Universitat Autònoma de Barcelona

Paper del receptor H_3 d'histamina en la neurotransmissió dopaminèrgica i el desig d'autoadministració de cocaïna.

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Memòria presentada per Santi Rosell Vilar, Llicenciat en Psicologia, per optar al grau de Doctor en Neurociències per la Universitat Autònoma de Barcelona.

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Director de la tesi	Doctorand
Dr. Jordi Ortiz de Pablo	Santi Rosell Vilar

Bellaterra, Octubre 2012

Cap nombre d'experiments,
per molts que siguin,
podran demostrar que tingui raó.
Només un experiment pot demostrar
que estic equivocat.

(Albert Einstein)

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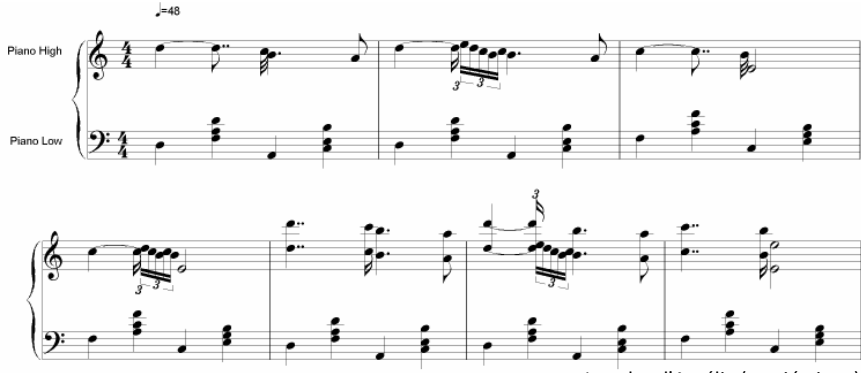
ABREVIATURES

AMPc	Adenosín monofosfato-3',5' cíclico
COMT	Catecol-O-metiltransferasa
DA	Dopamina
DAT	Dopamine Transporter
GABA	Àcid-aminobutíric
MAO	Monoaminooxidasa
NAc	Nucli accumbens
NET	Norepinephrine Transporter
NMDA	N-metil D-aspartat
PKA	Proteina Kinasa A
SERT	Serotonin Transporter
5-HT$_{1,2,3,4}$	Receptors de Serotonina
SN	Substància nigra
SNC	Sistema Nerviós Central
SNc	Substància nigra compacta
SNr	Substància nigra reticulata
VTA	Àrea tegmental ventral

INTRODUCCIÓ



La valse d'Amélie (versió piano)
Yann Tiersen

INTRODUCCIÓ

Revisió històrica de la dopamina

La L-Tirosina (del Grec *tyros,* significa formatge), va ser descoberta l'any 1846 pel químic alemany Justus von Liebig en el formatge, i la va obtenir a partir de la caseïna, una fosfoproteïna present a la llet i alguns derivats làctics (Rogers 2011).

La L-Dopa (3,4-dihidroxifenilalanina) va ser aïllada per primera vegada l'any 1913 a partir d'un tipus de faves **(Figura 1)** per Marcus Guggenheim (Fahn 2008). Dos anys abans Casimir Funk havia sintetitzat D,L-Dopa al laboratori. Ambdós investigadors van suposar que aquest aminoàcid devia ser un compost derivat de la adrenalina (Hornykiewicz 2002a).

Figura 1. *Planta Vicia Faba. Il·lustració de Thomé, Flora von Deutchland, 1885.*

La dopamina (DA) va ser sintetitzada per primera vegada als Wellcome laboratories de Londres l'any 1910 per George Barger i James Ewens. Poc després als mateixos laboratoris Henry Dale (Premi Nobel de fisiologia l'any 1936), va investigar l'activitat biològica de la DA i va concloure que tenia una funció simpaticomimètica com la adrenalina (Hornykiewicz 2002b).

L'enzim tirosina Hidroxilasa responsable de catalitzar la conversió de l'aminoàcid L-Tirosina a L-Dopa va resultar difícil d'aïllar però finalment és va aconseguir per primera vegada l'any 1964 en la medul·la adrenal de vaca (Fahn 2008).

El descobriment realitzat per Peter Holtz et al. l'any 1938 del amino-àcid-aromàtic-descarboxilasa (catalitza la descarboxil·lació de la L-dopa davant DA) en ronyons de mamífers va ser un punt d'inflexió en la recerca on Hermann Blaschko va postular la via metabòlica de la DA **(Figura 2).**

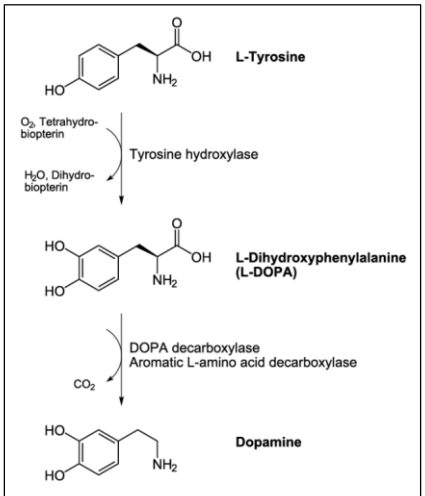


Figura 2. *Biosíntesis de la Dopamina.*

La DA o 3,4-dihidroxifeniletilamina **(Figura 3)** forma part del grup de les catecolamines, que son compostos químics formats per un nucli catecol (un anell benzè amb dos grups hidroxils) i una cadena d'etilamina o algun dels seus derivats.

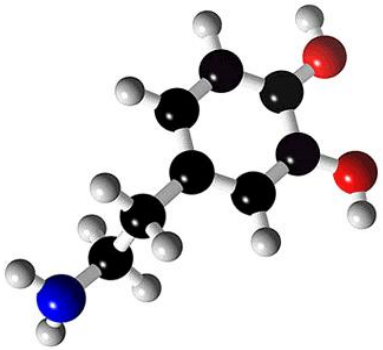


Figura 3. *Estructura tridimensional de la DA.*

El descobriment de la DA a l'encèfal com a neurotransmissor es remunta cap als anys seixanta per Arvid Carlsson (Carlsson, Falck et al. 1962). Posteriorment l'estudi dels seus mecanismes de transducció de senyal intracel·lular per Paul Greengard va valer per ambdós el premi Nobel de Medicina l'any 2000. Aquest neurotransmissor està implicat en moltes funcions cerebrals com pot ser l'aprenentatge, control de la locomoció, secreció neuroendocrina (Jaber, Robinson et al. 1996) i la recompensa al menjar. El seu desequilibri està implicat en varies malalties com el Parkinson (Stoessl 2011), Corea de Huntington (Seeman and Van Tol 1994), Trastorn Dèficit Atenció més Hiperactivitat (Brown, Biederman et al. 2011), Esquizofrènia (Raznahan, Greenstein et al.

2011) i la dependència a drogues com l'amfetamina o la cocaïna (Di Chiara, Bassareo et al. 2004) entre d'altres patologies. S'han postulat moltes funcions en les quals està implicada la DA en l'encèfal i a vegades no són del tot clares.

La DA va ser el primer neurotransmissor identificat que estava implicat en la modulació de la motivació (Ungerstedt 1971). La lesió de la via dopaminèrgica nigroestriatal produïa un dèficit en la ingesta del beure i del menjar en rates (Smith, Storhmayer et al. 1972; Ervin, Fink et al. 1977), així com també la lesió de la via dopaminèrgica mesolímbica produïa una disminució de l'activitat locomotora (Smith 1976). Amb el desenvolupament d'antagonistes selectius dopaminèrgics com la pimozida és va poder comprovar que administrats en rates produïen anhedònia i inhibien l'interés per buscar el menjar (Wise, Spindler et al. 1978).

És va poder comprovar que els neurolèptics atenuaven o inhibien els efectes de la recompensa obtinguda quan s'estimulava elèctricament l'hipotàlem lateral (Fouriezos, Hansson et al. 1978), s'administrava amfetamina endovenosa (Yokel and Wise 1975) o cocaïna (De Wit and Wise 1977). Aquesta atenuació dels efectes de la recompensa provocava que els animals augmentessin la cerca per la droga incrementant així el numero de palancades en les sessions d'autoadministració de cocaïna (Haney and Spealman 2008).

Regulació de la síntesi i l'alliberament de dopamina

La síntesi d'aquest neurotransmissor té lloc a les terminals dopaminèrgiques, on es troba en gran concentració l'enzim tirosina hidroxilasa (Freund, Powell et al. 1984).

Els treballs de Nagatsu (Nagatsu, Levitt et al. 1964) i Levitt (Levitt, Spector et al. 1965) van demostrar que la hidroxilació de l'aminoàcid L-tirosina era el punt limitant de regulació de la síntesi de les catecolamines en el SNC.

Regulació per autoreceptors

Diversos estudis tant *in vivo* com *in vitro* han demostrat que agonistes dopaminèrgics disminueixen la síntesi de DA (Hetey, Kudrin et al. 1985; Onali and Olianas 1989), actuant sobre autoreceptors localitzats a les terminals dopaminèrgiques (veure receptors de DA, pàg. 15). L'efecte inhibidor és bloquejat per antagonistes dopaminèrgics i es troba mediat per receptors que pertanyen a la família D_2. L'activació d' aquests receptors també inhibeix l'alliberament de DA (Boyar and Altar 1987; Herdon, Strupish et al. 1987).

Alguns estudis indiquen que dins la família D_2, el subtipus D_3 podria ser l'autoreceptor responsable de la regulació de la síntesi i l'alliberament de DA als somes i dendrites (Diaz, Pilon et al. 2000) però no a terminacions dopaminèrgiques estriatals (Koeltzow, Xu et al. 1998). Les terminals dopaminèrgiques contenen autoreceptors que pertanyen a la família D_2, que en activar-se, redueixen l'alliberament de DA (Dwoskin and Zahniser 1986; Watanabe, Suda et al. 1987).

Està demostrat que hi ha dues isoformes del receptor dopaminèrgic D_2: D_{2L} (long) (Raznahan, Greenstein et al. 2011) i D_{2S} (short) (Xu, Hranilovic et al. 2002). La isoforma $D_{2S,}$ es troba principalment en terminals presinàptiques, i la isoforma D_{2L}, està en terminals postsinàptiques (Lindgren, Usiello et al. 2003).

Regulació per heteroreceptors

Altres neurotransmissors poden modular la síntesi i l'alliberament de la DA activant receptors presents en les terminals dopaminèrgiques. La regulació de la síntesi de DA està mediada en part per receptors NMDA de glutamat. La qual cosa suggereix que en les terminals dopaminèrgiques estriatals hi ha aquests receptors localitzats presinapticament. D'acord amb aquesta hipòtesi, s'ha observat que els receptors NMDA de glutamat, estimulen l'activitat de la tirosina hidroxilasa estriatal (Arias-Montano, Martinez-Fong et al. 1992a).

Estudis anatòmics han demostrat que les neurones serotoninèrgiques innerven tant la SN com VTA (Moukhles, Bosler et al. 1997).

L'existència d'una relació funcional entre neurones dopaminèrgiques i serotoninèrgiques ha estat confirmada per una sèrie d'estudis que van demostrar la inhibició o l'alliberament de DA en presència d'agonistes o antagonistes de serotonina. L'acció de la serotonina a l'incubar-la amb estriat de rata va fer augmentar la DA alliberada, el que suggeria que la serotonina tenia una acció activadora sobre la DA mediada per 5-HT_4 (Bonhomme, De

Deurwaerdere et al. 1995). Sigui mediat per 5-HT_1 o 5-HT_4 la serotonina sempre sembla estimular l'alliberament. Estudis de microdiàlisis en rata van observar que l'administració a l'estriat d'un antagonista 5-HT_1 inhibia l'alliberament de DA (Benloucif and Galloway 1991). També s'ha observat que els receptors 5-HT_{2A} i 5-HT_3 de serotonina presents en VTA regulen l'activació de les neurones dopaminèrgiques (Adell and Artigas 2004).

Una característica de l'estriat és la densa població de neurones dopaminèrgiques i interneurones colinèrgiques (Zhou, Liang et al. 2001). La interacció i bona cooperació d'aquestes dues poblacions neuronals son importants per al bon funcionament del cos estriat (Di Chiara, Morelli et al. 1994; Saka, Iadarola et al. 2002).

Hi ha dos tipus de receptors d'acetilcolina: nicotínics i muscarínics.

Les interneurones colinèrgiques actuen a l'estriat a través dels receptors nACh regulant l'alliberament de la DA (Zhou, Liang et al. 2001). Hi ha una gran densitat de receptors nicotínics en SNc i VTA, els quals tenen un paper important en funcions fisiològiques com el control voluntari del moviment, memòria, atenció, ansietat, entre d'altres (Le Novere, Corringer et al. 2002). La nicotina injectada en VTA feia augmentar la DA en el NAc, així com l'administració d'antagonistes nicotínics en VTA bloquejava l'alliberament de DA (Nisell, Nomikos et al. 1994). Experiments electrofisiològics demostren que la nicotina és capaç d'augmentar l'alliberament de DA tant in vitro com in vivo (Pidoplichko, DeBiasi et al. 1997; Picciotto, Zoli et al. 1998). Les rates que se'ls va injectar nicotina al NAc, van mostrar sensibilització locomotora als efectes

estimulants de la nicotina administrada via endovenosa (Kita, Okamoto et al. 1992), mentres que les injeccions a l'estriat d'antagonistes nicotínics bloquejava les estereotípies produïdes per la amfetamina (Karler, Calder et al. 1996). Tots aquests resultats suggereixen que els receptors nicotínics (Nisell, Nomikos et al. 1994), podrien jugar un paper important en el control de la locomoció i en conductes relacionades amb l'abús de drogues.

Es conegut que els receptors M_5 estimulen l'alliberament de DA (Forster, Yeomans et al. 2002). Encara que també sembla haver un subtipus de receptors muscarínics implicats en l'inhibició de l'alliberament de DA, es creu que GABA tindria un paper fonamental en aquesta inhibició ja que les neurones GABAèrgiques tenen en l'estriat una gran densitat de receptors muscarínics (Kemel, Desban et al. 1989).

Està descrit que els ganglis basals tenen una alta densitat de receptors $GABA_B$ (Bowery, Parry et al. 1999). A més, aquests receptors modulen l'alliberament de DA en l'estriat (Smolders, De Klippel et al. 1995), així com també inhibeixen la seva síntesi (Arias-Montano, Martinez-Fong et al. 1991; Arias Montano, Martinez-Fong et al. 1992b). Els receptors $GABA_B$ poden modular l'excitabilitat de les neurones glutamatèrgiques i dopaminèrgiques a l'estriat (Bowery 1993).

En resum, està demostrat que les terminals dopaminèrgiques tenen receptors GABA, glutamat, acetilcolina i serotonina (Raiteri, Marchi et al. 1982; Roberts, McBean et al. 1982; Raiteri, Leardi et al. 1984). Estudis *in vivo* i *in vitro* han demostrat que l'alliberament

de DA és estimulat per l'activació de receptors glutamatèrgics NMDA (Romo, Cheramy et al. 1986; Jhamandas and Marien 1987; Martinez-Fong, Rosales et al. 1992) $GABA_A$ (Giorguieff, Kemel et al. 1978; Starr 1978) i colinèrgics (Lehmann and Langer 1982).

Per altre costat, també s' ha observat que l'alliberament de DA s' inhibeix quan s' estimulen receptors $GABA_B$ (Bowery, Hill et al. 1980; Reimann, Zumstein et al. 1982; Reimann 1983).

Transport de dopamina

VMAT2 son proteïnes de membrana vesicular encarregades de captar del citosol el neurotransmissor i portar-lo cap a l'interior de les vesícules sinàptiques mitjançant un transport actiu de tipus secundari (Zheng, Dwoskin et al. 2006). Aquest es realitza en contra gradient d'alta concentració utilitzant una ATP-asa de protons (Wimalasena 2011). Un cop dins es guardarà per a la utilització posterior mitjançant exocitosi. L'altre part del neurotransmissor que ha quedat al citosol serà degradat per l'enzim monoamino oxidasa (MAO). VMAT2 s'expressa exclusivament en totes les neurones aminèrgiques de l'encèfal (Eiden and Weihe 2011), encara que *Lohoff et al.* afirma que VMAT1 també hi és present (Lohoff 2010).

El transportador de DA (DAT) és una proteïna que és localitza a les membranes presinàptiques de les neurones dopaminèrgiques (Ciliax, Heilman et al. 1995), tant a les membranes varicoses i no varicoses, però no a les zones sinàptiques actives (Hersch, Yi et al. 1997). La seva funció és recaptar la DA de l'espai sinàptic per regular la duració de la seva acció (Mortensen and Amara 2003).

Una part de la DA és recaptada cap a l'interior de les neurones on es degradada per l'enzim MAO que es troba a la membrana mitocondrial externa, en canvi l'altre part és degradada per l'enzim catecol-O-metiltransferasa que es troba a la membrana neuronal postsinàptica, i col·labora amb la inactivació de l'acció del neurotransmissor, degradant-lo en l'exterior cel·lular (Cumming, Brown et al. 1992) **(Figura 4).**

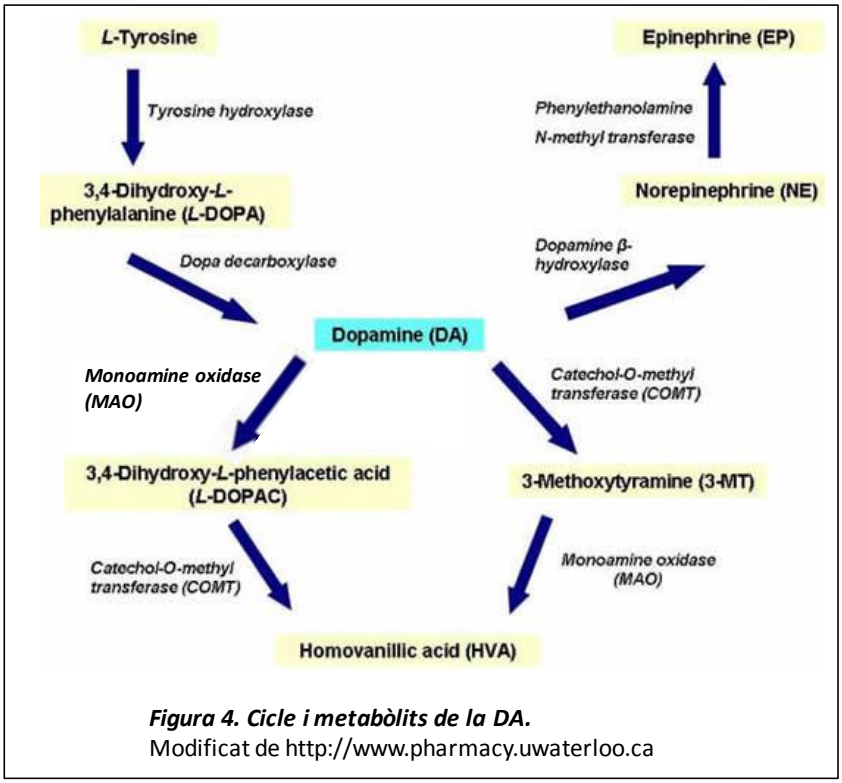


Figura 4. Cicle i metabòlits de la DA.
Modificat de http://www.pharmacy.uwaterloo.ca

Els psicoestimulants, com metilfenidat, amfetamina i cocaïna, exerceixen molts dels seus efectes al bloquejar el DAT (Koob and Caine 1999), evitant la seva recaptació i produint un augment de la DA extracel·lular (Williams and Galli 2006) la qual cosa augmenta l'activitat motora i l'eufòria associada al consum de drogues.

Receptors de dopamina

La DA quan és troba a l'espaï sinàptic pot interaccionar amb els seus receptors específics que és localitzen tant a la membrana presinàptica com postsinàptica. Estan formats per 7 dominis transmembrana i acoblats a proteïna G. Hi ha dues famílies de receptors: els $D_{1\text{-like}}$ i els $D_{2\text{-like}}$ **(Figura 5).**

Els primers estan formats pels receptors D_1 i D_5 que estimulen l'enzim adenilat ciclasa produint així AMPc, mentres que els receptors que pertanyen a la família dels D_2 agrupats pels receptors D_2, D_3 i D_4 la inhibeixen (Dal Toso, Sommer et al. 1989).

Receptor Subtype	D_1-like Receptors		D_2-like Receptors		
	D_1	D_5	D_2	D_3	D_4
G protein	G_s	G_s	$G_{i/o}$	$G_{i/o}$	$G_{i/o}$
Transduction Mechanism	↑ AC, ↑ PLC, ↑ L -type Ca^{2+} channels	↑ AC	↓ AC, ↑ $[Ca^{2+}]_i$, K^+ conductance, ↓ Ca^{2+} conductance	↓ AC, activation of MAP kinase	↓ AC, ↓ Ca^{2+} conductance
Localisation	Caudate, putamen, nucleus accumbens, olfactory tubercle	Hippocampus, hypothalamus	Caudate, putamen, nucleus accumbens, olfactory tubercle	Nucleus accumbens, olfactory tubercle, islands of Calleja	Frontal cortex, midbrain, amygdala, cardiovascular system
Likely Physiological Roles	Locomotion, reward, reinforcement, learning and memory, renin secretion	Learning and memory	Locomotion, reward, reinforcement, learning and memory	Locomotion, possible role in cognition and emotion	Mostly unknown - possible role in cognition and emotion, hypertension

Figura 5. *Classificació, localització i caracterització dels receptors de dopamina (www.tocris.com).*

Receptors $D_{1\text{-like}}$ de dopamina

Aquesta família està formada pels subtipus D_1 i D_5 els quals presenten una elevada similitud en els dominis transmembrana (80% d'identitat) i s' acoblen a proteïna $G\alpha_s$ per la qual activen Adenilat Ciclasa. Semblen ser receptors exclusivament postsinàptics i estan presents en neurones que reben innervació dopaminèrgica com les neurones estriatals de projecció.

El D_1 és el receptor dopaminèrgic més abundant al SNC (Missale, Nash et al. 1998). La seva distribució és molt variada, trobant-se una gran quantitat d'aquests subtipus de receptors ubicats en el tubercle olfactori, estriat, NAc, illes de Calleja, amígdala, nucli subtalàmic, cerebel i la substància nigra (compacta i reticulata). En menor quantitat hi són presents al còrtex frontal cerebral, tàlem i al globus pàl·lid (Jaber, Robinson et al. 1996).

El receptor D_5 s'expressa en menor intensitat que el D_1 i la seva localització es centra a l'hipocamp i als nuclis lateral mamil·lar i parafascicular del tàlem (Jaber, Robinson et al. 1996), encara que recentment s'ha descrit l'expressió de receptors D_5 en neurones dopaminèrgiques de VTA, fet que podria contribuir a la regulació dels receptors D_2 (Nimitvilai, Arora et al. 2012).

Receptors $D_{2\text{-like}}$ de dopamina

Son receptors acoblats a proteïna G_i per la que inhibeixen Adenilat Ciclasa. Existeixen tres subtipus de receptors anomenats D_2, D_3 i D_4.

El receptor D_2 té dues isoformes actives D_{2s} i D_{2L} (Xu, Hranilovic et al. 2002) amb propietats anatòmiques i farmacològiques diferents

(Lindgren, Usiello et al. 2003). Aquest receptor és molt abundant a l'estriat (neurones GABAèrgiques estriatopalidals), tubercle olfactori, NAc, illes de Calleja i VTA. De forma menys nombrosa estan presents a la substància nigra reticulada i la compacta (on s'expresa en neurones dopaminèrgiques com autoreceptor), còrtex cerebral, globus pàl·lidum, amígdala, tàlem i hipocamp (Jaber, Robinson et al. 1996).

El receptor D_3 es distribueix i és farmacològicament diferent als subtipus D_2 (en particular en afinitat per neurolèptics) (Sokoloff and Schwartz 1995). Hi ha una gran abundància dels receptors D_3 a la regió septal, illes de Calleja, nucli mamil·lar medial de l'hipotàlam i en les cèl·lules de Purkinje del cerebel. Paral·lelament la seva distribució és menor en VTA, substància nigra, còrtex parietal i temporal, bulb olfactori, neoestriat, NAc, amígdala i el nucli subtalàmic (Missale, Nash et al. 1998). El receptor D_3 és un autoreceptor present en el còrtex frontal, que regula l'alliberament de DA (Gobert, Lejeune et al. 1996; Whetzel, Shih et al. 1997).

El receptor D_4 va ser clonat l'any 1991 (Van Tol, Bunzow et al. 1991). Una característica interessant d'aquest receptor és la seva alta afinitat pel neurolèptic clozapina, considerat com a referència en la classe dels "atípics". Es localitza abundantment al còrtex frontal, al bulb olfactori, l'amígdala, al mesencèfal i la retina (Jaber, Robinson et al. 1996). A l'hipotàlem i a l'hipocamp es troba en menor quantitat.

Heteromerització del receptors $D_{1\text{-like}}$ i $D_{2\text{-like}}$

Està descrita la heteromerització en neurones postsinàptiques dels receptors D_1 i D_2 a diferents regions de l'encèfal com al NAc, Globus Pallidus i al caudat-putamen. L'activació simultània d'aquests dos receptors "sinergisme entre D_1 i D_2" (Dziedzicka-Wasylewska 2004), és necessària pel control motor (Hasbi, O'Dowd et al. 2011) i la mediació de la DA en processos de recompensa al NAc (White, Bednarz et al. 1988).

Hi ha evidències que els receptors D_1 i D_3 formen un heteròmer a les neurones GABAèrgiques de l'estriat (Marcellino, Ferre et al. 2008). En la interacció D_1-D_3, l'estimulació del receptor D_3 augmenta la unió del receptor D_1 pel lligand, fet que indica que l'estimulació del receptor D_3 podria potenciar els efectes de la DA endògena pel receptor D_1. En canvi, l'estimulació del receptor D_1 no modifica la unió del receptor D_3 pel lligand, la qual cosa indica que la interacció entre D_1-D_3 no és recíproca (Marcellino, Ferre et al. 2008).

Mitjançant experiments amb cèl·lules embrionàries de ronyó humanes es va demostrar que els receptors D_2-D_5 formen un heteròmer, provocant que es produeixi una estimulació en la senyalització del Ca^+ quan els dos receptors estan activats (So, Verma et al. 2009). Aquestes evidències donarien suport al model pel qual suggereix que perquè hi hagi una màxima activació del senyal, els dos receptors han de formar un dímer (Baneres and Parello 2003; Kniazeff, Bessis et al. 2004).

Experiments realitzats en cèl·lules COS-7 amb coimmuno-precipitació demostren que els receptors D_2 i D_3 formen un heterodímer funcional. La seva formació té propietats farmacològiques i fisiològiques diferents que l'activitat d'aquests dos receptors per separat (Scarselli, Novi et al. 2001).

El fenomen de la heteromerització es podria explicar a partir d'uns resultats no esperats sobre l'activitat dopaminèrgica en un model animal. Es va observar que administrant l'agonista D_3 7-OH-DPAT en rates s'induïa hipotèrmia a concentracions que només haurien d'activar el receptor D_3. No obstant, aquest efecte no només podia ser inhibit per antagonistes selectius D_3 sinó que també era revertit per un antagonista selectiu D_2 (Millan, Dekeyne et al. 2000).

