues and stress (2). Present report highlights the impact of opiate withdrawal-associated aversive stimuli on DG activity, which has a pivotal role in mediating memory consolidation and recall. Collectively, the present study determined the role of GCs (by using ADX animals) in the formation of morphine withdrawal-associated conditioned memory, as well as in the expression of morphine withdrawal-associated CPA. GCs have profound influence on different stages of opiate dependence, such as the relapse to opiate-seeking (26). However, opiate withdrawal, as such, did not reinstate drug-seeking behavior (27), thus highlights the essential role of withdrawal-related memories on drug reinstatement.

Drugs of abuse stimulate HPA axis during acute withdrawal (28,29). A GR antagonist alleviated the somatic signs of naloxone-induced opiate withdrawal, without altering plasma GCs concentration (30). Our current results indicate that brain GCs levels do not modulate the somatic expression of opiate withdrawal, thus highlighting the essential role for GR signaling but not for GCs levels on the expression of withdrawal-associated somatic signs. Increased withdrawal score observed in ADX+morphine rats on day 10 could underlie increased GR levels in stress-related areas, as described by our group (30). Naloxone injection to morphine-dependent rats decreased the levels of hippocampal GR mRNA (31,32,33). However, sham+morphine rats did not exhibit increased GR levels during conditioning, which could be explained by a lower naloxone dose used in our experiments.

On the other hand, enhanced GCs levels are known to impair memory retrieval (10). Importantly, sham or ADX animals implanted with morphine do not differ in plasma corticosterone levels from their controls during the test phase. Chronic opiate exposure did not alter GR mRNA in the DG (33). In contrast, we observed that ADX-animals exhibited enhanced GR levels during the test phase, which may be explained by the lack of endogenous GCs (34,35). Corticosterone secretion is required for the enhanced locomotor response to opiates (36,37). Our results show that withdrawal-induced behavioral sensitization occurred both, in sham and ADX-dependent animals, although we detected differences in

the room preference during the test phase. Thus, the reduced aversion in ADX+morphine rodents was not a result of nonspecific locomotor changes.

Abused drugs usurp the synaptic machinery of hippocampus circuits and may induce maladaptive plasticity (38). Hippocampal long-term potentiation (LTP) is a form of synaptic plasticity proposed as a cellular substrate for learning and memory (39). Previous studies have shown that chronic morphine can both reduce (40,41,42) or augment (43,44,45,46) LTP in rat hippocampal synapses. Our results demonstrate that morphine-dependent rats display associative learning through a LTP-dependent mechanism, with endogenous GCs playing a pivotal role.

Memory consolidation refers to the time-dependent stabilization process that leads to long-term storage of a newly acquired memory (15). Strengthening effects of GCs on memory consolidation are well known, but the underlying molecular mechanisms during CPA conditioning remain poorly understood. We uncovered that ADX-dependent group showed increased Egr-1 expression, while both sham and ADX-addicted animals displayed increased Arc levels during consolidation process. Corticosterone and GR are essential to control increased learning-dependent Arc (47,48,14), suggesting that GR-signaling should be active in ADX-rats. Arc and Egr-1, which are induced during memory consolidation (49,50,51), are required in late LTP and long-term memory (52,53). Thus, our results support that sham- and ADX morphine-dependent animals are able to encode the aversive experience trough LTP induction. Moreover, we detected that BDNF or pCREB levels remained unaltered during consolidation, also described for other conditioning tasks (54), but in contrast with increased pCREB-133 levels (14,55,54) and BDNF pathway recruitment (14) during fear training. We conclude that fear and morphine-withdrawal training only partially engage the same signaling pathways.

Post-transcriptional processes play essential roles in neuronal plasticity and contribute to addiction (56). We observed temporal dissociated miRNA regulation during the CPA protocol. miR-212 was downregulated in ADX groups, independently of the experimental phase. By contrast, daily injection of ACTH upregulated the expression of miR-212 in rat adrenal glands (57). A possible explanation to

this discrepancy is that ADX animals in our experiments displayed sustained high ACTH plasma levels, which could dysregulate the acute ACTH-induced miR-212 expression.

While advances towards understanding memory consolidation have been made, less is known about the molecular processes underlying plasticity during memory retrieval (58), a very rapid process in which a previously acquired memory trace is reactivated (59). Retrieval-dependent activation occurs in the dorsal hippocampus (60,58,8) and induces protein synthesis-dependent memory reconsolidation (61,62). We tested memory recall one day after the last conditioning session, since hippocampal GRs regulate essential mechanisms for long-term fear memory formation, without affecting short-term retention (14).

Compelling evidence shows that gene expression programs are, in part, orchestrated by epigenetic mechanisms. GCs induce the expression of miR-124, which in turn downregulates GR (63,64). In concordance, we observed a negative correlation between miR-124a and GR levels in sham+morphine rats during the test phase. Moreover, the extirpation of adrenal glands seems to lead to reduced miR-124a levels and increased GR expression. The negative relationship observed between miR-212 and GR raises the possibility that reduced miR-212 could participate in GR upregulation in ADX rats. Several genes related with synaptic plasticity and drug addiction have been described among miR-124 and mir-212 targets, such as CREB or BDNF (65,66,67,68). However, our results do not point to a direct regulation of BDNF or pCREB by these miRNAs during the CPA expression. Importantly, miRNAs can attract a variety of activities to their targets, even stimulating translation (69). It is noteworthy that miR-124 and miR-212 seem to promote Arc protein levels in sham+morphine animals.

Our results show that CPA expression in sham+morphine rats is accompanied by increased CREB activation, indicating that pCREB accompanies memory retrieval and plays a role in memory re-consolidation following retrieval (70). On the other hand, ADX animals show increased pCREB levels, which may be due to increased GR expression instead of memory-related mechanisms, since ADX-dependent animals do not display enhanced pCREB levels compared to their controls.

While GR–dependent memory-related pCREB molecular changes were nongenomic, Egr-1 or Arc induction required transcription (14). Thus, our results clearly show that IEG induction in the hippocampus during testing reflects the genomic activation of neurons relevant to the contextual memory retrieval (60). Previous reports demonstrated an increase in Egr-1 expression levels at the similar and more prolonged time-points (51,71,72). GR in the hippocampus enhance fear memory consolidation via Egr-1 activation (73,74). However, post-conditioning Egr-1 levels did not increase in sham+morphine rats, suggesting that morphine-withdrawal memory consolidation does not require Egr-1, whereas reconsolidation recruits Egr-1, as proposed by Lee and colleagues (72). Moreover, our results point out that homeostatic endogenous GCs are critical for retrieval-induced Egr-1 expression.

Similar data were obtained for Arc induction during fear-memory retrieval (51), although other reports failed to detect these signaling event at late time-points (75). Arc KO animals results in an unusual phenotype: short-term learning is normal but lasting memories cannot be formed (76). Late-phase LTP is considered to be the underlying mechanism of long-term memory, and LTP consolidation in the DG requires sustained period of Arc synthesis translation since Arc protein underlying LTP consolidation is rapidly degraded (77,78). Given that Arc learning-dependent expression in the hippocampus is controlled by corticosterone and GR (47,48,14), we propose that ADX-dependent animals are able to induce Arc-mediated LTP, but in the absence of withdrawal-induced corticosterone are unable to maintain LTP and form long-term memory. A role for Arc in reconsolidation following memory retrieval has been proposed (79). Our data indicated that Arc plays a pivotal role in morphine-withdrawal memory retrieval and reconsolidation: rats with higher Arc expression significantly exhibited enhanced aversion. Arc identifies genomically activated neurons as a result of retrieval, so if associative memory retrieval is reduced and reconsolidation processes are not invoked, then Arc expression might be selectively diminished, as occurs in ADX+morphine animals.

Increased GCs levels displayed consequent reduction of GR expression (34,35). Given that we detected a selective negative correlation between GR and Arc in sham+morphine rats, it is tempting to speculate that GCs levels during memory retrieval enhance Arc expression, as described by

McReynolds (47). Moreover, correlation analysis in all the experimental groups supports that pCREB regulates Arc expression (80). We observed that Arc was found in glutamatergic neurons of the GZ, which extends previous evidence (81). Moreover, expression of Arc in GAD65/67-positive neurons suggests that CPA retrieval and subsequent reconsolidation represented a strong-learning stimulus (81). On the other hand, associative learning is dependent on D1 signaling (82), which indicates that Arc induction occurs in the same neurons that become activated through D1-dependent learning.

In concordance with previous data (72), we demonstrate that reconsolidation does not recruit BDNF. It could be surprising that, while BDNF enhances LTP in the DG (83,84,85) and triggers protein synthesis-dependent LTP (86,87), we observed reduced BDNF levels in sham+morphine group after the CPA test. However, literature describes that chronic morphine treatment represses BDNF (88,89,90). On the other hand, opiates decrease the number/complexity of dendritic spines on hippocampus neurons (91,92,93). Given the role of BDNF in dendrite development (94,93), reduced BDNF levels could account for this effect. Similarly, intermittent stress decreases dendritic branching (95), raising the possibility that the lack of increased GCs and enhanced BDNF in ADX-dependent rats could revert this consequence. In addition, chronic opiate exposure negatively impacts subgranular zone (SGZ) neurogenesis (96,97,98,99), in direct opposition with BDNF (100). Thus, cognitive defects are described in animals chronically exposed to opiates (101,102,103), suggesting that reduced neurotrophic factors may mediate the morphine-induced cognitive deficit (101). However, our data indicate that sham+morphine rats can perform associative learning and recall long-term memory. It has been suggested that BDNF effects on memory are downstream or independent of Arc (14). We did not observe relationship between BDNF and Arc levels in morphine-dependent animals and immunofluorescence study revealed no colocalization between Arc and pTrkB.

In summary these experiments represent the first demonstration that Egr-1 and Arc expression in the DG are associated with morphine-withdrawal memory retrieval and suggest that these genes may contribute to plasticity and reconsolidation accompanying the retrieval process. Besides, GCs play an

essential role during the formation of long-term withdrawal-aversive memories, given that ADX-dependent animals exhibit reduced place aversion and decreased Egr-1 and Arc levels.

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## FINANCIAL DISCLOSURES

Mr García-Pérez reported no biomedical financial interests or potential conflicts of interest. Dr. Ferenczi reported no biomedical financial interests or potential conflicts of interest. Dr. Kovács reported no biomedical financial interests or potential conflicts of interest. Dr. Laorden reported no biomedical financial interests or potential conflicts of interest. Dr. Milanés reported no biomedical financial interests or potential conflicts of interest. Dr. Núñez reported no biomedical financial interests or potential conflicts of interest.

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**FIGURE LEGENDS**

**Figure 1.** Effects of adrenalectomy on the naloxone-induced conditioned place aversion in morphine dependent rats. (A) Schematic overview of the animals' treatment and behavioral experiments time course. (B) Time spent in the dot chamber during pretest phase. (C) Time spent in the stripes chamber during pretest phase. (D) Time spent in the naloxone-paired chamber during pretest phase. (E) Difference between the time spent in the naloxone-paired compartment after conditioning sessions minus the time spent in the same compartment before conditioning sessions. Morphine treatment: $F_{(1,48)} = 10.05$, $P = 0.0027$; Interaction adrenalectomy X morphine treatment: $F_{(1,48)} = 10.44$, $P = 0.0022$. Newman-Keuls' post hoc: ***$P < 0.001$ vs sham+pla; $^{++}P < 0.01$ vs sham+mor. (F) Number of entries to the dots chamber during pretest phase. (G) Number of entries to the stripes chamber during pretest phase. (H) Number of entries to the naloxone-paired chamber during pretest phase. (I) Percentage of total entries to the dots and stripes chambers during pretest phase. (J) Difference between the number of total entries after conditioning sessions minus the number of total entries before conditioning sessions. Morphine treatment: $F_{(1,48)} = 13.72$, $P = 0.0005$. Newman-Keuls' post hoc: *$P < 0.05$ vs sham+pla; $^{\#}P < 0.05$ vs ADX+pla. (K) Difference between the number of entries to the saline-paired chamber after conditioning sessions minus the number of entries to the same chamber before conditioning sessions. Morphine treatment: $F_{(1,48)} = 11.11$, $P = 0.0017$. Newman-Keuls' post hoc: **$P < 0.01$ vs sham+pla. (L) Difference between the number of entries to the naloxone-paired chamber after conditioning sessions minus the number of entries to the same chamber before conditioning sessions. Morphine treatment: $F_{(1,48)} = 11.68$, $P = 0.0013$. Newman-Keuls' post hoc: $^{\#\#}P < 0.01$ vs ADX+pla. (M) Correlation between the score and the number of entries to the naloxone-paired chamber. The number of entries to the opiate antagonist-paired chamber was positively correlated with the decrease of the score in the sham+mor group.

**Figure 2.** Effects of adrenalectomy on metabolic parameters and on HPA-axis activity in morphine dependent rats. (A) Body weight gain of rats 4 d after ADX or sham surgery. $t_{50} = 12.57$, $P < 0.0001$.

(B) Body weight gain of rats from the day of pellets implantation to the first day of conditioning sessions. Adrenalectomy: $F_{(1,48)} = 6.802$, $P = 0.0121$; Morphine treatment: $F_{(1,48)} = 62.98$, $P < 0.0001$; Interaction adrenalectomy X morphine treatment: $F_{(1,48)} = 11.52$, $P = 0.0014$. Newman-Keuls' post hoc: ***P < 0.001 vs sham+pla; $^{##}P < 0.01$ vs ADX+pla. (C) Body weight gain of rats during the days of conditioning sessions. **Day 8:** Morphine treatment: $F_{(1,48)} = 32.60$, $P < 0.0001$; Interaction adrenalectomy X morphine treatment: $F_{(1,48)} = 10.91$, $P = 0.0018$. Newman-Keuls' post hoc: $^{++}P < 0.01$ vs sham+mor; $^{###}P < 0.001$ vs ADX+pla. **Day 9:** Morphine treatment: $F_{(1,48)} = 41.99$, $P < 0.0001$. Newman-Keuls' post hoc: **P < 0.01 vs sham+pla; $^{###}P < 0.001$ vs ADX+pla. **Day 10** Morphine treatment: $F_{(1,48)} = 56.47$, $P < 0.0001$. Newman-Keuls' post hoc: ***P < 0.001 vs sham+pla; $^{###}P < 0.001$ vs ADX+pla. **Day 11:** Morphine treatment: $F_{(1,48)} = 60.27$, $P < 0.0001$. Newman-Keuls' post hoc: ***P < 0.001 vs sham+pla; $^{###}P < 0.001$ vs ADX+pla. **Sham+pla:** Day: $F_{(4,55)} = 22.21$, $P < 0.0001$. Newman-Keuls' post hoc: $^{\&\&\&}P < 0.001$ vs day 7; $^{\$}P < 0.05$, $^{\$\$\$}P < 0.001$ vs day 8; $^{@}P < 0.05$, $^{@@@}P < 0.001$ vs day 9. **Sham+mor:** ns. **ADX+pla:** Day: $F_{(4,55)} = 12.70$, $P < 0.0001$. Newman-Keuls' post hoc: $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ vs day 7; $^{\$\$}P < 0.01$ vs day 8; $^{@}P < 0.05$ vs day 9. **ADX+mor:** ns. (D) Body weight loss during conditioning sessions: **Day 7:** Morphine treatment: $F_{(1,48)} = 4.572$, $P = 0.0376$; **Day 8:** Morphine treatment: $F_{(1,48)} = 77.30$, $P < 0.0001$. Newman-Keuls' post hoc: ***P < 0.001 vs sham+pla; $^{###}P < 0.001$ vs ADX+pla; **Day 9:** ns; **Day 10:** Adrenalectomy: $F_{(1,48)} = 5.406$, $P = 0.0243$; Morphine treatment: $F_{(1,48)} = 131.8$, $P < 0.0001$. Newman-Keuls' post hoc: ***P < 0.001 vs sham+pla; $^{+}P < 0.05$ vs sham+mor; $^{###}P < 0.001$ vs ADX+pla. **Sham+pla:** ns; **Sham+mor:** Naloxone injection: $F_{(3,60)} = 43.30$, $P < 0.0001$. Newman-Keuls' post hoc: $^{\&\&\&}P < 0.001$ vs day 7 (saline); $^{@@@}P < 0.001$ vs day 9 (saline). **ADX+pla:** ns; **ADX+mor:** Naloxone injection: $F_{(3,44)} = 35.75$; $P < 0.0001$. Newman-Keuls' post hoc: $^{\&\&\&}P < 0.001$ vs day 7 (saline); $^{@@@}P < 0.001$ vs day 9 (saline). (E) Graded signs of opiate withdrawal in sham- and ADX-operated animals treated with morphine during 15 min after the conditioning sessions with naloxone. $^{++}P < 0.01$ vs sham+mor (day 10); $^{\$}P < 0.05$ vs ADX+mor (day 8). (F) Appearance of signs of morphine withdrawal in sham- and ADX-operated animals treated with morphine during 15 min after the conditioning sessions with naloxone. $^{++}P < 0.01$ vs sham+mor (day 10). (G) Global opiate withdrawal score in sham- and ADX-operated animals

treated with morphine during the conditioning sessions with naloxone. Day of conditioning session: $F_{(1,26)} = 5.387$, $P = 0.0284$. Interaction adrenalectomy X day of conditioning session: $F_{(1,26)} = 7{,}487$, $P = 0.0111$. Newman-Keuls' post hoc: $^{++}P < 0.01$ vs sham+mor (day 10); $^{\$}P < 0.05$ vs ADX+mor (day 8). (H) Corticosterone plasma levels 15 min after leaving the naloxone-paired compartment on day 10. Adrenalectomy: $F_{(1,21)} = 224.3$, $P < 0.0001$. Morphine treatment: $F_{(1,21)} = 244.2$, $P < 0.0001$; Interaction adrenalectomy X morphine treatment: $F_{(1,21)} = 236.9$, $P < 0.0001$. Newman-Keuls' post hoc: $^{***}P < 0.001$ vs sham+pla; $^{+++}P < 0.001$ vs sham+mor. (I) ACTH plasma concentration 1 hour after starting the testing phase. Adrenalectomy: $F_{(1,32)} = 219.2$, $P < 0.0001$. Newman-Keuls' post hoc: $^{***}P < 0.001$ vs sham+pla; $^{+++}P < 0.001$ vs sham+mor. (J) Corticosterone plasma concentration 1 hour after starting the testing phase.

**Figure 3.** miR, mRNA and protein expression during memory consolidation process. (A) miR-124a: ns. (B) miR-212: Adrenalectomy: $F_{(1,14)} = 17.06$, $P = 0.0010$. Newman-Keuls' post hoc: $^{*}P < 0.05$ vs sham+pla; $^{+}P < 0.05$ vs sham+mor. (C) GR protein: ns. (D) pCREB protein: ns. (E) Egr-1 mRNA: ns. (F) BDNF mRNA: ns. (G) Arc mRNA: Morphine treatment: $F_{(1,15)} = 17.53$, $P = 0.0008$. Newman-Keuls' post hoc: $^{*}P < 0.05$ vs sham+pla; $^{\#}P < 0.05$ vs ADX+pla. (H) Egr-1 protein: Morphine treatment: $F_{(1,22)} = 5.257$, $P = 0.0318$; Interaction adrenalectomy X morphine treatment: $F_{(1,22)} = 5.155$, $P = 0.0333$. Newman-Keuls' post hoc: $^{++}P < 0.01$ vs sham+mor; $^{\#}P < 0.05$ vs ADX+pla. (I) BDNF protein: ns. (J) Arc protein: Morphine treatment: $F_{(1,19)} = 16.07$, $P = 0.0008$. Newman-Keuls' post hoc: $^{*}P < 0.05$ vs sham+pla; $^{\#}P < 0.05$ vs ADX+pla.

**Figure 4.** miR, mRNA and protein expression during memory retrieval process. (A) miR-124a: Adenalectomy: $F_{(1,23)} = 6.621$, $P = 0.0170$. Newman-Keuls' post hoc: $^{+}P < 0.05$ vs sham+mor. (B) miR-212: Adenalectomy: $F_{(1,25)} = 16.83$, $P = 0.0004$. Newman-Keuls' post hoc: $^{*}P < 0.05$ vs sham+pla; $^{+}P < 0.05$ vs sham+mor. (C) Egr-1 mRNA: Adenalectomy: $F_{(1,23)} = 5.123$, $P = 0.0334$. Newman-Keuls' post hoc: $^{+}P < 0.05$ vs sham+mor. (D) BDNF mRNA: Interaction adrenalectomy X

morphine treatment: $F_{(1,24)} = 6.521$, $P = 0.0174$. (E) Arc mRNA: Adenalectomy: $F_{(1,24)} = 6.878$, $P = 0.0149$; Interaction adrenalectomy X morphine treatment: $F_{(1,24)} = 4.740$, $P = 0.0395$. Newman-Keuls' post hoc: $^{**}P < 0.01$ vs sham+pla; $^{+}P < 0.05$ vs sham+mor. (F-I) Correlation analysis between miR-124a and miR-212 and protein levels in sham+mor rats: significant negative correlations were observed between both miRs and GR protein levels; significant positive correlation was observed between miR-124a and Arc protein levels. (J) Correlation analysis between Egr-1, BDNF and Arc mRNAs in sham+mor animals: negative correlation between BDNF and Egr-1 or Arc mRNAs is observed. (K) GR protein: Adenalectomy: $F_{(1,28)} = 7.502$, $P = 0.0106$. (L) pCREB protein: Interaction adrenalectomy X morphine treatment: $F_{(1,26)} = 4.373$, $P = 0.0464$. Newman-Keuls' post hoc: $^{*}P < 0.05$ vs sham+pla. (M) Egr-1 protein: Adenalectomy: $F_{(1,28)} = 11.94$, $P = 0.0018$. Newman-Keuls' post hoc: $^{+}P < 0.05$ vs sham+mor. (N) BDNF protein: Interaction adrenalectomy X morphine treatment: $F_{(1,29)} = 6.208$, $P = 0.0187$. Newman-Keuls' post hoc: $^{+}P < 0.05$ vs sham+mor. (O) Arc protein: Adenalectomy: $F_{(1,28)} = 6.807$, $P = 0.0144$; Interaction adrenalectomy X morphine treatment: $F_{(1,28)} = 4.460$, $P = 0.0438$. Newman-Keuls' post hoc: $^{*}P < 0.05$ vs sham+pla; $^{++}P < 0.01$ vs sham+mor. (P-U) Correlation analysis between aversion score and the different proteins analysed. Only significant correlation between Arc protein and aversion score in sham+mor rats was observed.