Vies dopaminèrgiques

Com ja s'ha comentat abans, la DA és un neurotransmissor molt important del Sistema Nerviós Central (SNC) en els mamífers i està implicat en la regulació de diverses funcions com la conducta motora, les emocions, l'aprenentatge, la consolidació de la memòria, la comunicació neuroendocrina i les addiccions (Arias-Carrion, Stamelou et al. 2010). Les vies dopaminèrgiques han estat àmpliament estudiades mitjançant tècniques d'immuno histoquímica i fluorescència. Els grups neuronals han estat denominats des de A8 fins A17 segons la classificació de Fuxe (Fuxe 1965). Originàriament van ser anomenades com si fossin una continuació rostral de les poblacions noradrenèrgiques. Per aquest motiu la classificació comença amb el grup A8, població situada a la

zona retrorubral i que projecta cap a l'estriat. Aquestes neurones constitueixen una extensió caudal de la població A9 (en la SNc).

La població A9 i la A10 (en la VTA) donen lloc a dues vies de projecció implicades en la conducta motora, les emocions, aprenentatge i la memòria. D'acord amb aquesta classificació, les neurones dopaminèrgiques es poden agrupar en tres sistemes principals (Cooper 2003).

Sistema Ultracurt

Format per cèl·lules dopaminèrgiques del bulb olfactori (A16), i per neurones interflexiformes presents en les capes internes i externes de la retina (A17).

Sistema longitud intermedi

Inclou: a) el sistema tuberohipofisari amb origen als nuclis hipotalàmics (A12), b) neurones localitzades en l'hipotàlam dorsal i posterior (A13 i A14) i c) el grup periventricular medul·lar que inclou les neurones localitzades a la perifèria dels nuclis del tracte solitari i motor dorsal del nervi vague.

Sistema llarg

Aquest grup inclou els grups neuronals més importants **(Figura 6):**

> **La via nigroestriatal** té els cossos neuronals a la SN (A9) i és projecta principalment cap a l'estriat dorsal (caudat i putamen). Aquesta via està implicada en el control de la conducta motora així com l'aprenentatge dels hàbits motors (Janhunen and Ahtee 2007).

La via mesocorticolimbica s'origina a la VTA (A8, A10) i està implicada en la recompensa i el control de les emocions. S'anomena així perquè hi ha un solapament entre les vies mesolímbica i mesocortical (Janhunen and Ahtee 2007):

La via mesolímbica projecta els axons al Nac (Laviolette and van der Kooy 2003).

La via mesocortical projecta els axons cap al còrtex medial prefrontal i al còrtex cingulat anterior (Lapish, Kroener et al. 2007).

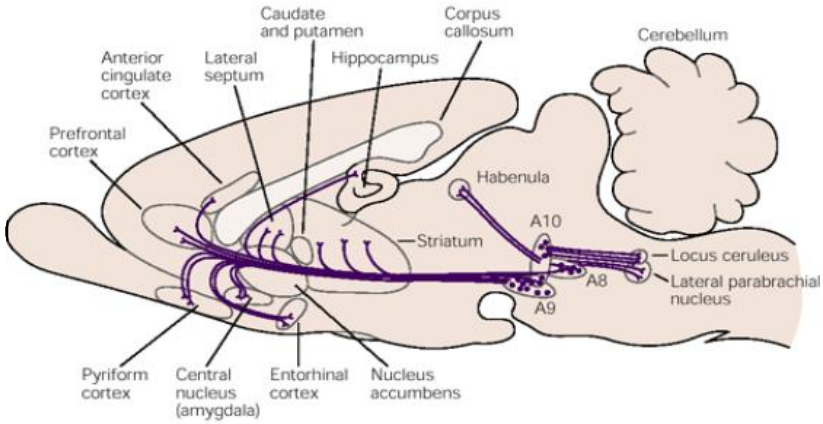


Figura 6. Principals vies dopaminèrgiques en un encèfal de rata. Esquema en un pla sagital. *(Kandel, Schwartz and jessell 2000). Neurones dopaminèrgiques dels grups A8, A9 i A10 projecten cap a l'estriat, sistema límbic i el mPFC.*

Encara que també s'han identificat un tipus de neurones amb propietats electrofisiològiques diferents a les conegudes fins ara en la via dopaminèrgica mesolímbica, amb descàrregues ràpides.

Aquestes neurones projecten selectivament del mesencèfal cap al còrtex prefrontal, l'amígdala basolateral i el nucli accumbens i son capaces de produir potencials d'acció en freqüències molt més ràpides que les neurones dopaminèrgiques "convencionals" (Lammel, Hetzel et al. 2008). Futurs estudis *"in vivo"* amb aquestes neurones seran necessaris per definir la seva relació entre l'activitat elèctrica i l'alliberament de DA.

Estructura i funció del ganglis basals

Els ganglis basals formen una estructura subcortical i la seva funció principal és la de connectar el còrtex cerebral amb els sistemes neuronals que afectaran la conducta. Està formada per nuclis com l'estriat (caudat, putamen i el NAc), el nucli subtalàmic, el globus pallidus (intern, extern i ventral) i la substància nigra (pars *compacta*, SNc, i pars *reticulata*, SNr).

Subestructures del cos estriat

L'estriat de la rata és una massa de substància gris que ocupa la part més profunda de l'hemisferi cerebral. La seva principal funció és la d'actuar com a filtre dels *inputs* corticals topogràficament organitzats. Està format per l'estriat dorsal i l'estriat ventral **(Figura 7).**

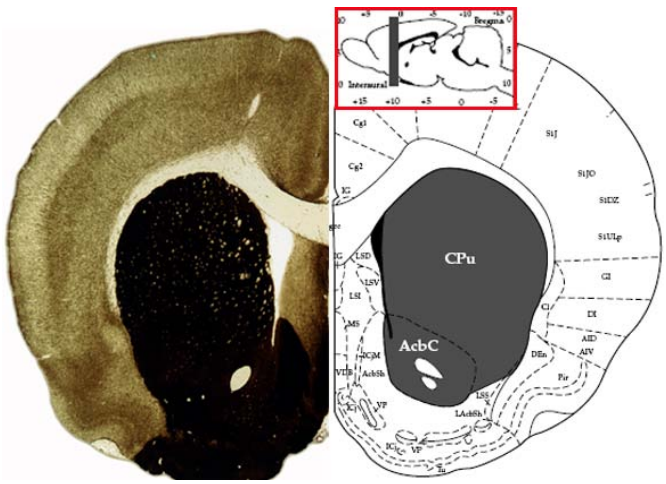


Figura 7. Localització de l'estriat dorsal i ventral en l'encèfal de rata. *Bregma 2.16mm (Paxinos and Watson, 2004). Cpu: Caudat-Putamen; AcbC: nuclli accumbens core.*

L'estriat dorsal o neoestriat és la part motora del cos estriat i està relacionada amb les funcions motores voluntàries. Participa en la iniciació i producció de la conducta motora així com també està implicat en les conductes addictives. Comprèn els nuclis caudat i putamen, que en rosegadors és una única estructura, el caudat-putamen. Està innervat principalment pel còrtex motor primari, anterior pre-motor i per neurones dopaminèrgiques de la SNc. Al seu torn projecta cap al Globus Pallidus i la SN (David, Ansseau et al. 2005).

L'estriat ventral està format principalment pel NAc, el qual és pot dividir en dues grans regions: el *core* (funció més motora) i la *Shell* (de característiques més límbiques). Constitueix la part límbica de l'estriat i actua com una interfase entre el sistema límbic i motor, ja

que te una funció molt important en les conductes de motivació així com en el desenvolupament i expressió de l'addicció.

Rep *inputs* de l' àrea prefrontal, hipocamp, amígdala així com també *inputs* dopaminèrgics de l' VTA. Envia les seves projeccions al Globus Pallidus ventral, SN, VTA i a l' hipocamp (David, Ansseau et al. 2005). Ambdues subdivisions anatòmiques són diferents en el tipus d'informació que processen i la topografia particular, si bé comparteixen nombroses característiques neuroquímiques i de citoarquitectura. Així en l'estriat dorsal i el ventral podem trobar neurones GABAèrgiques de projecció que son les neurones que és troben en major quantitat a l'estriat (la seva població és d'un 90-95% del total). Els seus cossos cel·lulars mesuren unes 10-20 µm de diàmetre i tenen llargues branques dendrítiques amb espines. Utilitzen l'àcid-aminobutíric (GABA) com a neurotransmissor. En base a les seves projeccions es poden diferenciar dues grans poblacions. Les neurones GABAèrgiques estriatopalidals expressen receptors D_2 de DA i A_2 de adenosina. Les seves projeccions constitueixen la "via indirecte" de control locomotor ja que projecten els cossos neuronals cap al Globus Pallidus i al nucli subtalàmic *(veure circuit motor dels ganglis basals)*. Les neurones GABAèrgiques estriatonigrals expressen receptors D_1 de DA i M_4 d'acetilcolina **(Figura 8)** i constitueixen la "via directa" de control locomotor.

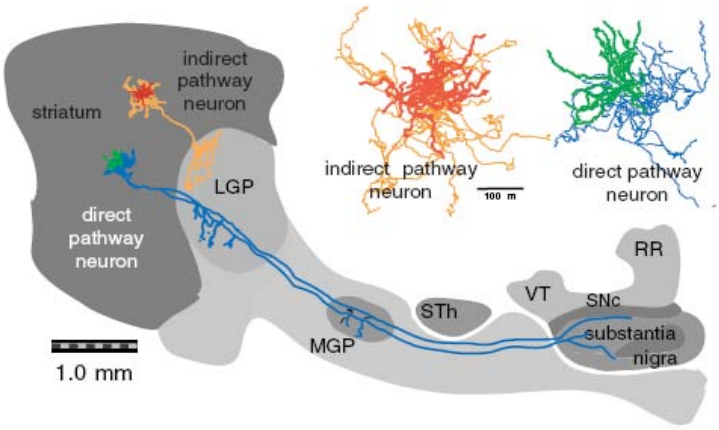


Figura 8. Vies de projecció de les neurones GABAèrgiques. *(Paxinos 2004). Localització de les vies directes i indirectes en un pla sagital.*

Subestructures de la substància nigra

La SN és l'àrea més estudiada dels ganglis basals. Està composta per diferents àrees funcionalment relacionades: la substància nigra pars compacta i la pars reticulata **(Figura 9).**

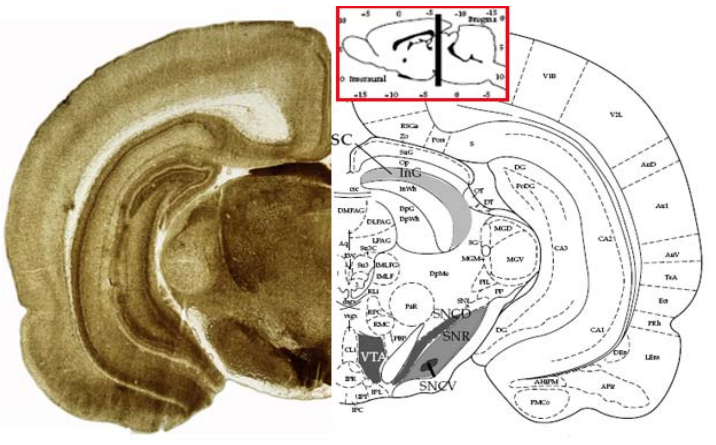


Figura 9. Localització de la substància nigra en l'encèfal de rata. *Bregma 5.04mm (Paxinos and Watson, 2004). SNCD i SNCV: substància nigra pars compacta dorsal i ventral, SNR: substància nigra pars reticulata; VTA: àrea tegmental ventral.*

Les neurones que formen la SN no és poden distingir morfològicament entre elles. Tenen un soma irregular del que projecten de 2 a 4 dendrites principals que és dividiran en branques secundàries i terciàries cobrint així una gran àrea. Aquestes neurones és poden diferenciar en funció del neurotransmissor que sintetitzin, en neurones dopaminèrgiques i GABAèrgiques. A la SNc, la població de cèl·lules que trobem principalment (88±2%) son neurones dopaminèrgiques (Margolis, Lock et al. 2006), encara que també és pot trobar una petita població a la SNr (8±5%) (Margolis, Lock et al. 2006). Es caracteritzen per tenir amplis potencials d'acció juntament amb una lenta conducció axonal (Fields, Hjelmstad et al. 2007). Projecten cap al caudat-putamen constituint així la via nigro-estriatal (Haber, Fudge et al. 2000).

Les neurones GABAèrgiques és troben principalment a la SNr i és caracteritzen per tenir un potencial d'acció més breu i una ràpida conducció axonal (Fields, Hjelmstad et al. 2007). Proporcionen sinapsis inhibidores a la SNc i la SNr i envien projeccions inhibidores cap al tàlem (Paxinos 2004). Tanmateix reben *inputs* inhibidors GABAèrgics del nucli estriat i del globus pallidus i *inputs* excitadors glutamatèrgics del nucli subtalàmic.

Circuit motor dels ganglis basals

Els ganglis basals suprimeixen els moviments no desitjats i preparen a les motoneurones superiors per a la iniciació dels moviments. Els components motors dels ganglis basals juntament

amb la substància nigra i el nucli subtalàmic formen un circuit subcortical **(Figura 10)** que connecta la major part de les regions del còrtex amb les motoneurones superiors en els còrtex premotor primari i el tronc encefàlic.

Les neurones d'aquest circuit responen durant la anticipació i execució dels moviments voluntaris de tal manera que el seu efecte sobre les motoneurones superiors és necessari per al desenvolupament normal dels moviments voluntaris (Purves 2004).

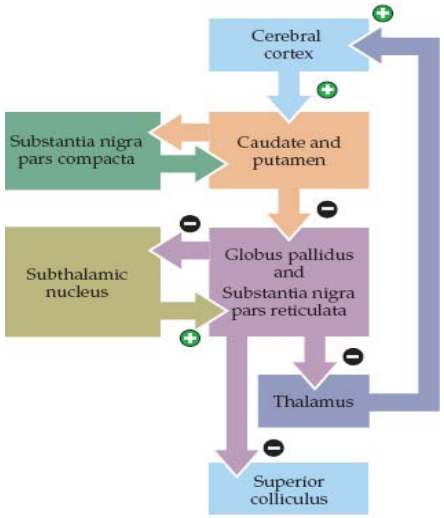


Figura 10. Circuit motor dels ganglis basals. *(Purves et al 2004). Esquema dels circuits dels ganglis basals. Els símbols (+) i (-) mostren les connexions excitadores i inhibitòries respectivament.*

Circuit límbic dels ganglis basals

El circuit límbic dels ganglis basals és responsable de la influència de la informació motivacional, emocional, contextual i afectiva

sobre el comportament (Pierce and Kumaresan 2006). Nuclis límbics externs als ganglis basals com l'amígdala, hipocamp i el còrtex medial prefrontal envien projeccions glutamatèrgiques al NAc **(Figura 11).** El NAc té dues projeccions GABAèrgiques que tenen similituds a les vies "directe" i "indirecte" del circuit motor. Aquestes és dirigeixen cap al ventral pallidum i la VTA/SN. Ambdues regions envien aferents GABAèrgiques cap al tàlem dorsal-medial que completa el circuit mitjançant una projecció glutamatèrgica cap al còrtex medial prefrontal.

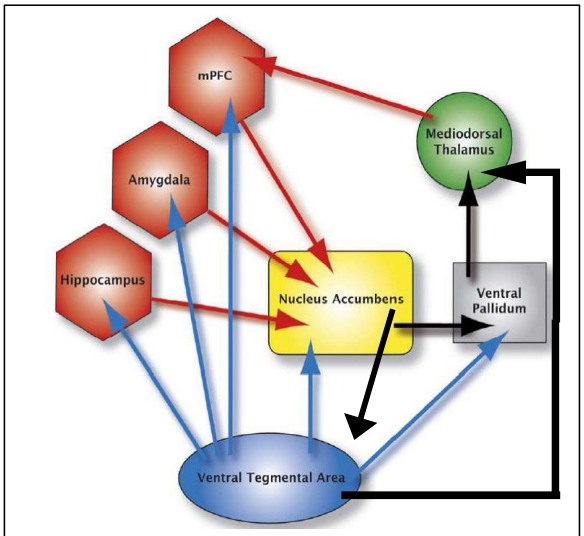


Figura 11. Circuit límbic dels ganglis basals. *Les fletxes blaves indiquen les vies dopaminèrgiques, vermelles glutamatèrgiques i les negres les GABAèrgiques. (modificada de Pierce and Kumaresan 2006).*

Les neurones dopaminèrgiques de la VTA innerven el NAc, hipocamp, amigdala, còrtex medial prefrontal i el ventral pallidum,

de tal manera que canvis en la neurotransmissió dopaminèrgica influeixen en el flux d'informació a través del circuit límbic composat pels nuclis interconectats.

Funcions de la dopamina

Tal i com ja s'ha comentat anteriorment, la DA intervé en una gran varietat de funcions al SNC com l'activitat locomotora, aprenentatge, reforços positius o la ingesta alimentària (Missale, Nash et al. 1998), encara que fora del SNC modula altres activitats fisiològiques. A nivell perifèric està implicada en la regulació de la motilitat intestinal (Li, Schmauss et al. 2006), la funció renal (Aperia 2000) i la visió (Witkovsky 2004) entre d'altres. A nivell central està implicada en patologies com la malaltia de Parkinson, una degeneració irreversible de les neurones dopaminèrgiques de la via nigroestriatal que es caracteritza per disfunció motora, bradicinèsia, bradipsíquia, tremolors incontrolats i rigidesa de les extremitats entre d'altres (Devine, Plun-Favreau et al. 2011), depressió (Guiard, El Mansari et al. 2009), alteracions de la son (Keating and Rye 2003), nivells d'energia i activitat (Knab and Lightfoot 2010), psicosis com l'esquizofrènia (Remington 2008), regulació de la secreció de les hormones estimuladores de l'alliberament de prolactina, renina i aldosterona sintetitzades a l'hipotàlem (Aperia 2000), Síndrome de Gilles Tourette (Steeves and Fox 2008), o el síndrome de PAP (Pèrdua d'Autoactivació Psíquica), que es produeix per lesions al còrtex frontal i ganglis basals i es caracteritza per la pèrdua de la motivació i

l'espontaneïtat (buit mental) que impedeix a la persona realitzar de forma voluntària activitats de la vida diària (desordre motivacional). Quan aconsegueix realitzar alguna activitat motora la seva execució és estereotipada i a vegades compulsiva (Engelborghs, Marien et al. 2000).

La dopamina en el circuit del reforç

En el circuit del reforç un estímul que predirà un benefici, provocarà una conducta de cerca d'aquest benefici. Constitueix el principi d'estímul-acció i es basa en una predicció de la recompensa a través d'un condicionament associat.

L'estímul que prediu un benefici està codificat per una activació de les neurones dopaminèrgiques mesolímbiques. La recompensa en canvi només activa les esmentades neurones si és imprevisible, ja que en cas de ser previsible les neurones s'activen per la predicció, però no per la recompensa. La predicció (o motivació) per la recompensa activa les neurones dopaminèrgiques fins i tot en absència de recompensa. Per l'activació dopaminèrgica s'allibera DA al Nac abans d'obtenir la recompensa. Es més, l'estimulació elèctrica del sistema mesolímbic és suficient per encetar la conducta d'autoadministració operant de cocaïna pressionant la palanca després d'haver après l'associació palanca-cocaïna (Phillips, Stuber et al. 2003; Schultz 2007). La hiperestimulació del sistema dopaminèrgic induït per drogues d'abús juga un paper fonamental en les addiccions, la qual es manifesta amb una gran

varietat de símptomes com son la hiperactivitat, eufòria, etc (Koob and Le Moal 2001).

Tanmateix no es creu que el dopaminèrgic sigui l'únic sistema de neurotransmissió implicat existent degut a la complexitat dels trastorns addictius. Per exemple, l'associació estímul-acció explica el reforç però no la pèrdua de control de l'addicte sobre la seva voluntat de regular el consum. Aquesta pèrdua de control esdevé característica de l'addicció. Es creu que el consum voluntari estaria regulat per àrees corticals que utilitzen glutamat com a neurotransmissor.

Altres neurotransmissors implicats en el circuit del reforç

El paper que juga la transmissió glutamatèrgica en els efectes conductuals i reforçants de la cocaïna son molt evidents, encara que aquesta droga no exerceix una acció directe sobre els receptors glutamatèrgics.

L'administració aguda o crònica de cocaïna en rates, incrementa els nivells extracel·lulars de glutamat en diverses àrees cerebrals, especialment en el sistema límbic. En concret, la neurotransmissió glutamatèrgica en el nucli accumbens sembla jugar un important paper en la recaiguda de l' autoadministració de cocaïna en rates.

Nombroses investigacions han confirmat el paper que juga el sistema glutamatèrgic en les accions de la cocaïna, així per exemple, l'administració endovenosa d'antagonistes glutamatèrgics bloquegen la sensibilització motora (Wolf and

Jeziorski 1993; Karler, Calder et al. 1994) i la adquisició de l'autoadministració de psicoestimulants (Schenk, Valadez et al. 1993).

Estudis de neuroimatge en addictes a la cocaïna han demostrat que les àrees de l'encèfal riques en glutamat, com son les regions corticals i límbiques, mostren fortes respostes metabòliques durant l'eufòria induïda per la cocaïna com també al procés cognitiu de desitjar intensament aquesta droga després un període d'abstinència (Dackis and O'Brien 2003). També és sap que la neurotransmissió glutamatèrgica està implicada en el desenvolupament i l'expressió de la sensibilització tant conductual com neuroquímica als opiacis i psicoestimulants (Pierce and Kalivas 1997; White and Kalivas 1998).

El sistema canabinoide ha estat implicat en moltes patologies neurològiques com l' esquizofrènia (Emrich, Leweke et al. 1997), Parkinson (Gubellini, Picconi et al. 2002) o l'addicció a drogues (Maldonado and Rodriguez de Fonseca 2002). La participació d'aquest sistema en l'addicció és probable que reflecteixi els seus efectes en el "circuit de la recompensa" (Berke and Hyman 2000).

L'estriat conté gran quantitat tant de receptors canabinoides CB_1 com de DA (Piomelli 2003). És per això una estructura ideal per estudiar la interacció entre aquests dos sistemes i els seus efectes en l'addicció a les drogues d'abús com la cocaïna.

L'autoadministració de tetrahidrocannabinol (compost psicoactiu de la marihuana) en micos és facilitat en gran mesura per la prèvia autoadministració de cocaïna (Tanda, Munzar et al. 2000).

D'altra banda l'administració crònica de canabinoides produeix sensibilització creuada no només als efectes locomotors dels opioïdes (Pontieri, Monnazzi et al. 2001) sinó també als de la amfetamina (Gorriti, Rodriguez de Fonseca et al. 1999). El bloqueig farmacològic dels receptors CB_1 evita la recaiguda en la cerca de la cocaïna, mentres que l'estimulació d'aquest la facilita (De Vries, Shaham et al. 2001).

El sistema dopaminèrgic mesolímbic ha estat implicat en la modulació dels efectes motivacionals dels opiàcids. L'injecció en VTA d'agonistes opioides de receptors μ incrementen l'alliberament de DA en el nucli accumbens, en canvi l'injecció dels antagonistes disminueixen l'alliberament de DA. Tanmateix si s'administren els lligands directament al nucli accumbens no es produeixen canvis (Spanagel, Herz et al. 1992).

Com a continuació explicaré, hi ha evidències que el sistema histaminèrgic podria jugar un paper inhibidor sobre diversos efectes provocats per les drogues d'abús.

Història de l'implicació de la histamina en el circuit del reforç

Entre el 1970 i 1980 els addictes a la heroïna, quan no podien aconseguir la droga, s'autoadministraven fàrmacs antihistamínics (antagonistes de receptors H_1 de HA) en combinació amb opioïdes poc potents que es podien aconseguir a la farmàcia sense recepta mèdica (Showalter 1980). La recompensa rebuda pels addictes per aquesta combinació de fàrmacs, arribava a ser molt semblant a la

produïda per l'heroïna. Estudis en humans i animals també indiquen que l'administració d'antihistamínics amb opioïdes habituals en els xarops per la tos potencien la recompensa produïda pels opioïdes (Suzuki, Masukawa et al. 1990).

També està descrit que la HA neuronal inhibeix potentment la recompensa produïda per la morfina (Suzuki, Takamori et al. 1995), i els efectes motors produïts per la metamfetamina i la cocaïna (Itoh, Nishibori et al. 1984; Ito, Onodera et al. 1997a) .

Tanmateix, l'administració de HA inhibeix el reforç produït per l'autoestimulació elèctrica intracranial en rates (Cohn, Ball et al. 1973). El conjunt d'aquestes evidències permet pensar que la potenciació del sistema histaminèrgic podria esdevenir d'utilitat terapèutica en el tractament d'addiccions.

Sistema histaminèrgic

La histamina 2-(4-imidazol) és una molècula que pertany al grup de les amines biògenes i està implicada en molt processos fisiològics com la resposta inflamatòria, secreció gàstrica, regulació son/vigília i la neurotransmissió (Passani and Blandina 2011). La presència d'aquesta molècula al SNC en mamífers ja fou descrita a la meitat del segle XX (Kwiatkowski 1943) i des de llavors s'han acumulat evidències de la seva funció al SNC i perifèric (Schwartz, Arrang et al. 1991; Brown, Stevens et al. 2001). El paper que juga l'histamina al SNC és molt diferent que al perifèric, ja que no pot traspassar la barrera hematoencefàlica i és el precursor, la L-histidina el que és captat pels transportadors d'aminoàcids de la barrera

hematoencefàlica. Un cop dins el SNC entra a les neurones histaminèrgiques i posteriorment la histamina és sintetitzada per descarboxilació de la L-histidina **(Figura 12).**

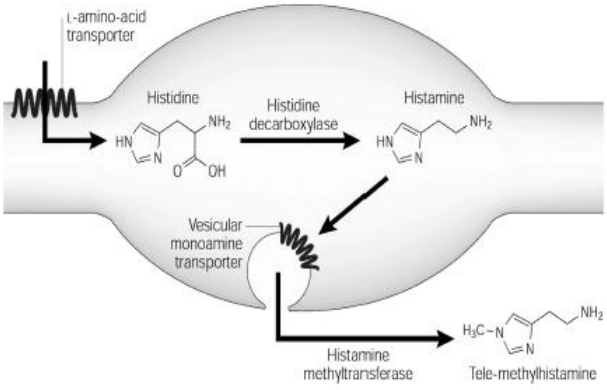


Figura 12. Metabolisme de la histamina *(Haas et al. 2008).*

Vies histaminèrgiques

Els somes de les neurones histaminèrgiques és localitzen als nuclis tuberomamilars de l'hipotàlems posterior (Burgess 2010).

Estudis inmunohistoquímics revelen que la majoria d'aquestes aquestes neurones tenen uns trets característics comuns com són un gran tamany (25-30µm), amb un nucli de forma rodona i un aparell de Golgi molt desenvolupat (Wouterlood, Sauren et al. 1986).

Les fibres nervioses es projecten quasi cap a tot l'encèfal per mitjà de dues vies ascendents i una descendent **(Figura 13).**

- Via ascendent cap a l'hipotàlam, septum, bulb olfactori, hipocamp i còrtex.

- Una altra via ascendent cap al tercer ventricle cap als ganglis basals, tàlem, hipocamp, amígdala i còrtex.
- La via descendent és projecta cap al cerebel i medul·la espinal.

Es de destacar la important innervació histaminèrgica d'àrees del sistema límbic.

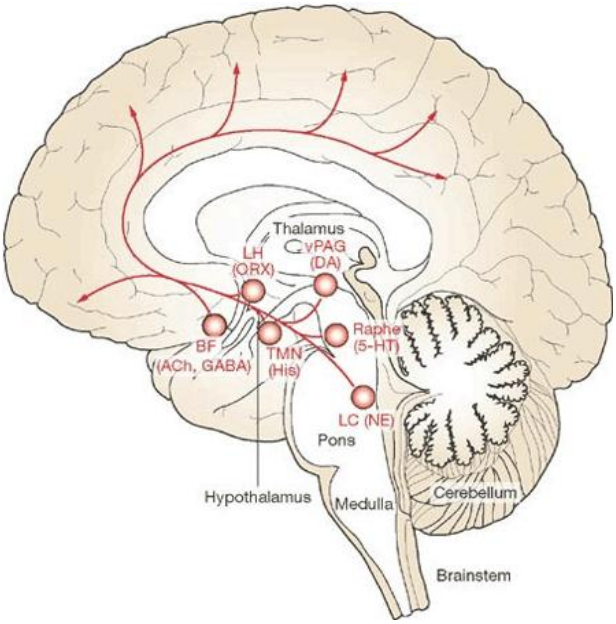


Figura 13. Principals vies histaminèrgiques en el SNC humà. *Esquema en un pla sagital. www.medscape.com*

Receptors d'histamina

Els receptors d'histamina es troben presents en les neurones postsinàptiques (heteroreceptors) i en el cas dels receptors H_3 també es poden trobar a les pròpies neurones histaminèrgiques (autoreceptors). Es classifiquen en quatre grups: H_1, H_2, H_3, H_4.

Cada un dels receptors té una expressió específica, encara que els receptors H_1, H_2, H_3 els podem trobar de manera abundant al SNC, els receptors H_4 tenen una expressió minoritària al SNC però que son abundants en cèl·lules del sistema immunològic (Mommert, Gschwandtner et al. 2011). El receptor H_3 és pot trobar tant a les neurones presinàptiques com postsinàptiques (Arrang, Garbarg et al. 1983).

Receptor H_1, H_2, H_4

Els receptors H_1 estan implicats en molts processos fisiològics com les reaccions al·lèrgiques (Siegel 1993), cicle son/vigília (Thakkar 2011) i la ingesta alimentària (Passani and Blandina 2011). Estàn àmpliament distribuïts per tot l'organisme: cor, cèl·lules endotelials, musculatura llisa i en l'encèfal més concretament el trobem al caudat-putamen, neocòrtex i al hipocamp (Martinez-Mir, Pollard et al. 1990). Sobre el mecanisme de transducció, el receptor està acoblat a proteïna $G_{\alpha q/11}$, això farà que quan un agonista s'uneixi al receptor H_1 aquest activarà les vies de senyalització de la fosfolipasa C, augmentant la concentració de Ca^+ intracel·lular (Haas, Sergeeva et al. 2008; Criado, Criado et al. 2010).

El receptor H_2 està acoblat a proteïna G_s i la seva activació produirà una estimulació de la proteïna de membrana Adenilat Ciclasa potenciant la transmissió sinàptica en l'hipocamp (Selbach, Brown et al. 1997). També podem trobar grans densitats d'aquest receptor als ganglis basals, còrtex i amígdala (Haas, Sergeeva et al.

2008). Fora del SNC el trobem a la cavitat gàstrica on modula la secreció d'àcid gàstric (Huang, Cao et al. 2010).

El receptor H_4 és el que ha estat clonat més recientment (Nakamura, Itadani et al. 2000) i té una homologia i característiques farmacològiques molt similars al H_3 (Gbahou, Rouleau et al. 2003). S'expressa principalment en cèl·lules i teixits com la sang, melsa, intestins i fetge (Breunig, Michel et al. 2007; Morgan, McAllister et al. 2007), encara que hi ha alguns estudis que indiquen que també el podem trobar al SNC (Lim, Adami et al. 2009).

Receptor H_3

L'existència del tercer receptor d'histamina fou establerta cap a la dècada del 80, on es va caracteritzar com autoreceptor, regulant l'alliberament d'histamina en el SNC (Arrang, Garbarg et al. 1983). Després es va observar que també regulava la seva síntesis (Arrang, Garbarg et al. 1987) i que no només estava en neurones histaminèrgiques sinó que també s'expressava en altres neurones on podria modul·lar diversos neurotransmissors com la serotonina (Schlicker, Betz et al. 1988), NA (Schlicker, Fink et al. 1989), Glutamat (Fink, Schlicker et al. 1994) i GABA (Arias-Montano, Floran et al. 2001). S'ha demostrat que els receptors H_3 d'histamina tenen activitat constitutiva, que és la capacitat d'activar-se en absència d'un agonista (Schwartz 2011). El receptor H_3 de rata té sis isoformes quatre de les quals son actives i

s'expressen en cervell amb variacions segons les regions cerebrals (Drutel, Peitsaro et al. 2001; Morisset, Sasse et al. 2001).

La seva expressió és més marcada en còrtex, tàlem i estriat (Lovenberg, Roland et al. 1999). Estudis més detallats amb radiolligands van observar la presència d'aquest receptor als nuclis olfactoris, còrtex, estriat, SNr, amígdala, tàlem i hipotàlem (Pillot, Heron et al. 2002).

El NAc té una alta densitat de receptors H_1 i H_2 de HA, i particularment en la shell del NAc és molt rica en receptors H_3. També estan presents en neurones estriatals GABAèrgiques de projecció (Pillot, Heron et al. 2002). Totes aquestes evidències suggereixen que un lloc de preferència d' acció dels lligands de receptors H_3 de HA administrats de forma sistèmica podria ser la shell del NAc, regió molt coneguda per la seva implicació en el reforç de drogues d'abús (Kalivas and Nakamura 1999).

Implicació dels receptors H_3 en el reforç

Per sí mateixos, els antagonistes H_3 no semblen produir autoadministració (Munzar, Tanda et al. 2004) ni condicionament de la preferència de lloc (Brabant, Charlier et al. 2005).

Munzar i col·laboradors van observar que el pretractament amb dosis baixes d' antagonistes H_3, disminuïa l'autoadministració de metamfetamina en rates. Tanmateix, els mateixos autors van observar que els antagonistes H_3 també potenciaven els efectes en dosis menors de metamfetamina que les rates que normalment no s' autoadministraven. Això suggereix que els antagonistes H_3

potencien els efectes reforçants de la metamfetamina, desplaçant a l'esquerre la corba dosi-resposta produïda per la droga, que té forma de campana. Sembla ser que aquest efecte es produiria per un augment de l'alliberament de DA provocat per antagonistes H_3. Aquestes evidències recolzen les observacions anteriorment esmentades en el sentit que el sistema histaminèrgic podria ser de gran importància com a inhibidor de respostes fisiològiques de recompensa. Per tot això es podria concloure que potser la HA jugaria un paper inhibidor de la recompensa a través de heteroreceptors H_3. Al bloquejar els receptors H_3 amb antagonistes es podria potenciar la recompensa produïda per determinades drogues. Contràriament, seria d'esperar que els agonistes de receptors H_3 podrien disminuir els efectes reforçadors de les drogues d' abús. Aquesta ha estat la hipòtesi central per orientar la present tesi doctoral.

També està descrit que els antagonistes de receptors H_3 d' HA disminueixen l'autoadministració d' etanol en rates seleccionades per la seva preferència per aquesta droga (rates Alko-Alcohol) (Lintunen, Hyytia et al. 2001). Els receptors H_3 són autoreceptors inhibidors en neurones histaminèrgiques, encara que també poden ser heteroreceptors en altres neurones. Així doncs, els resultats obtinguts per l'autor Lintunen es podrien donar, bé per un augment en l'alliberament de HA, o bé per efectes directes dels antagonistes H_3 sobre altres neurones.

S' ha comprovat que els heteroreceptors H_3 inhibeixen la síntesi (Molina-Hernandez, Nunez et al. 2000), i l'alliberament de DA

(Schlicker, Fink et al. 1993), suggerint que aquest tipus de receptors estan presents en terminals dopaminèrgiques.

Resultats del nostre laboratori, paral·lels a aquesta tesi, demostren l'existència en neurones dopaminèrgiques del receptor H_3 i tirosina hidroxilasa al VTA i SN tant mRNA com proteïna mitjançant hibridació in situ i immunohistoquímica (González-sepulveda 2012).

Està demostrat que diferents tipus de drogues addictives tenen en comú el fet d'estimular l'alliberament de DA en el NAc, i en el cas particular de la amfetamina, aquest alliberament pot ser modulat mitjançant antagonistes H_3 (Munzar, Tanda et al. 2004). En regions corticals relacionades sembla que els receptors H_3 també modularien l'alliberament de DA (Ligneau, Perrin et al. 2007).

Paper de la dopamina en conductes addictives

Reforç

El terme reforç fa referència a una associació d'estímuls que esdevenen gratificants i uns hàbits de resposta. La idea originàriament va esdevenir en el reforç instrumental en rates que permetia l'aprenentatge d'uns hàbits que conduïen a una recompensa. En canvi els animals no aprenien a pressionar la palanca per tal d'aconseguir menjar, aigua o els contactes sexuals si tenien les vies dopaminèrgiques lesionades (Wise and Schwartz 1981). D'altre banda, els animals ben entrenats deixaven de pressionar la palanca per tal d'aconseguir menjar quan es

bloquejava els receptors amb antagonistes dopaminèrgics (McFarland and Ettenberg 1995; Dickinson, Smith et al. 2000).
Tanmateix quan hi havia un bloqueig del sistema dopaminèrgic i sota unes condicions de no-reforç els animals deixaven de pressionar la palanca molt més ràpidament (Wise 1982).

Recompensa

És produeix després que un comportament s'hagi vist reforçat. Hi ha una probabilitat molt alta de respondre a estímuls que van associats a una recompensa, encara que no hi hagi aquesta recompensa. Quan un rata pressiona la palanca i rep un estímul intracranial que activa el circuit del plaer, després a la següent sessió pot haver un reforç en la conducta de l'animal, independentment de que la rata obtingui recompensa(Wetzel 1963; Gallistel, Stellar et al. 1974).
Es creu que la DA juga un paper molt important en la dependència a algunes drogues addictives (Fibiger 1978; Wise 1978). Les primeres versions d'aquesta hipòtesis suggereixen que la DA podria ser decisiva en la dependència a les drogues on s'obté una recompensa (Wise and Bozarth 1987; Di Chiara and Imperato 1988). Malgrat això la fenciclidina (coneguda comunament com a "Pols de l'àngel") (Carlezon and Wise 1996), morfina (Bechara, Harrington et al. 1992) i la nicotina (Laviolette and van der Kooy 2003) semblen activar circuits no dopaminèrgics, però els efectes reforçants sí que serien mediats per la DA. També és qüestionable si els efectes gratificants de les benzodiazepines, barbitúrics o la

cafeïna son dependents de la DA. Per tant, la DA és crucial pels efectes de la recompensa dels psicoestimulants, encara que és important però no indispensable pels efectes de la recompensa dels opiàcids, nicotina, cànnabis i alcohol (Wise 1988).

L'incentiu motivacional

Es la conseqüència obtinguda per la realització d'una conducta motivada. Tals conseqüències poden ser desitjades i conseqüentment generar una conducta dirigida cap a la consecució de l'incentiu o aversives i induir una conducta d'evitació (Chóliz Montañés 2004).

Un estímul neutre com pot ser el color groc de la pell del plàtan després, aquest color anirà associat al seu sabor i esdevindrà un estímul motivacional per a obtenir la recompensa produïda pel sabor. S'ha suggerit que la DA és essencial per la motivació per un incentiu (Roberts and Koob 1982).

Quan el sistema dopaminèrgic és bloqueja en un animal que està aprenent una tasca, deixa d'haver-hi un incentiu per aconseguir la recompensa. En canvi quan és bloquejat en animals que ja han après la tasca ja s'han establert predictors de recompensa però és perd l'incentiu motivacional per la tasca a realitzar.

Un estímul gratificant ha d'ésser eficaç com a reforç si és manté la seva associació al valor de l'incentiu. Si el sistema dopaminèrgic és bloqueja la primera vegada que l'animal obté una recompensa en un nou escenari, no hi haurà la capacitat de repetir la conducta en aquell nou ambient (Spyraki, Fibiger et al. 1982).

Un cop s'ha establert l'incentiu motivacional, els estímuls seran temporalment autònoms i podran impulsar a hàbits de resposta inclús quan el sistema dopaminèrgic s'ha bloquejat amb antagonistes (Dickinson, Smith et al. 2000).

Reforç condicionat

La recompensa que va associada a estímuls motivacionals en la conducta, no només enfortirà el comportament quan s'administra un agonista dopaminèrgic abans d'obtenir una resposta sinó que també pot servir com a reforç condicionat quan s'administra després d'una resposta. Per exemple les rates que tenen set aprenen a treballar amb la presentació d'una llum que està relacionada amb l'obtenció d'aigua.

Si se'ls injecta amfetamina al NAc és produirà un alliberament de DA millorant així la resposta cap a l'estímul lluminós (Taylor and Robbins 1984), mentres que lesionant aquesta àrea cerebral disminuirà la resposta cap a l'estímul (Taylor and Robbins 1986). Per tant la DA pot modular l'expressió del reforç condicionat, ademés de ser essencial per establir reforços condicionats (Horrocks and House 2002; Phillips, Stuber et al. 2003).

L'anhedònia

La hipòtesis de l' anhedònia (Wise 1982) postula que la DA és important per aconseguir el plaer. Es basa en l'evidència que dosis moderades d'antagonistes dopaminèrgics administrades a rates poden bloquejar la voluntat de l'animal per a realitzar les respostes instrumentals, sense que hi hagi una gran limitació en la capacitat

per a realitzar-les. La recompensa és normalment associada a una sensació subjectiva de plaer o eufòria. Estudis en imatges cerebrals indiquen que hi ha certa correlació entre l' eufòria i el grau d'alliberament de DA induïda per drogues (Laruelle, Abi-Dargham et al. 1995).

Es suposa que el plaer té una correlació amb el reforç.Hi ha evidències en animals que poden aprendre a treballar sota estímuls dolorosos (Kelleher and Morse 1968).

Hi ha un elevat numero de persones que han estat diagnosticades de depressió o trastorn de la personalitat que s'infligeixen dolor compulsivament. Els factors desencadenants d'aquesta conducta poden ser alteracions psiquiàtriques com son la depressió o trastorns de la personalitat o bé factors socials com la pobresa o l'atur (Horrocks and House 2002).

El plaer i la recompensa poden estar dissociats inclòs en drogues euforitzants: Hi ha una ràpida tolerància al plaer gratificant de les drogues com la cocaïna (Foltin and Fischman 1991), morfina (Lamb, Preston et al. 1991) i nicotina (Russell 1989). El fet que un subjecte respongui de manera constant davant una droga administrada per sota el llindar del reforç, indica que el plaer és subjectiu i que és pot sentir de manera subliminal (Volkow, Wang et al. 1999; Martinez, Broft et al. 2004). Tanmateix hi ha estudis que indiquen que la recompensa per aconseguir una solució glucosada es devalua amb l'administració de neurolèptics (Pecina, Berridge et al. 1997). Segons *Berridge et al*., el voler i l'agradar poden ser "preconscients" el que implica que la gent no és del tot

conscient del seus gustos i desitjos (Berridge 1995). L' actual evidència suggereix que els augments de DA en el cervell tenen una certa correlació amb el plaer subjectiu.

Paper del nucli accumbens

La hipòtesis que la DA juga un paper molt important en la recompensa té el seu origen en l'observació que la lesió del NAc atenua els efectes gratificants de la cocaïna (Roberts, Corcoran et al. 1977) i la amfetamina (Lyness, Friedle et al. 1979), però en canvi les lesions produïdes al sistema adrenèrgic no alteraven el desig per a obtenir recompensa. Tot i aquests estudis que impliquen al NAc una funció en l'obtenció del premi, no s'han de creure que és l'única àrea implicada, ja que lesions a la zona nigroestriatal causa una desmotivació per l'obtenció de menjar i beure en rates (Smith, Storhmayer et al. 1972).

L'addicció

És una malaltia crònica de l'encèfal que afecta tant la neurotransmissió com les interaccions entre les estructures de la recompensa. Hi ha factors genètics, psicosocials i ambientals que influeixen en el seu desenvolupament. Es caracteritza per episodis de descontrol sobre l'individu, consum complussiu, encara que comporti conseqüències adverses i distorsions del pensament. Tot això provoca una distorsió en la conducta de tal manera que la jerarquia de les prioritats de l'addicte s'alteren (ASAM 2010). Aquesta conducta pot o no estar relacionada amb la dependència a una substància com pot ser l'alcohol, heroïna, cocaïna, etc, o bé a

Internet, menjar, compres, mòbils, sexe, etc. Pot presentar una manca de cura per sí mateix o en les obligacions que ha de realitzar (APA 2000). Tanmateix tots els addictes tenen un patró de conducta que esdevé compulsiva per aconseguir el reforç.

El paper del DAT en el reforç a la cocaïna

La cocaïna és un inhibidor de la recaptació de DA (Heikkila, Orlansky et al. 1975) i els efectes gratificants de la cocaïna son dependents de la DA (Ettenberg, Pettit et al. 1982). La cocaïna té més afinitat pel transportador de DA (DAT) que per altres llocs d'unió (Ritz, Lamb et al. 1987). L'addicció a la cocaïna esdevé com a resultat de la unió de la cocaïna amb el DAT i la inhibició resultant de la recaptació de DA (De Wit and Wise 1977; Roberts, Corcoran et al. 1977). Tot i que és clar que l'acció de la DA és essencial per la recompensa de la cocaïna (Spyraki, Nomikos et al. 1987), s'ha comprovat que ratolins "knockout" de DAT no presenten una menor autoadministració de cocaïna i que els nivells de DA estan augmentats tant si hi ha cocaïna extracel·lular com si no (Rocha, Fumagalli et al. 1998). L'acció de la cocaïna en el DAT pot ser suficient per a l'obtenció de l'efecte gratificant però no del tot necessari. Experiments amb ratolins "knockout" on és bloqueja altres transportadors de monoamines demostren que aquests són aparentment importants. La cocaïna bloqueja els transportadors de serotonina (NET), noradrenalina (SERT) així com també el DAT, i cadascú d'aquests pot eliminar la DA local extracel·lular. Per exemple, la recaptació de DA en l'escorça prefrontal, un lloc de

recompensa de la cocaïna (Goeders and Smith 1983) és mediada principalment per NET, en canvi DAT escasseja en aquesta regió on NET té una gran afinitat per la DA (Moron, Brockington et al. 2002). Els ratolins "knockout" de DAT en el NAc presenten nivells molt alts de DA en aquesta zona, però en aquests animals encara els augmenta més la DA extracel·lular en presència de cocaïna (Carboni, Spielewoy et al. 2001). Això és presumiblement mediat per la inhibició de la cocaïna sobre altres transportadors monoaminègics.

D'altre banda altes concentracions de DA en el bulb olfactori en comptes del NAc podrien ser crucials (Ikemoto 2003), encara que no és clar quin transportador s'activa i elimina la DA de l'espaï sinàptic en el tubercle olfactori.

En tot cas, la cocaïna no és gaire gratificant en ratolins "knockout" de DAT i SERT (Sora, Hall et al. 2001). Per tant l'acció de la cocaïna en els recaptadors NET i/o SERT contribueix al reforç en presència de cocaïna en ratolins "knockout" de DAT.

En animals normals, l'acció de la cocaïna en NET i SERT sembla contribuir poc en el reforç de la droga (Loh and Roberts 1990).

"Voler" vs "agradar"

"Voler" és un terme que utilitzen varis autors per a denominar processos psicològics amb rellevància d'incentiu (Everitt and Robbins 2005; Berridge 2009). Els incentius s'atribueixen a les recompenses i els seus senyals de predicció ajuden a determinar el seu valor motivacional. Aquestes senyals es converteixen en

potents disparadors del “voler”. D’aquesta manera, els desitjos es poden activar només imaginant amb la vista, l’olfacte i el gust (Pelchat, Johnson et al. 2004). "Voler" és psicològica i neurològicament distingible del “agradar”, encara que sovint succeeixen al mateix temps. D'acord amb el concepte de rellevància d'incentius, “voler” és un procés mesolímbic que pot marcar certes representacions d'estímuls al cervell que tenen associacions *pavlovianes* amb la recompensa (Berridge 2009). Un problema pràctic és la cerca de substrats neuronals per poder mesurar el “agradar”. Afortunadament el terme “agradar” és un procés psicològic amb mecanismes neuronals diferents al “voler” i té marcadors objectius en el cervell i la conducta (Berridge and Kringelbach 2008). La majoria de manipulacions cerebrals en humans no es poden portar a terme per motius ètics excepte en animals. S’ha de ser capaç de reconèixer el terme “agradar” de manera fiable fins i tot en animals. Una manera de mesurar “l’agradar” és en les expressions facials per l’impacte hedònic dels sabors dolços en humans i primats. Els sabors dolços provoquen unes expressions facials positives, mentre que els sabors amargs provoquen expressions de desgrat. Afortunadament per a l’estudi del “agradar vs desagradar” les expressions facials son homòlegs entre humans i animals en les quals tenen desenvolupat a partir de la mateixa font d’evolució (Steiner, Glaser et al. 2001). Aquests autors argumenten que encara que la DA no és important per “l’agrat” a la recompensa (veure anhedònia més amunt) si que ho

és pel desig al premi com pot ser el desig de menjar quan hi ha "fam" (Berridge and Robinson 1998).

Recompensa o predicció de recompensa?

Una nova línia d'estudi relaciona les neurones dopaminèrgiques del mesencèfal als senyals d'error que estan implicats en l'aprenentatge d'algoritmes (Schultz and Dickinson 2000).

Aquestes neurones s' activen quan hi ha contacte amb una recompensa inesperada com pot ser el gust o el tacte (Schultz, Apicella et al. 1992).

Quan aquests esdeveniments és repeteixen i deixen de ser inesperats, les neurones dopaminèrgiques del mesencèfal s'activen abans de rebre la recompensa, en comptes de fer-ho al rebre l'estímul o premi. Aquesta troballa suggereix que la DA del cervell és més sensible a factors de predicció de recompensa que a la pròpia recompensa (Romo and Schultz 1990). Fins i tot el gust dels premis actua com a reforç condicionat (Rozin and Kalat 1971). El sabor del premi és un factor de predicció de les conseqüències post-ingesta del menjar, ja que deixa una petjada de records per l'associació dels aliments.

Els reforços tenen una acció dual sobre el comportament: els estats afectius si és combinen amb els estímuls ambientals poden influir en el comportament futur i provocar una millora en les associacions prèviament formades (Messier and White 1984). Per tant la predicció de recompensa a estímuls condicionats pot tenir dos funcions: de guia i de modulació de la conducta ja que deixa

una empremta en la memòria per les associacions que les van precedir (Di Ciano and Everitt 2003).

Consum de cocaïna en humans

La cocaïna ($C_{17}H_{21}NO_4$) **(Figura 14)** és un alcaloide que s'extreu d'una planta nomenada *Eritroxillum Coca*, arbust originari de Perú i Bolívia **(Figura 15).**

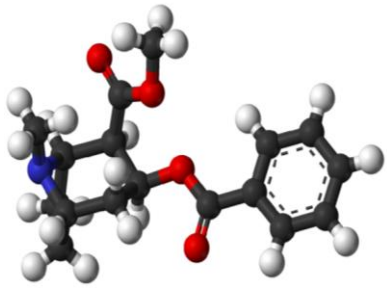


Figura 14. *Estructura tridimensional de la cocaïna.*

Figura 15. *Planta Eritroxillum coca.*

Segons un estudi de l'etnòleg anglès Antonil que va publicar l'any 1978, els orígens es remunten a començaments del període postglacial quan l'arbust *Eritroxillum Coca* es devia descobrir a les faldes orientals dels Andes centrals (per a la bona qualitat, aquest arbust necessita créixer entre els 1000 i 2000 metres sobre el nivell del mar) per un petit grup de nòmades que van començar a poblarla. Les proves arqueològiques més antigues del consum humà de la fulla de la coca daten del S.IV ac que pertany al període preceràmic (Bureau 1982).

Encara que les primeres referències escrites a europa sobre aquesta substància son del segle XVI pel metge i botànic espanyol Nicolás Bautista Monardes (1493-1588) **(Figura 16).**

Figura 16. Gravat de Nicolás Monardes. *Metge i botànic sevillà (1550).*

El seu treball més significatiu i conegut va ser "*Historia medicinal de las cosas que se traen de nuestras Indias Occidentales".*

En aquesta obra es va proposar l'estudi i l'experimentació amb productes i "medicines del Nou Món". Va conrear i descriure per primera vegada moltes espècies com la pebre, canella, tabac i la planta de la coca. (Viesca-Trevino 1989; Dewey 1992). Aquest alcaloide va ser aïllat per primer cop a europa l'any 1855 pel químic alemany Friedrich Gaedcke (1828-1890) (Zaunick 1956).

El químic francès Ange-François Mariani (1838-1914) va ser el creador d'un vi amb extractes de cocaïna, Vin Mariani (1863) **(Figura 17).** Va ser indirectament qui va introduir al públic en general al consum de cocaïna.

Mariani va importar tones d'aquesta planta a europa i els va utilitzar en una gran quantitat de productes.

Figura 17. *Rètol publicitari del "Vin Mariani". Litografia de Jules Chéret 1894.*

Aquest fet el va fer immensament ric, inclòs el Papa Lleó XIII el va condecorar amb una medalla d'agraïment (http://www.euvs.org 2008). Aquesta beguda es considera el precursor de la Coca-Cola®.

Després de la creació d'aquest vi tant popular, altres fabricants van comercialitzar begudes o "medicines" que contenien cocaïna o altres drogues psicoactives com la heroïna o l' opi. La cocaïna tòpica també era utilitzada com anestèsic local tant en cops com

pel dolor de queixals. El seu ús era recomanat tant per adults com per a nens quan es feien mal **(Figura 18).**

Figura 18. *Rètol de propaganda de la cocaïna (1900).*

L'any 1883 el físic militar Theodor Aschenbrandt va administrar cocaïna en gran puresa als soldats Bàvars durant unes maniobres. Va descriure l' augment de la capacitat dels soldats per a suportar el cansament.

El 1885 John Pemberton va crear una beguda mundialment coneguda com a Coca-Cola® a la seva farmàcia d'Atlanta. Amb una barreja de fulles i llavors de coca, va voler crear un remei que començà essent comercialitzat com a una "medecina" que disminuïa el mal de cap i les nàusees **(Figura 19).**

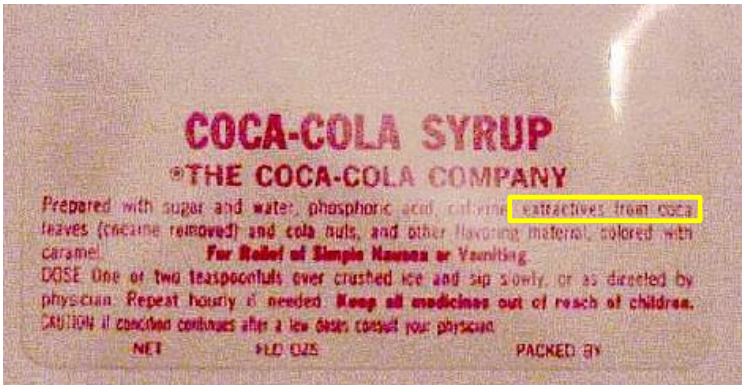


Figura 19. *Etiqueta dels ingredients de la beguda Coca-Cola® (1906). Amb color groc es mostra la utilització de la cocaïna com a ingredient.*

Cap als anys 1890 ja era coneguda les propietats de la cocaïna per a produir psicosis i crear addicció (Jones 1953). Durant anys la cocaïna va ser un component d'aquesta beguda, fins que a principis del segle XX es va retirar de la fórmula en previsió de les lleis que prohibirien la venda i ús de la cocaïna. Aquesta substància és va prohibir l'any 1914.