**Figure 5.** Characterization of dopaminoceptive, glutamatergic and GABAergic neurons in the DG from sham-operated animals treated with morphine after naloxone-induced CPA. (A) Representative confocal image showing DG coronal sections of rat immunostained for D1 in the polymorphic zone. (C-C''') D1 and D2 staining in the DG. While D1 expression was ubiquitous (molecular, granular, and polymorphic zone) (C), D2 signal was restricted to the granular zone (C'). (B) Schematic illustration showing the subdivisions of the DG (diagram modified from (20)). (D-E'') Representative confocal images showing DG rat coronal sections immunostained for GLS2 (green; D) and GAD (red; D'). Colocalization is shown by yellow neurons in the merged images (D''). High magnification images of GLS2 (E) and GAD (E') immunostaining, and merged (E'') in the DG. (F-F''') Representative

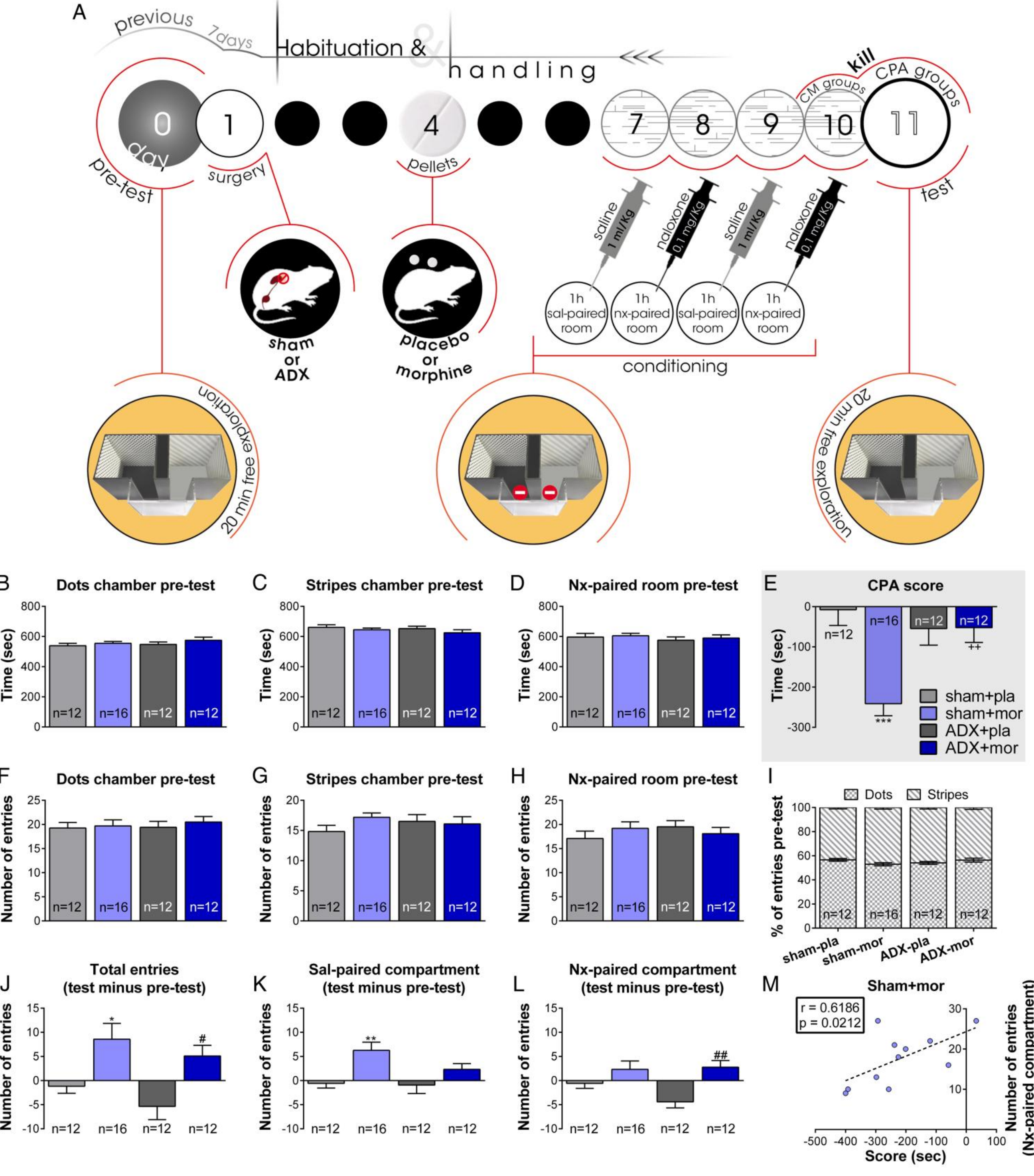
confocal images showing DG coronal sections of rat immunostained for D1 (green; F), GFAP (red; F'), merged (F'') and DAPI (nuclear stain, blue; F'''). (G-G''') Representative confocal images showing DG rat coronal sections immunostained for GLS2 (green; G), GFAP (red; G'), merged (G'') and DAPI (nuclear stain, blue; G'''). Scale bars, 100 μm.

**Figure 6.** Effects of naloxone-induced CPA on GR colocalization with pCREB, Egr-1, BDNF or pTrkB in the DG of sham- and ADX-operated morphine-dependent rats. Representative confocal images of GR (red) and pCREB (green) (A–B'), GR (red) and Egr-1 (green) (C–D'), GR (red) and BDNF (green) (E–F') and GR (red) and pTrkB (green) (G–H') immunostaining in the DG. Merged images are shown in A'',B'' (GR/pCREB), C'',D'' (GR/Egr-1), E'',F'' (GR/BDNF), and G'',H'' (GR/pTrkB). (I-J) Correlation between GR and pCREB protein levels in the DG after naloxone-induced CPA in sham- (I) and ADX-operated (J) morphine-dependent animals. GR protein levels were positively correlated with pCREB protein levels in the sham+mor group. (K-L) Correlation between GR and Egr-1 protein levels in the DG after naloxone-induced CPA in sham- (K) and ADX-operated (L) morphine-dependent rats. GR protein levels were positively correlated with Egr-1 protein levels in the ADX+mor group. (M-N) Correlation between GR and BDNF protein levels in the DG after naloxone-induced CPA in sham- (M) and ADX-operated (N) morphine-dependent animals. GR protein levels were negatively correlated with BDNF protein levels in the ADX+mor group. Scale bars, 100 μm.

**Figure 7.** Arc colocalization with D1, GLS2, GAD or GR in the DG of morphine dependent rats during the expression of naloxone-induced CPA. Representative confocal images of Arc (red) and D1 (green) (A,A'), Arc (red) and GLS2 (green) (B,B') and Arc (red) and GAD (green) (C,C'); DAPI immunostaining (nuclear stain, blue; A''–C''). Merged images are shown in A''' (Arc/D1), B''' (Arc/GLS2), C''' (Arc/GAD); merged images with DAPI are also depicted (A''''-C''''). Representative confocal images of Arc (red) and GR (green) (D-D') in sham+mor animals and Arc

(red; F) and GR (green; F') in ADX+mor rodents. Immunostaining for DAPI (D'',F''), merged (D''',F''') and merged with DAPI (D''''-F''''). (E-E'''') High magnification images from sham+mor group. (G-J) Correlation between Arc and GR protein levels in the DG after naloxone-induced CPA in sham-pla (G), sham-mor (H), ADX-pla (I), and ADX-mor (J) groups. Arc protein levels were negatively correlated with GR protein levels in the sham+mor group. Scale bars, 100 µm (A-D,F); 25 µm (E).

**Figure 8.** Effects of naloxone-induced CPA on Arc colocalization with pCREB, Egr-1, BDNF or pTrkB in the DG of sham- and ADX-operated morphine dependent rats. Representative confocal images of Arc (red) and pCREB (green) (A–B'), Arc (red) and Egr-1 (green) (C–D'), Arc (red) and BDNF (green) (E–F') and Arc (red) and pTrkB (green) (G–H') immunostaining in the DG. Merged images are shown in A'',B'' (Arc/pCREB), C'',D'' (Arc/Egr-1), E'',F'' (Arc/BDNF), and G'',H'' (Arc/pTrkB). (I-J) Correlation between Arc and pCREB protein levels in the DG after naloxone-induced CPA in sham- (I) and ADX-operated (J) morphine-dependent animals. Arc protein levels were positively correlated with pCREB protein levels in both sham- and ADX-operated morphine-treated group. (K-L) Correlation between Arc and Egr-1 protein levels in the DG after naloxone-induced CPA in sham- (K) and ADX-operated (L) morphine-dependent rats. (M-N) Correlation between Arc and BDNF protein levels in the DG after naloxone-induced CPA in sham- (M) and ADX-operated (N) morphine-dependent animals. . Scale bars, 100 µm.


A
previous 7days
Habituation & handling
kill
CM groups
CPA groups
0 day
1
4
7
8
9
10
11
pre-test
surgery
pellets
test
saline 1 ml/Kg
naloxone 0.1 mg/Kg
1h sal-paired room
1h nx-paired room
sham or ADX
placebo or morphine
conditioning
20 min free exploration
B Dots chamber pre-test
C Stripes chamber pre-test
D Nx-paired room pre-test
E CPA score
Time (sec)
n=12
n=16
sham+pla
sham+mor
ADX+pla
ADX+mor
F Dots chamber pre-test
G Stripes chamber pre-test
H Nx-paired room pre-test
I
Dots
Stripes
% of entries pre-test
Number of entries
sham-pla
sham-mor
ADX-pla
ADX-mor
J Total entries (test minus pre-test)
K Sal-paired compartment (test minus pre-test)
L Nx-paired compartment (test minus pre-test)
M Sham+mor
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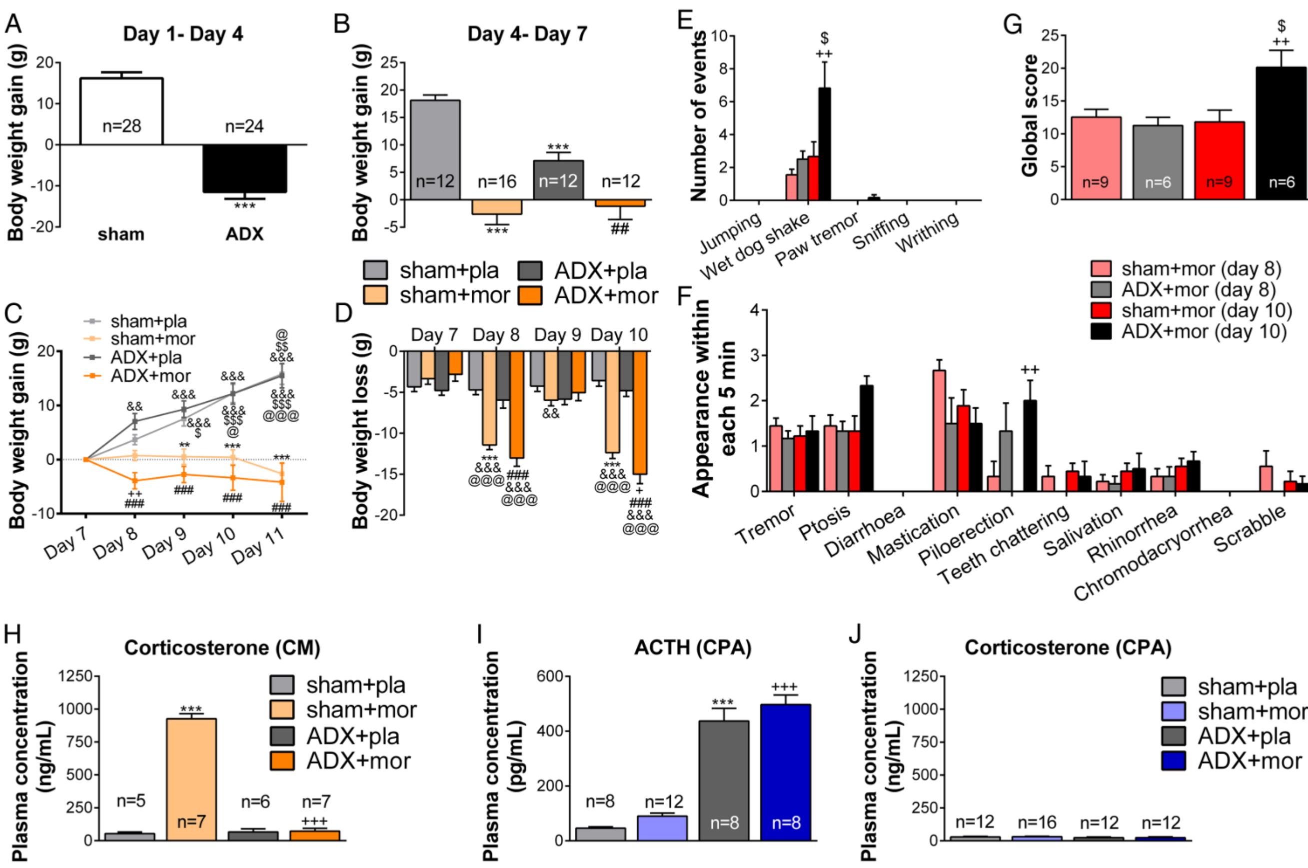
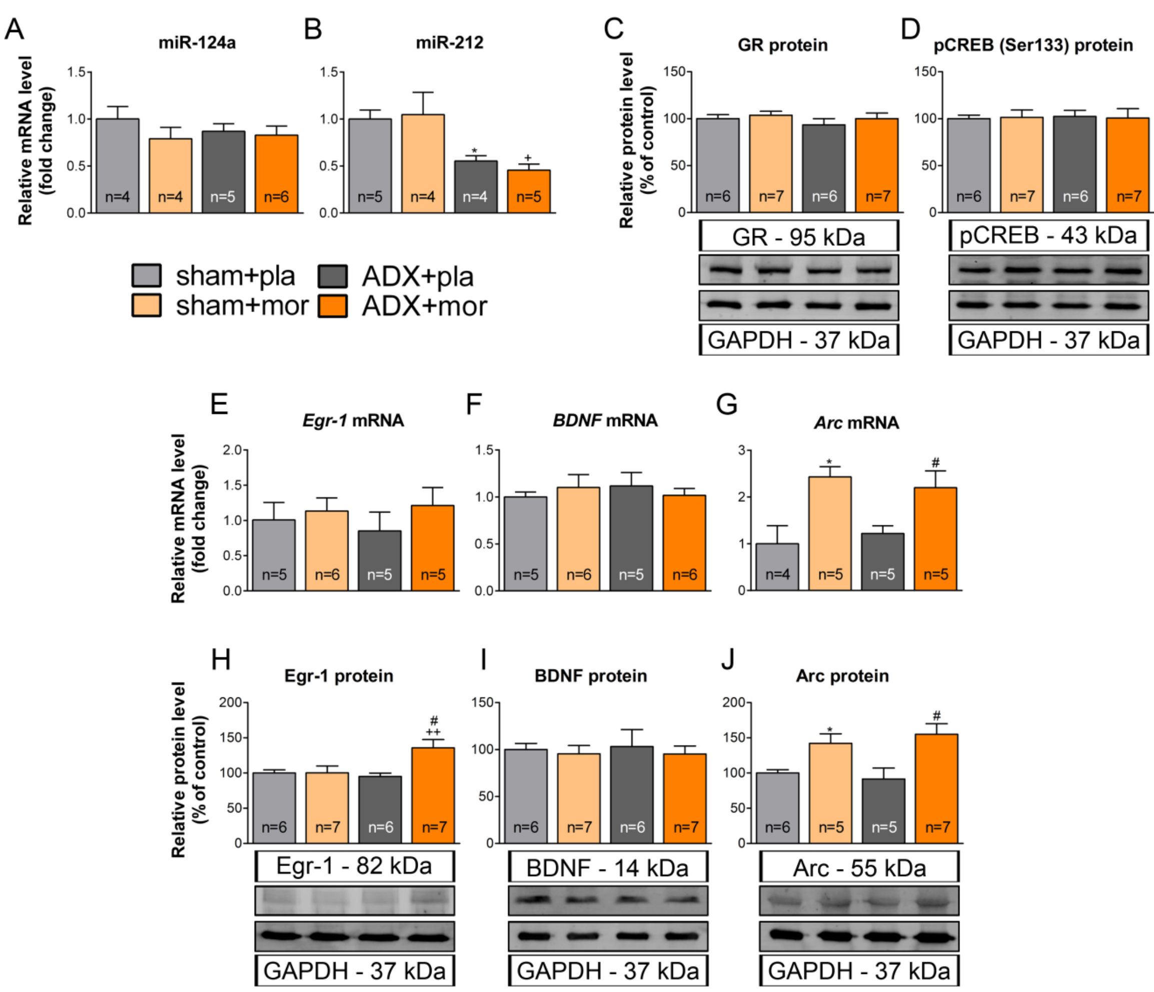
p = 0.0212
Score (sec)
Number of entries (Nx-paired compartment)



A
Day 1- Day 4
Body weight gain (g)
n=28
n=24
sham
ADX
B
Day 4- Day 7
n=12
n=16
n=12
n=12
sham+pla
ADX+pla
sham+mor
ADX+mor
C
Day 7
Day 8
Day 9
Day 10
Day 11
D
Body weight loss (g)
E
Number of events
Jumping
Wet dog shake
Paw tremor
Sniffing
Writhing
G
Global score
n=9
n=6
n=9
n=6
F
Appearance within each 5 min
sham+mor (day 8)
ADX+mor (day 8)
sham+mor (day 10)
ADX+mor (day 10)
Tremor
Ptosis
Diarrhoea
Mastication
Piloerection
Teeth chattering
Salivation
Rhinorrhea
Chromodacryorrhea
Scrabble
H
Corticosterone (CM)
Plasma concentration (ng/mL)
n=5
n=7
n=6
n=7
I
ACTH (CPA)
Plasma concentration (pg/mL)
n=8
n=12
n=8
n=8
J
Corticosterone (CPA)
n=12
n=16
n=12
n=12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miR-124a
Relative mRNA level (fold change)
B
miR-212
C
GR protein
Relative protein level (% of control)
D
pCREB (Ser133) protein
GR - 95 kDa
GAPDH - 37 kDa
pCREB - 43 kDa
GAPDH - 37 kDa
sham+pla
sham+mor
ADX+pla
ADX+mor
E
Egr-1 mRNA
F
BDNF mRNA
G
Arc mRNA
H
Egr-1 protein
I
BDNF protein
J
Arc protein
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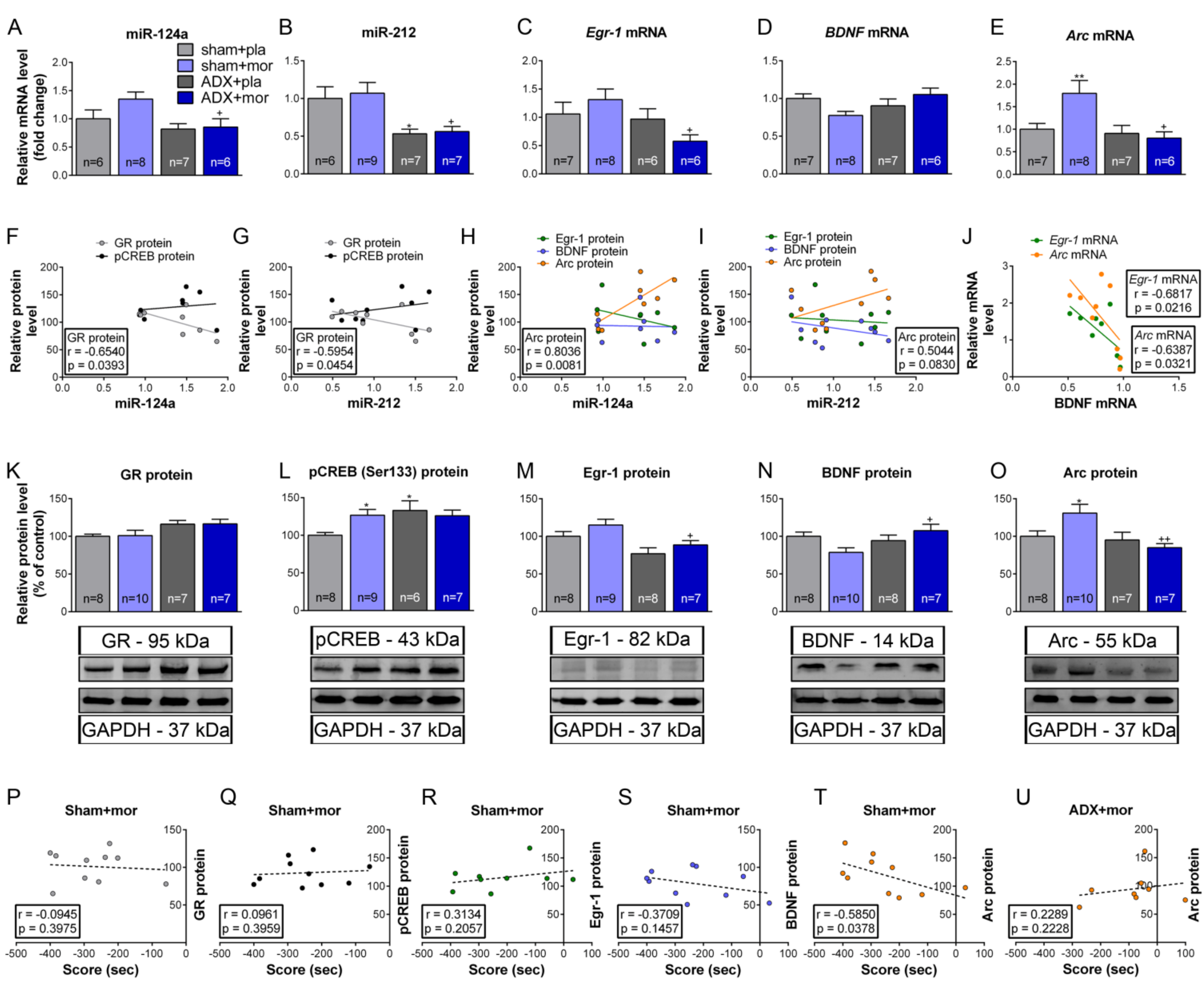
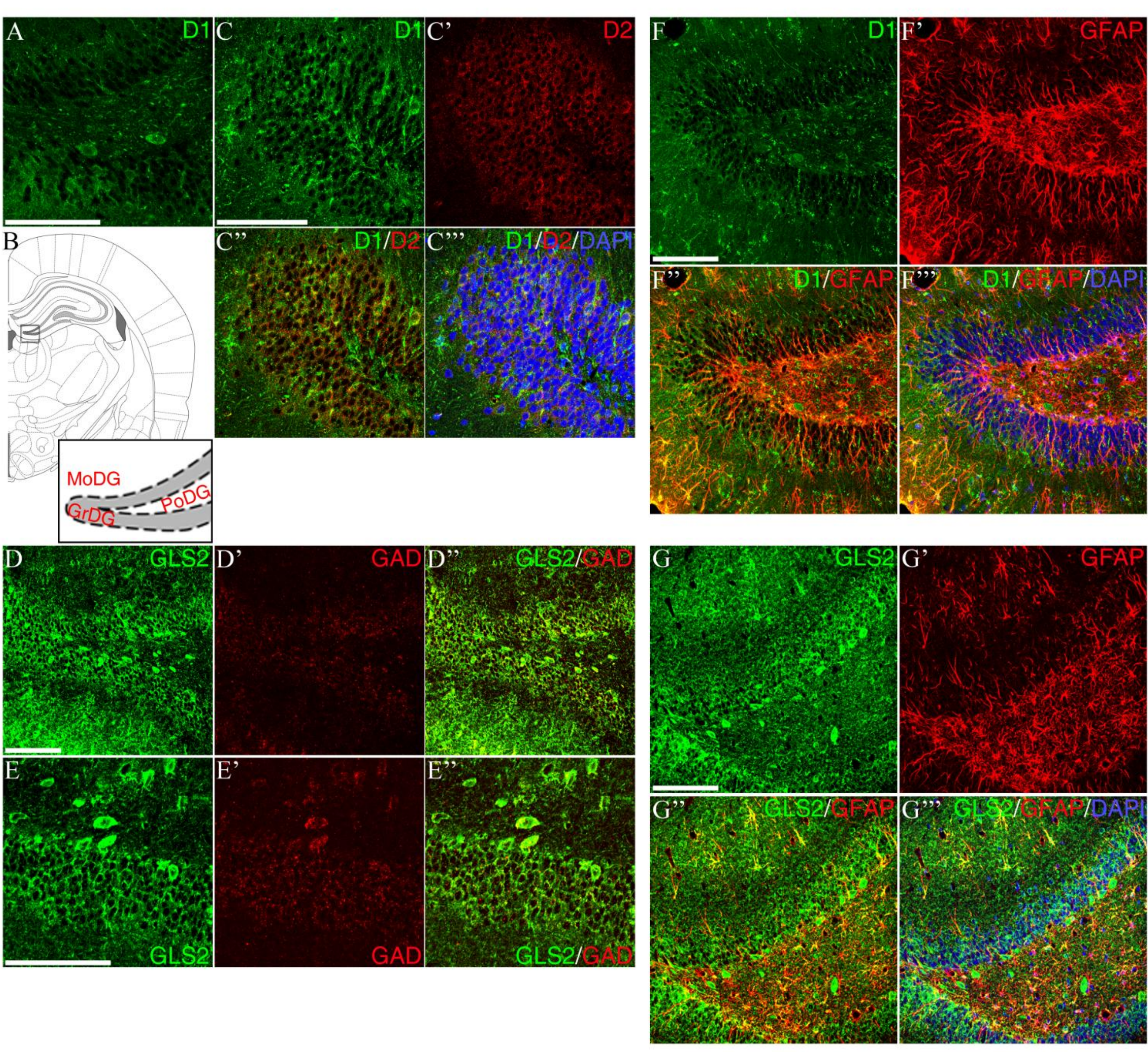
BDNF - 14 kDa
Arc - 55 kDa
GAPDH - 37 kDa