Un jove neuròleg vienès, Dr. Sigmund Freud va quedar fascinat per aquest informe i va experimentar amb aquesta droga inclòs injectant-se-la ell mateix. Alguns dels seus descobriments sobre la cocaïna, com el de ser una droga psicoactiva van quedar àmpliament confirmats en posteriors investigacions.

El consum de cocaïna ha augmentat en els últims 10 anys, i és la segona droga il·legal més consumida a Espanya després del cànnabis (De la Fuente, Barrio et al. 1998; OEDT 2010) i va en augment. La població entre 15 i 64 anys consumidora de cocaïna segons: "Enquesta domiciliària sobre drogues" es va incrementar

del 1,8% en el 1995 al 4,1% al 2010. Encara que les xifres de consum oscil·len molt d'un país a l'altre, son majors en països de l'Europa occidental.

Existeix una diversitat considerable entre els consumidors d'aquests substància, tant en les pautes de consum com en les variables sociodemogràfiques. En alguns països europeus, un numero considerable de persones han consumit cocaïna de forma experimental o només un cop o dos ocasions en la seva vida (Van der Poel, Rodenburg et al. 2009). Entre els consumidors més habituals d'aquesta droga, es poden distingir dos grans grups. El primer està format pels consumidors "socialment integrats" que consumeixen cocaïna els caps de setmana, festes o altres ocasions especials, a vegades en grans quantitats (consum compulsiu) o amb certa freqüència. També hi ha proves que molts d'aquests consumidors de cocaïna socialment integrats controlen el consum d'aquest droga imposant-se normes que limiten la quantitat, freqüència o el context del consum (Decorte 2000). Alguns d'ells poden patir problemes de salut relacionats amb el consum d'aquesta droga o adquirir pautes compulsives de consum que requereixin tractament.

Els estudis indiquen que una part important dels consumidors problemàtics de cocaïna poden recuperar-se sense necessitat de rebre tractament formal (Cunningham 2000). El segon grup està format per consumidors més «marginats» o «socialment exclosos», alguns dels quals també consumeixen o han consumit en el passat opiacis, consumeixen *crack* (es produeix barrejant la

cocaïna amb amoníac o bicarbonat sòdic, posteriorment s'escalfa i quan està fred es fuma) o s'injecten cocaïna. En aquest grup es troben els consumidors intensius de cocaïna i *crack* que pertanyen a grups socialment desafavorits, com treballadors/es del sexe o immigrants (Prinzleve, Haasen et al. 2004).

Encara que actualment no existeix cap farmacoteràpia efectiva, els pacients tractats per consum de cocaïna reben recolzament psicosocial i teràpies cognitivo-conductuals. Els fàrmacs que s'utilitzen son per alleugerar els símptomes de la dependència a la cocaïna. Fins ara, s'han avaluat molts fàrmacs per al tractament de la dependència a la cocaïna i cap d'ells ha sigut clarament eficaç, i ni la *Agència Europea del medicament* ni la *Food and Drug Administration* dels EEUU han aprovat un fàrmac per al tractament de la dependència a aquesta droga (Kleber, Weiss et al. 2007).

El 46% dels drogoaddictes son consumidors de cocaïna. Les dades oficials de usuaris que demanen començar tractament per tal de deixar la cocaïna son del 10% aproximadament. La proporció home-dona consumidors d'aquesta droga que inicien tractament és de 5:1 i l'edat mitjana és d'uns 32 anys.

L'edat mitjana que és comença a consumir cocaïna és als 22 anys. Des de l'inici del consum de cocaïna fins a l'inici del primer tractament que sol·liciten passen d'uns 9 anys. La majoria de pacients que reben tractament esnifen (63%), fumen (31%) i el 3% consumeix la droga via parenteral. Un estudi sobre els tractaments facilitats va revelar que el 63% dels pacients que iniciaven tractament eres policonsumidors de drogues (OEDT 2010).

El consum crònic de drogues d'abús pot portar a l'addicte a un augment de la inestabilitat emocional com pot ser: irritabilitat, agitació, atacs de pànic inclós els trastorns psicòtics com la paranoïa i l'esquizofrènia (NIDA 2008).

Model d'autoadministració de cocaïna en rates com a predictor de l'efecte en humans

L'addicció no es només el consum de drogues, sinó un ús continuat i compulsiu a pesar del perjudici que comporta (Deroche-Gamonet, Belin et al. 2004). L'estudi portat a terme per Deroche-Gamonet es va realitzar en rates i va servir per avaluar algun tipus de conducta que estigués sota criteri de diagnòstic d' addicció a les drogues en humans, segons el DSM-IV (APA 2000). De forma similar als símptomes de l'addicció en humans, aquest estudi posa de manifest que les rates que s'autoadministraven cocaïna endovenosa tenien dificultat per deixar de consumir-la o limitar el seu consum. Els subjectes estaven molt motivats per buscar la droga i aquesta era consumida contínuament encara que tenia conseqüències perjudicials (Deroche-Gamonet, Belin et al. 2004). Altres estudis d'autoadministració de cocaïna en animals com els cucs planària (Raffa and Valdez 2001), la Drosophila Melanogaster (Torres and Horowitz 1998), peixos com el "Goldfish" (Volkoff and Peter 2001) o els crancs de mar (Panksepp and Huber 2004), suggereixen que l'activació per cocaïna de les vies dopaminèrgiques formen part d'una conservació evolutiva en aquests animals, vertebrats superiors i l'ésser humà. La cocaïna

activa les vies implicades en els reforços naturals de manera intensa i confusa i dóna lloc a un plaer distorsionat fora del rang de les experiències reforçants naturals, com ara el menjar, sexe...etc.

Un cop experimentats els efectes de la cocaïna per l'animal, el desig per tornar–la a consumir pot iniciar-se per estímuls visuals, olfactius o auditius prèviament associats als efectes de la cocaïna, que bloquegen l'atenció de l'individu sobre ells (Childress, Mozley et al. 1999; Kalivas and McFarland 2003) de manera que pot arribar a ser molt més intens que les necessitats de menjar i beure.

S'ha observat en diversos experiments d' autoadministració, que la lliure disposició de cocaïna, pot arribar a ser preferida abans que el menjar o l'aparellament, i que amb freqüència condueix al deteriorament biològic i a la mort. Estudis amb animals han confirmat que els estímuls ambientals associats al reforç provoquen la cerca de la droga, provocant la recaiguda (Shalev, Grimm et al. 2002). En els èssers humans, l'exposició a contextos ambientals previament associats al consum de drogues sovint provoca la recaiguda, encara que hagin fet una bona teràpia d'extinció fora del seu ambient on consumien la droga (Crombag, Bossert et al. 2008). Els estímuls contextuals que prediuen la disponibilitat de la cocaïna provoquen la cerca per la droga en la fase d'extinció (Weiss 2010). Ademés, la busqueda de la droga induïda per estímuls mostra una gran resistència a l'extinció (Ciccocioppo, Angeletti et al. 2001), i en el cas de la cocaïna és pot observar després de diversos mesos d'abstinència (Weiss, Martin-Fardon et al. 2001).

L'estrès es un altre factor com a desencadenant de la recaiguda en el consum de drogues en els èssers humans (Kreek and Koob 1998). Situacions d'estrés emocional aplicats a animals de laboratori, faciliten la recaiguda en l'autoadministració de la droga o l'increment en la seva busqueda en rosegadors (Ramsey and Van Ree 1993). L'estrès físic s'aconseguia aplicant descàrregues elèctriques a les potes dels animals, provocant la recaiguda en el consum d'heroïna (Ahmed, Walker et al. 2000), etanol (Martin-Fardon, Ciccocioppo et al. 2000), nicotina (Buczek, Le et al. 1999) o cocaïna (Ahmed and Koob 1997). Es creu que totes aquestes alteracions son la base dels símptomes com l'ansietat, irritabilitat, excitació autònoma i una resposta exagerada als estímuls ansiògens que sorgeixen quan hi ha una interrupció del consum de la droga (McDougle, Black et al. 1994).

L'evidència creixent suggereix que els canvis neuroadaptatius duren més que l'abstinència i la desintoxicació (Kowatch, Schnoll et al. 1992). Per exemple, addictes a la cocaïna que han rebut tractament per desintoxicar-se presenten un augment d'ansietat i pànic (Goodwin, Stayner et al. 2002).

L'ansietat i altres símptomes com el desig a consumir, la mala regulació del cicle son vigília i altres simptomes somàtics fan predir un mal pronòstic (Kasarabada, Anglin et al. 1998).

Aquests simptomes fruit d'una "abstinència perllongada" que van ser descrits originariament en addictes als opiàcids (Martin and Jasinski 1969), també representen una complicació freqüent en

pacients que és recuperen de l'addicció a la cocaïna i a l'alcohol (Satel, Kosten et al. 1993; Meyer 1996; Kreek and Koob 1998).

Mètodes de determinació de la síntesi i l'alliberament de dopamina

L'esmentada relació de la DA amb les propietats addictives de la cocaïna fa que el sistema neurotransmissor dopaminèrgic cerebral sigui la principal diana terapèutica pel desenvolupament de fàrmacs pal·liatius pel tractament de l'addicció.

Els mètodes de determinació de síntesi i alliberament de DA poden ser molt rellevants en aquest cerca. Al llarg del anys les tècniques emprades per detectar DA en teixit han evolucionat molt. El terme cromatografia prové del grec *χρομα chroma "color"* i *γραφω graphos "escriure"*. La cromatografia de columna líquida va ser descoberta a principis del segle XX pel botànic Tsweet (Abraham 2004), però va passar desapercebuda durant 25 anys, fins que cap a l'any 1931 Lederer i Kuhn van utilitzar el mètode de Tsweett per a la separació de productes naturals. La tècnica de la cromatografia es va fer popular degut a la alta resolució i velocitat. La millora en el rendiment de la tècnica ha provocat que la seva utilització creixi molt a partir del anys setanta, convertint-se avui en dia com un dels principals mètodes d'anàlisi en moltes disciplines científiques, amb les sigles HPLC cromatografia líquida d'alt rendiment (o d'alta pressió).

Hi ha hagut almenys una dotzena de tècniques descrites per a la separació i purificació de catecolamines i els seus metabòlits. La

majoria de les tècniques es basen en l'intercanvi iònic, fase reversa o adsorció (Amberlite, alúmina).

La HPLC de fase reversa (RP-HPLC) consisteix en una fase estacionària apolar i una fase mòbil de polaritat moderada. Una de les fases estacionàries més comunes en aquest tipus de cromatografia és la columna C_{18}, que conté cadenes hidrofòbiques de 18 carbons de longitud. El temps de retenció del compost és major per a les molècules de naturalesa apolar, mentres que les molècules amb caràcter polar son arrossegades i eliminades més rapidament. El temps de retenció disminueix amb l'addició de disolvents polars a la fase mòbil i augmenta amb la utilització de disolvents més hidrofòbics. La cromatografia de fase reversa es basa en el principi de les interacions hidrofòbiques que provenen de les forçes de repulsió entre un disolvent relativament polar, un compost relativament apolar i una fase estacionària apolar (Freifelder 1981).

L'extracció de les mostres mitjançant la tècnica de l' alúmina és simple, econòmica, ràpida i adequada per preparar mostres i realitzar una cromatografia líquida amb detecció electroquímica. Aquesta extracció es basa en l'adsorció de les catecolamines sobre alúmina en un mitjà alcalí. La alúmina pot ser rentada amb aigua o una solució tampó, i les catecolamines difoses per acidificació. Els resultats de l'extracció d'adsorció son deguts a l'atracció entre l'alúmina i els dos grups hidroxils de l'anell benzè del nucli catecol. Com a resultat, tots els grups catecol i no només les catecolamines s'extreuen mitjançant l'alúmina (Krstulovic 1986).

La espectrometría de fluorescència o fluorometria utilitzada cap al 1950, consistia en aplicar a les mostres unes reaccions químiques: la primera desenvolupada per *Natelson et al*., consistia en condensar els grups catecol de les amines amb etilendiamina fet que produïa una fluorescència molt intensa. Amb aquesta tècnica es podia mesurar catecolamines, però era molt poc específica. La segona reacció va ser desenvolupada per Arvid Carlsson, guanyador del premi Nobel de Medicina l'any 2000. Consistia en una reacció per oxidacció de la conversió de la DA en un derivat indol i posteriorment era tractat amb una sol·lució àcida, fet que produïa fluorescència en la mostra (Carlsson 1959). Aquesta tècnica era més específica ja que és coneixia que la longitud d'ona de la DA era més curta que altres amines com la adrenalina o noradrenalina (Carlsson, Lindqvist et al. 1958).

Glowinski et al., incubaven els teixits amb L-3,5 ^{3}H-tirosina per després mesurar la ^{3}H-DA sintetitzada. La DA radiactiva tenia que ser separada per una cromatografia d'intercanvi iònic de la ^{3}H-tirosina. També es mesurava la ^{3}H-H_2O formada a partir de la biosíntesi de la ^{3}H-DA com a mesura indirecta, però més senzilla, de la síntesi (Besson, Cheramy et al. 1971). Una de les tècniques més utilitzades per estudiar la síntesi de DA en homogenats ha sigut el mètode de l'alliberament $^{3}H_2O$ formada a partir de l'incubació amb ^{3}H-tirosina amb l'absorció del sustrat isotòpic per una suspensió acuosa de carbó actiu (Reinhard, Smith et al. 1986).

Una altra tècnica emprada per determinar la síntesi de DA, consisteix en incubar el teixit amb L-[1-^{14}C] tirosina i monitoritzar la producció de $^{14}CO_2$ (Patrick and Barchas 1974).

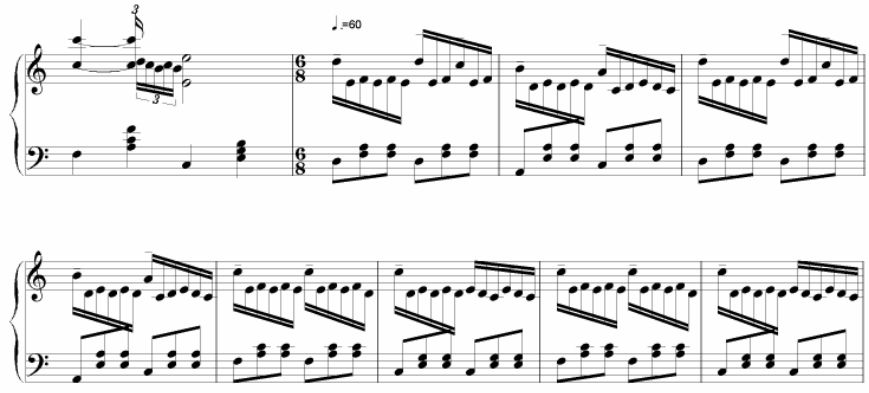
Amb la finalitat d'estudiar la síntesis de catecolamines també es pot determinar l' acumulació de L-DOPA després d'inhibir l'enzim dopa descarboxilasa mitjançant el NSD-1015. Després la L-DOPA es quantifica mitjançant la detecció electroquímica per HPLC (Lindgren, Xu et al. 2001).

Encara que amb aquest mètode es pot arribar a detectar concentracions fins a fentomols, no es possible estudiar els mecanismes relacionats amb l'inhibició per retroalimentació ja que degut a la inhibició de la descarboxilació de L-DOPA no es formarà DA (veure article a l'ANNEX, pàgina 203).

Per l'altre costat en els típics estudis de microdialisis in vivo, es pot mesurar l'alliberament de catecolamines però no s'acostuma a mesurar la seva síntesi (Herrera-Marschitz, Arbuthnott et al. 2010).

Una altre tècnica per determinar la DA alliberada consisteix en preincubar el teixit amb ^{3}H-DA durant uns 15 min. Després es realitzen rentats amb el tampó per eliminar la ^{3}H-DA no captada. Finalment per estimular l'alliberamenent de DA en el teixit, s'incuba amb algun agonista dopaminèrgic i les mostres son recollides on es determinarà la radioactivitat (Russell, de Villiers et al. 1998).

OBJECTIUS



OBJECTIUS

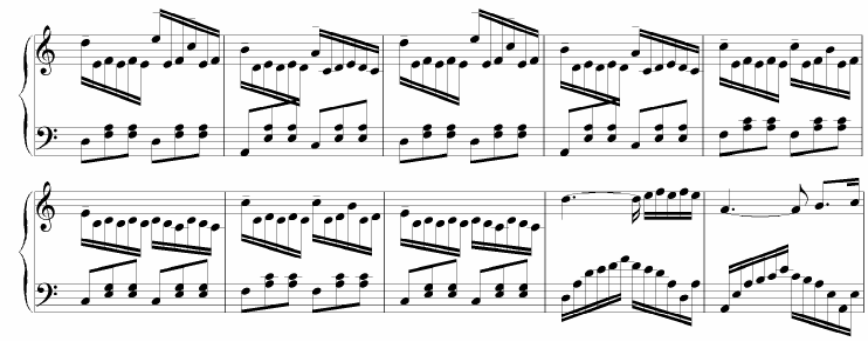
Actualment en la nostra societat s'ha incrementat considerablement el consum de substàncies psicoestimulants i, en concret, de cocaïna. Tanmateix, encara no existeix cap fàrmac efectiu per a poder combatre la dependència de la cocaïna. Donada la implicació del sistema dopaminèrgic en l'addicció a les drogues, el nostre grup s'ha proposat posar a punt un mètode senzill, acurat i sensible per a determinar la síntesi de DA en miniprismes d'estriat de rata per facilitar la cerca de fàrmacs útils per les addiccions.

Ja que seria possible que fàrmacs que actuïn sobre la síntesi o l'alliberament de DA puguin modular conductes addictives, es va voler observar si l'administració d'un agonista de receptors H_3 d'histamina alterava els patrons de conducta d' autoadministració de cocaïna. Com hem mencionat a la introducció, les neurones dopaminèrgiques semblen tenir receptors H_3 d'histamina. Per tant els objectius d'aquesta tesi són:

- Posar a punt i avaluar el nostre mètode per determinar la síntesi de DA *in vitro* respecte metodologies prèvies.
- Comprovar l'efecte de l'agonista H_3 imetit sobre la síntesi de DA en miniprismes d'estriat de rata.
- Avaluar l'efecte de l'agonista H_3 imetit sobre l'extinció de la conducta d' autoadministració de cocaïna en rates.
- Avaluar l'efecte de l'agonista H_3 imetit sobre l'autoadministració de cocaïna en rates.

- Avaluar l'efecte de la coadministració de l'antagonista H_3 tioperamida i de l'agonista imetit en una sessió d'autoadministració de cocaïna en rates.
- Avaluar l'efecte de l'agonista H_3 imetit en una sessió de raó progressiva sota reforç de cocaïna en rates.

MATERIALS I MÈTODES



MATERIAL I MÈTODES

Animals

Per a realitzar aquest treball experimental es van utilitzar rates mascle Sprague-Dawley (OFA) subministrades pel Servei d'estabulari de la UAB, amb una edat aproximada de 8 setmanes i un pes corporal que oscil·lava entre 250-300 gr. Els animals estaven estabulats en caixes transparents de 900 cm^3 (45.5x24x20) amb un cicle llum-foscor de 12:12, l'inici del qual era a les 8h. Els flocs de les caixes eren el model ultrasorb de Panlab®. La temperatura es mantenia constant a 22±2º C, i tant l'alimentació (pinso per a rosegadors A04, Panlab®) com l'aigua s'administrava *ad líbitum*, excepte durant l'entrenament d'autoadministració operant. Els procediments utilitzats en aquest estudi van realitzar-se conforme la directiva de la Comunitat Econòmica Europea en la regulació i ús d'animals de laboratori (86/609/CEE, de 24 de Novembre de 1986) i el decret de la Generalitat de Catalunya (DOGC 2450 7/8/1977) en el que es regula la utilització d'animals per a l'experimentació i per altres finalitats científiques. Tanmateix, el procediment experimental va ser aprovat per la Comissió d'Ètica en Experimentació Animal i Humana (CEEAH) de la Universitat Autònoma de Barcelona.

Reactius i material

Els estocs de [3,5-^{3}H] L-Tirosina van ser subministrats per Amersham Biosciences® (Espanya), les columnes C18 de fase-reversa (Tracer Extrasil ODS2, amb una longitud i diàmetre de 25 x

0,46 cm i un tamany de partícula de 5 µm) per Teknokroma (Espanya); el còctel de centelleig (Optiphase HiSafe III) per Wallac; el Thermomixer confort per la incubació de les mostres, així com els tubs corresponents van ser subministrats per Eppendorf (eppendorf Ibérica). Finalment, la Tirosina i altres reactius van ser obtinguts per Sigma Chemical Co (Espanya).

Purificació de ^{3}H-Tirosina i ^{3}H-dopamina

Per a realitzar la purificació hem utilitzat l'HPLC (*High Performance Liquid Chromatography),* que consta de: fase mòbil, fase reversa, bomba, injector, columna, detector i col·lector. Tot el sistema va connectat a un ordinador.

Previament a la realització dels experiments *in vitro*, es va purificar la L-[3,5-^{3}H] tirosina comercial per eliminar les possible impureses. La fase mòbil que vem emprar estava formada per tampó fosfat (NaH_2PO_4 0.1 M, 0.75 mM de octanosulfònic, 1 mM EDTA, metanol 1%, pH 3.4). La fase mòbil utilitzada per purificar la ^{3}H-DA, estava formada per tampó fosfat (NaH_2PO_4 0.1 M, 0.75 mM de octanosulfònic, 1 mM EDTA, metanol 12%, pH 5.0).

La bomba del HPLC manté en el sistema un flux constant de 1ml/min. La fase mòbil passa a través d'una columna de C18, que conté cadenes hidrofòbiques d'una longitud de 18 carbonis i que reté substàncies hidrofòbiques. La fase mòbil conté octanosulfònic, un compost amfipàtic, amb un extrem carregat negativament i una cadena hidrocarbonada que s'uneix a la columna convertint-la en una columna d'intercanvi catiònic.

Per tant, quan l'injector introdueix la ^{3}H-DA en el flux, i passa per la columna, queda retinguda parcialment degut a la càrrega positiva present al grup amino de la seva estructura.

L'espectrofotòmetre ultraviolat (A=285nm) detecta els patrons de tirosina i la dopamina, ja que el grup aromàtic absorbeix en aquesta longitud d'ona. Quan l'espectrofotòmetre detecta la presència d'aquest grup aromàtic, envia un senyal al col·lector que desvia el flux fins a un tub concret i així recull la mostra amb l'objectiu de quantificar la radioactivitat en forma de triti present a la molècula. Les dades de les absorbàncies eren registrades per l'ordinador, de manera que s'obtenien cromatogrames on s'hi observava un pic als 10 minuts corresponent al patró intern de la ^{3}H-DA . L'àrea dels pics era quantificada mitjançant un software específic (Borwin 1.5 JMBS Developpments, USA).

La quantitat de ^{3}H-DA formada durant la incubació amb el precursor radioactiu no és suficientment gran com per a poder detectar la seva presència mesurant l'absorbància, així com tampoc és quantificable la DA endògena de la mostra. El que realment s'observa és la DA del patró intern **(Fig. 1A capítol 1)**, que s'afegeix juntament amb l'àcid tricloroacètic per precipitar les proteïnes després de la incubació. La mesura de l'àrea del pic del patró intern de la DA ens permet calcular l'eficiència d'aquest procés, comparant-lo amb un patró extern de la mateixa concentració.

Quantificació de la síntesi i l'alliberament de DA en estriat de rata

El mètode utilitzat ja va ser estandarditzat amb histamina (Ortiz, Gomez et al. 2000). Part del treball d'aquesta tesi va consistir en aplicar a la DA el mètode emprat pel laboratori prèviament per determinar la síntesi i alliberament d'histamina.

Els animals es van sacrificar sense anestèsia per decapitació. El cervell va ser extret de la cavitat cranial i submergit en una solució de tampó Krebs-Ringer en fred (TKR: 120 mM NaCl, 0.8 mM KCl, 2.6 mM $CaCl_2$, 0.67 mM $MgSO_4$, 1.2 mM KH_2PO_4 27.5 mM $NaHCO_3$, 10 mM Glucosa, pH 7.4) per preservar el teixit. La dissecció del nucli estriat, juntament amb la retirada de les restes dels vasos sanguinis i leptomeninges es va realitzar a una càmbra freda a 4 ºC. El teixit va ser processat amb un *chopper* Mc Ilwain (Campden Instruments Ltd) per a obtenir miniprismes amb un gruix de 300 µm x 300 µm. Després van ser resuspesos en TKR i es van rentar tres cops amb el mateix tampó. Els miniprismes es van distribuir en alíquotes de 25 µl de mostra (contingut aproximat de 0.5-1 mg proteïna) i van ser preincubades durant 30 minuts, utilitzant un *Thermomixer* que permetia controlar en tot moment els paràmetres d'agitació, gasificació i temperatura de la mostra.

Els fàrmacs utilitzats en els experiments, van ser afegits als 15 minuts de la preincubació per afavorir la seva difusió i distribució pel teixit. Als 25 minuts de la preincubació, es va afegir [^{3}H]-tirosina, a una concentració final de 0.12 µM. Quan va finalitzar la preincubació de 30 minuts, es va afegir el tampó despolaritzant

(TKR) a les mostres que ho requerien fins a obtenir un volum final de 250 µl. Posteriorment es van incubar les mostres durant 10 minuts. En els experiments que voliem quantificar l'alliberament de DA, vem centrifugar les mostres durant 1 minut a 1000 rpm, obtenint aproximadament 150 µl de tampó que hi contenia la DA alliberada.

En una mostra centrifugada, es considera que en el tampó hi conté el neurotransmissor alliberat al medi extracel·lular, i que en el teixit hi resta la DA sintetitzada que no ha estat alliberada.

Aquesta DA alliberada es va posar en tubs nous, i s' hi va afegir a cada mostra, 35 µl d'una mescla d'àcid tricloroacètic (0.5%), DA (100 nM) i àcid ascòrbic (120 nM) per evitar l'oxidació de la DA (Hugh, Grennan et al. 1987). La DA afegida en la barreja anterior va ser utilitzada com a patró intern per a calcular l'eficàcia del posterior tractament de la mostra. En el teixit restant se li va afegir aproximadament 150 µl de TKR i 35 µl de la solució utilitzada abans d'àcid tricloroacètic, DA i àcid ascòrbic. Es va homogeneïtzar el teixit per sonicació en gel, per evitar el sobrecalentament de les mostres. Part d'aquest volum va ser utilitzat per a determinar la concentració de proteïna mitjançant el mètode Lowry. Posteriorment es van centrifugar les mostres a 1000 rpm's i 4ºC i es va recollir el sobrenadant desproteïnitzat. Les dpm's obtingudes van ser corregides pels blancs adients i per la recuperació de l'estàndard intern.

Procediment d'autoadministració de cocaïna en rates

Per a realitzar aquest treball experimental es van utilitzar 54 rates mascle OFA Sprague-Dawley, amb una edat aproximada de 8 setmanes i un pes corporal inicial entre 250-300 gr.

Aquestes rates van ser criades al Servei d'Estabulari de la Universitat Autònoma de Barcelona, sota les condicions prèviament descrites.

Caixes d'autoadministració operant

En aquest experiment es van utilitzar 8 caixes modulars d'autoadministració operant o gàbies d' Skinner (25 cm x 25 cm x 25 cm; LE1005, Panlab S.L., Barcelona, Espanya) **(Figura 20)**.

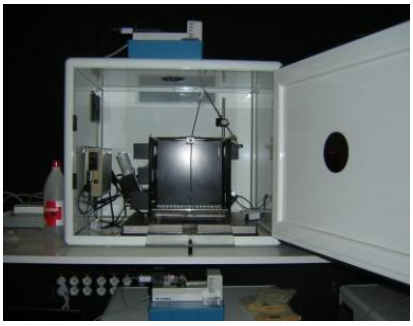


Figura 20. Gàbia d'Skinner. *Panlab®.*

Cadascuna de les gàbies estava col·locada dins d'una altra insonoritzada que contenia un aparell de ventilació.

Al centre de cada gàbia s'hi ubicava un recipient de 3.5 x 3.5 cm, a través del qual es subministraven els *pellets* de menjar mitjançant un dispensador situat a la part externa de la caixa. A uns 20 cm per

sobre el dispensador hi havia una llum de color blanc 2.4W, 24V que era la llum de la gàbia que s'encenia quan començava una sessió. A 4 cm del terra i a cada costat del dispensador de *pellets* en sobresortien dues palanques metàl·liques; en el moment dels l'experiments, la palanca designada com a activa era retràctil; mentres que la palanca inactiva era fixa (**Figura 21).**

Figura 21. Gàbia d'Skinner. *Palanca activa (esquerre), palanca inactiva (dreta).*

Sobre cadascuna de les palanques hi havia un disc de 4 cm de diàmetre que s'il·luminava mitjançant una llum blanca 2.4W, 24V i servia com a estímul discriminatori per assenyalar la presentació immediata del reforç. Les infusions de cocaïna s'administraven mitjançant un tub de plàstic que anava connectava a una xeringa de 20 ml situada a la part externa de la superfície de la caixa insonoritzada, controlada per una bomba d'infusió. L'altre extrem anava connectat al catèter de l'animal; aquest tub estava col·locat a l'interior d'un altre tub flexible metàl·lic (per evitar que l'animal

ho mossegués) que s'enrroscava a la part externa del catèter. L'aparell i la recollida de dades era controlat pel programa Packwin (Panlab S.L., Barcelona, Espanya).

Entrenament de reforç per sacarosa

Amb l'objectiu que els animals es familiaritzessin amb el funcionament de les gàbies de conducta, es va realitzar un entrenament de resposta a la palanca utilitzant sucre com a reforç. Les rates van ser entrenades a respondre per obtenir *pellets* de sacarosa (45 mg *pellets*; Bio-Serv, Frenchtown, NJ, USA). Aproximadament 7 dies abans de l'inici de l'entrenament de reforç per sacarosa, es va restringir la ingesta d' aliments als animals i es va reduir el seu pes fins al 85%, mantenint-lo durant tot l'entrenament.

Es van realitzar sessions diàries en les quals les rates tenien que pressionar la palanca designada com a "activa" per aconseguir el *pellet* de sacarosa. Les respostes a la palanca inactiva no tenien cap conseqüència però eren enregistrades. La sessió inicial va consistir en una sessió nocturna de 14 hores, a l'inici de la qual s'administraven 10 *pellets* no contingents (1 cada 50 segons). La sessió començava amb l'aparició de la palanca retràctil (activa). Si la rata apretava la palanca activa se li administrava un *pellet* a través del dispensador, i s'encenia la llum situada sobre la palanca activa (s'encenia durant 5 segons). Immediatament després, tant la llum de la palanca activa com la de la càmera s'apagaven durant 10 segons: aquest període l'anomenàvem "time out" (15 segons).

Durant el time out les respostes de l'animal a qualsevol palanca no produïen cap conseqüència, però eren enregistrades per ser analitzades posteriorment.

Vem utilitzar un programa d'entrenament de raó fixa 1 (FR1+15 segons "time out"). Quan acabava el període de time out, s'encenia la llum de la gàbia i la seqüència començava altre cop. Aquesta sessió inicial de 14 hores es repetia com a màxim 3 vegades, en les quals si la rata no s'administrava 80 *pellets* era eliminada de l'experiment. Durant totes les sessions les rates tenien accés a aigua *ad libitum*. Normalment les rates necessitaven una única sessió per aprendre les contingències i posteriorment es realitzaven dues sessions de dos hores de durada, durant la fase de llum del cicle. Aquestes sessions d'entrenament seguien els mateixos paràmetres definits anteriorment i finalitzàven quan la rata aconseguia 100 *pellets* en tres sessions consecutives.

Autoadministració de cocaïna

Després de l'entrenament de reforç, a les rates se'ls hi va implantar un catèter endovenós seguint el procediment de cirurgia establert (Colby, Whisler et al. 2003) **(Figura 22).**

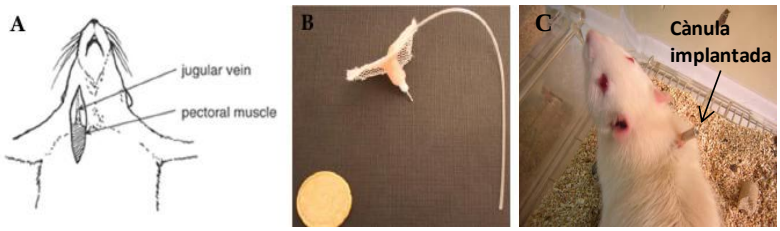


Figura 22. Implantació quirúrgica del catèter. A) *L'incisió a la vena jugular dreta permet canalitzar el catèter.* ***B)*** *Catèter abans de ser insertat. C) La cànula que sobresurt és protegida amb una rosca per evitar la seva obstrucció.*

Les sessions d'autoadministració es van dur a terme durant 5-6 dies a la setmana i van tenir una durada de 2 hores diàries per animal. La cocaïna, en forma de clorhidrat (cedida pel Ministeri de Sanitat) es va dissoldre en 0.9% de sèrum fisiològic estèril a una concentració de 5 mg/ml.

Inicialment, les rates pressionaven la palanca per obtenir una infusió endovenosa de cocaïna de 0.5mg/kg en un volum de 0.1ml administrada sota un programa de reforç de raó fixa (FR1), de manera que una resposta a la palanca activa resultava d'una infusió endovenosa de 100 µl de solució de cocaïna durant 5 segons a través del catèter. Les respostes a la palanca inactiva van ser registrades però no van produir cap conseqüència. La llum de la gàbia s' il·luminava a l'inici de la sessió. Quan l'animal apretava la palanca activa rebia una infusió de cocaïna, s'encenia la llum sobre la palanca activa durant 5 segons, i després s'apagaven tots els estímuls lluminosos durant 10 segons (període 'time out'). Després d'aquest període es tornava a encendre la llum de la gàbia i s' iniciava altre cop la seqüència. També es van registrar les respostes

en la palanca activa durant els períodes 'd'infusió' i 'time out', encara que no provocaven cap conseqüència **(Figura 23).**

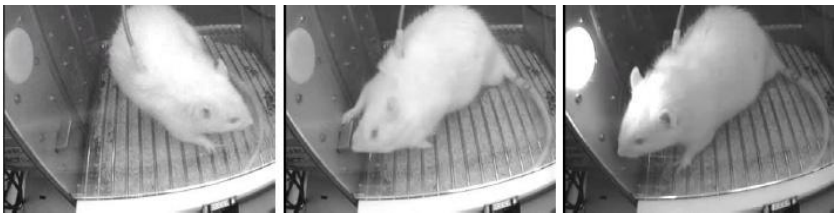


Figura 23. Autoadministració de cocaïna. *Després d'un període de latència (esquerre), la rata comença a pressionar la palanca activa (centre) fins que aconsegueix el reforç de cocaïna, que va associat al llum encès (dreta) i un període de "time out".*

Les rates van estar en el programa de FR1 durant 7 dies. Si la rata s' autoadministrava 10 o més infusions en les sessions de 2 hores durant un mínim de 5 dies, la raó fixa s' augmentava a FR3 el vuitè dia i posteriorment a FR5 durant els següents 5 dies (del 9 al 13).

S'entén com a raó fixa (FR) el numero de palancades que ha de prémer la rata per tal d'obtenir una infusió endovenosa de cocaïna (FR1 una dosi, FR3 tres dosis, FR5 cinc dosis).

Les sessions finalitzaven al cap de 2 hores o quan l'animal s' administrava un màxim de 50 infusions, per tal d' evitar la sobredosificació. En la **Figura 24** es mostra dos patrons de dues sessions d'autoadministració de cocaïna: patró regular de consum i patró irregular o de no consum.

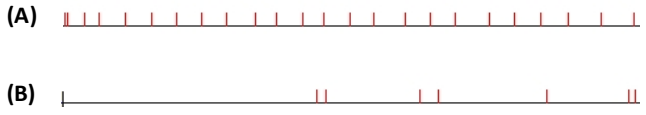


Figura 24. Patrons d'autoadministració de cocaïna. ***(A)*** *Regular,* ***(B)*** *Irregular. Les línies verticals corresponen a reforços en FR5. Les línies horitzontals corresponen al temps de la sessió (2h).*

Efectes de fàrmacs sobre l'extinció de la conducta d'autoadministració de cocaïna

El grup que es va utilitzar per a realitzar l'experiment d'extinció va ser de 10 subjectes. Un cop els subjectes eren estables a FR5 (±15% de reforços en 3 dies) es van iniciar els tractaments amb els fàrmacs i el procediment d'extinció. En aquestes sessions d'extinció, els animals estaven connectats al "swivel" i s'encenien tots els estímuls lluminosos al prémer les palanques encara que no s'administraven res. Es van realitzar cinc sessions d'extinció de dues hores cadascuna. Els subjectes van ser assignats a l'atzar a un dels grups de tractament: l'agonista de receptors H_3 d' histamina imetit 3mg/kg i salí via subcutània. El fàrmac va ser injectat una hora abans de començar la sessió d'extinció per afavorir la seva distribució. Després de les sessions les rates van tornar al protocol d' autoadministració diari a FR5. Al cap de set dies, quan les rates ja eren altre cop estables a FR5, es repetia l'experiment a la inversa: les rates prèviament injectades amb salí se'ls administrava imetit 3mg/kg i a la resta salí via subcutània. Després les rates van

tornar al protocol d' autoadministració a FR5. Entre els grups imetit i salí no hi van haver diferències significatives en reforços en FR5.

Passat un mes d'aquest procés experimental, es va realitzar amb els mateixos subjectes un experiment on tots els subjectes s'autoadministraven cocaïna durant 1 hora (segons el protocol explicat anteriorment). Després d' aquesta hora eren retirades de la gàbia de Skinner i assignades a l'atzar a un dels grups de tractaments: imetit 3mg/kg i salí via endovenosa. Després de l'administració del fàrmac les rates van tornar a la gàbia de Skinner per continuar les sessions d'extinció **(Figura 25).**

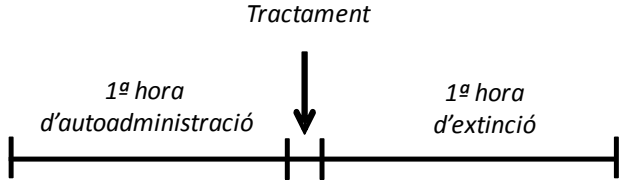


Figura 25. *Procediment experimental per evaluar l'efecte de l'imetit en una sessió d'extinció.*
La línia horitzontal correspon al temps de la sessió (2h).

Després d' aquesta sessió les rates van tornar al protocol d' autoadministració diari a FR5. Al cap de set dies, quan les rates ja eren de nou estables a FR5, es repetia l'experiment a la inversa: les rates prèviament injectades amb salí se'ls administrava imetit 3mg/kg i a la resta salí via endovenosa. Després un altre cop les rates van tornar al protocol d' autoadministració a FR5.

Al cap d'un mes del procés experimental es va tornar a dur a terme l'experiment però incrementant la concentració de imetit a

10mg/kg. Els experiments que es van dur a terme amb el fàrmac imetit 3mg/kg i imetit 10mg/kg es van utilitzar els mateixos subjectes. Entre els grups salí no hi van haver diferències significatives.

Efectes de fàrmacs sobre la conducta d'autoadministració de cocaïna

El grup que es va utilitzar per a realitzar l'experiment va ser de 9 subjectes. Un cop els subjectes eren estables a FR5 (±15% de reforços en 3 dies) es van iniciar els tractaments amb els fàrmacs i el procediment d'autoadministració.

El dia de l'experiment, tots els subjectes s'autoadministraven cocaïna durant 1 hora (segons el protocol explicat anteriorment). Després d' aquesta hora eren retirades de la gàbia de Skinner i assignades a l'atzar a un dels grups de tractaments: imetit 10mg/kg i salí via endovenosa. Després de l'administració del fàrmac les rates van tornar a la gàbia de Skinner per continuar les sessions d'autoadministració **(Figura 26)**.

Després d' aquesta sessió les rates van tornar al protocol d' autoadministració diari a FR5. Al cap de set dies, quan les rates ja eren de nou estables a FR5, es repetia l'experiment a la inversa: les rates prèviament injectades amb salí se'ls administrava imetit 10mg/kg i a la resta salí via endovenosa.

Després un altre cop les rates van tornar al protocol d' autoadministració a FR5. Entre els grups salí no hi van haver diferències significatives.

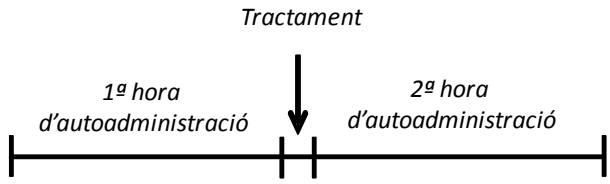


Figura 26. *Procediment experimental per evaluar l'efecte de l'imetit en una sessió d'autoadministració.*
La línia horitzontal correspon al temps de la sessió (2h).

En una altre sessió d'autoadministració de cocaïna és va testar l'acció de l'antagonista H_3 tioperamida 6mg/kg via endovenosa (vem utilitzar la mateixa dosi que als estudis portats a terme pel nostre grup a Dallas, encara que es va canviar la via d'administració) seguit de l'administració de l'agonista imetit 10mg/kg via endovenosa. El procediment experimental va ser el mateix que el descrit anteriorment. L'agonista imetit és va administrar 2 minuts després de l'antagonista **(Figura 27).**

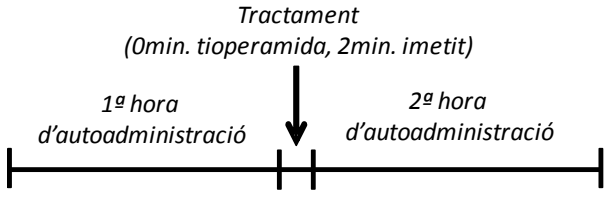


Figura 27. *Procediment experimental per evaluar l'efecte de l'imetit i la tioperamida en una sessió d'autoadministració.*
La línia horitzontal correspon al temps de la sessió (2h).

Efectes de fàrmacs sobre la conducta en raó progressiva de cocaïna

El grup que es va utilitzar per a realitzar l'experiment va ser de 6 subjectes. Un cop els subjectes eren estables a FR5 (±15% de reforços en 3 dies) es van iniciar els tractaments amb el fàrmac i el procediment de raó progressiva. En aquest procediment la raó necessària per rebre el reforç s'incrementa en l'ordre següent: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492 i 603. És a dir, en el primer reforç el rebrà en FR1, però per obtindre el segon haurà de realitzar un FR2 i així successivament. La sessió finalitzava a les 4 hores o quan l'animal feia 1 hora que no havia aconseguit rebre cap reforç.

El dia de l'experiment, prèviament a l' inici de la sessió de raó progressiva (RP) les rates van ser assignades a l'atzar a un dels grups de tractaments: salí i imetit 10mg/kg via endovenosa. Després de l'administració del fàrmac les rates eren distribuïdes a les gàbies de Skinner per iniciar la sessió d'autoadministració de cocaïna **(Figura 28)**.

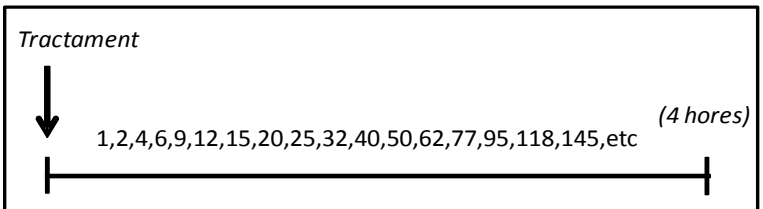


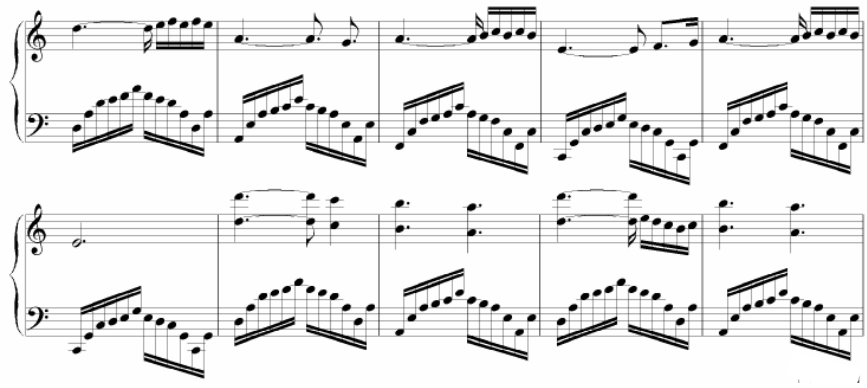
Figura 28. *Procediment experimental per evaluar l'efecte de l'imetit en una sessió de raó progressiva. La línia horitzontal correspon al temps de la sessió.*

Després d' aquesta sessió les rates van tornar al protocol d' autoadministració diari a FR5. Al cap de set dies, quan les rates ja eren de nou estables a FR5, es repetia l'experiment a la inversa: les rates prèviament injectades amb salí se'ls administrava imetit 10mg/kg i a la resta salí via endovenosa. Després un altre cop les rates van tornar al protocol d' autoadministració a FR5. Entre els grups salí no hi van haver diferències significatives. . El "breaking point" del grup imetit va ser de 794 ± 111 palancades, en canvi del grup control 603 ± 101 palancades.

Criteris d'exclusió

En aquests experiments es van utilitzar un total de 40 animals, 21 d'ells van ser exclosos del procediment experimental i de l'anàlisi estadístic. 8 subjectes no van adquirir el criteri d'autoadministració de resposta a la palanca (prèviament establert), 12 van patir complicacions post-quirúrgiques (infeccions, extracció accidental del catèter, mort sobtada) o bé no van superar el test de pentotal sòdic (indicatiu de l'estat del catèter). Aquesta prova consistia en administrar pel catèter una solució de pentotal sòdic 20mg/ml/kg al finalitzar la sessió d'autoadministració, provocant una sedació temporal en l'animal i així comprovar que el catèter era permeable i no estava obstruït. 1 subjecte va morir de sobredosi mentres s'autoadministrava cocaïna.

RESULTATS



RESULTATS

Chapter 1. Dopamine synthesis and release determination in brain tissue based on automated HPLC purification of ^{3}H-dopamine (in preparation).

Resum.

Encara no existeix un fàrmac eficaç per tractar l'addicció a la cocaïna. Per poder estudiar l'efecte de possibles nous fàrmacs en teixit cerebral "ex vivo", en el nostre grup de recerca, ens vem proposar posar a punt una metodologia per a determinar la síntesi i l'alliberament de DA de manera rapida, sencilla i acurada.

Objectius.

- Posar a punt i avaluar el nostre mètode per determinar la síntesi de DA *in vitro* respecte metodologies prèvies.

Resultats.

DOPAMINE SYNTHESIS AND RELEASE DETERMINATION IN BRAIN TISSUE BASED ON AUTOMATED HPLC PURIFICATION OF ^{3}H-DOPAMINE.

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ABSTRACT

Tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of catecholamines. This enzyme is extremely regulated by factors such as phosphorylation and end product inhibition by catecholamines. Some techniques for the determination of tyrosine hydroxylase activity in tissue are not simple, and others such as those based in the detection of L-DOPA accumulation disregard crucial regulation mechanisms like end

product inhibition. We have developed a straightforward radioisotopic determination of dopamine synthesis in brain striatal tissue preparations. Brain miniprisms are incubated with ^{3}H-tyrosine, and ^{3}H-dopamine formed is purified by HPLC. We tested our method with prototypical stimulators (dbcAMP, okadaic acid) and inhibitors (3-I-tyrosine, GABA) of tyrosine hydroxylase activity as well as with dopamine D_2 autoreceptor agonists. Dopamine release to the incubation buffer was simultaneously determined in some experiments in order to better characterize dopaminergic neuronal mechanisms. We tested our method on determinations of ^{3}H-dopamine release after K^{+} depolarization, inhibition of dopamine uptake or metabolism. Effects of psychotropic drugs nicotine and cocaine are also described.

The simultaneous determination of dopamine synthesis and release makes of this a versatile technique. Automated HPLC allows to process a high number of samples with minimal treatment.

INTRODUCTION

Dopaminergic neurons are involved in important physiological functions such as the regulation of goal-directed movement and reward (Kurian, Gissen et al. 2011). The functions of dopaminergic neurons require finely tuned regulatory mechanisms based in rapid induction and suppression. Suppression is mainly controlled by two processes triggered by dopamine: D_2 autoreceptor activation and

end-product feedback inhibition of dopamine synthesis. Dopamine D_2 receptors are known as major targets for the treatment of psychoses, Parkinson's disease and drug addiction (Missale, Nash et al. 1998). D_2 receptors are highly expressed in the striatum where they participate in the regulation of tyrosine hydroxylase (Lindgren, Usiello et al. 2003). D_2 receptor activation, due to high dopamine release, activates G_i protein, reducing cAMP levels and PKA activation, so it finally decreases tyrosine hydroxylase phosphorylation and activity. Tyrosine hydroxylase catalyzes the first and rate-limiting step of catecholamine biosynthesis, the conversion of tyrosine into L-DOPA, and afterwards dopamine is synthesized by L-amino acid aromatic decarboxylase (Daubner, Le et al. 2010).

Once synthesized, dopamine exerts end-product feedback inhibition on tyrosine hydroxylase, which defines the basal levels of dopamine synthesis (Gordon, Quinsey et al. 2008).

Several classical techniques have been used in order to study dopaminergic neuronal activity. Dopamine synthesis in homogenates has been studied with the tritiated water release method (Reinhard, Smith et al. 1986) based upon the release of ^3H_2O formed from ^{3}H-[3,5]-L-tyrosine with adsorption of the isotopic substrate (and its metabolites) by an aqueous slurry of activated charcoal. Another technique used to determine the synthesis of dopamine consisted on incubating tissue with L-[1-^{14}C] tyrosine and monitoring the production of $^{14}CO_2$ (Patrick and Barchas 1974).

Cathecolamines can be quantified by HPLC and electrochemical detection as they are oxido-reductable compounds. In tissue, total catecholamine amount reflects mainly catecholamines stored in vesicles between synthesis and release processes. Catecholamine synthesis determinations have commonly relied on HPLC determination of L-DOPA accumulation after impairment of L-amino acid decarboxylase with NSD-1015. L-DOPA is then quantified by HPLC coupled to electrochemical detection (Lindgren, Xu et al. 2001). This method can achieve a detection limit around pmols of L-DOPA. However, by using this technique it is not possible to study tyrosine hydroxylase mechanisms related to feedback inhibition because dopamine synthesis is abolished in order to accumulate L-DOPA.

In another common application of cathecolamine determinations by HPLC, in vivo mycrodialysis studies typically measure how release of cathecolamines are regulated, but not synthesis (Jing, Chen et al. 2007).

In this work our main aim was to develop a highly sensitive and repetitive radioisotopic technique to determine simultaneously dopamine synthesis and release in slice preparations. We used HPLC with UV detection as a simple and efficient way to automatically purify [^{3}H]-dopamine formed in the slices. We compare the results of [^{3}H]-dopamine synthesis obtained with the classical method of L-DOPA accumulation. Incubation with several prototypical dopaminergic activators and inhibitors was tested, as well as the effects of some psychotropic drugs.

MATERIAL AND METHODS

Chemicals

Opti-Phase HiSafe-3 liquid scintillation cocktail and tritium-labeled tyrosine were supplied by PerkinElmer Wallac (Turku, Finland). [3,5-^{3}H]L-tyrosine (50 Ci mmol/1) was purified by high-performance liquid chromatography (HPLC) before use as described (Purification of [^{3}H]-tyrosine). db-cAMP was obtained from Biolog Life Science Institute (Bremen, Germany). Okadaic acid was purchased from Merck Biosciences (Darmstadt, Germany). NSD-1015, 3-Iodo-tyrosine, EDTA, HPLC standards, and other reagents were purchased from Sigma/RBI (Steinheim, Germany).

Purification of [^{3}H] tyrosine

Standards of ring-labeled [3,5-3H]L-tyrosine (40–60 Ci/mmol) typically show a decomposition rate lower than 1 % per month during the first six months, but this may increase after longer storage. Such radiolysis generates unwanted by-products that should be separated from [^{3}H]-tyrosine. The main goal of purification is to maintain a high degree of purity and to control the specific activity of the [^{3}H]-tyrosine before and after storage.

The system used for HPLC purification consisted of a reverse-phase C_{18} column (Tracer Extrasil ODS2, 5µm particle size, 25 x 0.46 cm; Teknokroma, Spain) and a mobile phase 100 mM sodium phosphate buffer, 1mM EDTA, 0.75 mM octanosulfonic acid and 1% (v/v) methanol (pH 3.4). The flow rate was 1 ml/min. Under these conditions, tyrosine eluted at 9–10 min.

In each purification, 0.4mCi of [3,5-3H]L-tyrosine (2 nmol) was injected into the HPLC and the whole tyrosine fraction (0.5–1 ml) was collected. The amount of [^{3}H]-tyrosine was quantified against an external standard calibration curve of nonradiolabeled tyrosine by UV absorbance at 285 nm. An aliquot of the purified fraction was subjected to liquid scintillation counting, so that the specific activity of the purified product could be obtained by dividing total dpm in the purified fraction by the amount of tyrosine detected by UV.