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C
D1
C'
D2
B
C''
D1/D2
C'''
D1/D2/DAPI
MoDG
GrDG
PoDG
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D1
F'
GFAP
F''
D1/GFAP
F'''
D1/GFAP/DAPI
D
GLS2
D'
GAD
D''
GLS2/GAD
E
GLS2
E'
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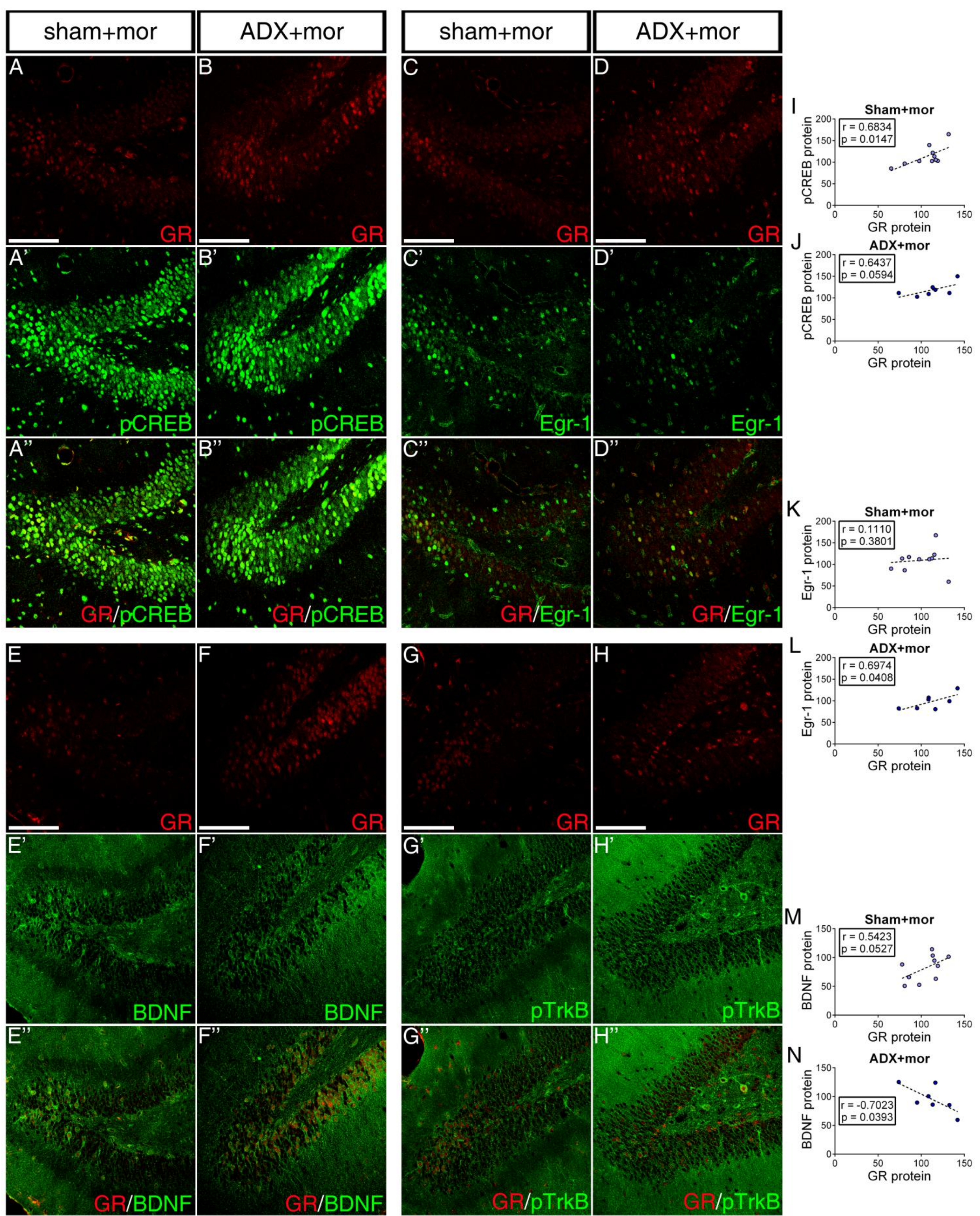
GLS2/GFAP/DAPI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ADX+mor
sham+mor
ADX+mor
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B
C
D
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B'
C'
D'
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A''
B''
C''
D''
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GR/Egr-1
E
F
G
H
E'
F'
G'
H'
BDNF
pTrkB
E''
F''
G''
H''
GR/BDNF
GR/pTrkB
I
Sham+mor
r = 0.6834
p = 0.0147
pCREB protein
GR protein
J
ADX+mor
r = 0.6437
p = 0.0594
K
Sham+mor
r = 0.1110
p = 0.3801
Egr-1 protein
L
ADX+mor
r = 0.6974
p = 0.0408
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BDNF protein
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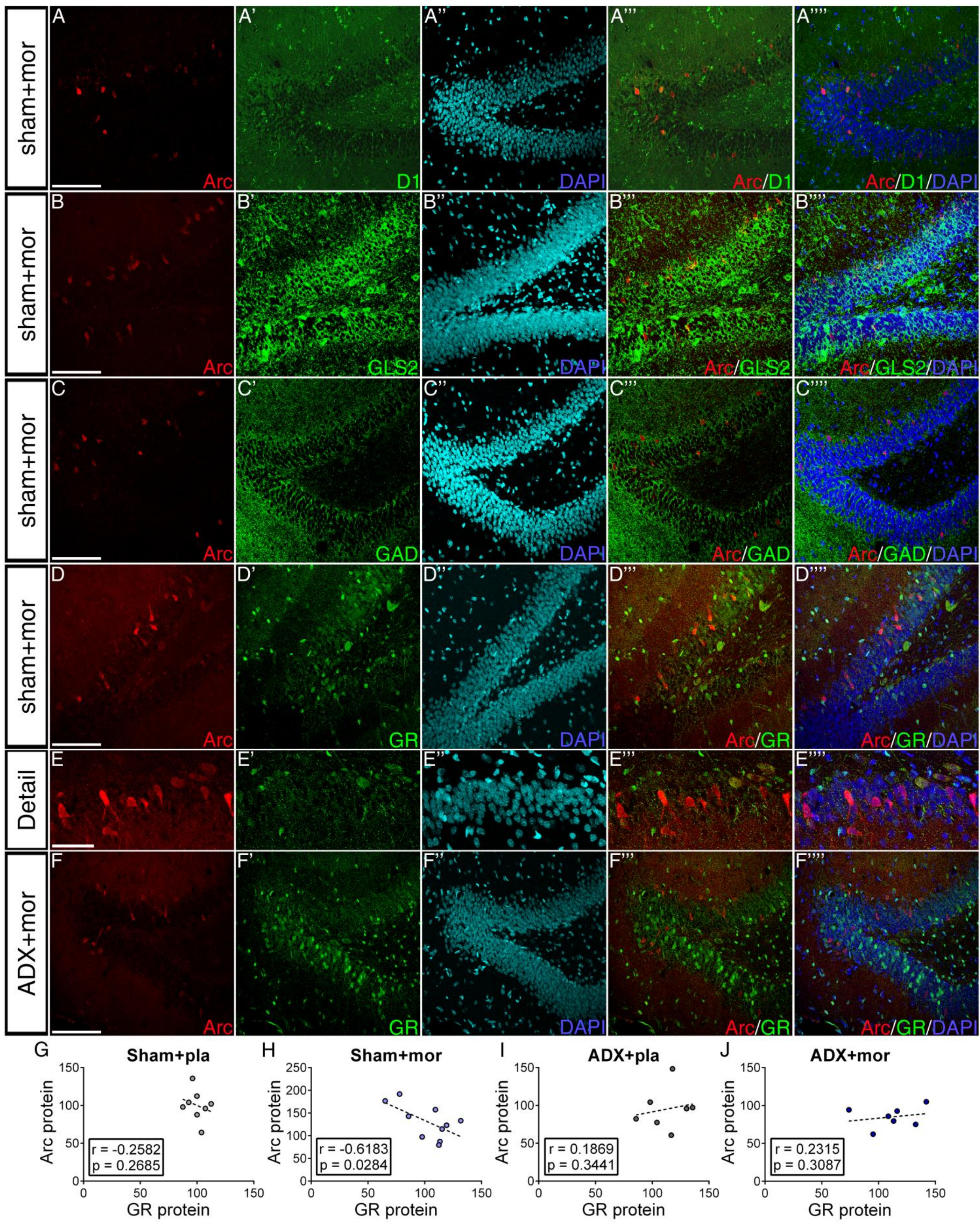
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sham+mor
sham+mor
sham+mor
Detail
ADX+mor
Arc
D1
DAPI
Arc/D1
Arc/D1/DAPI
GLS2
Arc/GLS2
Arc/GLS2/DAPI
GAD
Arc/GAD
Arc/GAD/DAPI
GR
Arc/GR
Arc/GR/DAPI
Sham+pla
r = -0.2582
p = 0.2685
Sham+mor
r = -0.6183
p = 0.0284
ADX+pla
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ADX+mor
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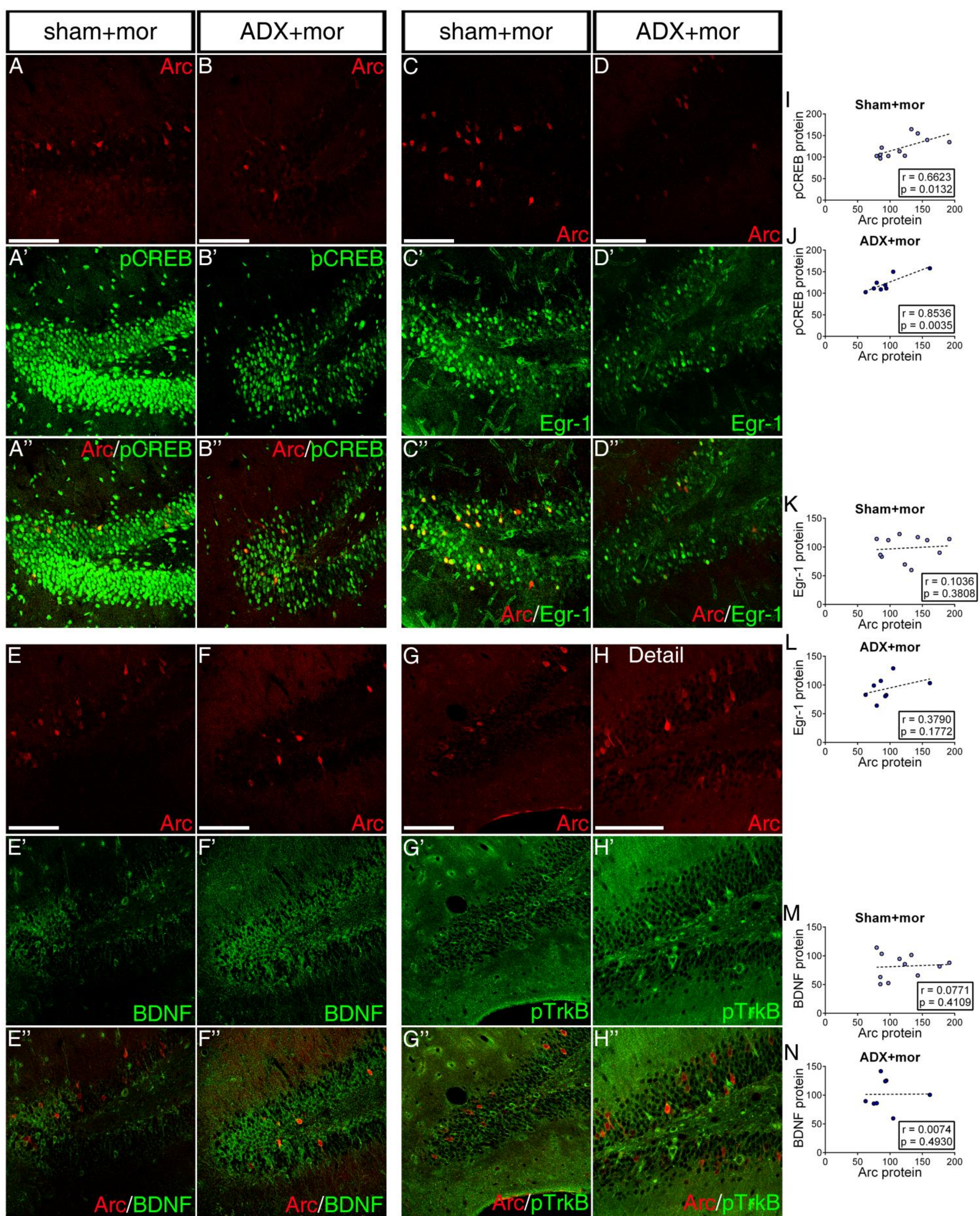
Arc protein
GR protein


sham+mor
ADX+mor
sham+mor
ADX+mor
A Arc
B Arc
C Arc
D Arc
A' pCREB
B' pCREB
C' Egr-1
D' Egr-1
A'' Arc/pCREB
B'' Arc/pCREB
C'' Arc/Egr-1
D'' Arc/Egr-1
E Arc
F Arc
G Arc
H Detail Arc
E' BDNF
F' BDNF
G' pTrkB
H' pTrkB
E'' Arc/BDNF
F'' Arc/BDNF
G'' Arc/pTrkB
H'' Arc/pTrkB
I Sham+mor
pCREB protein
Arc protein
r = 0.6623
p = 0.0132
J ADX+mor
pCREB protein
Arc protein
r = 0.8536
p = 0.0035
K Sham+mor
Egr-1 protein
Arc protein
r = 0.1036
p = 0.3808
L ADX+mor
Egr-1 protein
Arc protein
r = 0.3790
p = 0.1772
M Sham+mor
BDNF protein
Arc protein
r = 0.0771
p = 0.4109
N ADX+mor
BDNF protein
Arc protein
r = 0.0074
p = 0.4930

# SUPPLEMENTAL INFORMATION

## SUPPLEMENTAL METHODS AND MATERIALS

### Subjects

All surgical and experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE), and were approved by the local Committees for animal research (REGA ES300305440012). Male Wistar rats (n = 78, Harlan, Barcelona, Spain) initially weighting 220–240 g were housed (2-3/cage) on arrival in a room with controlled temperature (22 ± 2°C) and humidity (50 ± 10%), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light-dark cycle (lights on: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments.

### Adrenalectomy

Rats were bilaterally adrenalectomized (ADX) via a dorsal approach under 100 mg/kg ketamine chlorhydrate (Imalgene 1000, Merial, Lyon, France) and 8 mg/kg xylazin (Xilagesic® 2%, Lab. Calier S.A., Barcelona, Spain) intraperitoneally anaesthesia, and implanted subcutaneously (s.c.) with slow-release corticosterone pellets at surgery. The composition of steroid pellets (25 mg corticosterone plus 75 mg cholesterol) was chosen to provide stable corticosterone concentration corresponding to circadian nadir up to 20 days after implantation (1). ADX rats with corticosterone replacement (ADX plus corticosterone) do not mount a challenge-induced increase of plasma corticosterone. After surgery, ADX plus corticosterone rats had free choice to drink isotonic saline (0.9% NaCl) to replace depleted sodium secondary to the loss of aldosterone because of adrenalectomy. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation. Sham and ADX plus corticosterone rats were allowed to recover from surgery for 3 days before morphine or placebo pellet

implantation. Successful bilateral adrenalectomy was confirmed by plasma concentration of corticosterone and ACTH and by post-mortem examination of the ADX animals.

**Induction of Opiate Dependence**

Morphine base was supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Morphine dependence was induced by subcutaneous implantation (lower back) under light ether anaesthesia of two slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base). Full dependence on morphine has been previously operationally defined in this model by using a complete naloxone dose effect on various behavioral parameters (spontaneous locomotor activity, operant responding for food, intracranial self-stimulation threshold, conditioned place aversion) (2), and the rating of abstinence signs showed that opiate dependence was achieved 24 h after implantation of the morphine pellets and remained constant for 15 d (3). Placebo-pelleted rats received lactose pellets also implanted subcutaneously under anaesthesia. There were four experimental groups: sham + placebo pellets, sham + morphine pellets, ADX + placebo pellets and ADX + morphine pellets.

**Drugs**

Naloxone hydrochloride (N-7758, Sigma Chemical Co, St Louis, MO, USA) was dissolved in sterile saline (0.9% NaCl; ERN Laboratories, Barcelona, Spain) and injected subcutaneously. Naloxone was administered at a dose of 0.1 mg/kg, 1 ml/kg body weight. Naloxone HCl doses are expressed as the weight of the salt. Sterile 0.9% saline was also injected subcutaneously at a dose of 1 ml/kg. Note that a slightly higher naloxone dose (0.120 mg/kg) has previously been shown to be without effect on place aversion conditioning in placebo control rats (4).

**Conditioned Place Aversion Paradigm**

Briefly, the conditioned place aversion apparatus (Panlab, Barcelona, Spain) used to induce a reliable aversion consists in a box with two equally sized chambers (40 *L* x 34 *W* x 45 *H* cm) interconnected by a rectangular corridor (25 *L* x 13 *W* x 45 *H* cm). Distinctive visual and tactile cues distinguish the compartments: the motifs painted on the walls (either black dots or grey stripes), the floor coloring (black or grey) and the floor texture (smooth or rough). The sensory cues combination that produces a balanced choice are for walls and floor coloring and texture, respectively: (A) black dots, black smooth floor; (B) grey stripes, grey rough floor. Transparent walls are also used to minimize the time the animal spent in the corridor. The weight transducer technology (and PPCWIN software) allows for detection and analysis of animal position throughout the test and the number of entries in each compartment. The experimental protocol consists of three distinct phases: a preconditioning phase, a conditioning phase, and a testing phase. The weight gain of the rats was checked during the entire protocol to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (5,6). In addition, the animals were observed for body weight loss and opioid withdrawal behaviours for 15 min after the conditioning phase.

**Pre-testing Phase**

In the pre-testing phase (day 0), animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. Animals showing strong unconditioned aversion (less than 40% of the session time) or preference (more than 60% of the session time) for any compartment were discarded. For each rat, one room was randomly chosen to be paired with naloxone and the other chamber to vehicle. Importantly, after the compartment assignments were completed, there were no significant differences between time spent in the naloxone-paired and the vehicle-paired compartments during the

preconditioning phase. This is an important step in the experimental procedure that avoids any preference bias prior to conditioning. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

**Conditioning Phase**

In the second phase (conditioning), rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. In addition, opioid withdrawal behaviours on sham or ADX animals receiving naloxone on days 8 and 10 were measured for 15 min after the conditioning phase. A cohort of animals, conditioning memory rats (CM), was decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

**Testing Phase**

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). The difference ($\Delta D = D - D0$) between the time spent in the naloxone-paired compartment after conditioning ($D$) minus the time spent in the same compartment before conditioning (preconditioning test $D0$) reflects the change of preference induced by opiate withdrawal. A negative score indicates a place aversion; a positive score indicates a place preference. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

**Measurement of the withdrawal syndrome**

Experiments were carried out in a quiet room. The observer was unaware of the drug combination used. Sham + mor and ADX + mor rats were individually placed into transparent plastic cages after

the conditioning phase and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 15 min on days 8 and 10 (naloxone injection). Subsequently, previously identified behavioural characteristics of the rat opiate abstinence syndrome (7) were evaluated, including jumping, wet-dog shakes, paw tremor, sniffing, writhing, tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble. The number of jumping, wet-dog shakes, paw tremor, sniffing and writhing was counted as the number of events occurring during the total test time period (graded signs). Tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble were scored 1 for appearance or 0 for non-appearance within each 5 min time. To obtain a comprehensive index of the severity of somatic opiate withdrawal including all the signs examined, a global withdrawal score was calculated for each animal by giving each individual sign a relative weight as previously reported (8): jumping, x0.8; wet-dog shakes, x1; paw tremor, x0.35; sniffing, x0.5; writhing, x0.5; tremor, x1.5; ptosis, x1.5; diarrhoea, x1.5; mastication, x1.5; piloerection, x1.5; teeth chattering, x1.5; salivation, x1.5; rhinorrhea, x1.5; chromodacryorrhea, x1.5; and scrabble, x1.5. Body weight loss was determined as the difference between the weight determined immediately before naloxone injection and that determined 1 hour later, after the conditioning phase.

**Preparation of tissue extract**

Rats were decapitated on days 10 and 11 (between 10:00-12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed, and stored immediately at -80°C until use for high performance liquid chromatography (HPLC), quantitative real-time PCR (qPCR) and Western blot analysis. Brains were sliced on a cryostat and kept at -20°C until each region of interest comes into the cutting plane (Fig. 3A). For DG study, three consecutive 500-µm coronal slides were made corresponding to approximately -2.9 to -4.4 mm from bregma, according to the atlas of (9). Tissues of interest were dissected using a punching device with a 1-mm internal diameter. The anatomical locations and boundaries of each region were determined using the rat brain Atlas of (9).

Bilateral punches of the DG were collected into Eppendorf tubes, according to the method of (10). A second set of animals from each treatment group was used for immunofluorescence staining.

**RNA extraction and quantitative real-time PCR (qPCR)**

One punch from the DG was placed in an Eppendorf tube containing 30 µl of Trizol® reagent (Invitrogen Corp., Carlsbad, CA, USA) and rapidly stored at -80°C. Frozen brain tissue samples were homogenized in Trizol® reagent (Invitrogen Corp., USA) and total RNA was isolated with QIAGEN miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination DNase I treatment were used and 100 µl Rnase-free DNase I (1 unit DNase) (Thermo Scientific, USA) solution was added. Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific, USA). Amplification was not detected in the RT-minus controls. The cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for the comparative Ct experiments were designed by Primer Express 3.0 Program. The primers (Microsynth, Balgach) were used in the Real-Time PCR reaction with Fast EvaGreen® qPCR Master Mix (Biotium, USA) on ABI StepOnePlus instrument was listed. The reverse transcription for microRNA assays were carried out by TaqMan MicroRNA Reverse Transcription Kit according the manufacturer's instruction. The microRNA expressions were detected using TaqMan MicroRNA assays.

List of the genes: gapdh, arc, egr-1, bdnf

List of the miRNA assays: U6 snRNA (cat.: 001973, 4427975), miR-212 (cat.: 002551, 4427975), miR-124a (cat.: 001182, 4427975)

The gene expression was analyzed by ABI Step One 2.1 program. The amplicon was tested by Melt Curve Analysis on ABI Step OnePlus Instrument. Experiments were normalized to gapdh (glyceraldehyde-3-phosphate dehydrogenase) and U6 snRNA expressions.

gapdh for ACAGCCGCATCTTCTTGTGC

gapdh rev GCCTCACCCCATTTGATGTT

arc for CCCCCAGCAGTGATTCATAC

arc rev CAGACATGGCCGGAAAGACT

egr-1 for CACCTGACCACAGAGTCCTTTT

egr-1 rev ACCAGCGCCTTCTCGTTATT

bdnf for AAACGTCCACGGACAAGGCA

bdnf rev TTCTGGTCCTCATCCAGCAGC

U6 snRNA cat:001973, 4427975

mmu-miR-212 cat: 002551, 4427975

mmu-miR-124a cat: 001182, 4427975

**Western Blotting**

Four bilateral punches from VTA were placed in homogenization buffer. The Eppendorf tubes contained 100 µl of homogenization buffer. The tubes were frozen immediately on dry ice and stored

at −80°C until assaying. Samples were sonicated, vortexed and sonicated again prior to centrifugation (6000× g; 10 minutes at 4°C). Samples containing equal quantities of total proteins (20–40 mg, depending on the protein of interest) were separated by 10% or 12% SDS-PAGE (depending on the molecular weight of the protein of interest) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS containing 0.15 % Tween-20 (TBS-T), 1% BSA for 90 minutes at room temperature (RT), and incubated overnight at 4ºC with the primary antibody diluted in 1% BSA in TBS-T. The primary antibodies used are referenced in Table S1. Blots were then washed and incubated for 90 min at RT in TBS-T with 1% BSA with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies: goat anti-rabbit (1:5000; sc-2004, Santa Cruz Biotechnology) or goat anti-mouse (1:5000; sc-2005, Santa Cruz Biotechnology). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). We used GAPDH as our loading control. Before reprobing, blots were stripped by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37°C. Blots were subsequently reblocked and probed with GAPDH. The ratios of protein of interest/GAPDH were plotted and analyzed. Protein levels were corrected for individual levels.