Preparation and preincubation of brain striatal miniprisms

Protocols for animal handling were previously approved by the Ethics Committee for Human and Animal Research (Universitat Autònoma de Barcelona) in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Male Sprague-Dawley rats weighing 200-250 gr. (Animal Service, Universitat Autònoma de Barcelona, Barcelona, Spain) were sacrificed by decapitation. Brains were chilled immediately in modified Krebs-Ringer-bicarbonate medium with the following composition: 120 mM NaCl, 0.8 mM KCl, 2.6 mM $CaCl_2$, 0.67 mM $MgSO_4$, 1.2 mM KH_2PO_4, 27.5 mM $NaHCO_3$, and 10 mM glucose, pH 7.4 previously gassed with 95% O_2, 5% CO_2. In a 4ºC chamber, striata from each hemisphere were dissected and sliced in a McIlwain tissue chopper to obtain miniprisms of 0.3-0.3 mm/side. The miniprisms were suspended in ice-cold Krebs Ringer bicarbonate medium and washed by centrifugation and

resuspension in order to remove debris of damaged cells. Striatal tissue from a single rat allowed to obtain up to 28 aliquots (25 µl each) of the settled slice suspension corresponding to 24 incubations and 4 blank samples. Blank tubes were kept on ice and the rest were distributed into 2ml polypropylene tubes and incubated at 37 ºC in an Eppendorf Thermomixer (5 Prime, Inc., Boulder, CO) under 95% O_2, 5% CO_2 atmosphere. Samples were preincubated for 30 min in order to equalize metabolism of the different samples.

^{3}H-Dopamine synthesis and release

Previously purified [^{3}H]-tyrosine was added to all samples during the last 5 min of the preincubation to a final concentration of 0.12 µM. In experiments where drugs were needed, they were added during preincubation period. Final volume was 250µl considering drugs and buffer added. Afterwards we incubated the samples for 10 min more to synthesize and release [^{3}H]-dopamine.

To stop incubations two different protocols were followed depending on whether the aim of the experiment was to measure only dopamine synthesis or dopamine synthesis and release. When dopamine synthesis was only tested a deproteinizing mixture (containing trichloroacetic acid to make a 0.5% final concentration, plus 100 nmol dopamine per tube as internal standard and 120 nmol ascorbic acid) was added to the samples before homogenization in a Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA). An aliquot was taken for protein quantification

by the Lowry method to take into account the variability of tissue amounts inside each tube. Tissue homogenates were then centrifuged (10,000 g, 10 min, 4 ºC), and all supernatants were processed for [^{3}H]-dopamine purification by HPLC. When dopamine synthesis and release were simultaneously determined, samples were immediately centrifuged (1 min, 4,000 g) after incubation period to separate buffer and tissue. Buffer and released compounds were extracted and placed in new tubes containing deproteinizing mixture.

The same amount of deproteinizing mixture was also added to the tissue fractions.Buffer volume extracted was replaced by fresh ice-cold Krebs-Ringer-bicarbonate buffer. Afterwards, tissue samples were homogenized and manipulated as explained previously.

^{3}H-Dopamine Purification by HPLC-UV

[^{3}H]-Dopamine formed during the incubation reaction was separated from [^{3}H]-tyrosine and purified by HPLC. The chromatography system consisted of a reverse-phase C_{18} column (Tracer Extrasil ODS2, 5-µm particle size, 25 x 0.46 cm; Teknokroma, Spain) and an ion-pair mobile phase, made up of 0.1 M sodium phosphate buffer , 1mM EDTA, 0,75 mM octanesulfonic acid plus 12% (v/v) methanol (pH 5). The flow rate was 1 ml/min. This HPLC system completely separated standards of tyrosine and dopamine that could be detected by UV absorbance at 285 nm (Fig. 1A). Dopamine retention time was 10 min and depended heavily on methanol concentrations. Samples contained equivalent

amounts of internal standard dopamine than external standards injected for comparison to obtain internal standard recovery. Relative to the internal standard, the samples contained amounts undetectable by UV absorbance of radiolabeled tyrosine and dopamine as well as endogenous tyrosine and dopamine. Thus, a typical UV profile of a sample consisted largely of one front with undesired compounds and one peak corresponding to the internal dopamine standard (100 nmol per sample, corrected for recovery; Fig. 1B).The radioactivity profile showed that [^{3}H]-tyrosine and [^{3}H]-dopamine were well resolved in experimental samples, so that [^{3}H]-dopamine could be collected with negligible radioactivity contamination from [^{3}H]-tyrosine.

The recovery of the internal standard in each sample (internal/external standard peak area) was quantified from dopamine HPLC peak areas, obtained using Kontron data system 450 software. The dopamine eluate fraction was automatically collected in a scintillation vial by a fraction collector (Gilson Fc 203B) at peak detection. Dopamine eluates were mixed with Optiphase HiSafe III cocktail (Wallac), and [^{3}H]-dopamine was quantified in a liquid scintillation counter.

Calculations and statistical analysis

Dpm obtained from each HPLC eluate were corrected by internal standard recovery and dpm in blank samples. Dpm of [^{3}H]-dopamine obtained were then expressed as function of protein content in each incubate and incubation time. Results were then

expressed as % of controls in each experiment. Controls were usually obtained from a minimum of N=4 miniprism incubations in each experiment. In release experiments, extracellular [^{3}H]-dopamine was quantified from dpm obtained from the dopamine purification of incubation buffer. Synthesized [^{3}H]-dopamine in a single incubation was the sum of dpm present in the buffer plus tissue samples.

Release was then expressed as the % of extracellular vs. total (synthesized) dopamine (% released) in that particular miniprism incubation. Statistical significance of differences between groups was assessed by analysis of variance (ANOVA) followed by Bonferroni post hoc tests. Statistical significance was set at $p< 0.05$ prior to the experiments.

Estimation of tyrosine hydroxylase activity in situ by L-DOPA quantification by HPLC with electrochemical detector.

Miniprism samples were treated as Preparation and preincubation of brain striatal miniprisms and they were treated with NSD-1015 (100 µM) to inhibit L-aminoacid aromatic decarboxylase. After 30 min of incubation the accumulation of L-DOPA was quantified by HPLC-EQ analysis as described by (Lindgren, Xu et al. 2001).

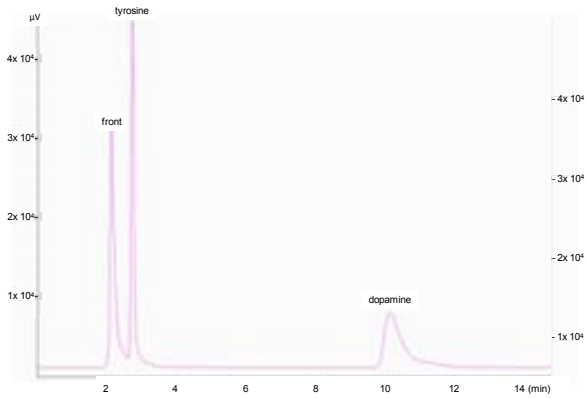
The stationary phase consisted in a reverse-phase C_{18} column (2.5µm particle Fortis C_{18}, 250 x 4.6 mm , Sugelabor, Spain) and an ion-pair mobile phase, made up of 0.1 M sodium phosphate buffer, 1mM EDTA, 5 mM octanesulfonic acid plus 1% (v/v) methanol (pH 2.5). The flow rate was 1 ml/min. This HPLC system completely

separated standards of tyrosine, L-DOPA and dopamine. L-DOPA external standards with different concentrations were injected in every experiment in order to quantify L-DOPA amount in miniprism samples by linear regression.

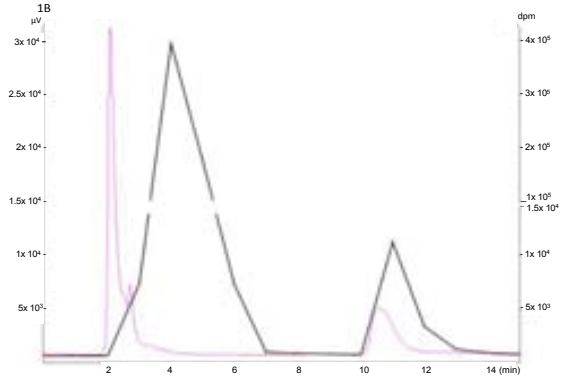
RESULTS

In Fig 1-A an HPLC chromatogram of non-radiolabelled tyrosine/dopamine standard mixture is shown using the HPLC conditions described in *^{3}H-Dopamine Purification*. Both peaks were completely resolved, with tyrosine observed at 3 minutes right after the front peak, and dopamine observed at 10 minutes by UV absorbance. Rat brain striatal miniprims were incubated in presence of ^{3}H-tyrosine to synthesize ^{3}H-dopamine. Under the same chromatographic conditions, radiactivity corresponding to ^{3}H-dopamine and ^{3}H-tyrosine was also completely resolved as presented in Fig. 1-B and C. In samples, the only peaks clearly detected by UV absorbance were the front peak and internal standard dopamine at 10 minutes. Non-incubated blanks were compared with incubated samples. In blanks, deproteinizing mixture was added before ^{3}H-tyrosine. As expected, we could not find any significant radiactive signal at dopamine retention time in blanks, while they contained only ^{3}H-tyrosine according to radiactivity eluting at 4-5 minutes.

1A



1B



1C

	Desintegration per minute (DPM)		
Time (min)	Standars	Blank	Sample
0 - 1	46	103	33
1 - 2	45	55	38
2 - 3	31	788	7675
3 - 4	57	**255392**	**406907**
4 - 5	38	**223954**	**113150**
5 - 6	35	3908	7558
6 - 7	42	906	423
7 - 8	38	165	275
8 - 9	50	100	188
9 - 10	35	59	155
10 - 11	35	57	**12031**
11 - 12	39	57	**3106**
12 - 13	24	54	724
13 - 14	31	59	330
14 - 15	39	52	190

Figure 1. (A) Chromatographic UV 285nm profile of a standard sample containing 100nmol of tyrosine and dopamine using the HPLC conditions described in *^{3}H-Dopamine Purification*. (B) Chromatographic UV 285nm profile of a problem sample containing 100nmol of dopamine as internal standard. (C) Radiactivity profile of the different samples injected to the HPLC system. Standard sample does not contain significant radioactivity (<50 dpm/ml is considered background). Blank sample only contains the radioactivity corresponding to [^{3}H]-tyrosine but has not [^{3}H]-dopamine. The problem sample contains two maximums of radioactivity corresponding to [^{3}H]-tyrosine initially added and [^{3}H]-dopamine synthesized during incubation time.

To check the validity of our radioisotopic technique we carried out several series of experiments with TH activators and inhibitors. We incubated rat brain striatal miniprisms using the radioisotopic method described in this work. Incubation was performed in presence of okadaic acid (a potent phosphatase inhibitor), (Ishihara, Martin et al. 1989) or dbcAMP (a potent kinase A activator), (Navarro, Punzon et al. 1998) as they are known stimulators of dopamine synthesis. As shown in Fig. 2A, 1 µM okadaic acid increased dopamine synthesis by 65%. The incubation with the kinase activator dbcAMP at 1mM also increased dopamine synthesis by 90%. We compared our technique with the classic method based on % L-DOPA accumulation. Briefly, rat brain striatal miniprisms were incubated in presence of okadaic acid 1 µM or dbcAMP 1 mM. Under these conditions the results appeared similar to our method: The presence of okadaic acid or dbcAMP increased the % L-DOPA accumulated to a similar extent (Fig. 2B).

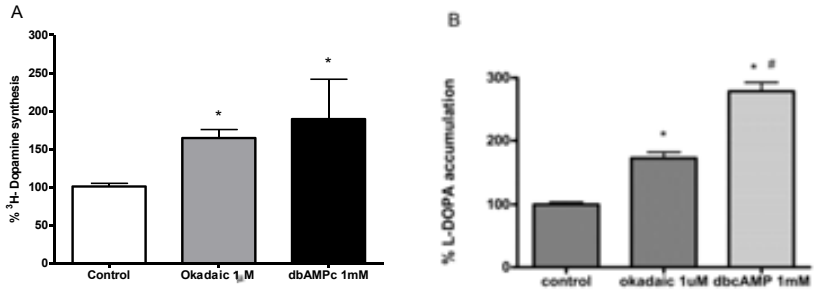


Figure 2. The Ser/Thr protein phosphatases inhibitor, okadaic acid (1μM) as well as the PKA activator, dbcAMP (1mM), increased ^{3}H-dopamine synthesis (A) and L-DOPA accumulation (B). Date are expressed as % of controls as function of protein content and incubation time (In A, control values were 101 dpm/mg prot·h) . Results are means ± SEM of N=7 incubations per condition. * P<0.001 versus control; # P<0.001 versus okadaic acid, one-way ANOVA followed by Bonferroni post-hoc test.

To test how our technique can measure % of ^{3}H-dopamine synthesis under inhibitory conditions we used GABA, 3-I-tyrosine (a potent tyrosine Hydroxylase inhibitor) (Parker and Cubeddu 1986) or dopamine (an end-product that retroactively inhibits the enzyme) (Gordon, Quinsey et al. 2008) in the rat brain stiatal miniprisms (Fig 3A). Also the classic method of L-DOPA accumulation was performed for comparison (Fig. 3B). It can be observed that GABA at 100 μM decreased to a similar extent (around 25%) the synthesis of both, ^{3}H-dopamine and L-DOPA accumulation. Similarly, striatal minisprisms incubated with dopamine at 10 μM showed a decrease of around 75% of both ^{3}H-dopamine and L-DOPA accumulation. The most extreme case was observed with 3-I-tyrosine. Under our technique conditions a complete inhibition of ^{3}H-dopamine was observed, nonetheless using the classic technique we could detect only 75% of inhibition.

To account for this difference, it is possible that residual L-DOPA present in tissue was measured.

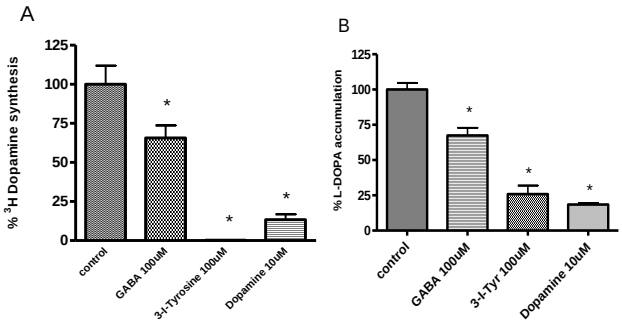


Figure 3. GABA (100mM) and dopamine (10mM) decrease dopamine and L-DOPA synthesis in rat brain striatal miniprisms. Tyrosine hydroxilase inhibitor 3-I-tyrosine completly inhibits dopamine synthesis using our radioisotopic methodology, but only partially impairs L-DOPA synthesis using electochemical detection. Date in A are expressed as % of controls calculated as DPM's ^{3}H-DA as function of protein content in incubated time. N=12 (control), N=6 (GABA), N=3 (3I-Tyr), 16 (DA). (In A, control values were 140 dpm/mg·prot·h). Results are means ±SEM. * P<0.05 vs control 2mM K+; one-way ANOVA followed by Bonferroni post-hoc test.

Given that D_2 autoreceptors inhibit DA synthesis in dopaminergic neurons (Benoit-Marand, Borrelli et al. 2001), we tested if D_2 agonists were able to inhibit DA synthesis following our methodology. Were treated striatal miniprisms with D_2 agonists bromocriptine and quinpirole at 100 nM (Fig. 4A). It was observed that bromocriptine decreased synthesis of DA up to 60%. The agonist quinpirole also decreased the synthesis of DA significantly under this concentration. Dose-response curve of quinpirole vs ^{3}H-dopamine (Fig 4B) shows a maximal effect of 1 µM quinpirole that inhibits ^{3}H-dopamine synthesis around 40%.

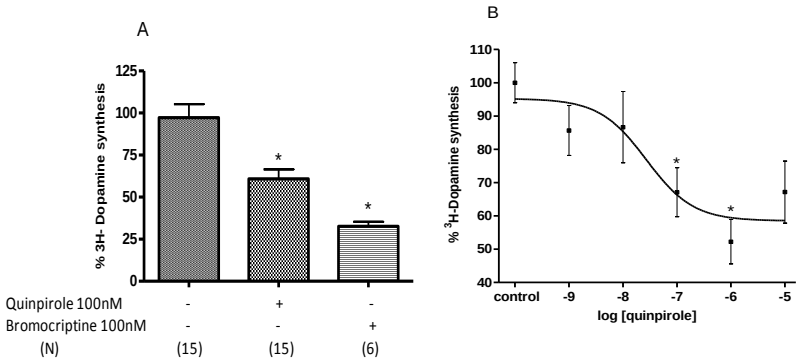


Figure 4. The D2/3 dopamine receptor agonists quinpirole and bromocriptine decrease dopamine synthesis in rat brain striatal miniprisms. Date are expressed as % of controls calculated as DPM's of 3H-DA as function of protein content in incubated time. (97 dpm/mg prot·h in control). Results are means ± SEM. In B, N=6 incubations per condition. P<0.001 versus control, one-way ANOVA followed by Bonferroni's test.

In a second series of experiments, ^{3}H-dopamine release was measured simultaneously to ^{3}H-dopamine synthesis by separating buffer and tissue immediately after incubations. Drugs were added to incubations 10 minutes previously to ^{3}H-tyrosine.

Miniprisms were depolarized for 10 minutes after 5 minutes of ^{3}H-tyrosine incubations. In Figure 5 it can be observed the higher [K^{+}], the higher is the release of DA as expected. This result agrees with the known fact that depolarization of neurons by increment of [K^{+}] drives a greater DA release. We incubated and depolarized the miniprisms at different [K^{+}] to find the best concentration for the following experiments. In Fig 5A we observed that the optimal [K^{+}] that yield the maximal DA release is 30mM under our conditions. However we observed in Fig 5 an interesting phenomena: DA synthesis decreased at higher K^{+} concentrations (Fig 5A, bottom line) in the same samples (see *Discussion*).

We also tested GBR12909 a highly selective dopamine reuptake with a K_i=1nM (Andersen 1989). We used this molecule to quantify, using our method, extracellular DA in presence of GBR12909. As show (Fig.5B) once [GBR] exceeds Ki value, DA released is present in a higher amount in the extracellular space.

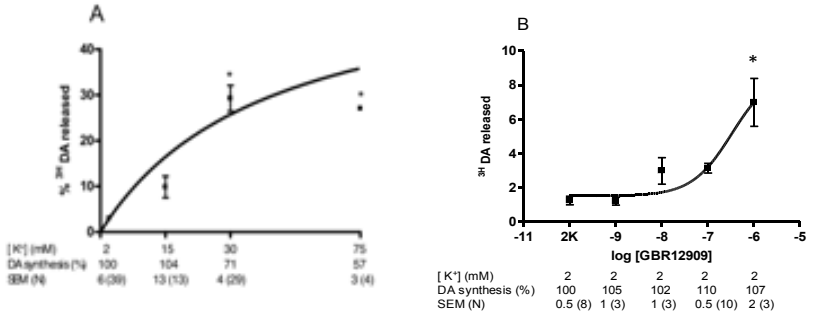
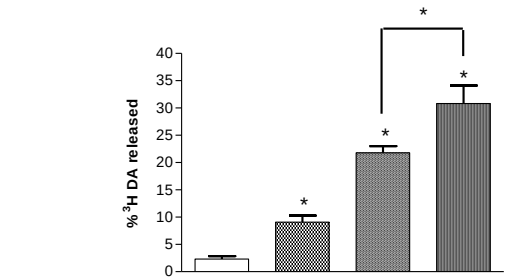


Fig. 5. (A) Potassium Increased the dopamine release in rat brain striatal miniprismes. (B) GBR increase the DA release dose dependently. Results are expressed as percent of basal release (means ± SEM of values obtained from at least N= 4 incubations per condition). P<0.0001 (*) versus control. One-way ANOVA followed by Bonferroni's test.

Inhibition of MAO-A/B (Youdim and Bakhle 2006) and COMT (Mannisto, Kaakkola et al. 1988) increases DA levels. To test whether our technique is able to measure increases of ^{3}H-dopamine in presence of MAO-A/B and COMT inhibitors, we depolarized miniprisms using 30 mM of K^+ and added trans-2-phenylcyclopropylamine hydrochloride at 25 µM (tranylcypromine, an unspecific inhibitor of MAO-A/B), and 3,5-dinitrocatechol at 25 µM (a COMT inhibitor) (Nissinen, Linden et al. 1988). Results in Fig. 6 agree with Youdim and Bakhle: When MAO-A/B and COMT are simultaneously inhibited, an increment in extracellular ^{3}H-

dopamine is detected. The effects were clearer under depolarizing conditions.



[K+] (mM)	2	2	30	30
[MAO A/B inhibitor], µM	-	25	-	25
[COMT inhibitor], µM	-	25	-	25
DA Synthesis (%)	100	119	67	69
SEM (N)	1 (6)	3 (6)	3 (6)	8 (6)

Fig.6. The inhibition of MAO A/B and COMT enzymes increased the extracelular dopamine. We incubated the striatal minisprisms with Trans-2-Phenylcyclopropylamine at 25 µM to inhibit MAO A/B and COMT Inhibitor 3,5-Dinitrocatechol at 25 µM. Results are means ±SEM. * P<0.05 vs control 2mM K+; one-way ANOVA followed by Bonferroni post-hoc test.

To evaluate the usefulness of this technique to study the effects of psychotropic drugs, we quantified the % ^{3}H-dopamine released in miniprisms depolarized with [K^{+}] 15 mM. The effects of incubations with 100nM of nicotine (acetylcholine nicotinic receptor agonist) (Zhang, Zhang et al. 2009) or 10 µM of cocaine (DAT inhibitor) (Giros, Jaber et al. 1996) were tested. Depolarization by 15 mM K^{+} increased DA release to 5% of total ^{3}H-dopamine formed (Fig. 7). In the presence of nicotine or cocaine a significative increment in % DA released was obtained. The effect of nicotine (100 nM) should be attributed to a higher depolaritzation due to activation of nicotine acetilcholine receptors (nAChR) (Fig. 7A). Experiments using cocaine (10µM) elicited a higher % DA released consistent with DAT blockade (Fig. 7B).

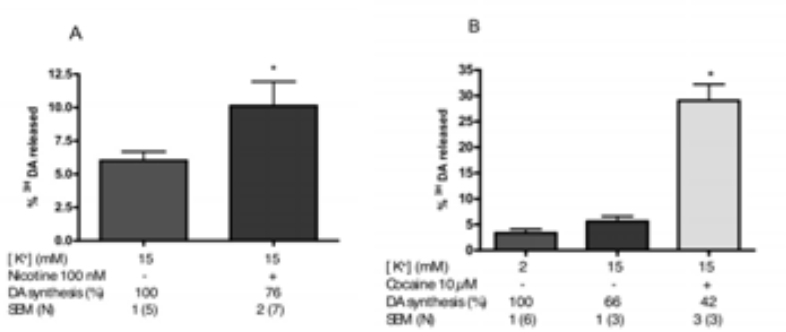


Fig. 7. (A) Nicotine increases dopamine release in rat brain striatal miniprisms incubated in 15 mM potassium. Results are means ± SEM of N=5 incubations per condition. P<0.01 versus control, Student t test. (B) Cocaine increases dopamine release in rat brain striatal miniprisms. Results are means ± SEM of N=6 incubations per condition P<0.01 versus 15K, one-way ANOVA followed by Bonferroni's test.

DISCUSSION

We developed a new technique to determine dopamine synthesis and release in rat brain preparations with easy manipulation of samples. Both synthesis and release of dopamine can be measured quickly in the same experiment. This technique is quite sensitive: is necessary around 1 mg of brain striatal tissue to carry out one incubation, obtaining 28 incubations from striatal tissue from one rat. Our method determines a short in time what changes are happening in catecholamine terminals after preincubation with different drugs. This makes ours methodology accurate and reliable to investigate effects of psychotropic drugs. A disadvantage of our method is that we have to handle samples with tritium.

In addition it is advisable to purify ^{3}H-tyrosine to eliminate impurities and confirm specific activity before carrying the experiments. HPLC purifications of ^{3}H-dopamine and ^{3}H-tyrosine are extremely simple techniques that can be performed with any routine HPLC materials. Standard washing of HPLC injectors efficiently removes tritium, although nitric acid diluted to 0.1 N facilitates cleaning of injection systems.

In most of the in vitro studies on striatal and nigral dopamine release the technique of measuring release of exogenously applied ^{3}H-dopamine has been used (Geffen, Jessell et al. 1976). This classic technique has, however, some drawbacks: ^{3}H-dopamine is taken up and released not only from dopaminergic neurons but also from other neuronal elements notably in the substantia nigra (Kelly, Jenner et al. 1985). In addition, ^{3}H-dopamine metabolites or impurities cannot be differentiated from authentic ^{3}H-dopamine. Our method improves both aspects, as the ^{3}H-dopamine we measured has to be synthesized in cathecolaminergic terminals expressing tyrosine hydroxylase. Also, our HPLC purification of ^{3}H-dopamine ensures we are measuring authentic ^{3}H-dopamine formed from ^{3}H-tyrosine.

Dopamine synthesis measured with our radioisotopic method is affected by regulation and compensatory mechanisms to the same extent as previously described, so it is possible to identify and study such mechanisms contributing to a better knowledge of dopaminergic neuronal system and its pharmacology.

In the classic method most commonly used to determine changes in dopamine synthesis the accumulation of L-DOPA after inhibiting DDC is measured. We know that dopamine synthesized does feedback on tyrosine hydroxylase in brain (Gordon, Quinsey et al. 2008). However, dopamine feedback is disregarded with the classic method of L-DOPA accumulation. Moreover, Cumming et al. suggest that 75% to 90% of endogenously formed DOPA in rat striatum normally is committed to DA synthesis in these structures. After DDC inhibition, traces of OMe-^{3}H-DOPA were detected in striatum (Cumming, Ase et al. 1998). Thus, methodologies based on L-DOPA accumulation disregard the significant amounts of L-DOPA that may follow alternative pathways instead of DA formation.

Our technique, in contrast can also be adapted to tyrosine hydroxylase estimations by ^{3}H-L-DOPA accumulation as shown by Gonzalez-Sepulveda et al (in preparation) with minor changes in HPLC mobile phase.

Depolarization of miniprisms by potassium increases extracellular dopamine released. Unexpectedly, ^{3}H-dopamine synthesis decreased rather than increased when K^{+} stimulated ^{3}H-dopamine release (El Mestikawy, Glowinski et al. 1983).

The increase in tyrosine hydroxylase activity mediated by K^{+} depolarization was described in homogenates, not in relatively "intact" tissue miniprisms. Thus, we confirmed that tissue homogenization after different K^{+} depolarization clearly increased tyrosine hydroxylase activity (see table below).

3H-TH activity			
K+	2K	30K	60K
(%)	100	276	300
SEM	188	650	665
N	5	6	5

Thus inhibitory mechanisms must be present in dopaminergic terminals that prevent ^{3}H-dopamine synthesis increase by K^+ despite tyrosine hydroxylase activation in homogenates. One of such mechanisms can be feedback by reuptaken dopamine on TH enzyme. An alternative mechanisms is D_2 autoreceptor stimulation. We have confirmed that activation of D_2 receptor by agonist like quinpirole or bromocriptine decrease dopamine release (see table below), indication that released dopamine activates D_2 autoreceptors.

DA release as % of 2K				
K+	2K	15K	15K+ quinpirole (100nM)	15K+ bromocriptine (100nM)
%	100	203	52	55
SEM	0.5	2	2	3
N	4	5	5	5

However, blockade of D_2 receptors with 100 nM haloperidol potentiated the decrease of dopamine synthesis elicited by depolarization with 30 mM K^+. This suggests that dopamine acting on D_2 autoreceptors does not explain the decrease of dopamine synthesis elicited by 30 mM K^+ (see table below).