**Table S1. Primary antibodies used in this study**

| **Antibody** | **Host** | **Supplier** | **Catalog Number** | **WB dilution** | **IF dilution** |
|---|---|---|---|---|---|
| GR | Rabbit | Santa Cruz Biotechnology | sc-1004 | 1:500 | 1:600 |
| pCREB | Rabbit | Millipore | # 06-519 | 1:2,000 | 1:3,000 |
| Egr-1 | Rabbit | Santa Cruz Biotechnology | sc-189 | 1:500 | 1:400 |
| BDNF | Rabbit | Santa Cruz Biotechnology | sc-546 | 1:500 | 1:400 |
| Arc | Mouse | Santa Cruz Biotechnology | sc-17839 | 1:500 | 1:400 |

| GAPDH | Rabbit | Cell Signaling Technology Inc. | #2118 | 1:5,000 | -- |
|---|---|---|---|---|---|
| GR | Mouse | Abcam | ab2768 | -- | 1:600 |
| pTrkB | Rabbit | Abcam | ab81288 | -- | 1:600 |
| DRD1 | Goat | Santa Cruz Biotechnology | sc-1434 | -- | 1:200 |
| DRD2 | Mouse | Santa Cruz Biotechnology | sc-5303 | -- | 1:200 |
| GLS2 | Rabbit | Abcam | ab113509 | -- | 1:1,000 |
| GAD-65/67 | Goat | Santa Cruz Biotechnology | sc-7513 | -- | 1:200 |
| GFAP | Mouse | Santa Cruz Biotechnology | sc-33673 | -- | 1:400 |

**Immunofluorescence study**

Another set of rats was deeply anaesthetized with an overdose of pentobarbital (100 mg/kg i.p.) and perfused transcardially with 250 ml 0.9% saline following by 500 ml cold fixative solution containing paraformaldehyde (4% paraformaldehyde in 0.1 M borate buffer, pH 9.5). After removal of the perfused brains, they were post fixed in the same fixative solution containing sucrose (30%) for 3 h and stored at 4ºC in PBS containing 30% sucrose until coronal sections (30-mm thickness) were cut rostrocaudally on a freezing microtome (Leica, Nussloch, Germany). The atlas of Paxinos and Watson (2007) (19) was used to identify different brain regions. The sections were cryoprotected and stored at -20ºC until use.

Brain sections were rinsed in PBS and an antigen retrieval procedure was applied by treating sections with citrate buffer (10 mM citric acid in 0.05% Tween 20, pH 6.0) at 60°C for 20 min. Non-specific Fc binding sites were blocked with 3% normal horse serum/0.3% Triton-X-100 in PBS for 1 h at RT, and the sections were incubated for 72 h (4ºC, constant shaking) with primary antibodies described in Table S1. Alexa Fluor 488 donkey anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 488 donkey anti-goat IgG (1:1000; A-11055, Invitrogen), Alexa Fluor 594 donkey anti-goat IgG (1:1000; A-11058, Invitrogen) and Alexa Fluor 594 donkey anti-mouse IgG (1:1000; A-

21203, Invitrogen) labelled secondary antibodies were applied for 4 h. After washing, sections were incubated in 4, 6-diamino-2-phenylindole (DAPI, 1:100,000) for 1 min and the sections were mounted in ProLong® Gold antifade reagent (Invitrogen). Specificity for D1 and d2 antibodies was tested in rat cortex, where both receptors are known to display only partial overlapping signal (Fig. S1A). Moreover, given that we required two different antibodies raised against GR, we tested if both of them displayed the same immunofluorescence signal (Fig. S1B-C).

**Confocal analysis**

The brain sections were examined using a Leica DMIRE2 confocal microscope and Leica Confocal Software (Leica Microsystems). Images from the DG were captured from low magnification to high magnification (40X to 63X oil objective). Confocal images were obtained using 405-nm excitation for DAPI, 488-nm excitation for Alexa Fluor 488 and 594-nm excitation for Alexa Fluor 594. Emitted light was detected in the range of 450-460 nm for DAPI, 515-530 nm for Alexa Fluor 488 and 610-630 nm for Alexa Fluor 594. Every channel was captured separately to avoid spectral crosstalking. Series of optical sections were performed determining an upper and lower threshold using the Z/Y position for Spatial Image Series setting. The optical series covered 20 µm of thickness through the tissue. The confocal microscope settings were established and maintained by Leica and local technicians for optimal resolution. Final confocal images can be illustrated as they appear throughout the stack of sections as a simple layer or as a transparency of all layers merged together.

**Radioimmunoassay**

Blood was collected on days 10 and 11 into ice-cooled tubes containing 5% EDTA and was then centrifuged (500 g; 4ºC; 15 min). Plasma was separated, divided into two aliquots and stored at -80ºC until assayed for corticosterone or ACTH. Plasma concentration of corticosterone and ACTH were quantified using specific corticosterone and ACTH antibodies for rats ([$^{125}$I]-CORT and [$^{125}$I]-ACTH

RIA; MP Biomedicals, Orangeburg, NY, USA). The sensitivity of the assay was 7.7 ng/mL for corticosterone and 5.7 pg/mL for ACTH.

**Reagents**

Protease inhibitors were purchased from Boehringer Mannheim, (Mannheim, Germany); phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany); HPLC reagents were purchased from Sigma Chemical Co.

**Data Analysis**

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test to determine specific group differences. Student's *t*-test was used when comparisons were restricted to two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

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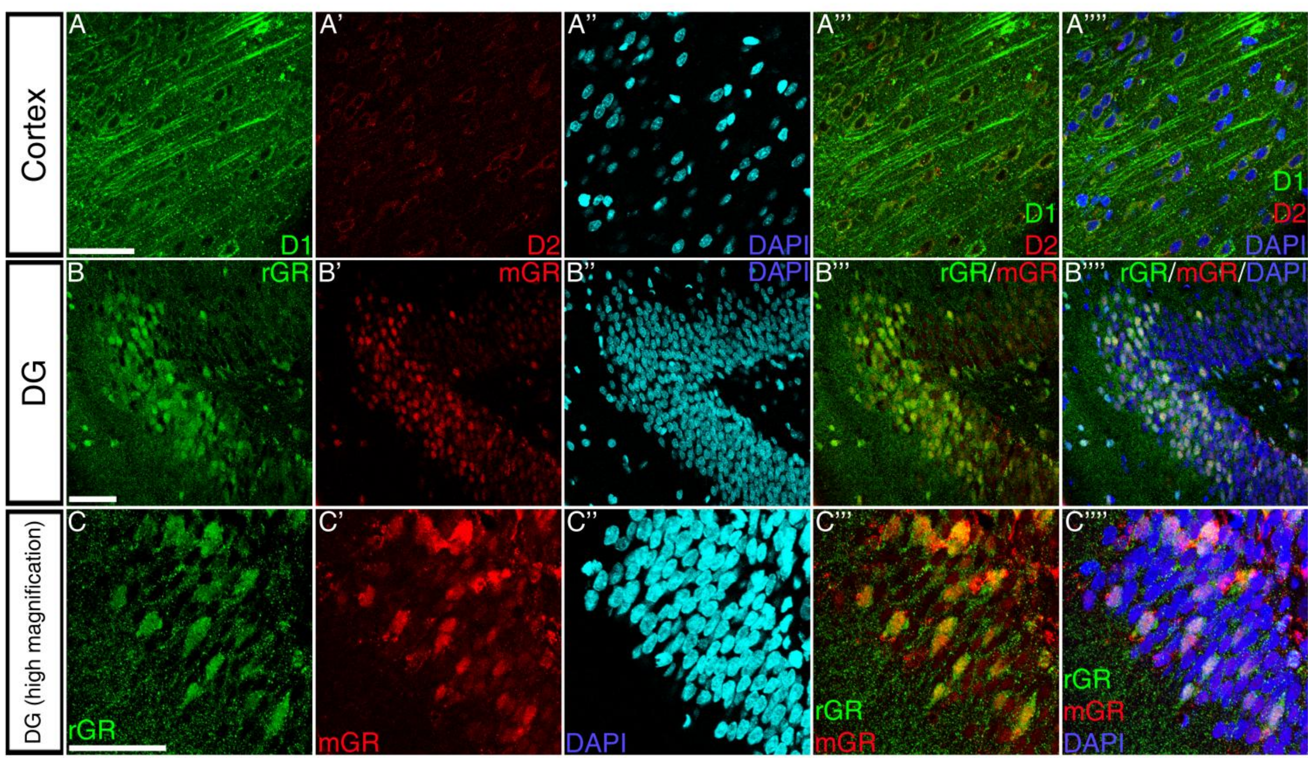
## LEGENDS TO SUPPLEMENTAL FIGURES

**Supplemental Figure 1.** (A-A'''') Representative confocal images showing cortex coronal sections of rat immunostained for D1 (green; A) and D2 (red; A'), DAPI (nuclear stain, blue; A''), merged (A''') and merged with DAPI (A''''). (B-B'''',C-C'''') Low and high magnification confocal images showing GR overlapping signal between an antibody originated in rabbit (green; B,C) and an antibody originated in mouse (red; B',C'). DAPI (nuclear stain, blue; B''C''), merged (B'''C''') and merged with DAPI (B'''',C''''). Scale bars, 50 µm.

**Suplemental Figure 2.** Correlation of GR protein levels with pCREB (A,B), Egr-1 (C,D) or BDNF (E,F) protein levels in the DG of sham- and ADX-operated control animals. (A) Correlation between GR and pCREB protein levels in the sham+pla group. (B) Correlation between GR and pCREB protein levels in the ADX+pla group. (C) Correlation between GR and Egr-1 protein levels in the sham+pla group. GR protein levels were negatively correlated with Egr-1 protein levels. (D) Correlation between GR and Egr-1 protein levels in the ADX+pla group. GR protein levels were negatively correlated with Egr-1 protein levels. (E) Correlation between GR and BDNF protein levels in the sham+pla group. (F) Correlation between GR and BDNF protein levels in the ADX+pla group.

**Supplemental Figure 3.** (A-A'''') Representative confocal images showing DG coronal sections of rat immunostained for Arc (red; A) and GR (green; A'), DAPI (nuclear stain, blue; A''), merged (A''') and merged with DAPI (A''''). (B-B''') Representative confocal images showing DG coronal sections of rat immunostained for Arc (red; B) and pCREB (green; B'), merged (B'') and merged with DAPI (B'''). (C-C') High magnification images of Arc and Arc/pCREB immunostaining. (D-D'''') Representative confocal images showing DG coronal sections of rat immunostained for Arc (red; D) and Egr-1 (green; D'), DAPI (nuclear stain, blue; D''), merged (D''') and merged with DAPI (D''''). Scale bars, 50 µm.

**Suplemental Figure 4.** Correlation of Arc protein levels with pCREB (A,B), Egr-1 (C,D) or BDNF (E,F) protein levels in the DG of sham- and ADX-operated control animals. (A) Correlation between Arc and pCREB protein levels in the sham+pla group. Arc protein levels were positively correlated with pCREB protein levels. (B) Correlation between Arc and pCREB protein levels in the ADX+pla group. Arc protein levels were positively correlated with pCREB protein levels. (C) Correlation between Arc and Egr-1 protein levels in the sham+pla group. Arc protein levels were positively correlated with Egr-1 protein levels. (D) Correlation between Arc and Egr-1 protein levels in the ADX+pla group. (E) Correlation between Arc and BDNF protein levels in the sham+pla group. Arc protein levels were negatively correlated with pCREB protein levels. (F) Correlation between Arc and BDNF protein levels in the ADX+pla group. Arc protein levels were positively correlated with BDNF protein levels.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DG (high magnification)
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B
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B'
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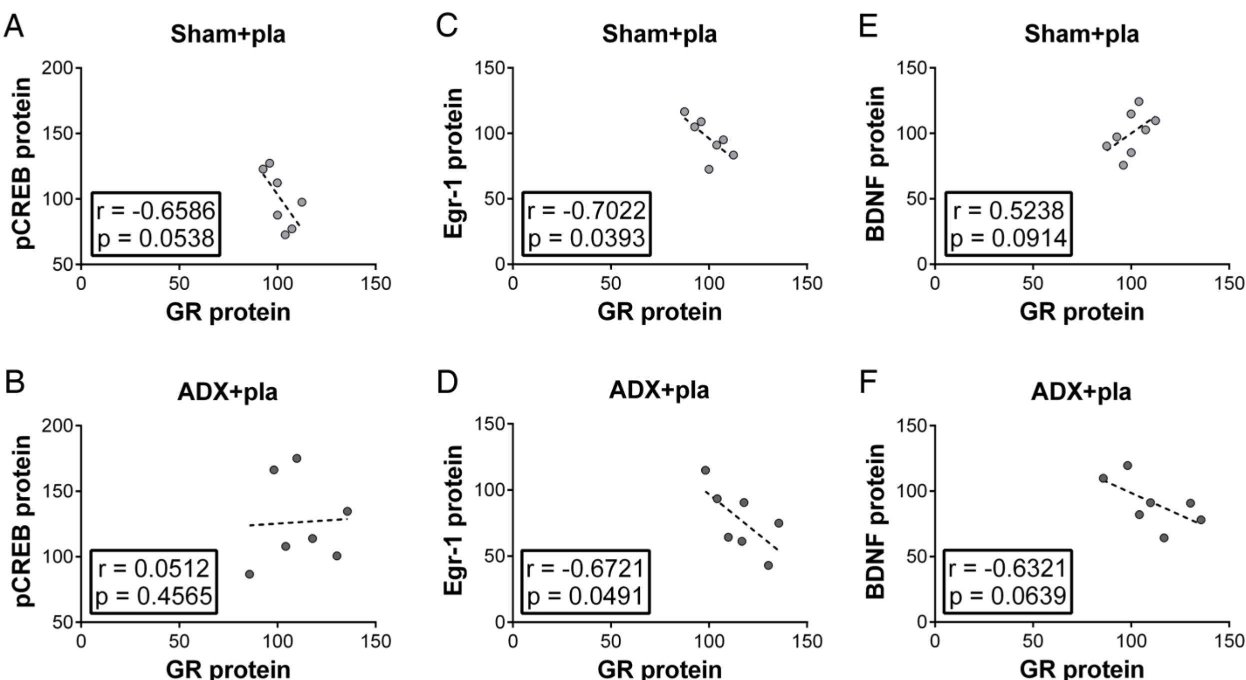
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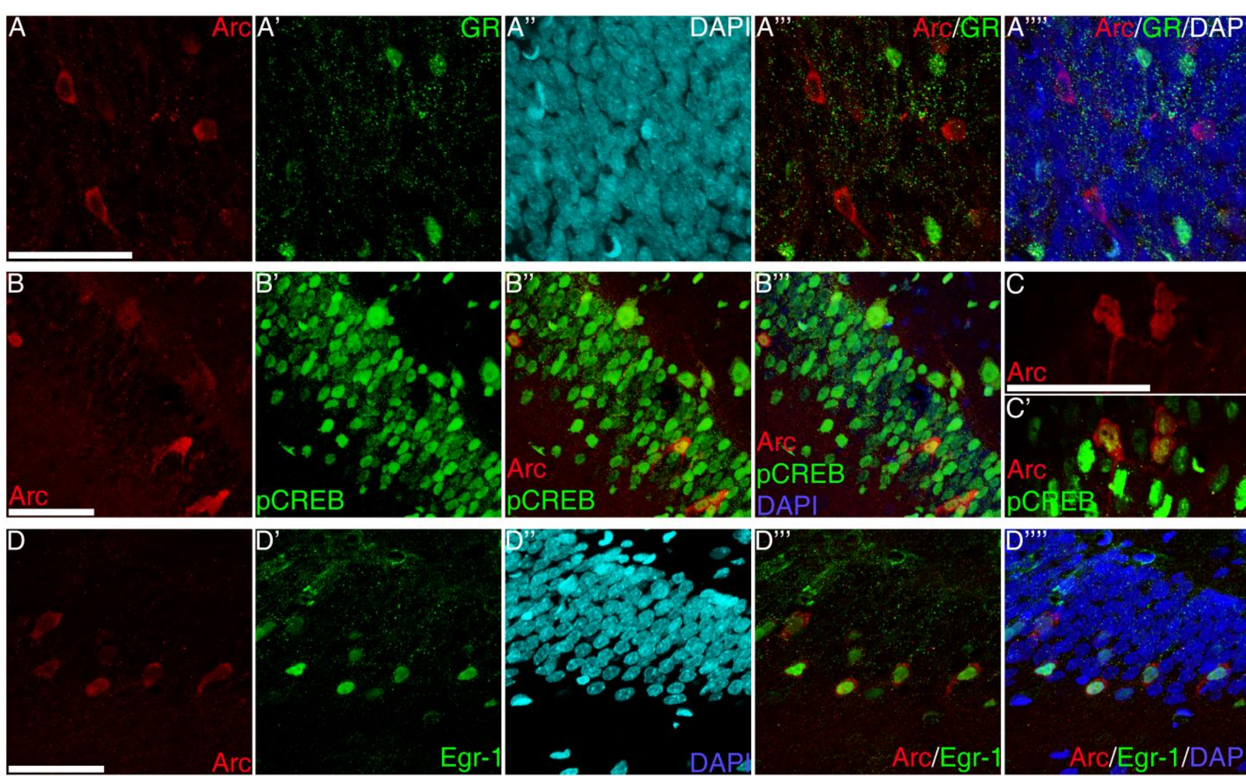
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C
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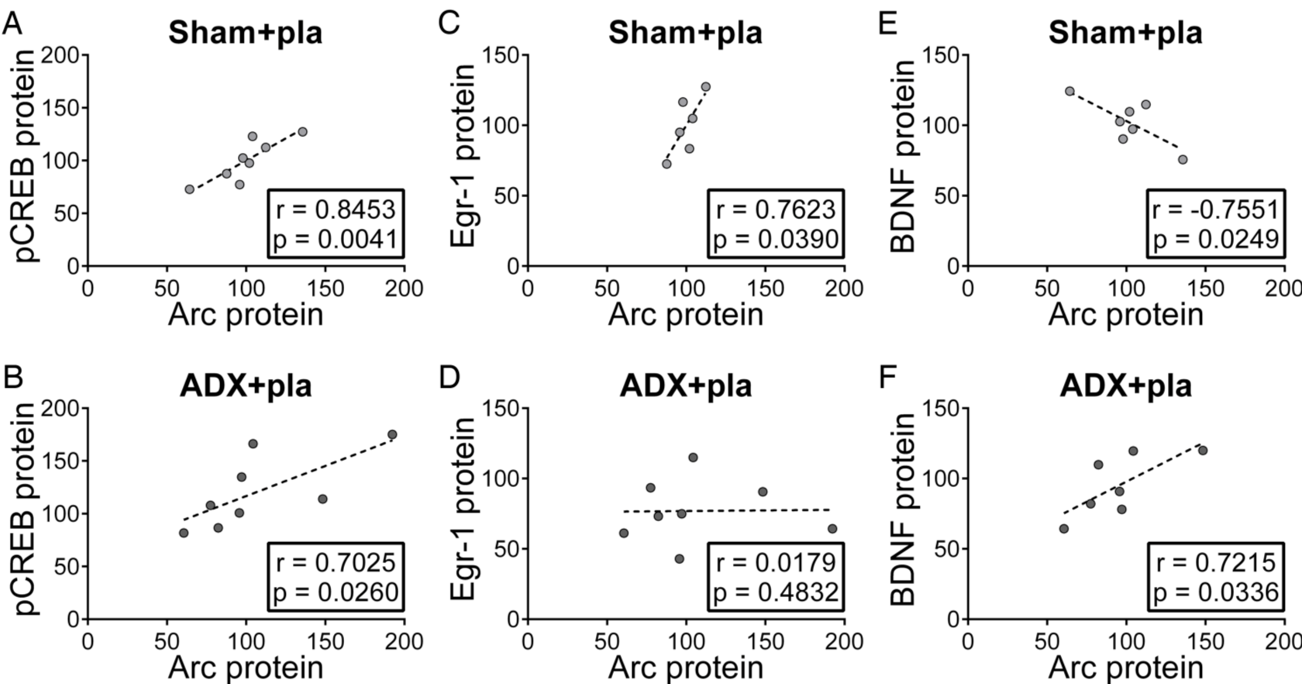
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ADX+pla
Egr-1 protein
Arc protein
r = 0.0179
p = 0.4832
F
ADX+pla
BDNF protein
Arc protein
r = 0.7215
p = 0.0336

**Addiction Biology**

# Different contribution of glucocorticoids in the basolateral amygdala to the formation and expression of opiate withdrawal-associated memories

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|---|---|
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| Keywords: | Adrenalectomy, conditioned place aversion , memory consolidation and retrieval |
| Abstract: | Drug-withdrawal aversive memories generate a motivational state leading to compulsive drug taking, with plasticity changes involved in the formation of aversive memories. The basolateral amygdala (BLA) is involved in aversive motivational learning. We examined the influence of glucocorticoids (GCs) on memory consolidation and retrieval-reconsolidation for a morphine-withdrawal-paired environment through conditioned place aversion (CPA) paradigm, along with plasticity changes in the BLA, in sham-operated and adrenalectomized (ADX) animals. We demonstrated that sham+morphine animals robustly expressed CPA. In contrast, ADX-dependent animals lacked the affective-like signs of opiate withdrawal but displayed increased somatic signs of withdrawal. Following the conditioning phase, ADX-animals displayed reduced phosphorylated cAMP response element binding protein (pCREB). The consolidation process was accompanied by increased early growth response 1 (Egr-1) mRNA, activity-regulated cytoskeletal-associated protein (Arc) mRNA and protein both, in sham-dependent and ADX-dependent animals. Moreover, glucocorticoid receptor (GR) levels were positively correlated with pCREB, Egr-1 and Arc mRNA only in sham+morphine animals upon naloxone-induced corticosterone secretion. In contrast, memory retrieval elicited increased pCREB levels in sham+morphine and ADX animals, and enhanced Arc levels in sham+morphine, but not in ADX+morphine animals. Arc was mainly expressed in glutamatergic neurons. Corticosterone levels were unaltered following memory retrieval, and consequently GR no longer regulated pCREB or Arc. However, we uncovered that pCREB levels positively correlated Arc mRNA/protein expression following memory recall. In conclusion, context-withdrawal associations are accompanied by |

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|---|---|
| | structural and functional plasticity in the BLA, which are, in part, regulated by GR signaling. These findings might have important implications for drug-seeking behavior. |
| | |

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# Different contribution of glucocorticoids in the basolateral amygdala to the formation and expression of opiate withdrawal-associated memories

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**Running head:** Withdrawal memory, amygdala

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## Abstract

Drug-withdrawal aversive memories generate a motivational state leading to compulsive drug taking, with plasticity changes involved in the formation of aversive memories. The basolateral amygdala (BLA) is involved in aversive motivational learning. We examined the influence of glucocorticoids (GCs) on memory consolidation and retrieval-reconsolidation for a morphine-withdrawal-paired environment through conditioned place aversion (CPA) paradigm, along with plasticity changes in the BLA, in sham-operated and adrenalectomized (ADX) animals. We demonstrated that sham+morphine animals robustly expressed CPA. In contrast, ADX-dependent animals lacked the affective-like signs of opiate withdrawal but displayed increased somatic signs of withdrawal. Following the conditioning phase, ADX-animals displayed reduced phosphorylated cAMP response element binding protein (pCREB). The consolidation process was accompanied by increased *early growth response 1* (*Egr-1*) mRNA, activity-regulated cytoskeletal-associated protein (Arc) mRNA and protein both, in sham-dependent and ADX-dependent animals. Moreover, glucocorticoid receptor (GR) levels were positively correlated with pCREB, *Egr-1* and *Arc* mRNA only in sham+morphine animals upon naloxone-induced corticosterone secretion. In contrast, memory retrieval elicited increased pCREB levels in sham+morphine and ADX animals, and enhanced Arc levels in sham+morphine, but not in ADX+morphine animals. Arc was mainly expressed in glutamatergic neurons. Corticosterone levels were unaltered following memory retrieval, and consequently GR no longer regulated pCREB or Arc. However, we uncovered that pCREB levels positively correlated Arc mRNA/protein expression following memory recall. In conclusion, context-withdrawal associations are accompanied by structural and functional plasticity in the BLA, which are, in part, regulated by GR signaling. These findings might have important implications for drug-seeking behavior.

**Key words:** Adrenalectomy; conditioned place aversion; memory consolidation and retrieval; miRNAs; morphine dependence; tanscription factors.

## Introduction

Evidence supports that some of the most important features of addiction reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology (Robbins et al. 2008). One factor responsible for this vulnerability is reactivation of withdrawal memories, which consists in somatic and affective withdrawal signs elicited in the presence of cues that were previously paired with drug withdrawal (O'Brien et al. 1977). The conditioned-place aversion (CPA) paradigm is a highly sensitive animal model for measurement of the negative affective component of drug withdrawal as well as to investigate neural substrates underlying the aversive memory associated with drug withdrawal (Myers et al. 2012; Stinus et al. 2000). CPA triggers association between negative affective consequences of withdrawal with context (aversive memory formation or consolidation). When the animals are re-exposed to the paired environment in a drug-free state, they avoid the paired environment due to the association between the context and aversive memories of drug withdrawal (aversive memory retrieval). Besides, reconsolidation is the process by which memories are re-stabilized and maintained after retrieval, the disruption of which can result in the permanent loss of a previously established memory (Nader et al. 2000).

Re-exposure to a withdrawal-paired environment induces conditioned responses in a specific limbic circuit, which can be partially dissociated from the structures involved in acute withdrawal (Frenois et al. 2005b). The amygdala is a heterogeneous brain region critically involved in processing emotional response (LeDoux 2000), which includes the basolateral amygdala (BLA), a brain area implicated in addictive behavior. Importantly, BLA neuronal activity is correlated with the acquisition and recall of opiate-related associative memories (Frenois et al. 2005a). On the other hand, it is well known that glucocorticoids (GCs) effects on memory are mediated by the BLA. Infusions of a glucocorticoid receptor (GR) agonist into

the BLA enhance memory consolidation (Roozendaal and McGaugh 1997), whereas activation of GC pathway in the BLA is important in the impairing effects of stress on memory retrieval (de Quervain et al. 1998).

CPA develops through associative learning and requires synaptic plasticity (Hou et al. 2009). It has been demonstrated that the reconsolidation of memories during retrieval requires both transcription and translation in the amygdala in a manner analogous to that seen during the initial consolidation of learning to form long-term memories (Miller and Matzel 2000;Nader et al. 2000). However, the molecular processes underlying plasticity during opiate-withdrawal memory consolidation and retrieval have been little studied. Converging evidence supports a role for cAMP response element binding protein (CREB), a transcription factor that becomes activated when phosphorylated on Ser 133 residue, in both consolidation and reconsolidation of fear (Bourtchuladze et al. 1994;Dash et al. 1990;Hall et al. 2001b;Kida et al. 2002;Mamiya et al. 2009;Yin et al. 1994). It is therefore important to examine the specific role for CREB-regulated signaling in amygdala-dependent aversive-withdrawal memory processes. On the other hand, inmediate early genes (IEGs) are inducible genes which can be divided into two functional classes: a) regulatory transcription factors, such as early growth response 1 (Egr-1), and b) “effector” proteins, such as activity-regulated cytoskeletal-associated protein (Arc) (Guzowski et al. 2001). Egr-1 and Arc are required in learning and long-term memory (Guzowski et al. 2000;Jones et al. 2001), and are necessary for the consolidation and reconsolidation of a variety of amygdala-dependent memory tasks (Hellemans et al. 2006;Maddox and Schafe 2011;Plath et al. 2006;Ploski et al. 2008). Importantly, pCREB, Egr-1 and Arc have been proposed to be targeted by activation of GRs, which leads to fear memory consolidation (Finsterwald and Alberini 2014). In addition, microRNAs (miRNAs) are involved in the development of synaptic connections and

plasticity, direct dendrite formation in neurons (Schratt et al. 2006), and have an important role in the development of addiction-related behaviors (Chandrasekar and Dreyer 2011).

To date, despite the fact that GCs modulate memory consolidation and retrieval (Roozendaal and McGaugh 2011), the role of GCs in the BLA in the context of morphine-induced CPA has not been assessed. Therefore, we hypothesized that GCs might modulate context-drug associations and could affect memory-associated plasticity in the BLA, a brain region known to mediate the negative motivational component of opiate withdrawal. The present study was undertaken to test this hypothesis by examining morphine-withdrawal aversive memories in sham-operated and adrenalectomized (ADX) rats, and changes in miR-124a, GR, pCREB, Egr-1 and Arc during consolidation and retrieval-reconsolidation memory.

## Materials and methods

Details are provided in Supplementary Materials and Methods.

**Animals**

Experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE), and were approved by the local Committees for animal research (REGA ES300305440012). Male Wistar rats (n=78) were used.

**Adrenalectomy**

Rats were bilaterally ADX as described in (García-Pérez et al. 2012) and implanted subcutaneously (s.c.) with slow-release corticosterone pellets at surgery. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation.

**Induction of Opiate Dependence**

Morphine dependence was induced by s.c. implantation (lower back) under light ether anaesthesia of two, slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base). Placebo-pelleted rats received lactose pellets also implanted subcutaneously under anaesthesia.

**Conditioned Place Aversion Protocol**

**Pre-testing Phase**

Animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. For each rat, one room was randomly chosen to be paired with naloxone and the

other chamber to vehicle. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

**Conditioning Phase**

Rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. Opiate withdrawal behaviors were measured for 15 min after the conditioning phase. A cohort of animals, named conditioning memory rats (CM), were decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

**Testing Phase**

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). A negative score indicates a place aversion; a positive score indicates a place preference. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

**Tissue processing**

Brains were rapidly removed, and stored immediately at -80°C until use for quantitative real-time PCR (qPCR) and Western blot analysis. For BLA study, three consecutive 500-μm coronal slides were made corresponding to approximately -1.9 to -3.4 mm from bregma, according to the atlas of (Figure S1) (Paxinos and Watson 2007). Tissue samples of interest

were dissected using a punching device with a 1-mm internal diameter. A second set of animals from each treatment group was used for immunofluorescence staining.

**RNA extraction and qPCR**

qPCR was performed as previously described (Polyák et al. 2014).

List of the genes: *gapdh, arc, egr-1*

List of the miRNA assays: U6 snRNA, miR-124a

The gene expression was analyzed by ABI Step One 2.1 program. The amplicon was tested by Melt Curve Analysis on ABI Step OnePlus Instrument. Experiments were normalized to gapdh (glyceraldehyde-3-phosphate dehydrogenase) and U6 snRNA expressions.

gapdh for ACAGCCGCATCTTCTTGTGC

gapdh rev GCCTCACCCCATTTGATGTT

arc for CCCCCAGCAGTGATTCATAC

arc rev CAGACATGGCCGGAAAGACT

egr-1 for CACCTGACCACAGAGTCCTTTT

egr-1 rev ACCAGCGCCTTCTCGTTATT

U6 snRNA cat:001973, 4427975

mmu-miR-124a cat: 001182, 4427975

**Western Blotting**

Western Blotting protocol (García-Pérez et al. 2015) and a list of the primary antibodies used in this study can be found at Supplementary Materials and Methods. We used GAPDH as our loading control.

**Immunofluorescence study and Confocal analysis**

The tissue for immunofluorescence was processed as previously described (García-Pérez et al. 2015) (see details in the Supplementary Materials and Methods). Equipment and settings are detailed in the SI. Final confocal images can be illustrated as they appear throughout the stack of sections as a simple layer or as a transparency of all layers merged together.

**Data Analysis**

Data are presented as mean ± SEM. Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test. Student's *t*-test was used when comparisons were restricted to two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

# Results

### Effect of GCs on opiate withdrawal-associated somatic and affective behavior

The sequence of events during the CPA assay can be conceptualized as follows: (1) the morphine-dependent animal is administered naloxone and develops naloxone-precipitated morphine withdrawal [an unconditioned stimulus (US)], (2) experiences a subjective aversive dysphoric effect [an unconditioned response (UR)], (3) learns to associate this UR with a specific environmental context [a conditioned stimulus (CS)], and when subsequently presented with the CS, (4) the animal exhibits avoidance behavior [a conditioned response (CR)] to the earlier withdrawal-paired CS. Importantly, before the conditioning sessions, the different experimental groups used in this study did not exhibit different locomotor activity (Figure 1A), spent similar amount of time in the naloxone-paired room (Figure 1B) and did not display natural preference for neither the dots or stripes chambers (Figure 1C).

Opiate withdrawal is characterized by both somatic and motivational behavioral signs upon cessation of drug use, leading to the emergence of an aversive state that can be conditioned to a specific environment (Frenois et al. 2005b). As expected, our results showed that naloxone administration to sham- or ADX-operated rats dependent on morphine evoked an increase of the body weight loss ($P<0.001$; Figure 1E) when compared to their control groups also receiving naloxone dose or compared to the same experimental groups after saline injection. As well, opiate antagonist administration to sham- or ADX-operated rats dependent on morphine elicited the appearance of a number of behavioral symptoms characteristic of opiate withdrawal (Figure 1F). It is noteworthy that, on day 10, the ADX+morphine group exhibited an enhancement of the withdrawal score when compared with both sham+morphine (day 10; $P<0.01$) and ADX+morphine (day 8; $P<0.05$) groups (Figure 1F).

Since CPA expression occurs on a naloxone-free test day, the behavior is dependent upon the retrieval of the association between the environment and the affective state produced by the drug (White and Carr 1985). Therefore, behavior in the place aversion method depends upon both the affective and the memory improving properties of the reinforcers under test (White and Carr 1985). During the test, morphine-dependent rats spent less time in the naloxone-paired compartment than controls ($P<0.001$; Figure 1G). However, adrenalectomy diminished the naloxone-induced CPA in opiate-dependent animals ($P<0.01$). Additionally, in the test phase the total entries were higher in animals receiving opiates ($P<0.05$; Figure 1H), which might indicate an increased locomotor activity of the morphine-dependent rats.

**Gene expression and protein synthesis in the BLA during memory consolidation and retrieval-reconsolidation processes**

We focused our study on the BLA, because among the limbic areas that are likely to mediate motivational features of opiate dependence, the BLA represents a critical neural substrate (Frenois et al. 2005a;Lucas et al. 2008). Furthermore, the BLA is part of an integrated circuit of neural structures implicated in the formation and storage of CS–US associations and in the processing of emotional events in relation to environmental stimuli that guide motivated behaviour (Everitt et al. 2003). Moreover, it is well known that GC effects on memory are mediated by the BLA (Schwabe et al. 2012).

We studied GR expression in the amygdala, given that the BLA is enriched in GRs, which are known to play critical roles in encoding, processing and retaining the information of emotional events (Finsterwald and Alberini 2014). Western blot analysis did not reflect any change on GR protein between the different groups after the last conditioning session (Figure

2D). In contrast, after memory recall, two-way ANOVA revealed a main effect of surgery ($P<0.05$, Figure 2E) on GR expression, although no differences between groups were observed with the *post hoc* test. In addition, no correlation was observed between GR induction and the aversive score (Figure 2F).

miRNAs and post-transcriptional processes have an important role in the development of addiction-related behaviors (Chandrasekar and Dreyer 2011). Here we directly assessed the specific role of amygdalar miR-124a in memory consolidation and retrieval-reconsolidation. Our results show that during the memory consolidation process (day 10), miR-124a levels were not modified (Figure 2A). In contrast, during the test phase, miR-124a levels were significantly diminished in sham animals treated with morphine ($P<0.05$; Figure 2B), whereas adrenalectomy reversed this effect. Furthermore, we observed that miR-124a was positively correlated with the aversion score in the sham+morphine group (Figure 2C). Thus, rats that expressed higher aversion in the CPA test and spent less time in the naloxone-paired room, displayed the lowest miR-124a levels.

We next have investigated whether activation of the transcription factor CREB is induced in the BLA during the formarion and recall of Pavlovian cued morphine-withdrawal associations. We uncovered that the lack of adrenal glands resulted in decreased pCREB levels during memory consolidation ($P<0.05$; Figure 3A). On the other hand, after memory retrieval, pCREB levels were increased both in sham+morphine animals ($P<0.05$; Figure 3B) and ADX animals ($P<0.05$). However, pCREB levels showed no direct relationship with the aversive index (Figure 3C). Furthermore, we performed an immunofluorescence study to investigate if CREB activation after memory retrieval occurred in $GR^+$ cells. As depicted in Figure 3D-E, we found that $pCREB^+$ neurons were also $GR^+$ cells, although some cells that expressed GR did not exhibit CREB activation.

To extend our results, the present study aimed to assess the cellular reactivity and plasticity of amygdala nuclei during the opiate withdrawal conditioning process. Egr-1 and Arc IEGs are required in long-term potentiation (LTP) and long-term memory (Guzowski et al. 2000;Jones et al. 2001). For this, we have quantified Egr-1 and Arc expression using PCR and Western-blot analysis, following the last training session and after re-exposure to the withdrawal-paired environment. During the consolidation process, we detected that sham or ADX morphine-dependent rats exhibited increased *Egr-1* mRNA levels ($P<0.05$), although these changes were not observed at the protein level (Figure 4A,D). Following retrieval, ADX+placebo animals exhibited decreased Egr-1 mRNA levels ($P<0.01$; Figure 4B). However, no differences in Egr-1 protein were observed at the same time-point (Figure 4E). In addition, immunofluorescence analysis demonstrated that Egr-1 immunoreactivity was widely distributed throughout the BLA, and double-labeling with anti-GR showed that Egr-1 is expressed on a high percentage of $GR^+$ neurons (Figure 4G-H).

BLA tissue from sham+morphine ($P<0.01$) and ADX+morphine ($P<0.05$) animals showed an increase in the expression of the plasticity-related Arc gene during conditioning (Figure 5A), and these changes were paralleled in Arc protein ($P<0.05$; Figure 5D). Interestingly, following memory retrieval, ADX groups showed a significant reduction in Arc mRNA expression ($P<0.05$ or 0.01; Figure 5B). Arc protein was also increased in sham-dependent rodents by re-exposure to the withdrawal-paired environment ($P<0.05$), although addicted animals that had been adrenalectomized displayed a significant reduction in protein levels ($P<0.05$; Figure 5E). Correlation analysis between the different IEGs mRNA or protein content and CPA index failed to detects any significant correlation. As shown in Figure 5G-H, according to our results, sham+morphine animals displayed a higher number of Arc positive neurons compared to ADX-dependent subjects. In addition, double immnunofluorescence study revealed that Arc protein was expressed in a reduced number of $GR^+$ cells.

**Distinct GR-dependent non-genomic and genomic regulation following conditioning and testing**

Importantly, mineralocorticoid receptors (MRs) have a tenfold higher affinity for GCs than GRs and are largely occupied by the ligand in basal conditions, whereas GRs occupation highly depends on increases in glucocorticoid levels following stress response (de Kloet et al. 2005). Thus, we studied the activity of the hypothalamic-pituitary-adrenal (HPA) axis throughout the CPA paradigm. Our results revealed that naloxone administration to morphine dependent rats before conditioning induced an increase in GCs plasma levels ($P<0.001$) which was not observed in ADX animals due to adrenalectomy ($P<0.001$; Fig. 6A). In contrast, following memory retrieval of aversive withdrawal memories did not alter the corticosterone blood levels (Figure 6B). On ligand binding, GRs undergo conformational changes that lead to their homodimerization and nuclear translocation, or their binding to other cytoplasmic proteins. In the nucleus, GRs can bind to specific sequences and directly activate transcription of target genes (Zalachoras et al. 2013). In addition, GRs can control rapid cellular responses by mechanisms that are independent of nuclear translocation and gene expression regulation, but instead occur through genomic-independent actions (Groeneweg et al. 2011). During the consolidation process, we detected that GR levels were positively correlated with pCREB, *Egr-1* and *Arc* mRNA only in sham+morphine animals (Figure 6C-E), indicating that genomic and non-genomic GR-dependent regulation during memory consolidation depends on increased GCs levels. In contrast, in other experimental groups with basal GC levels, correlation analysis revealed that GR might exert inhibitory genomic regulation (Figure 6F-H,O). Following memory retrieval, we observed a lack of GR regulation of the same targets, except for ADX+morphine animals, where GR seemed to negatively regulate pCREB levels (Figure 6I-N,P).

Given the absence of positive GR regulation of Arc, we next compared pCREB induction with that of Arc, the expression of which can be regulated by pCREB (Kawashima et al. 2009). We confirmed the co-localization of pCREB and Arc in the BLA after the CPA in sham- and ADX-operated morphine-dependent rats (Figure 7A-B). In addition, the present results showed that CREB activation correlated with increased Arc mRNA and protein in sham-dependent animals, while in ADX+morphine animals pCREB did only correlate Arc protein levels, but not Arc mRNA (Figure 7E-I). However, greater numbers of nuclei showed induction of pCREB than of Arc, suggesting that pCREB may regulate the expression of other IEGs. Indeed, the CRE consensus sequence is present in Egr-1 promoter (Changelian et al. 1989), raising the possibility that CREB activation may regulate Egr-1 during reconsolidation. Nonetheless, we observed no relationship between pCREB and Egr-1 levels after the reactivation test (data not shown). On the other hand, Egr1 can also regulate Arc (Li et al. 2005). Although we observed that Egr-1 and Arc were expressed on the same cells (Figure 7C-D), our results reject that Egr-1 controls Arc induction during memory retrieval-reconsolidation (Figure 7J).

**Characterization of Arc-expressing neurons**

The BLA mainly contains glutamate output neurons (GAD-) together with GABA interneurons (GAD+) (Pitkänen et al. 1997). We performed a phenotypic analysis to characterize the neuronal populations involved in Arc expression to better understand the cellular events underlying these processes. We uncovered that the majority of Arc-positive cells were glutamatergic neurons (Figure 8A). Nevertheless, Arc was also stained in few GABAergic neurons (Figure 8B).

## Discussion

Somatic and affective components of the opiate withdrawal syndrome are dissociable at different levels of analysis (Frenois et al. 2002;Mucha 1987). Our findings of a clear-cut dissociation of affective-like and somatic signs of opiate withdrawal in sham and ADX rats provide robust evidence in support of the latter notion. It has been previously demonstrated that CRF1−/− mice, which displayed basal and stress-related plasma corticosterone deficits (Smith et al. 1998;Timpl et al. 1998), showed increased somatic expression of opiate withdrawal (Papaleo et al. 2007). Moreover, intake of nonstressful amounts of corticosterone reduced the exacerbated somatic reactions of CRF1−/− mice to opiate withdrawal (Papaleo et al. 2007). Thus, the use of adrenalectomy also allowed us to unravel a HPA axis-dependent modulatory (inhibitory) role for GCs upon the somatic expression of opiate withdrawal. These findings clearly indicate that failure to adequately activate stress-responsive HPA axis may lead to exaggerated somatic reactions to opiate withdrawal. In contrast, CRF1−/− mice lack the negative affective states of opiate withdrawal (Contarino and Papaleo 2005)). Together, our results indicate that a fully functional HPA system is required for the negative affective properties of opiate withdrawal.

The lack of affective-like behaviors in ADX-dependent animals cannot be attributed to decreased levels of somatic opiate withdrawal. Alternatively, GCs activate regions enriched in GRs, which play critical roles in encoding, processing, and retaining the information of emotional events (Finsterwald and Alberini 2014). Thus, we next focused on the molecular processes accompanying withdrawal memory consolidation and retrieval-reconsolidation in the BLA for several reasons: GC effects on memory are mediated by the BLA (Schwabe et al. 2012), GRs are highly expressed in this brain area, and the BLA is a neural region strongly involved in emotional learning and affective conditioning (LeDoux 2000). In the hippocampus, several long-lasting molecular modifications required for fear memory

consolidation are dependent on GR activation. Specifically, GRs control the rapid non-genomic learning-dependent increase of pCREB (Chen et al. 2012) and the genomic expression of the IEGs Arc and Egr-1 (Chen et al. 2012;Revest 2005;Revest et al. 2010). However, GRs actions highly depend on increases in GCs levels (de Kloet et al. 2005;Lupien et al. 2007). In agreement, we uncovered that GR expression correlated with pCREB, Egr-1 and Arc induction during aversive-withdrawal memory consolidation only in sham+morphine animals. Since delayed genomic GR actions suppress competing information processing after learning and hence promote memory consolidation (Schwabe et al. 2012), GCs secretion and GR-signaling are likely to mediate the differences in the CPA index between sham- and ADX-dependent animals.

CREB plays a critical role in memory consolidation (Bourtchuladze et al. 1994;Dash et al. 1990;Yin et al. 1994). In the context of opiate-withdrawal CPA paradigm, it has been previously reported that despite a global inhibition of *c-fos* expression in the BLA after conditioning session, a subpopulation of output neurons was activated by conditioned withdrawal (Frenois et al. 2005b). In concordance, in the present study sham+morphine rodents did not display a net increase in pCREB levels during memory formation, but the fact that training-dependent GR activation seemed to be coupled to CREB phosphorylation suggests that pCREB signaling is engaged in a subset of BLA cells during memory consolidation. Furthermore, our data demonstrate that adrenalectomy mediates CREB hipoactivation within the BLA, which might underlie the impairment of withdrawal-memories consolidation in ADX+morphine animals.

In addition, synaptic plasticity within the BLA is critical in the process of a CS becoming associated with a US during conditioning (Blair et al. 2005). We tested this hypothesis by assessing Arc gene expression, which has been widely implicated in synaptic plasticity, LTP and memory formation (Korb and Finkbeiner 2011). Importantly,Arc has been

extensively described in the consolidation phases of a variety of memory tasks (Guzowski et al. 2000;Plath et al. 2006;Ploski et al. 2008). Our findings are consistent with previous data suggesting a strong increase in the number of output neurons expressing *Arc* mRNA (Lucas et al. 2008) and enhanced Arc protein expression (Hou et al. 2009) in the BLA following withdrawal pairing. Particularly, late-phase LTP is considered to be the underlying mechanism of long-term memory, and LTP consolidation requires sustained period of Arc synthesis translation since Arc protein underlying LTP consolidation is rapidly degraded (Bramham et al. 2010;Messaoudi et al. 2007). Actually, evidence points to a second wave of Arc induction hours after the degradation of the first wave of Arc (Ramírez-Amaya et al. 2005), which may indicate the reactivation of circuits during consolidation. Besides, Arc learning-dependent expression is controlled by corticosterone and GR (Chen et al. 2012;McReynolds 2010;Molteni et al. 2010). Accordingly, in sham+morphine animals, we observed that activated GR lead to genomic activation of Arc gene, which would lead to sustained Arc transcription required for consolidation. Because of the lack of endogenous GCs in ADX+morphine animals, GR did not mediate genomic activation. It is likely that enhanced Arc mRNA levels in ADX+morphine animals are induced by neural activity (Abraham et al. 1993;Worley et al. 1993). Together, our data suggest that ADX-dependent animals are able to induce Arc-mediated LTP, but in the absence of withdrawal-induced corticosterone are unable to maintain LTP and form long-term memory.

Memory retrieval additionally engages a protein synthesis-dependent re-consolidation of memories (Nader et al. 2000). However, the transcriptional mechanisms necessary for initial memory consolidation and stability after retrieval differ. For instance, stress is known to have an inhibitory effect on memory retrieval. Activation of GC pathway in the BLA is important in the impairing effects of stress on memory retrieval (de Quervain et al. 1998;Roozendaal et al. 2004). Moreover, the severity of memory impairments has been

correlated with the concentration of circulating plasma corticosterone at the time of testing (de Quervain et al. 1998;Diamond et al. 1999). In agreement, we showed that GRs acted as transcriptional regulators specifically during memory consolidation upon activation by GCs, but not after memory retrieval, when GCs levels remained at basal levels.