3H-DA synthesis			
K+	2K	30K	30K+ Haloperidol (100nM)
(%)	100	69	38
SEM	1.3	1.8	1.9
N	6	6	6

One alternative possibility could be that dopamine released by depolarization is reuptaken and then it elicits a negative feedback on tyrosine hydroxylase (Gordon, Quinsey et al. 2008). Finally, it is also possible that part of ^{3}H-dopamine released is degraded by MAO and COMT. Thus, the % of ^{3}H-dopamine released is increased by simultaneous MAO and COMT inhibition (Fig. 7). However, the use of inhibitors of degradation of released ^{3}H-dopamine did not appear to be required for ^{3}H-dopamine release determinations, and it did not affect ^{3}H-dopamine synthesis.

Finally we carried out experiments with psychotropic drugs to contrast the results with the literature. The incubation of striatal miniprisms with nicotine increase dopamine release (Wonnacott, Kaiser et al. 2000; Zhang, Zhang et al. 2009). Furthermore the incubation of miniprisms with cocaine also increase the dopamine release (Patrick and Barchas 1976) as already described in literature.

In conclusion, our method is valid, simple and reliable to determine dopamine synthesis and release in fresh brain. In our experience, similar methodologies can be used to detect synthesis and release of other amines or metabolites (Moreno-Delgado, Gomez-Ramirez et al. 2009).

This method could be used to analyse biological samples from patients. However we would expect much worse results on frozen samples where synapses can lose functionality, difficulting its application to clinical specimens.

Our method does not determine dopamine levels that can be "pictured" at a given time. Instead, its reflects the working of dopamine machinery during a short time. The minimal amounts of tissue needed allow to test several conditions in one brain, comparing drug effects at several concentrations for pharmacological studies. It can be used for a fast screening of molecules influencing dopaminergic neurotransmission. Variability among incubations likely reflect heterogeneity of striatal tissue. We think this method will provide a short, fast test of pharmacological agents of great use before testing in vivo, minimizing the use of animals and experimental work.

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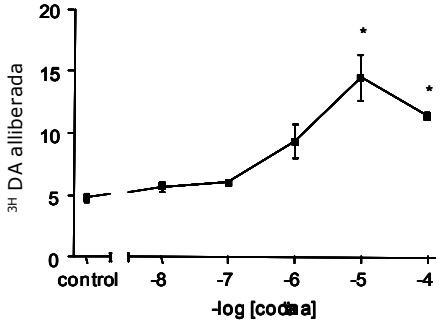
Resultats complementaris.

Per tal de corroborar amb el nostre mètode com actuen substàncies psicoactives com la cocaïna, vem voler realitzar un experiment incubant els miniprismes a diferents concentracions. Es pot observar **(Figura 1)** que el màxim d'alliberament de DA es produeix a 10µM i després ja comença a disminuir aquest alliberament.



[K+](mM)	2	2	2	2	2	2
Síntesis DA (%)	100	114	140	99	156	61
SEM(N)	0.5(3)	0.5(3)	0.5(3)	1.5(3)	2(3)	0.5(3)

Figura 1. L'incubació de miniprismes a diferents concentracions de cocaïna, augmenta la DA extracel·lular. Es van incubar amb cocaïna durant un temps de 20 minuts i sense condicions despolaritzants (2mM K^+). Es va mesurar l'alliberament de ^{3}H-DA per HPLC. Es representen els valors mitjos ±SEM. (N=3). (*) P<0.05 respecte 2mM K^+ ANOVA d'un factor, post hoc de Bonferroni.

Conclusions.

- El nostre protocol permet quantificar la síntesi i l'alliberament de DA en un mateix experiment amb l'automatització i l'eficàcia pròpia de l'HPLC millorant tècniques anteriors.
- Molècules addictives com la cocaïna i la nicotina faciliten l'alliberament de DA en estriat de rata.

Chapter 2. HISTAMINE H_3 RECEPTOR AGONISTS DECREASE COCAINE SEEKING (in preparation).

Resum.

Hi ha evidències que suggereixen que el sistema histaminèrgic podria jugar un paper inhibidor sobre efectes produïts per drogues d'abús. En base a totes aquestes evidències ens vem plantejar avaluar els efectes de l'agonista H_3 imetit sobre la motivació de consumir cocaïna en un model de rosegadors.

Objectius.

- Comprovar l'efecte de l'agonista H_3 imetit sobre la síntesi de DA en miniprismes d'estriat de rata.
- Avaluar l'efecte de l'agonista H_3 imetit sobre l'extinció de la conducta d' autoadministració de cocaïna en rates.
- Avaluar l'efecte de l'agonista H_3 imetit en una sessió d'autoadministració de cocaïna en rates.
- Avaluar l'efecte de l'antagonista H_3 tioperamida i de l'agonista imetit en una sessió d'autoadministració de cocaïna en rates.
- Avaluar l'efecte de l'agonista H_3 imetit sobre una conducta de reforç de cocaïna en raó progressiva en rates.

Resultats.

HISTAMINE H_3 RECEPTOR AGONISTS DECREASE COCAINE SEEKING

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ABSTRACT

Cocaine dependence has no pharmacological treatment. Relapse models in animals are used as a screen for new medications. Histamine H_3 receptors are relatively abundant in brain respect to other tissues, and particularly in the nucleus accumbens and striatum. Previous studies suggest that H_3 antagonists/inverse agonists could facilitate dopamine release, reinforcement and/or

the subjective effects of psychostimulants. Here we show that histamine H_3 receptor agonists decrease cocaine seeking in tests of extinction and reinstatement of cocaine self-administration. Rats were subjected to cocaine self-administration under a FR5 schedule, followed by a reinforcement dose-response, progressive ratio, extinction and reinstatement tests elicited by two doses of cocaine priming. Pretreatment with the standard histamine H_3 agonist imetit decreased responding in extinction and reinstatement tests. No effects of H_3 agonists were found under cocaine reinforcement, except for a delay in starting lever press. The histamine precursor L-histidine (which is transformed into histamine in brain) mimciked most of imetit effects. Locomotion tests performed in naive animals showed a small, but significant decrease of cocaine-induced hyperlocomotion in imetit and L-histidine treated animals. We conclude that histamine H_3 receptor agonists could be interesting cadidates for clinical trials where relapse into cocaine abuse is determined. Furthermore, the aminoacid L-histidine could also have beneficial effects.

INTRODUCTION

Cocaine dependence has no pharmacological treatment despite provoking high rates of relapse. Rat models of cocaine self-administration mimmick some core symtoms of addiction including vulnerability to relapse (Deroche-Gamonet et al., 2004). Relapse models are accepted as a screen for new medications, although

proof of their predictive (or criterion) validity needs further research (Epstein et al., 2006). Several lines of evidence show that dopaminergic neurotransmission can be necessary to associate rewards with motivational salience, contributing to reward prediction and participating in relapse during abstinence (Berridge and Robinson, 1998; Shaham et al., 2003; Schultz, 2007). Thus, agents modifying mesolimbic dopamine responses could have effects in cocaine-seeking behavior and should be investigated as potential new treatments for cocaine dependence.

Brain histaminergic neurons have their cell bodies in the hypothalamic tuberomammillary nuclei from where they innervate limbic regions participating in arousal, cognition and drug reinforcement. Histamine actions are mediated by four types of receptors termed H_1 to H_4. Among these, histamine H_3 receptors are relatively abundant in brain respect to other tissues, and particularly in brain regions such as the nucleus accumbens, striatum and cortex (Pillot et al., 2002).

H_3 receptors are inhibitory autoreceptors in histaminergic neurons, as well as heteroreceptors in other cell types. In some tissues H_3 receptors have constitutive activity that can be suppressed by ligands called inverse agonists (Arrang et al., 2007). H_3 receptor antagonists/inverse agonists are being investigated as new treatments for cognitive, sleep and feeding disorders (Passani et al., 2007; Lin et al., 2008).

Administration of histamine H_3 receptor antagonists/inverse agonists increase dopamine release in the cortex, measured by

microdialysis (Ligneau et al., 2007). In contrast in the nucleus accumbens shell H_3 antagonists/inverse agonists do not appear to increase dopamine release by themselves, although they do potentiate methamphetamine-stimulated dopamine release (Munzar et al., 2004). In a striking parallelism, behavioral experiments of self-administration and conditioned place preference showed that H_3 antagonists/inverse agonists potentiate the effects of low doses of psychostimulants, but they did not appear to elicit these behaviors when given alone. In fact, H_3 antagonists/inverse agonists appeared to displace to the left bell-shaped curves of dose responses to psychostimulants, decreasing the dose threshold to elicit the behavior and responses to highest doses as well (Munzar et al., 2004; Brabant et al., 2005; Hyytia et al., 2003). These findings suggest that H_3 antagonists/inverse agonists could facilitate dopamine release, reinforcement and/or the subjective effects of psychostimulants (Brabant et al., 2010).

In this work we were interested in testing whether, conversely to previous findings, stimulation of histamine H_3 receptors would decrease behavioral effects of cocaine. At first we used the histamine precursor L-histidine as a non-specific stimulator of histaminergic neurotransmission. We also used two specific histamine H_3 receptor ligands: the prototypical H_3 agonist agonist imetit and the inverse agonist thioperamide. Particularly we focused on models of cocaine seeking during extinction and reinstatement due to their suggested predictive value as models of cocaine craving and relapse.

MATERIALS AND METHODS.

Behavioral procedures

Male Sprague-Dawley rats of 250 - 300 g were obtained from Charles River or from the Animal Service, Universitat Autonoma de Barcelona, Spain. All animal protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and European Communities Council Directive 86/609/EEC and were approved by local Animal Care and Ethics Committees. Rats were housed individually in a climate controlled environment at 21 ºC on a 12 h light / dark cycle. Food and water was aivailable *ad libitum*, except during initial sucrose training when feeding was restricted to maintain body weight around 85-90 % of theoretical weight. Rats were trained to press a lever in Skinner chambers (Med Associates or PANLAB SLU) to obtain sucrose pellets (45 mg Bio-Serv, Frenchtown, NJ, USA). Sucrose training was accomplished when 100 pellets were obtained in three sessions (first session overnight and two more in maximum 2 h). Rats were then feed ad libitum and implanted an i.v. catheter into the jugular vein. Catheters were flushed with heparin/tridodecylmethylammonium chloride complex (Polysciences Europe) and saline before implantation. The catheter (Silastic, Dow Corning) passed under the skin to reach out at the animal's back, where an incision permitted the exit of a 24 gauge cannula (Plastics One Inc) embedded in dental cement (Heraeus) supported by a round mesh platform (Mersilene®, Johnson and

Johnson, Intl). Implanted catheters were flushed daily with a heparin (10 UI/ml) and gentamicin (0.4 mg/ml) in 0.9% saline solution. After 3-4 days of recovery, the animals were subjected to daily sessions of i.v. cocaine self-administration in the same Skinner chambers, obtaining cocaine (0.5 mg/kg/injection through the catheter) for pressing the active lever. Pressing a second inactive lever in the chamber had no consequences, but it was recorded. Reinforcement was signalled by a light above the lever. After cocaine injection a 15 sec timeout followed meanwhile lever press was not rewarded. Self-administration sessions lasted for 2 h daily, 5-6 days per week on a fixed-ratio 1 (FR1) schedule until rats discriminated active and inactive levers and showed stability in drug intake (<15 % variability in 3 consecutive days). Then they were changed successively to FR2, FR3 and FR5 schedules of reinforcement and allowed to stabilize again. Rats fulfilling all these criteria within three weeks were selected for testing the effects of histaminergic agents on cocaine reinforcement (self-administration) and drug seeking (extinction and reinstatement procedures). Pretreatment with histaminergic agents was performed as indicated in the Results section, either 1 h (via i.p. or s.c.) or immediately before (i.v.) the test session of either reinforcement or extinction. In progressive ratio sessions the schedule of reinforcement increased following the sequence: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492 i 603. Progressive ratio sessions lasted for 4 h or ended if the animal did not obtain a reinforcement in 1 h.

Behavioral tests of extinction and progressive ratio were typically performed by dividing rats in two groups (saline controls and histaminergic-pretreated animals). After the test session, rats underwent their normal routine self-administration protocols gaining stability in cocaine intake in about 1 week. Then a second test session was performed in a counterbalanced manner, e.g., with saline group now pretreated with histaminergic agents and viceversa. Saline groups performed in different days did not differ statistically.

Cocaine-induced horizontal hyperlocomotion was recorded in naive rats. Circular test chambers with a 12 cm wide runway and 1.95 m perimeter were set in the dark and equipped with four pairs of photocells located at 90-degree intervals. After a 90-minute habituation to the circular chamber rats received a pretreatment with histaminergic agents, and 60 minutes later a single dose of 15 mg/kg cocaine i.p.

Dopamine synthesis determination

A fresh brain obtained from a naive rat was chilled immediately in modified Krebs-Ringer-bicarbonate medium with the following composition: 120 mM NaCl, 0.8 mM KCl, 2.6 mM CaCl2, 0.67 mM MgSO4, 1.2 mM KH2PO4, 27.5 mM NaHCO3, and 10 mM glucose, pH 7.4 previously gassed with 95% O2, 5% CO2. In a 4ºC chamber, striata from each hemisphere were dissected and sliced in a McIlwain tissue chopper to obtain miniprisms of 0.3-0.3 mm/side. The miniprisms were suspended in the same buffer and washed by

centrifugation and resuspension in order to remove debris of damaged cells. Striatal tissue from one rat allowed to obtain 28 aliquots (25 µl each) of the settled slice suspension corresponding to 24 incubations and 4 blank samples. Blank tubes were kept on ice and the rest were distributed into 2ml polypropylene tubes and incubated at 37 ºC in an Eppendorf Thermomixer (5 Prime, Inc., Boulder, CO) under 95% O2, 5% CO2 atmosphere. Samples were preincubated for 30 min. Then HPLC purified [^{3}H]-tyrosine was added to all samples at the end of preincubation to a final concentration of 0.12 µM in a final volume of 250µl. Samples were incubated for 10 min more to synthesize [^{3}H]-dopamine. To stop incubations, a deproteinizing mixture (containing trichloroacetic acid to make a 0.5% final concentration, plus 100 nmol dopamine per tube as internal standard and 120 nmol ascorbic acid) was added. Samples were homogenized in a Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA). An aliquot was taken for protein quantification by the Lowry method to take into account the variability of tissue amounts inside each tube. Tissue homogenates were then centrifuged (10,000 g, 10 min, 4 ºC), and all supernatants were processed for [^{3}H]-dopamine purification by HPLC. [^{3}H]-Dopamine formed during the incubation reaction was separated from [^{3}H]-tyrosine in a HPLC system that consisted of a reverse-phase C18 column (Tracer Extrasil ODS2, 5-µm particle size, 25 x 0.46 cm; Teknokroma, Spain) and an ion-pair mobile phase, made up of 0.1 M sodium phosphate buffer , 1mM EDTA, 0,75 mM octanesulfonic acid plus 12% (v/v) methanol (pH 5). The

flow rate was 1 ml/min. This HPLC system completely separated standards of tyrosine and dopamine that could be detected by UV absorbance at 285 nm. Dopamine retention time was 10 min. Samples contained equivalent amounts of internal standard dopamine than external standards injected for comparison to obtain internal standard recovery. Relative to the internal standard, the samples contained amounts undetectable by UV absorbance of radiolabeled tyrosine and dopamine as well as endogenous tyrosine and dopamine. Thus, a typical UV profile of a sample consisted largely of one front with undesired compounds and one peak corresponding to the internal dopamine standard (100 nmol per sample, corrected for recovery). Recovery of the dopamine internal standard in each sample (internal/external standard peak area) was quantified from dopamine HPLC-UV peak areas. The dopamine eluate fraction was automatically collected in a scintillation vial by a fraction collector (Gilson Fc 203B) at peak detection. Dopamine eluates were mixed with Optiphase HiSafe III cocktail (Wallac), and [^{3}H]-dopamine was quantified in a liquid scintillation counter. Dpm obtained were corrected by internal standard recovery, dpm in blank samples and protein concentration.

Statistical analyses

Statistical significance of differences between groups was assessed by analysis of variance (ANOVA) unless otherwise indicated. Non-parametric statistics were used when variance was different

between groups. Statistical significance was set at $p< 0.05$ prior to the experiments.

Drugs

Histaminergic agents, HPLC standards, and other reagents were purchased from Sigma/RBI (Steinheim, Germany) or Tocris (UK). [3,5-^{3}H]L-tyrosine (50 Ci mmol/l) and Opti-Phase HiSafe-3 liquid scintillation cocktail were supplied by PerkinElmer Wallac (Turku, Finland).

RESULTS.

Effects on cocaine reinforcement.

Rats maintaining cocaine self-administration for a minimum of three weeks were used. All rats had reached a FR5 schedule of reinforcement previously to the experiment. One hour before their daily session of cocaine reinforcement rats were divided into four groups and pretreated with the histamine H_3 receptor agonist imetit (3 mg/kg s.c.), the antagonist/inverse agonist thioperamide (3 mg/kg s.c.), the histamine precursor L-histidine (500 mg/kg i.p.) or saline respectively. The four groups had showed similar levels of cocaine reinforcement in the three previous days (saline: 43±10; imetit:41±5; thioperamide:41±7: histidine:40±7; mean±S.D. of cocaine injections of 0.5mg/kg in 2 h).

Pretreatment effects on cocaine reinforcement were tested in five consecutive days using four decreasing doses of cocaine and finally saline (1, 0.3, 0.1, 0.03 and 0 mg/kg/self-injection).

All groups showed characteristic bell-shaped dose-response curves of reinforcement. Pretreatment with histaminergic agents did not modify cocaine reinforcement in the descending limb of the curve. However pretreatment with the histaminergic agonists imetit and L-histidine decreased responses in the ascending limb of the curve corresponding to the lowest cocaine dose near the threshold of cocaine self-administration (0.03 mg/kg/self-injection) and saline self-administration (0 mg/kg/self-injection, in fact an extinction session; $p<0.05$, two-tailed Student's t-test versus respective saline pretreatment; **(Figure 1).** Therefore we concluded that the observed effects could be more related to cocaine seeking under extinction conditions than to changes in reinforcement. The decrease in responding elicited by imetit (a specific histamine H_3 receptor agonist) and L-histidine (a precursor metabolized to histamine in brain) was strikingly similar. In contrast, pretreatment with the histamine H_3 receptor antagonist/inverse agonist thioperamide had no effect.

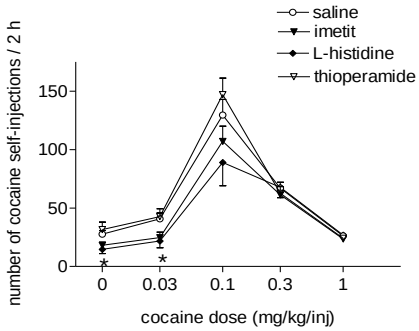


Figure 1. Cocaine reinforcement is not significantly modified by three histaminergic agents. However, the histamine H_3 receptor agonist imetit and the histamine precursor L-histidine decreased responding at low, non-reinforcing cocaine doses. Dose-response was assayed in consecutive days, decreasing from high to low doses. The zero dose is an extinction session. Values are means + SEM of N= 10-12 rats/group. * $p<0.05$ vs. saline, two-tailed Student t-test. Pretreatments were administered 1 h before the start of the session at the doses of 3 mg/kg s.c. (imetit and thioperamide) and 500 mg/kg i.p. (L-histidine).

Effects on cocaine-induced reinstatement.

To substantiate whether histaminergic agonists actually had effects on cocaine seeking rats were subjected to reinstatement tests.

Prior to reinstatement, rats underwent a mimimum of 6 extinction sessions (2 h) in consecutive days to extinguish lever presses to less than 20 in the second hour. During extinction sessions there was no reward or cues for pressing the lever. Then rats were subjected to 4 h reinstatement sessions, which were initiated with a 3 h extinction session followed by the automatic delivery of one saline or cocaine priming injection. Three reinstatement tests were carried out in random order for each rat, delivering a single non-contingent injection of 0 mg/kg (saline), 0.5 or 2 mg/kg cocaine priming i.v. through the catheter after 3 h of extinction. A dose-response of reinstatement by cocaine priming was obtained **(Figure 2).** One hour before priming rats received pretreatment with saline, imetit (3 mg/kg s.c.), thioperamide (3 or 6 mg/kg s.c.) or L-histidine (500 mg/kg i.p.). As shown in **Figure 2A**, pretreatment with either imetit or L-histidine decreased reinstatement of lever press elicited by the 0.5 mg/kg cocaine dose ($p<0.05$, Mann-Whitney U test versus pretreatment with saline). The effects of both histaminergic agonists were similar in

magnitude and they did not occur at the highest cocaine dose (2 mg/kg). In contrast, the histamine H_3 receptor antagonist/inverse agonist thioperamide did not elicit significant effects at either dose **(Figure 2B).**

According to dose-responses obtained in reinstatement and reinforcement tests, our results support that stimulation of histamine H_3 receptors decreases cocaine seeking behavior elicited by low cocaine doses.

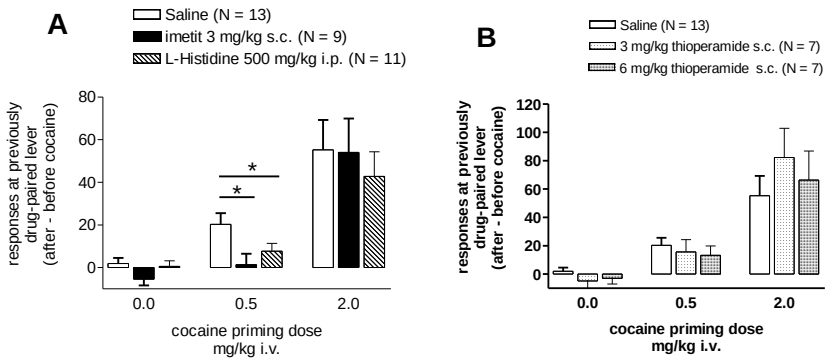


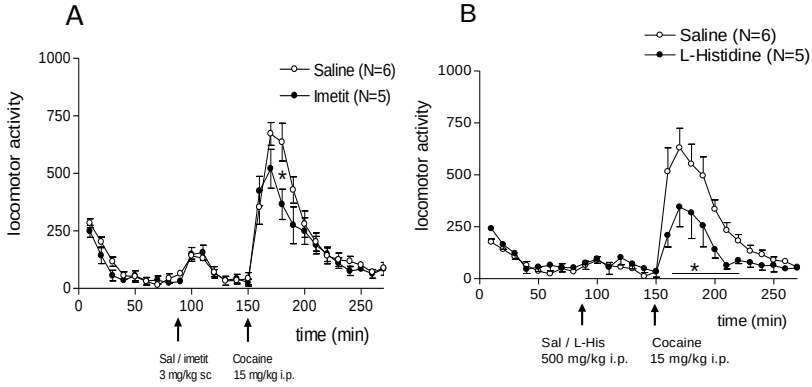
Figure 2. Effects of histaminergic agents on reintatement of cocaine seeking after extinction of the behavior. A, Reinstatement elicited by priming with a moderate dose of cocaine (0.5 mg/kg i.v.) is reduced by pretreatment with either the histamine H_3 receptor agonist imetit or with the histamine precursor L-histidine. B, The histamine H_3 receptor inverse agonist thioperamide had no effect. As expected non-contingent cocaine priming (white bars) elicited a dose-dependent increase in responding. Results are means $\pm$ SEM. * $p<0.05$ versus saline-pretreatment, Mann-Whitney U-test.

Effects on locomotion.

To investigate the possibility that locomotor effects underlyed the effects observed, naive rats were subject to tests of horizontal locomotion and injected with 15 mg/kg cocaine i.p. under the

effects of histaminergic agents **(Figure 3).** Pretreatment with L-histidine (500 mg/kg i.p., 1 h before) decreased cocaine-induced locomotion in naive rats, in agreement with Ito et al (1997).

The histamine H_3 receptor agonist imetit (3 mg/kg s.c. 1 h before) significantly decreased cocaine-induced locomotion at a single time point, 30 min after cocaine. In contrast, pretreatment with the histamine H_3 receptor inverse agonist thioperamide (3 mg/kg s.c. 1 h before cocaine) increased cocaine-induced locomotion, but its effects were only evident during the late phase of cocaine effects, from 70 to 100 min after cocaine. Therefore, it was possible that locomotor effects of histaminergic agents could contribute to some extent to the observed effects in cocaine-seeking behavior. However, given that histaminergic agents did not affect (1) basal locomotion before cocaine injection in naive rats, or (2) operant behavior in rats trained for cocaine self-administration **(Figure 1)**, we concluded that histaminergic agents did not cause a general inhibition of behavior.



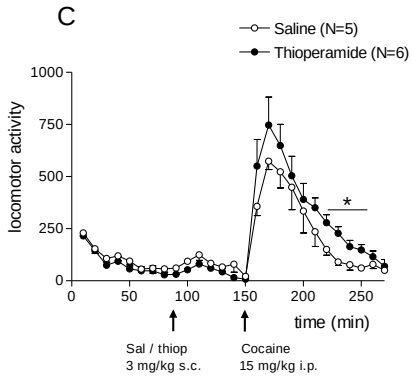


Figure 3. Effects of histaminergic agents in cocaine-induced locomotion. The histamine H_3 agonist imetit (A) and the histamine precursor L-Histidine (B) decreased cocaine-induced locomotion, while the H_3 inverse agonist thioperamide increased cocaine-induced locomotion (C). Results are means $\pm$ SEM of locomotor counts obtained from N=5-6 naive rats. *, $p<0.05$ Student's t-test vs. saline pretreatment at the indicated times (in A, time=180 min; in B, area under the curve from time=220 to 250 min; in C, area under the curve from time=160-220 min).

Effects on extinction tests.

To consolidate evidence of histaminergic effects in the absence of reinforcing doses of cocaine we designed experiments to test cocaine seeking during repeated extinction sessions of increasing predictability. Extinction tests were carried out in rats previously stable in a FR5 schedule. When extinction tests were performed 24 h after the last cocaine self-administration, pretreatment with the histamine H_3 receptor agonist imetit (3 mg/kg s.c., 1 h before) delayed the initiation of lever press on the first extinction session, as compared with saline pretreatment in the same rats **(Figure 4A).** However, this effect was statistically significant only on the first day of extinction, and it was not observed in following days.

Total lever presses during extinction sessions did not differ between saline and imetit pretreated groups any day tested (data

not shown). Imetit effects were restricted to the first extinction session, which was characterized by unpredictability and high responding as compared to the rest of extinction sessions. Thus we sought further evidence of imetit effects under unpredictable extinction conditions. We tested imetit effects on the high lever press responding during an unexpected extinction session carried out immediately after a cocaine self-administration session. In this case imetit was administered through the i.v. canula to obtain quick effects in between self-administration and the unexpected extinction sessions. In line with the previous finding, in this experiment imetit also increased latency to restart lever press in a dose-dependent manner **(Figure 4B).** However, the dose of imetit needed was higher than in previous experiments, perhaps due to the higher expectancy of cocaine by the rats due to unpredictability.