Emerging evidence supports a role for miRNAs in different steps of addiction. Chronic cocaine suppresses miR-124 levels throughout the brain, including the BLA (Chandrasekar and Dreyer 2009). Our mir-124a findings are particularly important for several reasons. First, a role for mir-124a in addicted behavior has been proposed. For instance, miR-124 is suppressed after cocaine CPP and during the reinstatement, and miR-124 over-expression attenuates cocaine CPP (Chandrasekar and Dreyer 2011). Second, our data indicated that miR-124a downregulation occurred selectively following a re-exposure to withdrawal context in sham-dependent animals, but was reversed by adrenalectomy. Third, miR-124a is likely to play a pivotal role in morphine-withdrawal memory retrieval and reconsolidation since lesser miR-124a expression significantly correlated with enhanced aversion.

GR no longer regulated pCREB during memory retrieval. However, CREB can be phosphorylated on Ser 133 by different kinases (De Cesare and Sassone-Corsi 2000;Shaywitz and Greenberg 1999), which are required in the amygdala for reconsolidation of fear or drug-paired cues (Duvarci et al. 2005;Sanchez et al. 2010;Tronson et al. 2006). Previous studies have demonstrated that CREB is required for reconsolidation of contextual and cued fear memories (Kida et al. 2002;Mamiya et al. 2009). Furthermore, it has been postulated that CREB activity in the BLA alone is required for reconsolidation of auditory memories, but is not required for retrieval or extinction of fear (Tronson et al. 2012). The present study extends these findings, and given that CREB activation promotes *c-fos* transcription (Mayr and Montminy 2001), is in agreement with the notion that the re-exposure to a withdrawal-paired

environment increased *c-fos* expression of most BLA output neurons (Frenois et al. 2005b). ADX-animals also showed increased pCREB levels. By all means, pCREB levels in ADX+morphine rats do not seem to be due to memory-related mechanisms, since ADX-dependent animals do not display enhanced pCREB levels compared to their controls.

Our findings contrast with studies showing that cue-induced opiate withdrawal memories undergo Egr-1 protein synthesis-dependent reconsolidation in the BLA (Hellemans et al. 2006). Given that Egr-1 expression following retrieval is described to be more prolonged than that of Arc (Besnard et al. 2014), our observations of unaltered Egr-1 levels during memory reactivation are not likely due to a time-course effect. It could be argued that Egr-1 within the BLA is only involved in the reconsolidation of cue-induced, but not context-induced, aversive withdrawal memories (Hellemans et al. 2006). However, Egr-1-activation in the BLA after re-exposure to CS is described in a great variety of training tasks, such as contextual and cued fear learning (Hall et al. 2001a;Hoffman et al. 2015;Lee et al. 2005), withdrawal-aversive (Hellemans et al. 2006) or appetitive (Lee et al. 2005;Lee et al. 2006;Thomas et al. 2003) drug-associated memories. A possible explanation is that Egr-1 activation evolves as a function of the number of pairings. After the last (second) withdrawal conditioning, we observed that opiate-dependent animals exhibited increased Egr-1 mRNA but unaltered Egr-1 protein levels, which raises the possibility that additional pairings resulted in increased strength of the association and Egr-1 protein levels. Actually, Egr-1 activation during opiate-withdrawal memory reconsolidation was detected after five conditioning sessions (Hellemans et al. 2006).

The increased plasticity within the BLA may contribute not only to the consolidation of CS–US associations, but also to facilitate BLA reactivation by conditioned environmental stimuli in order to initiate appropriate behavioral responses. While *Arc* induction is not necessary for memory retrieval, it likely identifies neurons genomically activated as a result

of retrieval (Zhang et al. 2005). When analyzing the expression of the plasticity-related *Arc* IEG after aversive withdrawal-memory retrieval, we observed that the group of animals that exhibited robust place aversion also displayed increased Arc levels, in agreement with (Lucas et al. 2008), suggesting that sustained Arc-induced BLA plasticity during the consolidation phase may well participate in the retrieval of learned associations in sham+morphine animals. This notion is supported by data showing that the same neurons in the BLA are activated both by learning and by memories retrieval in a fear conditioning paradigm (Reijmers et al. 2007). As expected, Arc mRNA and protein levels were significantly decreased within the BLA in the ADX+mor group. As proposed above, in the absence of GCs, GR do not activate sustained Arc induction during the consolidation phase, and subsequently exposure CS does not reactivate BLA neurons to facilitate memory retrieval.

The expression of CRE-driven genes after CREB phosphorylation is described to be essential for the stability of conditioned memories after retrieval (Kida et al. 2002), and miR-124 inhibition is crucial for long-term synaptic facilitation by regulating CREB (Chandrasekar and Dreyer 2011;Rajasethupathy et al. 2009). Together, our observations suggest that miR-124 downregulation may enhance the maladaptive drug-associated memories in sham+morphine animals, in line with the findings that miR-124 inhibition act as the switch necessary to convert short-term facilitation into long-term facilitation (Rajasethupathy et al. 2009). Moreover, our results suggest that sustained CREB phosphorylation following retrieval mediates Arc expression. This observation is of particular interest given the prominent role of Arc in fear memory reconsolidation (Maddox and Schafe 2011). Thus, dysregulation of CREB signaling in the amygdala may enhance reconsolidation, resulting in the maintenance of excessive aversive states.

In agreement with previous studies (Frenois et al. 2005b;Lucas et al. 2008), double-labeling showed that the majority of Arc-positive neurons during the re-exposure to withdrawal-paired stimuli were glutamatergic neurons, while very few $Arc^{+}/GAD^{+}$ neurons were detected. Plasticity in glutamate output neurons in the amygdala is of great interest since the apparent variety of functions of the amygdala are based on its diverse extended connections with cortical and subcortical structures (Noori et al. 2012). Thus, our results suggest that plasticity processes within BLA output neurons during the consolidation and reconsolidation of withdrawal aversive memories may participate in increasing the BLA reactivity to conditioned stimuli, which could in turn (by the control of downstream nuclei) reinforce and drive the motivational properties of withdrawal over drug consumption.

Defining the differential molecular mechanisms of consolidation and retrieval-reconsolidation allows for the possibility of separately targeting both of these processes to reduce persistent memories and maladaptive behavioral responses. Our data provide the first evidence that GCs mediate transcriptional events required for morphine-withdrawal memory consolidation and influence epigenetic mechanisms following retrieval in the BLA, supporting the idea that targeting GCs in this amygdalar area may provide important insights into the role of essential signaling cascades mediating aversive drug memories. Together, disrupting these processes might lead to effective treatments in drug addiction (Milton and Everitt 2010;Tronson and Taylor 2007)thereby rapidly and persistently reducing invasive memories and subsequent drug-seeking.

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## Figure legends

**Figure 1. Experimental procedures and behavioral parameters.** (A) Diagram showing the experimental protocol: after 7 habituation and handling days, on day 0 animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. For each rat, one room was randomly chosen to be paired with naloxone and the other chamber to vehicle. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4. In the second phase (conditioning), rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). **(B) Number of entries during pre-test phase:** Two-way ANOVA: surgery (F (1, 48) = 0,1450; P = 0,7050), treatment (F (1, 48) = 0,7319; P = 0,3965), interaction (F (1, 48) = 0,2800; P = 0,5991). **(C) Time in naloxone-paired room during pre-test phase:** Two-way ANOVA: surgery (F (1, 48) = 0,7422; P = 0,3932), treatment (F (1, 48) = 0,3054; P = 0,5831), interaction (F (1, 48) = 0,01515; P = 0,9026). **(D) % of time in dots or stripes rooms during pre-test phase:** Two-way ANOVA: surgery (F (1, 48) = 0,8195; P = 0,3699), treatment (F (1, 48) = 1,906; P = 0,1738), interaction (F (1, 48) = 0,1204; P = 0,7301). **(E) Body weight loss during conditioning sessions: Day 7 (saline):** Two-way ANOVA: surgery (F (1, 48) = 0,004669; P = 0,9458), treatment (F (1, 48) = 4,572; P = 0,0376), interaction (F (1, 48) = 0,4938; P = 0,4856). Post hoc test: ns. **Day 8 (naloxone):** Two-way ANOVA: surgery (F (1, 48) = 3,246; P = 0,0779), treatment (F (1, 48) = 77,30; P < 0,0001), interaction (F (1, 48) = 0,05720; P = 0,8120). Post hoc test: $^{***}$P < 0.001 versus sham+pla; $^{###}$P < 0.001 versus ADX+pla. **Day 9 (saline):** Two-way ANOVA: surgery (F (1, 48) = 0,1954; P = 0,6604), treatment (F (1, 48) = 0,3916; P = 0,5344), interaction (F (1, 48) = 2,762; P = 0,1030). **Day 10 (naloxone):** Two-

way ANOVA: surgery (F (1, 48) = 5,406; P = 0,0243), treatment (F (1, 48) = 131,8; P < 0,0001), interaction (F (1, 48) = 0,7116; P = 0,4031). Post hoc test: $^{***}P < 0.001$ versus sham+pla; $^{+}P < 0.05$ versus sham+mor; $^{\#\#\#}P < 0.001$ versus ADX+pla. **Sham+pla:** One-way ANOVA: acute injection (F (3, 44) = 0,5766; P = 0,6335). **Sham+mor:** One-way ANOVA: acute injection (F (3, 60) = 43,30; P < 0,0001). Post hoc test: $^{\&\&\&}P < 0.001$ versus day 7 (saline); $^{@@@}P < 0.001$ versus day 9 (saline). **ADX+pla:** One-way ANOVA: acute injection (F (3, 44) = 0,7106; P = 0,5509). **ADX+mor:** One-way ANOVA: acute injection (F (3, 44) = 35,75; P < 0,0001). Post hoc test: $^{\&\&\&}P < 0.001$ versus day 7 (saline); $^{@@@}P < 0.001$ versus day 9 (saline). **(F) Withdrawal score after conditioning sessions:** Two-way ANOVA: day (F (1, 26) = 5,387; P = 0,0284), surgery+treatment (F (1, 26) = 3,964; P = 0,0571), interaction (F (1, 26) = 7,487; P = 0,0111). Post hoc test: $^{++}P < 0.01$ versus sham+mor same day; $^{\$}P < 0.05$ versus ADX+mor day 8. **(G) Aversion score:** Two-way ANOVA: surgery (F (1, 48) = 3,789; P = 0,0574), treatment (F (1, 48) = 10,05; P = 0,0027), interaction (F (1, 48) = 10,44; P = 0,0022). Post hoc test: $^{***}P < 0.001$ versus sham+pla; $^{++}P < 0.01$ versus sham+mor. **(H) Number of entries during test phase:** Two-way ANOVA: surgery (F (1, 48) = 0,08893; P = 0,7668), treatment (F (1, 48) = 14,09; P = 0,0005), interaction (F (1, 48) = 0,0006481; P = 0,9798). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{\#}P < 0.05$ versus ADX+pla.

**Figure 2. miR-124a and GR regulation during the conditioning and test phase. (A) miR-124a expression during the conditioning phase:** Two-way ANOVA: surgery (F (1, 18) = 1,819; P = 0,1942), treatment (F (1, 18) = 0,7172; P = 0,4082), interaction (F (1, 18) = 3,137; P = 0,0934). **(B) miR-124a expression during the test phase:** Two-way ANOVA: surgery (F (1, 25) = 0,3312; P = 0,5701), treatment (F (1, 25) = 4,068; P = 0,0546), interaction (F (1, 25) = 4,306; P = 0,0484). Post hoc test: $^{*}P < 0.05$ versus sham+pla. **(C) Correlation analysis between aversion score and miR-124a. (D) GR expression during the conditioning**

**phase:** Two-way ANOVA: surgery (F (1, 22) = 0,1916; P = 0,6659), treatment (F (1, 22) = 0,0006138; P = 0,9805), interaction (F (1, 22) = 0,01153; P = 0,9155). **(E) GR expression during the test phase:** Two-way ANOVA: surgery (F (1, 29) = 4,628; P = 0,0399), treatment (F (1, 29) = 1,411; P = 0,2446), interaction (F (1, 29) = 0,2657; P = 0,6102). Post hoc test: ns. **(F) Correlation analysis between aversion score and GR.**

**Figure 3. pCREB regulation during the conditioning and test phase and identification of pCREB-expressing cells during conditioned place aversion (CPA) paradigm. (A) pCREB expression during the conditioning phase:** Two-way ANOVA: surgery (F (1, 20) = 16,15; P = 0,0007), treatment (F (1, 20) = 0,01732; P = 0,8966), interaction (F (1, 20) = 0,3543; P = 0,5584). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{+}P < 0.05$ versus sham+mor. **(B) pCREB expression during the test phase:** Two-way ANOVA: surgery (F (1, 28) = 2,725; P = 0,1100), treatment (F (1, 28) = 1,664; P = 0,2076), interaction (F (1, 28) = 5,629; P = 0,0248). Post hoc test: $^{*}P < 0.05$ versus sham+pla. **(C) Correlation analysis between aversion score and pCREB. (D-E)** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (GR), green (pCREB) and blue (DAPI, nuclear stain); scale bar, 50 µm. Merged images are also depicted.

**Figure 4. Egr-1 mRNA and protein regulation during the conditioning and test phase and identification of Egr-1-expressing cells during conditioned place aversion (CPA) paradigm. (A) Egr-1 mRNA expression during the conditioning phase:** Two-way ANOVA: surgery (F (1, 17) = 0,06232; P = 0,8059), treatment (F (1, 17) = 15,78; P = 0,0010), interaction (F (1, 17) = 0,1141; P = 0,7397). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{\#}P < 0.05$ versus ADX+pla. **(B) Egr-1 mRNA expression during the test phase:** Two-way ANOVA: surgery (F (1, 25) = 8,343; P = 0,0079), treatment (F (1, 25) = 4,229; P =

0,0503), interaction (F (1, 25) = 5,999; P = 0,0217). Post hoc test: $^{**}P < 0.01$ versus sham+pla; $^{\#\#}P < 0.01$ versus ADX+pla. **(C) Correlation analysis between aversion score and Egr-1 mRNA. (D) Egr-1 protein expression during the conditioning phase:** Two-way ANOVA: surgery (F (1, 20) = 0,01518; P = 0,9032), treatment (F (1, 20) = 0,9763; P = 0,3349), interaction (F (1, 20) = 0,5218; P = 0,4784). **(E) Egr-1 protein expression during the test phase:** Two-way ANOVA: surgery (F (1, 29) = 0,2768; P = 0,6028), treatment (F (1, 29) = 0,01433; P = 0,9055), interaction (F (1, 29) = 0,7424; P = 0,3959). **(F) Correlation analysis between aversion score and Egr-1 protein. (G-H)** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (GR), green (Egr-1) and blue (DAPI, nuclear stain); scale bar, 50 µm. Merged images are also depicted.

**Figure 5. Arc mRNA and protein regulation during the conditioning and test phase and identification of Arc -expressing cells during conditioned place aversion (CPA) paradigm. (A) Arc mRNA expression during the conditioning phase:** Two-way ANOVA: surgery (F (1, 17) = 0,4034; P = 0,5338), treatment (F (1, 17) = 20,01; P = 0,0003), interaction (F (1, 17) = 0,6690; P = 0,4247). Post hoc test: $^{**}P < 0.01$ versus sham+pla; $^{\#}P < 0.05$ versus ADX+pla. **(B) Arc mRNA expression during the test phase:** Two-way ANOVA: surgery (F (1, 26) = 16,96; P = 0,0003), treatment (F (1, 26) = 1,044; P = 0,3162), interaction (F (1, 26) = 0,1477; P = 0,7039). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{++}P < 0.01$ versus sham+mor. **(C) Correlation analysis between aversion score and Arc mRNA. (D) Arc protein expression during the conditioning phase:** Two-way ANOVA: surgery (F (1, 19) = 2,625; P = 0,1216), treatment (F (1, 19) = 14,14; P = 0,0013), interaction (F (1, 19) = 0,03367; P = 0,8563). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{\#}P < 0.05$ versus ADX+pla. **(E) Arc protein expression during the test phase:** Two-way ANOVA: surgery (F (1, 29) = 1,591; P = 0,2172), treatment (F (1, 29) = 0,7737; P = 0,3863), interaction (F (1, 29) = 7,545; P =

0,0102). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{+}P < 0.05$ versus sham+mor. **(F) Correlation analysis between aversion score and Arc protein. (G-H)** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (**Arc**), green (GR) and blue (DAPI, nuclear stain); scale bar, 50 μm. Merged images are also depicted.

**Figure 6. Corticosterone secretion and correlation analysis between GR and pCREB, Egr-1 mRNA and Arc mRNA during the conditioning and test phase. (A) Corticosterone levels during the conditioning phase:** Two-way ANOVA: surgery ($F (1, 21) = 224,3$; $P < 0,0001$), treatment ($F (1, 21) = 244,2$; $P < 0,0001$), interaction ($F (1, 21) = 236,9$; $P < 0,0001$). Post hoc test: $^{***}P < 0.001$ versus sham+pla; $^{+++}P < 0.001$ versus sham+mor. **(B) Corticosterone levels during the test phase:** Two-way ANOVA: surgery ($F (1, 48) = 1,732$; $P = 0,1944$), treatment ($F (1, 48) = 0,02623$; $P = 0,8720$), interaction ($F (1, 48) = 0,01722$; $P = 0,8962$). **(C-H) Correlation analysis during the conditioning phase.** GR was positively correlated with pCREB, Egr-1 mRNA and Arc mRNA in sham+morphine animals, whereas a trend for negative correlation was observed in ADX+morphine group. **(I-N) Correlation analysis during the test phase.** No relationship was observed, except a negative correlation between GR and pCREB in ADX+morphine rodents. **(O-P) Table summarizing the results obtained in the different experimental groups during the conditioning phase (O) and test phase (P).**

**Figure 7. Identification of Arc in pCREB- and Egr-1-expressing cells and correlation analysis between pCREB or Egr-1 and Arc mRNA/protein during the test phase. (A-B)**

Representative confocal microphotographs showing coronal sections of rats immunostained in: red (Arc), green (pCREB) and blue (DAPI, nuclear stain); scale bar, 50 µm. Merged images are also depicted. Confocal images demonstrated that all Arc neurons in sham or ADX morphine-dependent animals were pCREB positive. **(C-D)** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (Arc), green (Egr-1) and blue (DAPI, nuclear stain); scale bar, 50 µm. Merged images are also depicted. Immunofluorescence experiments showed that Arc-expressing neurons in sham or ADX morphine-dependent animals were Egr-1$^{+}$. **(E-I) Correlation analysis between pCREB and Arc mRNA/protein levels.** Positive correlation between pCREB and Arc mRNA was observed only in sham+morphine animals, whereas Arc protein was positively correlated in both morphine-dependent groups. **(J) Correlation analysis between Egr-1 and Arc mRNA/protein levels.** No relationship was detected.

**Figure 8. Identification of different cellular subtypes that express Arc during memory retrieval.** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (Arc), green [GLS2 **(A)** or GAD **(B)**] and blue (DAPI, nuclear stain); scale bar, 50 µm. Merged images are also depicted. **(A)** The majority of Arc-positive cells were glutamatergic neurons. **(B)** Arc was stained only in few GABAergic neurons. White arrows: Arc$^{+}$ and GLS2$^{+}$ or GAD$^{+}$ neurons. Orange arrows: Arc$^{+}$ and GLS2$^{-}$ or GAD$^{-}$ neurons. Blue arrows: Arc$^{-}$ and GAD$^{+}$ neurons.

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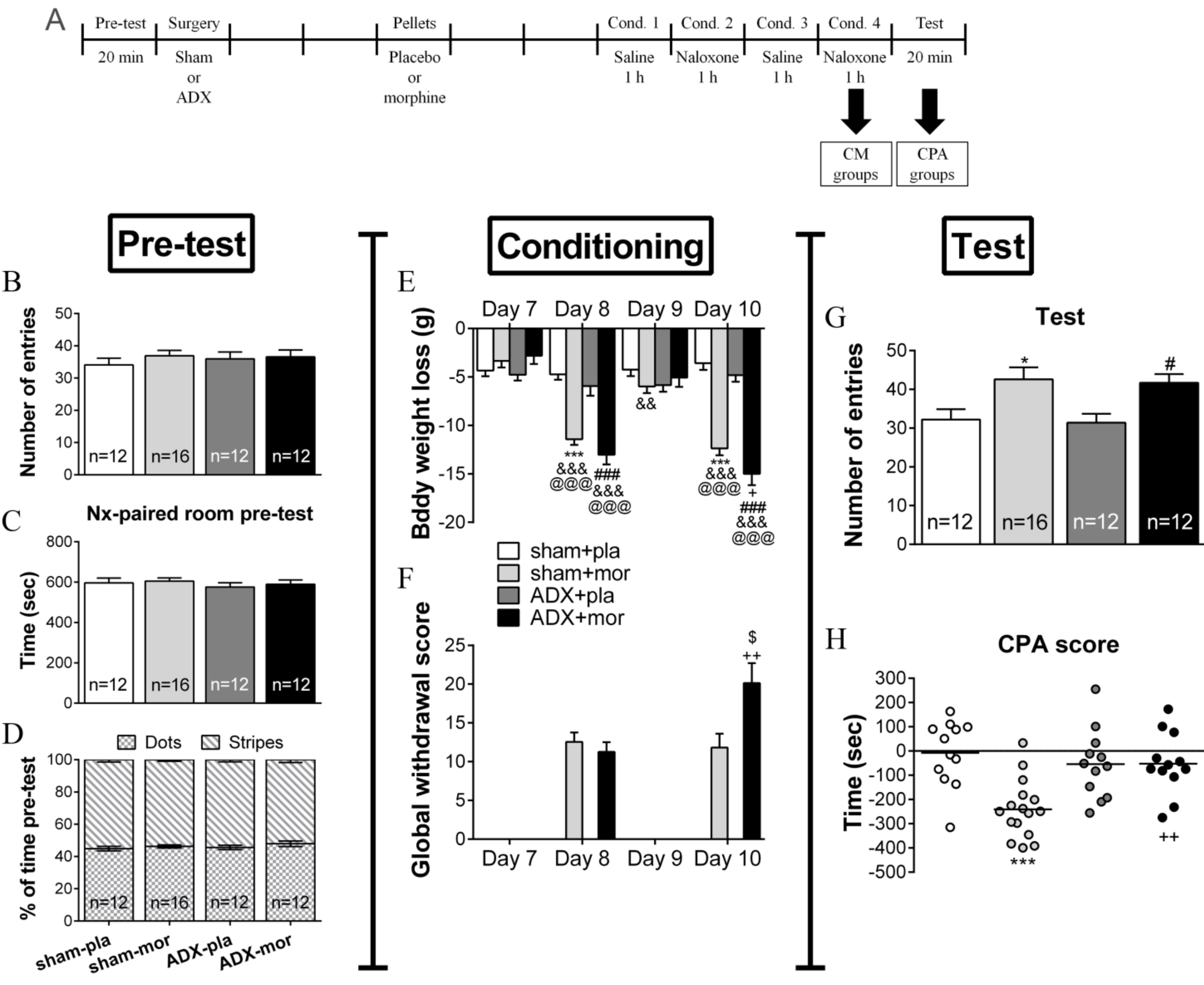
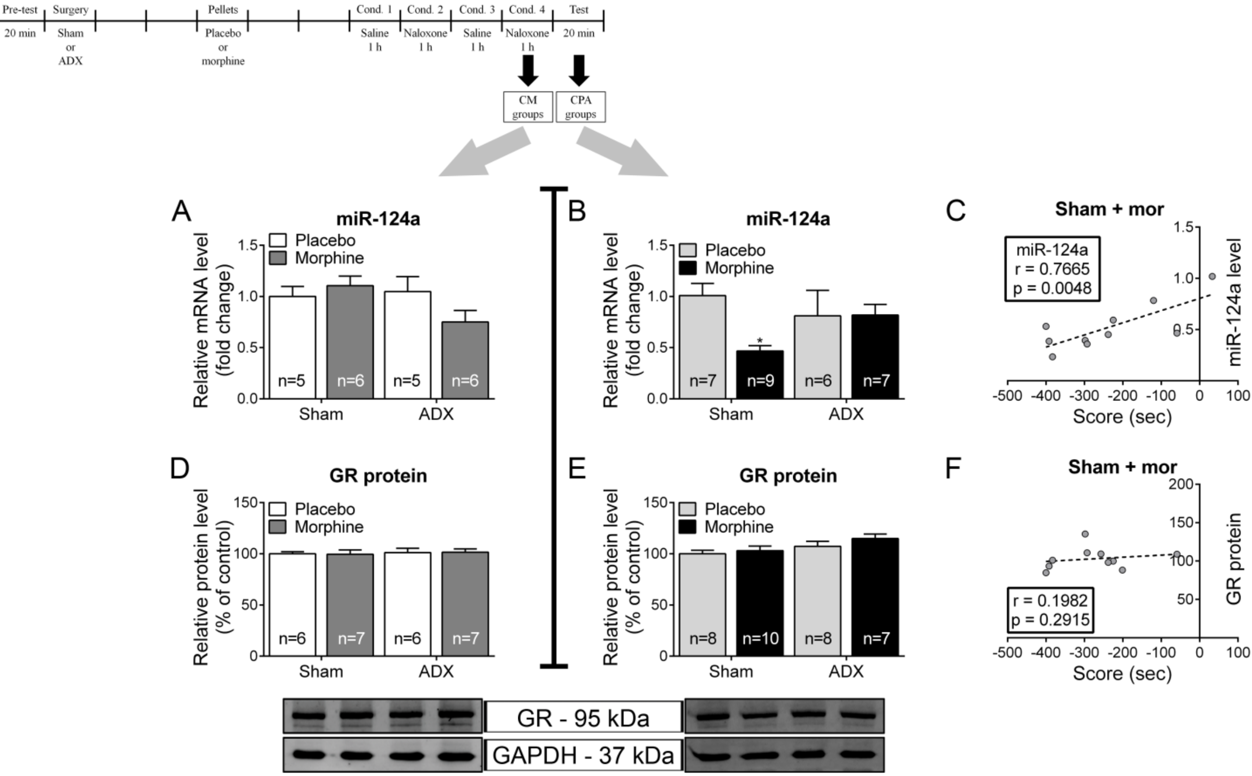
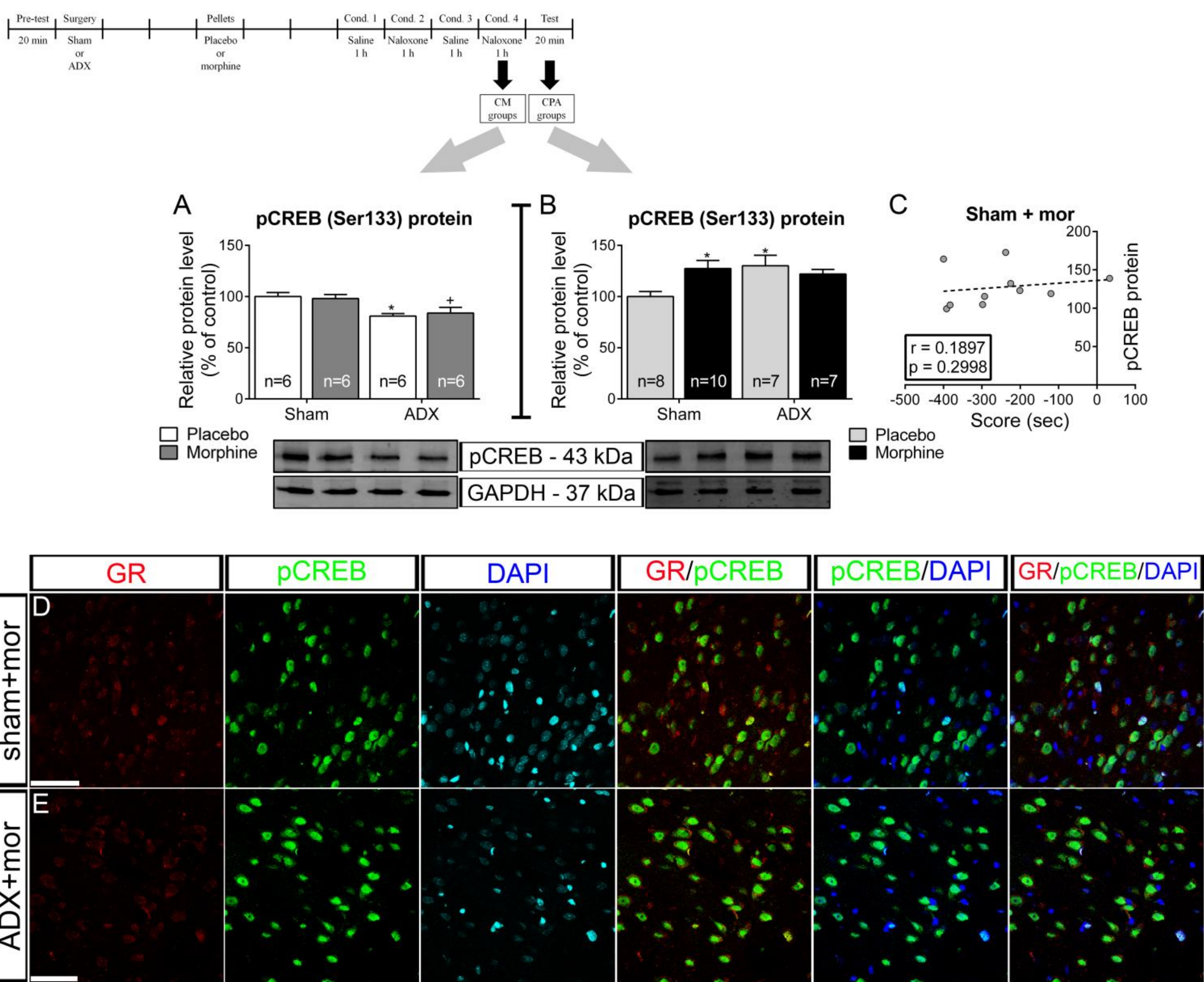
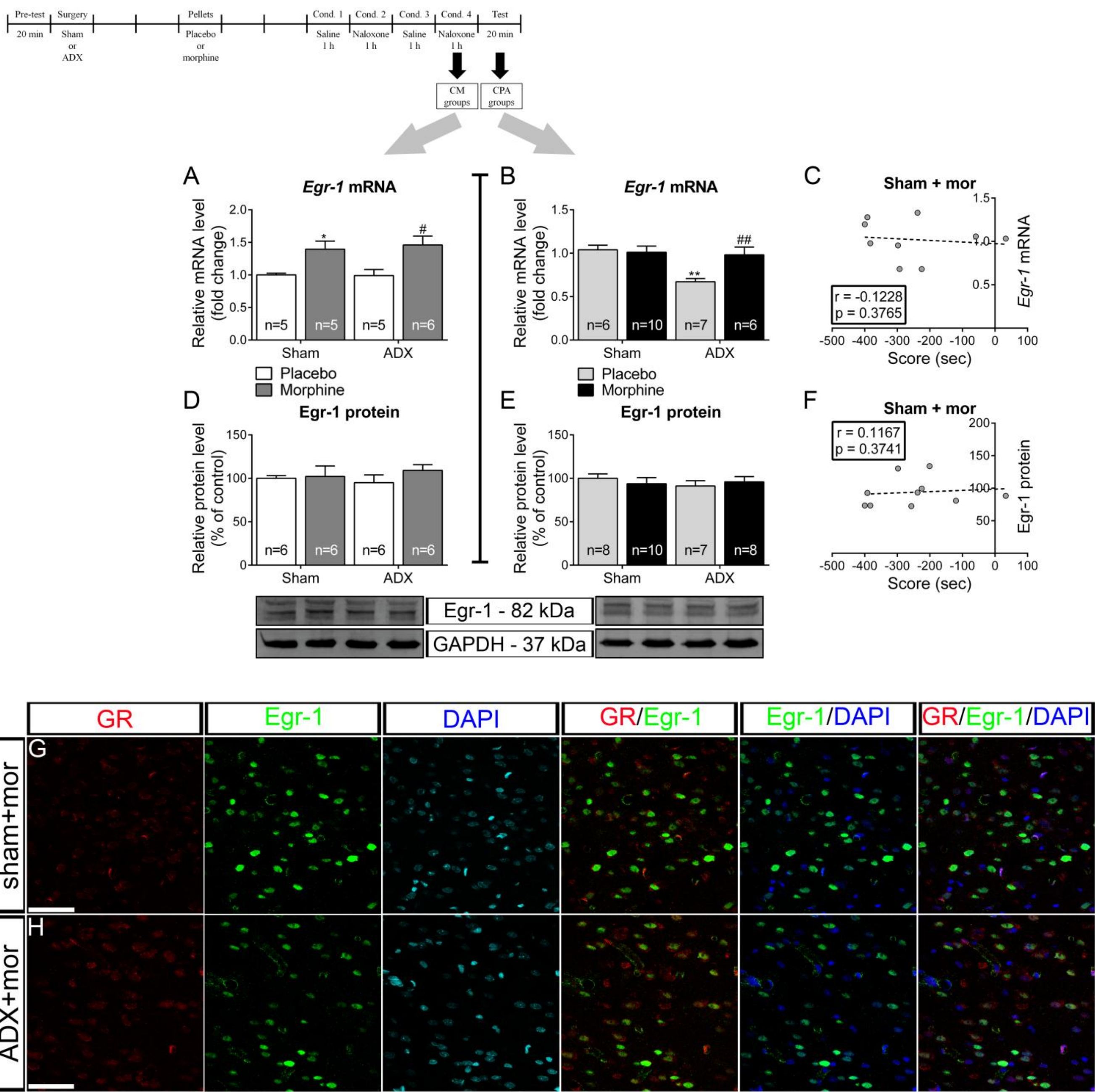
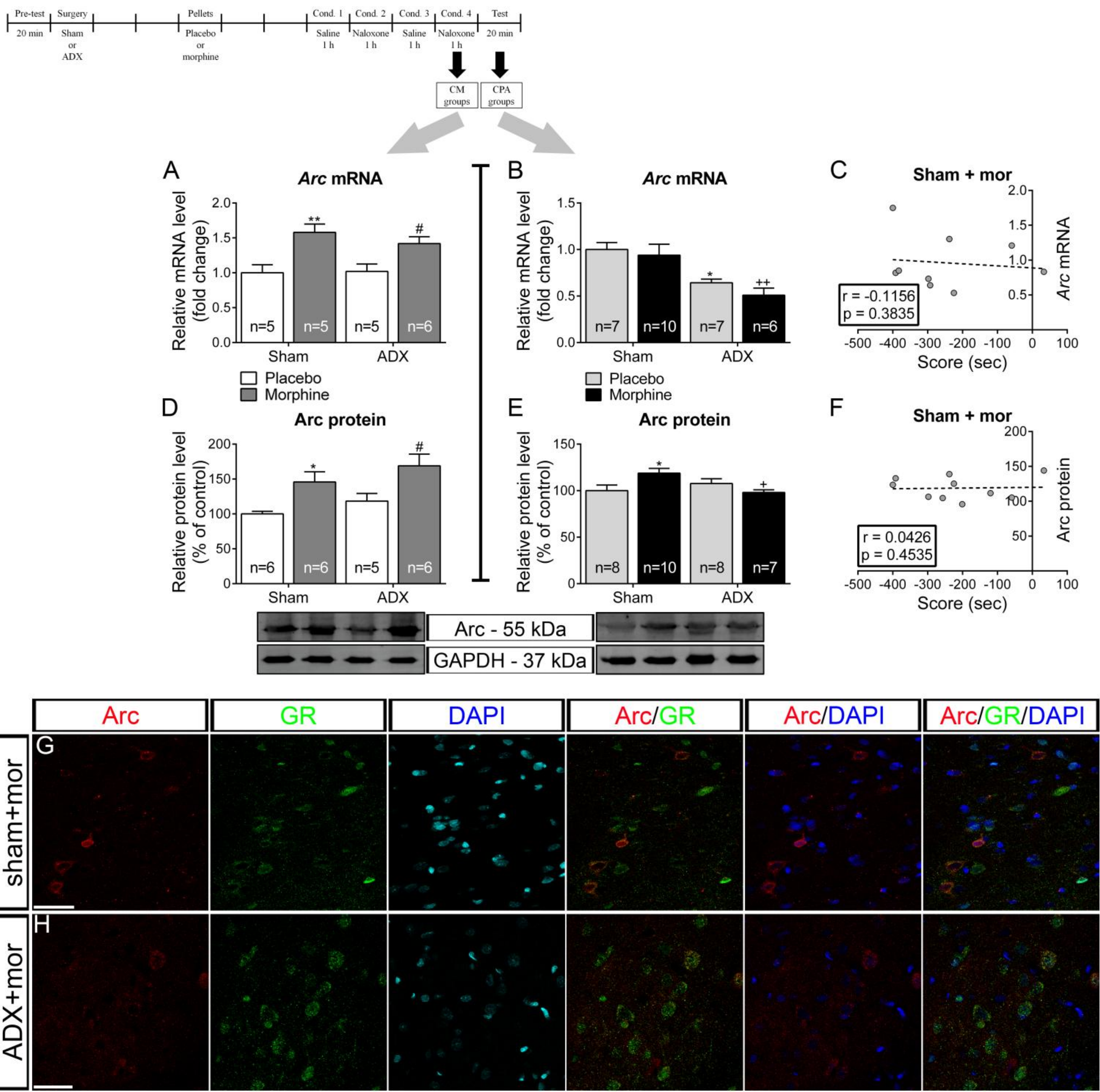
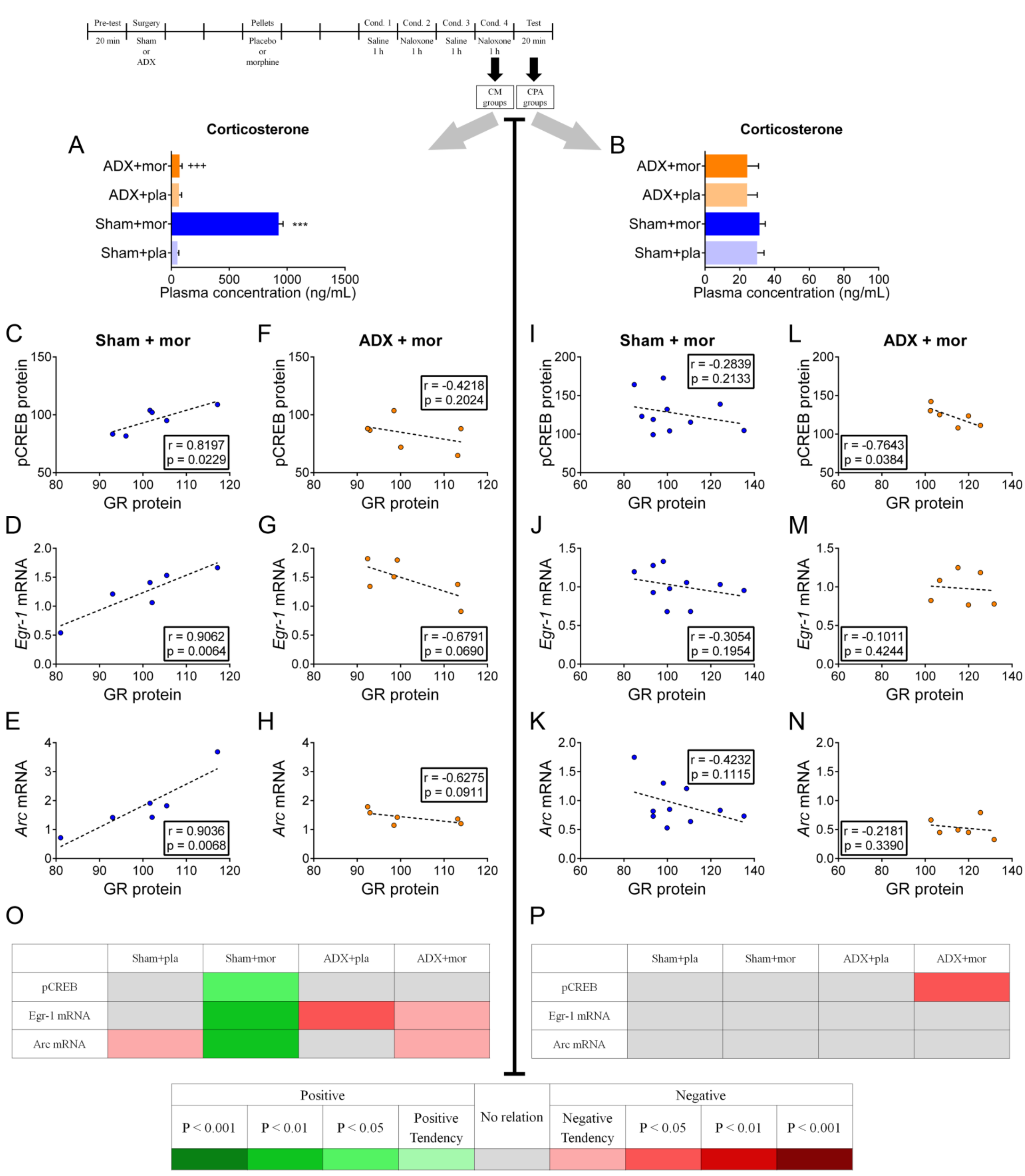

A
Pre-test
20 min
Surgery
Sham
or
ADX
Pellets
Placebo
or
morphine
Cond. 1
Saline
1 h
Cond. 2
Naloxone
1 h
Cond. 3
Saline
1 h
Cond. 4
Naloxone
1 h
Test
20 min
CM
groups
CPA
groups
Pre-test
Conditioning
Test
B
Number of entries
C
Nx-paired room pre-test
Time (sec)
D
Dots
Stripes
% of time pre-test
sham-pla
sham-mor
ADX-pla
ADX-mor
E
Day 7
Day 8
Day 9
Day 10
Bddy weight loss (g)
sham+pla
sham+mor
ADX+pla
ADX+mor
F
Global withdrawal score
G
Test
Number of entries
H
CPA score
Time (sec)


Pre-test
20 min
Surgery
Sham
or
ADX
Pellets
Placebo
or
morphine
Cond. 1
Saline
1 h
Cond. 2
Naloxone
1 h
Cond. 3
Saline
1 h
Cond. 4
Naloxone
1 h
Test
20 min
CM
groups
CPA
groups
A
miR-124a
Placebo
Morphine
Relative mRNA level
(fold change)
n=5
n=6
n=5
n=6
Sham
ADX
B
miR-124a
Placebo
Morphine
Relative mRNA level
(fold change)
n=7
n=9
n=6
n=7
Sham
ADX
C
Sham + mor
miR-124a
r = 0.7665
p = 0.0048
miR-124a level
Score (sec)
D
GR protein
Placebo
Morphine
Relative protein level
(% of control)
n=6
n=7
n=6
n=7
Sham
ADX
E
GR protein
Placebo
Morphine
Relative protein level
(% of control)
n=8
n=10
n=8
n=7
Sham
ADX
F
Sham + mor
r = 0.1982
p = 0.2915
GR protein
Score (sec)
GR - 95 kDa
GAPDH - 37 kDa

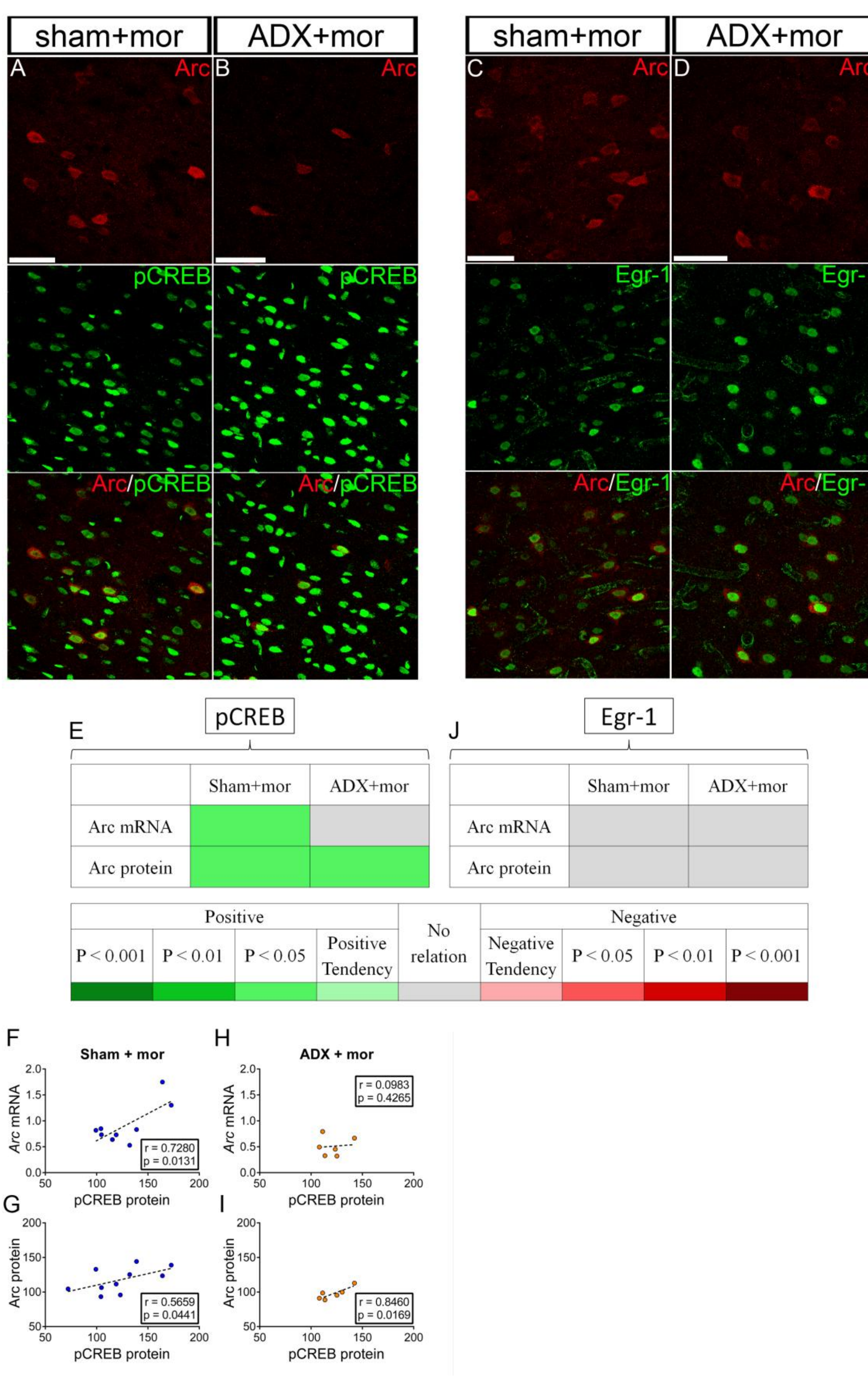

Pre-test
Surgery
Pellets
Cond. 1
Cond. 2
Cond. 3
Cond. 4
Test
20 min
Sham or ADX
Placebo or morphine
Saline 1 h
Naloxone 1 h
Saline 1 h
Naloxone 1 h
20 min
CM groups
CPA groups
A
pCREB (Ser133) protein
Relative protein level (% of control)
Sham
ADX
n=6
Placebo
Morphine
B
pCREB (Ser133) protein
n=8
n=10
n=7
C
Sham + mor
r = 0.1897
p = 0.2998
pCREB protein
Score (sec)
pCREB - 43 kDa
GAPDH - 37 kDa
GR
pCREB
DAPI
GR/pCREB
pCREB/DAPI
GR/pCREB/DAPI
D
E
sham+mor
ADX+mor

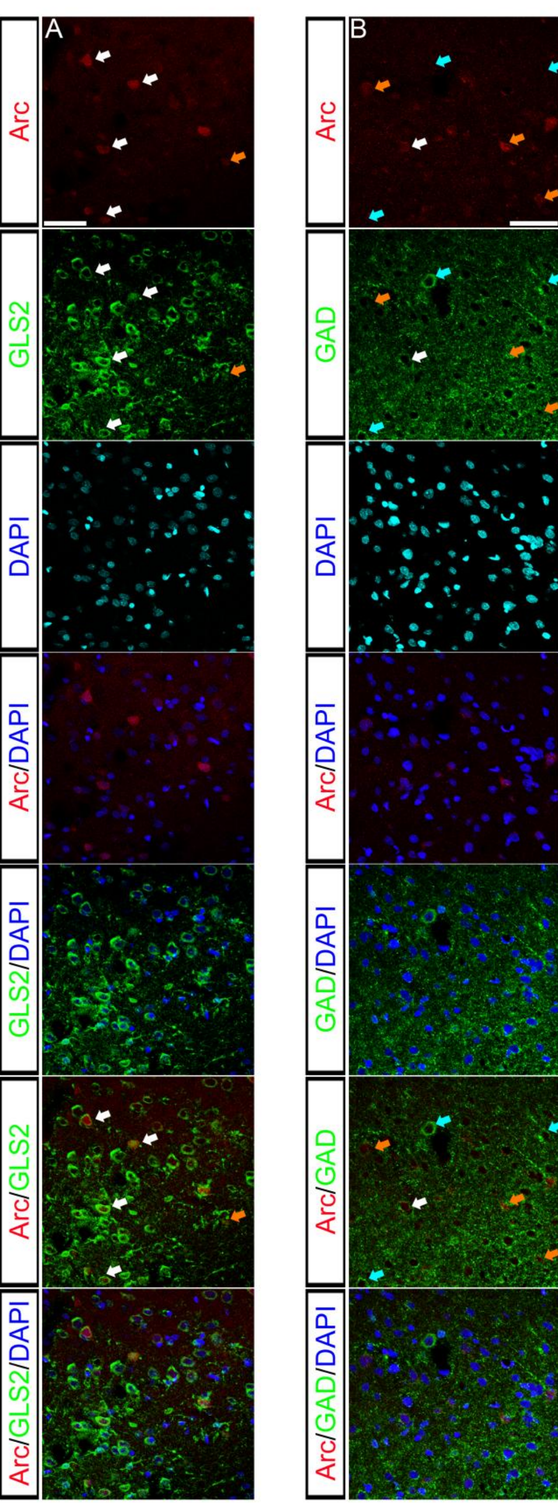

Pre-test
20 min
Surgery
Sham or ADX
Pellets
Placebo or morphine
Cond. 1
Saline 1 h
Cond. 2
Naloxone 1 h
Cond. 3
Saline 1 h
Cond. 4
Naloxone 1 h
Test
20 min
CM groups
CPA groups
A
Egr-1 mRNA
Relative mRNA level (fold change)
Sham
ADX
Placebo
Morphine
B
Egr-1 mRNA
C
Sham + mor
r = -0.1228
p = 0.3765
Egr-1 mRNA
Score (sec)
D
Egr-1 protein
Relative protein level (% of control)
E
Egr-1 protein
F
Sham + mor
r = 0.1167
p = 0.3741
Egr-1 protein
Score (sec)
Egr-1 - 82 kDa
GAPDH - 37 kDa
GR
Egr-1
DAPI
GR/Egr-1
Egr-1/DAPI
GR/Egr-1/DAPI
G
H
sham+mor
ADX+mor


Pre-test
20 min
Surgery
Sham or ADX
Pellets
Placebo or morphine
Cond. 1
Saline 1 h
Cond. 2
Naloxone 1 h
Cond. 3
Saline 1 h
Cond. 4
Naloxone 1 h
Test
20 min
CM groups
CPA groups
A
Arc mRNA
Relative mRNA level (fold change)
Sham
ADX
Placebo
Morphine
B
Arc mRNA
C
Sham + mor
r = -0.1156
p = 0.3835
Score (sec)
Arc mRNA
D
Arc protein
Relative protein level (% of control)
E
Arc protein
F
Sham + mor
r = 0.0426
p = 0.4535
Arc protein
Arc - 55 kDa
GAPDH - 37 kDa
Arc
GR
DAPI
Arc/GR
Arc/DAPI
Arc/GR/DAPI
G
sham+mor
H
ADX+mor



Pre-test
20 min
Surgery
Sham or ADX
Pellets
Placebo or morphine
Cond. 1
Saline 1 h
Cond. 2
Naloxone 1 h
Cond. 3
Saline 1 h
Cond. 4
Naloxone 1 h
Test
20 min
CM groups
CPA groups
A
Corticosterone
ADX+mor
ADX+pla
Sham+mor
Sham+pla
+++
***
Plasma concentration (ng/mL)
B
Corticosterone
C
Sham + mor
pCREB protein
GR protein
r = 0.8197
p = 0.0229
F
ADX + mor
r = -0.4218
p = 0.2024
I
Sham + mor
r = -0.2839
p = 0.2133
L
ADX + mor
r = -0.7643
p = 0.0384
D
Egr-1 mRNA
r = 0.9062
p = 0.0064
G
r = -0.6791
p = 0.0690
J
r = -0.3054
p = 0.1954
M
r = -0.1011
p = 0.4244
E
Arc mRNA
r = 0.9036
p = 0.0068
H
r = -0.6275
p = 0.0911
K
r = -0.4232
p = 0.1115
N
r = -0.2181
p = 0.3390
O
P
Sham+pla
Sham+mor
ADX+pla
ADX+mor
pCREB
Egr-1 mRNA
Arc mRNA
Positive
P < 0.001
P < 0.01
P < 0.05
Positive Tendency
No relation
Negative
Negative Tendency
P < 0.05
P < 0.01
P < 0.001


sham+mor
ADX+mor
A
Arc
B
C
D
pCREB
Egr-1
Arc/pCREB
Arc/Egr-1
E
pCREB
J
Egr-1
Sham+mor
ADX+mor
Arc mRNA
Arc protein
Positive
No relation
Negative
P < 0.001
P < 0.01
P < 0.05
Positive Tendency
Negative Tendency
F
Sham + mor
H
ADX + mor
G
I
Arc mRNA
Arc protein
pCREB protein
r = 0.7280
p = 0.0131
r = 0.0983
p = 0.4265
r = 0.5659
p = 0.0441
r = 0.8460
p = 0.0169


A
Arc
GLS2
DAPI
Arc/DAPI
GLS2/DAPI
Arc/GLS2
Arc/GLS2/DAPI
B
Arc
GAD
DAPI
Arc/DAPI
GAD/DAPI
Arc/GAD
Arc/GAD/DAPI

# SUPPLEMENTAL INFORMATION

## Supplemental materials and methods

### Animals

All surgical and experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE), and were approved by the local Committees for animal research (REGA ES300305440012). Male Wistar rats (n = 78, Harlan, Barcelona, Spain) initially weighting 220–240 g were housed (2-3/cage) on arrival in a room with controlled temperature (22 ± 2°C) and humidity (50 ± 10%), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light-dark cycle (lights on: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments.

### Adrenalectomy

Rats were bilaterally adrenalectomized (ADX) via a dorsal approach under 100 mg/kg ketamine chlorhydrate (Imalgene 1000, Merial, Lyon, France) and 8 mg/kg xylazin (Xilagesic® 2%, Lab. Calier S.A., Barcelona, Spain) intraperitoneally anaesthesia, and implanted s.c. with slow-release corticosterone pellets at surgery. The composition of steroid pellets (25 mg corticosterone plus 75 mg cholesterol) was chosen to provide stable corticosterone concentration corresponding to circadian nadir up to 20 days after implantation (Kovács et al. 2000). ADX rats with corticosterone replacement (ADX plus corticosterone) do not mount a challenge-induced increase of plasma corticosterone. After surgery, ADX plus corticosterone rats had free choice to drink isotonic saline (0.9% NaCl) to replace depleted

sodium secondary to the loss of aldosterone because of adrenalectomy. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation. Sham and ADX plus corticosterone rats were allowed to recover from surgery for 3 days before morphine or placebo pellet implantation. Successful bilateral adrenalectomy was confirmed by plasma concentration of corticosterone and ACTH and by post-mortem examination of the ADX animals.

**Induction of Opiate Dependence**

Morphine base was supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Morphine dependence was induced by s.c. implantation (lower back) under light ether anaesthesia of two slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base). Full dependence on morphine has been previously operationally defined in this model by using a complete naloxone dose effect on various behavioral parameters (spontaneous locomotor activity, operant responding for food, intracranial self-stimulation threshold, conditioned place aversion) (Schulteis et al. 1994), and the rating of abstinence signs showed that opiate dependence was achieved 24 h after implantation of the morphine pellets and remained constant for 15 d (Gold et al. 1994). There were four experimental groups: sham + placebo pellets, sham + morphine pellets, ADX + placebo pellets and ADX + morphine pellets.

**Drugs**

Naloxone hydrochloride (N-7758, Sigma Chemical Co, St Louis, MO, USA) was dissolved in sterile saline (0.9% NaCl; ERN Laboratories, Barcelona, Spain) and injected subcutaneously. Naloxone was administered at a dose of 0.1 mg/kg, 1 ml/kg body weight. Naloxone HCl doses are expressed as the weight of the salt. Sterile 0.9% saline was also injected subcutaneously at a dose of 1 ml/kg. Note that a slightly higher naloxone dose (0.120 mg/kg) has previously been shown to be without effect on place aversion conditioning in placebo control rats (Frenois et al. 2002).

**Conditioned Place Aversion Apparatus**

Briefly, the conditioned place aversion apparatus (Panlab, Barcelona, Spain) used to induce a reliable aversion consists in a box with two equally sized chambers (40 *L* x 34 *W* x 45 *H* cm) interconnected by a rectangular corridor (25 *L* x 13 *W* x 45 *H* cm). Distinctive visual and tactile cues distinguish the compartments: the motifs painted on the walls (either black dots or grey stripes), the floor colouring (black or grey) and the floor texture (smooth or rough). The sensory cues combination that produces a balanced choice are for walls and floor colouring and texture, respectively: (A) black dots, black smooth floor; (B) grey stripes, grey rough floor. Transparent walls are also used to minimize the time the animal spent in the corridor. The weight transducer technology (and PPCWIN software) allows for detection and analysis of animal position throughout the test and the number of entries in each compartment. The experimental protocol consists of three distinct phases: a preconditioning phase, a conditioning phase, and a testing phase. The weight gain of the rats was checked during the entire protocol to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (Houshyar et al. 2004; Núñez et al. 2009). In addition, the animals were

observed for body weight loss and opioid withdrawal behaviours for 15 min after the conditioning phase.

**Conditioned Place Aversion Protocol**

**Pre-testing Phase**

In the pre-testing phase (day 0), animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. Animals showing strong unconditioned aversion (less than 40% of the session time) or preference (more than 60% of the session time) for any compartment were discarded. For each rat, one room was randomly chosen to be paired with naloxone and the other chamber to vehicle. Importantly, after the compartment assignments were completed, there were no significant differences between time spent in the naloxone-paired and the vehicle-paired compartments during the preconditioning phase. This is an important step in the experimental procedure that avoids any preference bias prior to conditioning. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

**Conditioning Phase**

In the second phase (conditioning), rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. In addition, opioid withdrawal behaviours on sham or ADX animals receiving naloxone on days 8 and 10 were measured for 15 min after the conditioning phase. A cohort of animals, named conditioning memory rats (CM), were decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

**Testing Phase**

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). The difference ($\Delta D = D - D0$) between the time spent in the naloxone-paired compartment after conditioning ($D$) minus the time spent in the same compartment before conditioning (preconditioning test $D0$) reflects the change of preference induced by opiate withdrawal. A negative score indicates a place aversion; a positive score indicates a place preference. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

**Measurement of the withdrawal syndrome**

Experiments were carried out in a quiet room. The observer was unaware of the drug combination used. Sham + mor and ADX + mor rats were individually placed into transparent plastic cages after the conditioning phase and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 15 min on days 8 and 10 (naloxone injection). Subsequently, previously identified behavioural characteristics of the rat opiate abstinence syndrome (Lu et al. 2000) were evaluated, including jumping, wet-dog shakes, paw tremor, sniffing, writhing, tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble. The number of jumping, wet-dog shakes, paw tremor, sniffing and writhing was counted as the number of events occurring during the total test time period (graded signs). Tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble were scored 1 for appearance or 0 for non-appearance within each 5 min time. To obtain a comprehensive index of the severity of somatic opiate withdrawal including all the signs

examined, a global withdrawal score was calculated for each animal by giving each individual sign a relative weight as previously reported (Maldonado et al. 1996): jumping, x0.8; wet-dog shakes, x1; paw tremor, x0.35; sniffing, x0.5; writhing, x0.5; tremor, x1.5; ptosis, x1.5; diarrhoea, x1.5; mastication, x1.5; piloerection, x1.5; teeth chattering, x1.5; salivation, x1.5; rhinorrhea, x1.5; chromodacryorrhea, x1.5; and scrabble, x1.5. Body weight loss was determined as the difference between the weight determined immediately before naloxone injection and that determined 1 hour later, after the conditioning phase.

**Preparation of tissue extract**

Rats were decapitated on days 10 and 11 (between 10:00-12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed, and stored immediately at -80°C until use for quantitative real-time PCR (qPCR) and Western blot analysis. Brains were sliced on a cryostat and kept at -20°C until each region of interest comes into the cutting plane. For BLA study, three consecutive 500-µm coronal slides were made corresponding to approximately -1.9 to -3.4 mm from bregma, according to the atlas of (Figure S1) (Paxinos and Watson 2007). The anatomical locations and boundaries of each region were determined using the rat brain Atlas of (Paxinos and Watson 2007). Bilateral punches of the BLA were collected into Eppendorf tubes, according to the method of (Leng et al. 2004).

**RNA extraction and quantitative real-time PCR (qPCR)**

One punch from the BLA was placed in an Eppendorf tube containing 30 µl of Trizol® reagent (Invitrogen Corp., Carlsbad, CA, USA) and rapidly stored at -80°C. Frozen brain

tissue samples were homogenized in Trizol® reagent (Invitrogen Corp., USA) and total RNA was isolated with QIAGEN miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination DNase I treatment were used and 100 µl Rnase-free DNase I (1 unit DNase) (Thermo Scientific, USA) solution was added. Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific, USA). Amplification was not detected in the RT-minus controls. The cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for the comparative Ct experiments were designed by Primer Express 3.0 Program. The primers (Microsynth, Balgach) were used in the Real-Time PCR reaction with Fast EvaGreen® qPCR Master Mix (Biotium, USA) on ABI StepOnePlus instrument was listed. The reverse transcription for microRNA assays were carried out by TaqMan MicroRNA Reverse Transcription Kit according the manufacturer's instruction. The microRNA expressions were detected using TaqMan MicroRNA assays.

**Western Blotting**

Four bilateral punches from VTA were placed in homogenization buffer. The Eppendorf tubes contained 100 µl of homogenization buffer. The tubes were frozen immediately on dry ice and stored at −80°C until assaying. Samples were sonicated, vortexed and sonicated again prior to centrifugation (6000× g; 10 minutes at 4°C). Samples containing equal quantities of total proteins (20–40 mg, depending on the protein of interest) were separated by 10% or 12% SDS-PAGE (depending on the molecular weight of the protein of interest) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS containing 0.15 % Tween-20 (TBS-T), 1% BSA for 90 minutes at room temperature (RT), and incubated overnight at 4ºC with the primary antibody diluted in 1% BSA in TBS-T. The primary antibodies used are referenced in Table S1. Blots were then washed and incubated for

90 min at RT in TBS-T with 1% BSA with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies: goat anti-rabbit (1:5000; sc-2004, Santa Cruz Biotechnology) or goat anti-mouse (1:5000; sc-2005, Santa Cruz Biotechnology). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). We used GAPDH as our loading control. Before reprobing, blots were stripped by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37°C. Blots were subsequently reblocked and probed with GAPDH. The ratios of protein of interest/GAPDH were plotted and analyzed. Protein levels were corrected for individual levels.

**Table S1. Primary antibodies used in this study**

| Antibody | Host | Supplier | Catalog Number | WB dilution | IF dilution |
|---|---|---|---|---|---|
| GR | Rabbit | Santa Cruz Biotechnology | sc-1004 | 1:500 | 1:600 |
| pCREB | Rabbit | Millipore | # 06-519 | 1:2,000 | 1:3,000 |
| Egr-1 | Rabbit | Santa Cruz Biotechnology | sc-189 | 1:500 | 1:400 |
| Arc | Mouse | Santa Cruz Biotechnology | sc-17839 | 1:500 | 1:400 |
| GAPDH | Rabbit | Cell Signaling Technology Inc. | #2118 | 1:5,000 | -- |
| GR | Mouse | Abcam | ab2768 | -- | 1:600 |
| GLS2 | Rabbit | Abcam | ab113509 | -- | 1:1,000 |
| GAD-65/67 | Goat | Santa Cruz Biotechnology | sc-7513 | -- | 1:200 |
| CRF | Rabbit | Provided by Wylie W. Vale | -- | -- | 1:500 |

**Immunofluorescence study**

Another set of rats was deeply anaesthetised with an overdose of pentobarbital (100 mg/kg i.p.) and perfused transcardially with 250 ml 0.9% saline following by 500 ml cold fixative solution containing paraformaldehyde (4% paraformaldehyde in 0.1 M borate buffer, pH 9.5). After removal of the perfused brains, they were post fixed in the same fixative solution containing sucrose (30%) for 3 h and stored at 4ºC in PBS containing 30% sucrose until coronal sections (30-mm thickness) were cut rostrocaudally on a freezing microtome (Leica, Nussloch, Germany). The atlas of Paxinos and Watson (2007) (Paxinos and Watson 2007) was used to identify different brain regions. The sections were cryoprotected and stored at -20ºC until use.

Brain sections were rinsed in PBS and an antigen retrieval procedure was applied by treating sections with citrate buffer (10 mM citric acid in 0.05% Tween 20, pH 6.0) at 60°C for 20 min. Non-specific Fc binding sites were blocked with 3% normal horse serum/0.3% Triton-X-100 in PBS for 1 h at RT, and the sections were incubated for 72 h (4ºC, constant shaking) with primary antibodies described in Table S1. Alexa Fluor 488 donkey anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 488 donkey anti-goat IgG (1:1000; A-11055, Invitrogen), Alexa Fluor 594 donkey anti-goat IgG (1:1000; A-11058, Invitrogen) and Alexa Fluor 594 donkey anti-mouse IgG (1:1000; A-21203, Invitrogen) labelled secondary antibodies were applied for 4 h. After washing, sections were incubated in 4, 6-diamino-2-phenylindole (DAPI, 1:100,000) for 1 min and the sections were mounted in ProLong® Gold antifade reagent (Invitrogen).

**Confocal analysis**

The brain sections were examined using a Leica DMIRE2 confocal microscope and Leica Confocal Software (Leica Microsystems). Images from the BLA were captured from low magnification to high magnification (40X to 63X oil objective). Confocal images were obtained using 405-nm excitation for DAPI, 488-nm excitation for Alexa Fluor 488 and 594-nm excitation for Alexa Fluor 594. Emitted light was detected in the range of 450-460 nm for DAPI, 515-530 nm for Alexa Fluor 488 and 610-630 nm for Alexa Fluor 594. Every channel was captured separately to avoid spectral crosstalking. Series of optical sections were performed determining an upper and lower threshold using the Z/Y position for Spatial Image Series setting. The optical series covered 20 µm of thickness through the tissue. The confocal microscope settings were established and maintained by Leica and local technicians for optimal resolution.

**Radioimmunoassay**

Blood was collected on days 10 and 11 into ice-cooled tubes containing 5% EDTA and was then centrifuged (500 g; 4ºC; 15 min). Plasma was separated, divided into two aliquots and stored at -80ºC until assayed for corticosterone or ACTH. Plasma concentration of corticosterone and ACTH were quantified using specific corticosterone and ACTH antibodies for rats ([$^{125}$I]-CORT and [$^{125}$I]-ACTH RIA; MP Biomedicals, Orangeburg, NY, USA). The sensitivity of the assay was 7.7 ng/mL for corticosterone and 5.7 pg/mL for ACTH.

**Reagents**

Protease inhibitors were purchased from Boehringer Mannheim, (Mannheim, Germany); phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany); HPLC reagents were purchased from Sigma Chemical Co.

**Data Analysis**

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test to determine specific group differences. Student's *t*-test was used when comparisons were restricted to two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

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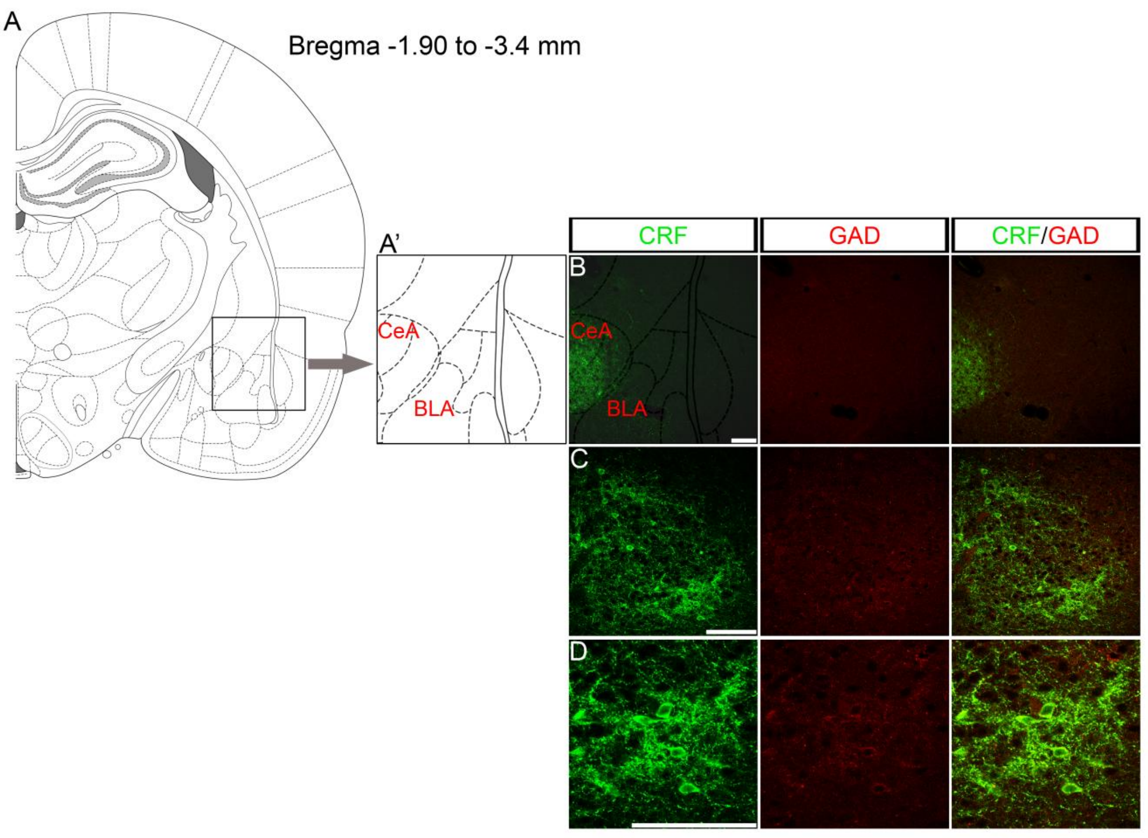
## Legends to supplemental figures

**Figure S1: (A-A')** The analysed region within BLA is schematically illustrated (diagram modified from Paxinos and Watson, 2007); coordinate is mm from Bregma. **(B-D)** Example of low and high magnification micrographs showing coronal section of rats immunostained for CRF (green) and glutamic acid decarboxylase 65/67 (GAD-65/67; red); scale bar, 100 µm. Merged images are also depicted. CRF neurons, which are selectively expressed in the CeA, were immunolabelled to serve as a guide to study the BLA (BLA is located lateral to the CeA). There are two forms of GADs that are found in the brain: GAD-65 and GAD-67. GAD-65 and GAD-67 are members of the group II decarboxylase family of proteins and are responsible for catalyzing the rate limiting step in the production of GABA from L-glutamic acid. GAD-67 is responsible for the basal levels of GABA synthesis. In the case of a heightened demand for GABA in neurotransmission, GAD-65 will transiently activate to assist in GABA production. The antibody that we used is recommended for detection of GAD-65 and GAD-67 of rat origin, thus is a good marker for GABAergic neurons. In addition, we observed that $CRF^+$ neurons co-expressed GAD, as previously described in the literature (Meister et al. 1988; Day et al. 1999; Bajo et al. 2008).


A
Bregma -1.90 to -3.4 mm
A'
CeA
BLA
CRF
GAD
CRF/GAD
B
C
D

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