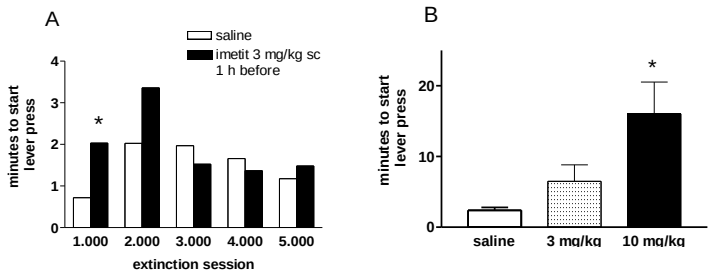


Figure 4. Pretreatment with the histamine H_3 receptor agonist imetit increases latency to first lever press in extinction tests. A, imetit delays lever press of first extinction session 24 h after last self-administration. In subsequent daily extinction sessions imetit had no effect. B, imetit delays lever press of a extinction session immediately after a self-administration session. In A imetit was administered s.c. 1 h before the session while in B administration was given i.v. through the catheter immediately before the extinction session. Results are means $\pm$ SEM of N=6 rats/group in A and N=10 rats/group in B. $p<0.05$ versus controls, Wilcoxon matched pairs test in A and one way ANOVA and Dunnett post-hoc test.

Moreover, the i.v. bolus of the higher dose of 10 mg/kg imetit completely suppressed the initial high responding that characterizes the start of an unexpected extinction **(Figure 5A).** This finding was strikingly clear, and it was accompanied by our personal observation of rats being less agitated when treated with imetit. Such agitation normally precedes cocaine self-administration sessions, and can be considered a sign of expectancy or anticipation of cocaine availability.

If instead of extinction we restarted self-administration after the 10 mg/kg i.v. bolus, rats delayed restart of lever press as shown before (confirming data in Figure 4B) but they progressively recovered their normal rate of self-administration **(Figure 5B).** This result also confirmed that the higher dose of imetit used did not impair operant behavior and cocaine reinforcement, as previously observed **(Figure 1)**.

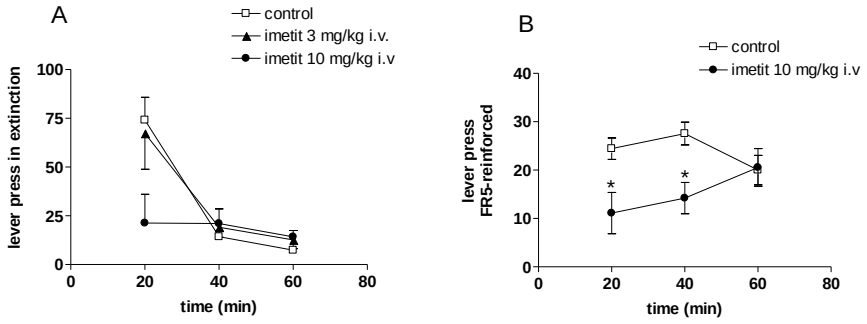


Figure 5. The histamine H_3 receptor agonist imetit suppresses high levels of cocaine seeking at the start of an unexpected extinction session. In saline-pretreated rats the initial 20 min block is characterized by high levels of impulsive cocaine seeking (A). However, reinforced operant behavior is not impaired (B). In both cases In both cases imetit was administered i.v. after a previous 1 h session of self-administration and immediately before the test session. Results are means $\pm$ SEM of N=10 rats/group. Asteriscs denote $p<0.01$ two-way ANOVA, time x treatment interaction and Bonferroni post tests.

Effects on a progressive ratio test.

Similarly to previous experiments, an i.v. bolus of 10 mg/kg imetit delayed restart of lever press in progressive ratio tests. After the initial delay, rats worked for 0.5 mg/kg cocaine under a progressive ratio schedule of reinforcement. Total drug earned and progressive ratio breaking point did not significantly differ between saline- and imetit-pretreated conditions (data not shown), suggesting that when cocaine is used as a reinforcer motivation to obtain the drug is not changed by the histaminergic agent. We could then conclude that the presence of reinforcing doses of cocaine overrides the effects of the H_3 histaminergic agonist **(Figure 6).**

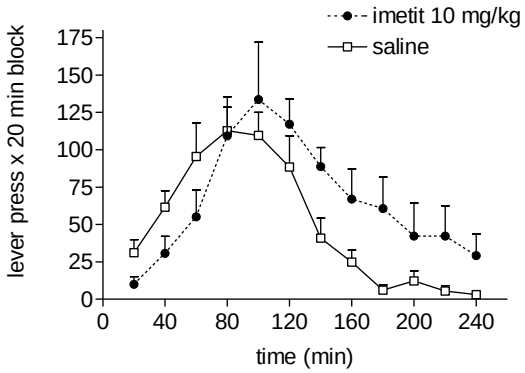


Figure 6. Lever press during a progressive ratio schedule of reinforcement is not changed by pretreatment with an i.v. bolus of the histamine H_3 receptor agonist imetit. As in previous experiments imetit delayed start of lever press, but reinforcement or breaking point were not significantly changed. Data are means ± SEM of N=6 rats run twice in the saline condition and only once under imetit pretreatment.

Functional effects of histamine H_3 receptors in dopaminergic neurons

Since agents modifying mesolimbic dopamine responses could have effects in cocaine-seeking behavior, we sought evidence of H_3 receptor modulation of dopaminergic responses in rat brain striatal tissue. In line with previous observations (Ligneau et al., 2007; Munzar et al., 2004), histamine H_3 receptors did not appear to modulate dopamine release (data not shown). However, a clear inhibition of dopamine synthesis was obtained in brain striatal slices from a naive rat using the H_3 agonist imetit. IC50 obtained (20 nM) in good agreement with previous data (Molina-Hernandez et al., 2000) **(Figure 7).**

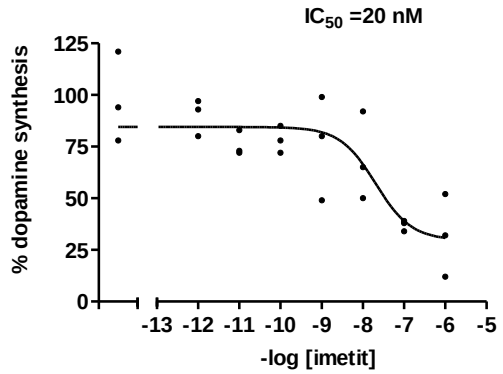


Figure 7. Dopaminergic effects of histamine H_3 receptors in brain striatal slices from a naive rat. Incubation of striatal miniprisms with the histamine H_3 receptor agonist imetit decreases DA synthesis with an IC_{50} consistent with receptor-ligand affinity. Data represent values of dopamine synthesis in individual incubations obtained from a single rat striatum (see methods).). N=5 incubations per condition. $P<0.01$ versus control, one-way ANOVA followed by Bonferroni's test.

DISCUSSION

Our results show that histamine H_3 receptors modulate cocaine seeking in behavioral models of extinction and relapse. We found coincident effects of the specific H3 receptor agonist imetit and of the non-specific histaminergic stimulator L-histidine in some of the tests. However when reinforcing doses of cocaine can be earned, operant behavior is not altered by these histaminergic agents.

Several lines of evidence support a histaminergic inhibition of reinforcement (see Brabant et al., 2010 for a review). Classic experiments show that histamine elevates the threshold for intracraneal self-stimulation (Cohn et al., 1973). Histamine H_1 receptors may decrease reward, as H_1 antagonists potentiate opioid reward in humans and rats (Showalter, 1980; Suzuki et al., 1990) and cocaine reinforcement in monkeys (Wang & Woolverton, 2009). However, the abuse liability of antihistamines is not unequivocally attributed to H_1 receptor blockade. Actions on targets other than H_1 receptors may contribute by facilitating dopaminergic neurotransmission and reinforcement (Bergman and Spealman, 1988; Halpert et al., 2002; Tanda et al., 2008). Administration of the histamine precursor L-histidine (500 mg/kg i.p.) has been shown to increase brain histamine levels, potentiating physiological histamine effects (Prell et al., 1996).

We used this dose under the hypothesis that L-histidine could decrease cocaine reinforcement by histaminergic stimulation of H_1 and/or H_3 receptors.

However we found no effect of L-histidine at cocaine doses near to those used to train the animals (0.3 and 1 mg/kg/ cocaine injection during testing vs. 0.5 mg/kg during training). We could speculate that increases of histamine synthesis by L-histidine may be irrelevant in the presence of cocaine, which also elevates histaminergic neurotransmission (Ito et al., 1997). In fact, effects of L-histidine were observed at smaller cocaine doses (0.03 mg/kg; Figure 1A). Unlike reinforcing cocaine doses, which elicit a regular pattern of self-administration, lever press for 0.03 mg/kg cocaine dose or saline is irregular and may be attributed to drug seeking and habits formed by previous conditioning. In our experimental protocol each cocaine dose was assayed in consecutive days in decreasing order. Thus, sessions with 0.03 and 0 mg/kg cocaine could rather be considered "extinction" sessions, where a too low dose of cocaine or saline can be obtained. In these conditions, stimulation of histaminergic neurotransmission with L-histidine could be decreasing cocaine seeking, rather than cocaine reinforcement. Our reinstatement data agreed with this interpretation. After repeated extinction sessions had decreased responding, L-histidine significantly decreased cocaine-induced reinstatement of lever press (Figure 2A).

In this test, rats that had accomplished extinction criteria were subjected to i.v. cocaine priming with 0.5 mg/kg or 2 mg/kg cocaine. L-histidine pretreatment only decreased lever press at the smallest cocaine dose.

This confirmed that L-histidine was ineffective when the rat was exposed to strong cocaine effects, as observed during reinforcement. This observation also indicates that L-histidine pretreatment did not blunt operant behavior, so its effects can not be attributed to a non-specific suppression of operant responding. L-histidine can be considered an indirect and non-specific histaminergic receptor agonist, provided it undergoes decarboxylation in brain giving histamine. After sistemic L-histidine pretreatment, it has been questioned whether decarboxylation only occurs in histaminergic nerve endings and mastocytes by their enzyme histidine decarboxylase (Prell et al., 1996). It is possible that part of L-histidine excess is decarboxylated in non-histaminergic nerve endings by L-aminoacid decarboxylase, an enzyme that has a much higher Km for L-histidine (about 20 mM; Prell et al., 1996). It is also possible that histamine formed and released from nerve terminals interacts with all types of histaminergic receptors. Among the possible receptors involved, we were interested specifically in the H_3 subtype because these receptors have a high relative expression in limbic brain nuclei related with reinforcement and locomotion (Pillot et al., 2002).

In addition H_3 ligands are quite selective if we exclude their actions on H_4 receptors expressed more abundantly out of the brain. In fact, the H_3 receptor agonist imetit mimicked L-histidine actions, as: (1) it was devoid of statistically significant effects at high reinforcing cocaine doses (Figure 1); (2) it decreased lever press at lower cocaine doses suggestive of an effect on drug seeking (Figure

1); and (3), imetit also decreased reinstatement of drug seeking by the low, but not the high cocaine dose (Figure 2A). These results show that L-histidine effects are mimicked by a specific H_3 receptor agonist, and therefore they could be mediated by the release of newly formed histamine acting on H_3 receptors. In contrast, opposite effects were not observed after pretreatment with the H_3 receptor inverse agonist thioperamide (Figure 1 and 2B). Two thioperamide doses were assayed (3 and 6 mg/kg, s.c.) which should occupy around 80 - 90 % of brain histamine H_3 receptors (Le et al., 2009). We did not find a potentiation of cocaine reinforcement although the same dose of thioperamide has been shown to potentiate methamphetamine self-administration (Munzar et al., 2004). The differences between methamphetamine and cocaine may relate to the particular neurochemical mechanisms activated by these drugs (Banks et al., 2009). We do not have a clear explanation of why the H_3 antagonist/inverse agonist thioperamide did not elicit opposite effect to those of the H_3 agonist imetit in our tests, but we could speculate that the complex neuronal mechanisms involved could hardly be further activated through this receptor type.

It has been reported that thioperamide and other relatively hydrophobic molecules containing a imidazole ring can inhibit P450 enzymes, altering pharmacokinetics of some coadministered drugs including cocaine (Brabant et al., 2009).

Although imetit and L-histidine contain imidazole rings, a pharmacokinetic interaction with cocaine does not explain their

effects. Firstly, imidazole inhibition of P450 could potentially increase cocaine concentrations. Higher cocaine levels would have displaced to the left of the dose-response curve of reinforcement. Such effect was not observed (Figure 1) with neither of three imidazole-containing molecules: L-histidine, imetit and thioperamide. Secondly, some of the effects of L-histidine and imetit were evident in the absence of cocaine since the previous day (Figure 1, 0 mg/kg cocaine dose; and Figure 4A). These effects can not be explained by changes in cocaine pharmacokinetics, and they fit better with changes in the expectancy of cocaine availability, or in other words, their conditioning by the environment of self-administration. In support of this, we have observed that imetit administration calms down the typical agitation of rats before their daily cocaine self-administration session (unpublished observation). In human cocaine addicts, agitation and anxiety are typical symptoms of short-term withdrawal (Erb, 2009). Short-term cocaine withdrawal is anxiogenic, and it has been reported diazepam reduces this type of anxiety as measured in the elevated plus maze test (Paine et al., 2002).

In rats, imetit calmed down initially high cocaine seeking elicited by unexpected extinction (Figures 4 and 5). It could be speculated that the H_3 agonist exerted a sort of sedative effect.

H_3 agonists have sedative properties similarly to classical brain-penetrating H_1 antagonists: They potentiate pentobarbital-induced narcosis, but they do not elicit narcosis by themselves (McLeod et

al., 1998). Although sedation caused by H_1 antagonists does not appear useful to treat anxiety (Davis et al., 2009), the role of H_3 receptors in animal models of anxiety needs clarification (Perez-Garcia et al., 1999; Bongers et al., 2004). Further studies will be needed to determine the role of H_3 receptors in cocaine withdrawal-induced anxiety. Moreover, it is also apparent that the presence of sufficient doses of cocaine totally overrides histaminergic effects, which are then limited to a delay of reinforcement behavior (Figures 1, 5B and notably Figure 6). This supports our view that H_3 receptor stimulation modifies cocaine seeking behavior, but not reinforcement to a significant extent.

The small locomotor effects of histaminergic agents in the presence of cocaine (Figure 3) may be relevant, but in our opinion they can not fully explain histaminergic effects on cocaine-seeking behavior. It has been shown that intracerebroventricular administration of histamine elicits a fast hypokinetic effect through H_3 receptor actication, and later hyperactivity mediated by H_1 receptors (Chiavegatto et al., 1998).

In addition, stimulation of H_3 receptors decreases L-dopa-induced turning behavior in 6-hydroxydopamine lesioned rats (Huotari et al., 2000).

It is possible that H_3 receptor stimulation transiently decreases GABA release by direct pathway neurons, where H_3 receptors and D_1 receptors have opposite effects (Moreno et al., 2011). Additional effects of H_3 receptors on excitatory inputs to the striatum should also be considered. However, a direct inhibitory

effect of H_3 receptors expressed by dopaminergic neurons (Figure 7 and Gonzalez-Sepúlveda et al., submitted) could contribute to explain both the locomotor and motivational effects observed in this paper. Regarding H_3 receptor effects on dopaminergic neurotransmission, at present it is uncertain why H_3 receptors appear to modulate better striatal dopamine synthesis Molina-Hernandez et al., 2000; and Figure 6) rather than release (Ligneau et al., 2007 and confirmed by our unpublised observations). We can hypothesize that the localization of H_3 receptors in dopaminergic neurons may be perisynaptic, away from the active release zone. However proof of this localization has yet to be obtained.

In conclusion, tested histaminergic agents (1) reduce cocaine seeking in behavioral models of extinction and relapse; (2) delay cocaine reinforcement, and (3) modulate cocaine-induced locomotion. Stimulation of histamine H_3 receptors present in dopaminergic neurons may account for these effects.

ACKOWLEDGEMENTS

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Resultats complementaris.

Ens va interesar provar si l'antagonista contrarrestava els efectes de l'agonista. En l'experiment hi va haver una sessió prèvia d'autoadministració de cocaïna, després se'ls va administrar l'antagonista histaminèrgic H_3 tioperamida seguit de l'agonista H_3 imetit. Després de l'administració dels fàrmacs, els animals van realitzar una altre sessió d'autoadministració. El temps de latència no va ser significativament inferior en els animals tractats conjuntament amb l'antagonista i l'agonista histaminèrgic.

El grup que se'ls va administrar imetit si van augmentar la latència a prèmer la palanca respecte al grup control **(Figura 1A).** En la Figura on es mesura el numero de palancades per blocks de 20 minuts es pot veure com en els blocs de 20, 40 i 60 minuts els animals tractats realitzen poques palancades respecte al grup control. **(Figura 1B).**

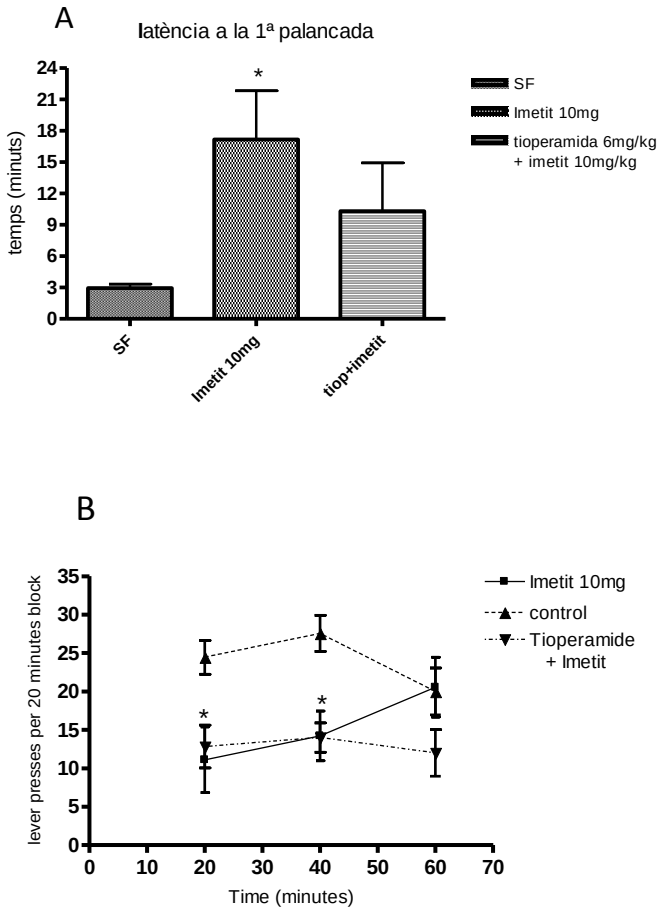
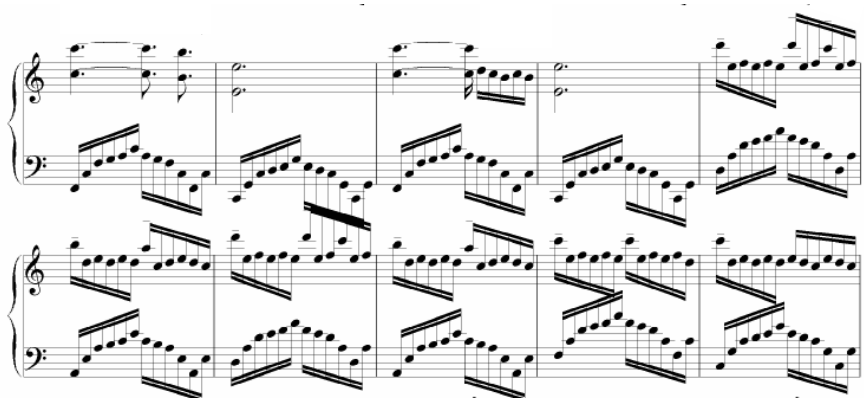


Figura 1. Efectes dels fàrmacs tioperamida i imetit sobre el patró d'autoadministració de cocaïna. A) La dosi de tioperamida emprada no reverteix significativament l'augment de la latencia produit per imetit. **B)** Aparentment els animals tractats amb tioperamida presenten un menor nombre de respostes però no hi ha significació estadística. L'antagonista tioperamida es va administrar a una concentració de 6mg/kg (via endovenosa) i l'agonista imetit a 10mg/kg (endovenosa), aquest fàrmac és va injectar 2 minuts després que l'antagonista. L'experiment és va portar a terme 24 hores després de l'ultima sessió d'autoadministració. ANOVA de dos factors, post hoc de Bonferroni. Es representen els valors mitjos ±SEM. (N=10 en grup control i imetit, N=6 en grup tioperamida). (*) P<0.05 respecte control.

Conclusions.

- L'agonista H_3 imetit disminueix la síntesi de DA en estriat de rata.
- El tractament amb l'agonista histaminèrgic H_3 imetit a una concentració de 10mg/kg, fa augmentar la latència a la primera palancada en una sessió d'extinció.
- El tractament amb l'agonista histaminèrgic H_3 imetit a una concentració de 10mg/kg, no produeix canvis en la cerca per la droga en una sessió d'autoadministració.
- El tractament amb l'agonista histaminèrgic H_3 a una concentració de 10mg/kg, no provoca canvis en un programa de raó progressiva sota reforç.
- El pretractament amb l'antagonista histaminèrgic H_3 tioperamida 6mg/kg no disminueix de forma estadisticament significativa l'increment de la latència a la primera palancada provocada per l'agonista H_3 imetit 10mg/kg en una sessió d'autoadministració de cocaïna.

DISCUSSIÓ GENERAL



DISCUSSIÓ GENERAL

Tal i com hem explicat anteriorment a la introducció, hi ha evidències que suggereixen que el sistema histaminèrgic podria jugar un paper inhibidor sobre efectes produïts per drogues d'abús.

Es sap que durant el reforç hi ha una activació de neurones dopaminèrgiques mesolímbiques que projecten cap al nucli accumbens, zona molt rica en receptors H_3 d'histamina. En base a aquestes evidències ens vem plantejar avaluar els efectes de l'agonista H_3 imetit sobre la motivació de consumir cocaïna en rosegadors. Aquest ha estat l'objectiu principal d'aquest tesi. Degut al llarg temps requerit per dur a terme els experiments conductuals, vem considerar convenient fer estudis previs "in vitro" d'efectes del fàrmac partint de la hipòtesi de que fàrmacs que frenin la síntesi i/o alliberament de DA poden ser útils pel tractament de les addiccions.

Prèviament a l'estudi *in vivo,* vem avaluar els efectes d'aquest fàrmac *in vitro,* per corroborar les dades ja publicades que indicaven l'acció del receptor H_3 sobre la síntesi de DA (Molina-Hernandez, Nunez et al. 2000). Per poder realitzar-ho vem adaptar un mètode de purificació de DA per HPLC, basant-nos en l'experiència del grup amb la síntesi i alliberament d'histamina i en resultats de la literatura (Bioulac, Cheramy et al. 1979; Arrang, Garbarg et al. 1983; Ortiz, Gomez et al. 2000).

La metodologia clàssica per quantificar la síntesi i l'alliberament de DA no permetia fer-ho de forma simultània i senzilla alhora. La manera més comuna per determinar efectes de fàrmacs sobre la síntesi de DA és inhibint la seva descarboxilació i mesurant l'acumulació de L-DOPA (Bioulac, Cheramy et al. 1979). En canvi per quantificar l'alliberament de DA es pot utilitzar mètodes *in vivo*, com la microdiàlisis (Kleijn, Wiskerke et al. 2012) o bé *in vitro,* incubant *slices* de cervell amb [^{3}H] DA (Fernandes, Massensini et al. 2004). Nosaltres hem optat per utilitzar [^{3}H] tirosina en slices de cervell per poder mesurar síntesi i alliberament de DA, en un mateix experiment. La nostra tècnica millora les existents ja que aporta l'automatització i l'eficàcia pròpia de l'HPLC per la purificació de la [^{3}H] dopamina formada en la incubació.

Els resultats obtinguts inhibint la tirosina hidroxilasa, induint la despolarització amb K^+ i utilitzant els inhibidors MAO-A/B i COMT, concorden amb els resultats obtinguts pels autors que utilitzen les metodologies clàssiques (Chowdhury, Steardo et al. 1987) **(Capítol 1, figures 3A, 5A i 6).**

També vem voler incubar miniprismes amb drogues psicotròpiques per aprofundir en l'estudi de la validesa del mètode respecte resultats de la bibliografia. En particular ens hem interessat per dos qüestions importants en el camp de l'addicció: l'efecte de la cocaïna i la nicotina sobre l'alliberament de DA, que es considera responsable de les seves propietats reforçants. Al realitzar la incubació dels miniprismes a diferents concentracions de cocaïna, vem observar que a 10 µM, es produïa l'alliberament màxim de DA

extracel·lular pel bloqueig del DAT (Povlock and Schenk 1997) i en canvi a 100 µM, començava a disminuir l'alliberament. Està descrit que la cocaïna a concentracions de (IC_{50}) 30µM bloqueja els canals de Ca^+ dependents de K^+ (Premkumar 2005; Hu 2007). Això podria explicar que a partir d'aquesta concentració, disminuís l'alliberament de DA provocat pel bloqueig dels DAT per la cocaïna **(capítol 1, figura 7B i resultats complementaris capítol 1, figura 1).**

Hi ha evidències que indiquen que la cooperació entre el sistema dopaminèrgic i colinèrgic son importants per al bon funcionament de l'estiat (Di Chiara, Morelli et al. 1994; Zhou, Liang et al. 2001). Està descrita la presència de receptors nicotínics en les terminals dopaminèrgiques de l'estriat (Wonnacott 1997) i que l'activació d'aquests receptors augmenten l'alliberament de DA (Livingstone and Wonnacott 2009). Per això vem voler utilitzar la nicotina en el nostre mètode per veure si es reproduiria els mateixos resultats ja descrits. L'incubació de miniprismes d'estriat de rata amb nicotina a 100 nM fan augmentar la DA alliberada tal i com està descrit a la bibliografia citada **(Capítol 1, figura 7A).**

Donat que els receptors D_2 són autoreceptors inhibidors de la síntesi de DA en neurones dopaminèrgiques, vem voler provar si el nostre mètode ens permetria observar el mateix que hi ha descrit a la literatura. Està comprovat que hi ha dues isoformes del receptor dopaminèrgic D_2: D_2L (long) i D_2S (short) (Xu, Hranilovic et al. 2002). La isoforma D_2S, es troba principalment en terminals presinàptiques, i la isoforma D_2L, està en terminals postsinàptiques (Lindgren, Usiello et al. 2003).

Al realitzar la inhibició de la síntesi de DA en miniprismes d'estriat de rata, vem observar que l'agonista dopaminèrgic de receptors D_2/D_3 bromocriptina (Tadori, Forbes et al. 2011) inhibia més la síntesi de DA que no pas l'agonista D_2 quinpirole **(Capítol 1, figura 4A).**

Com ja està descrit, l'afinitat de la bromocriptina (5.3nM/7.3nM) i el quinpirole (8nM/5.1nM) pels receptors D_2/D_3 és molt semblant. Podria ser que la disminució de la síntesi de DA per part de la bromocriptina no fos un efecte directe sobre receptors dopaminèrgics sinó que estigués mediat per receptors de 5HT, ja que està descrit que la bromocriptina també és un agonista serotoninèrgic. Més concretament, està descrit que l'activació del receptor $5HT_{2c}$ disminueix la síntesi de DA (Di Matteo, Di Giovanni et al. 2008). D'altre banda també és podria interpretar com la presència d'autoreceptors D_2/D_3 a l'estriat en forma de heteròmer, fet que produiria un sinergisme d'aquest dos receptors i una inhibició més gran de la síntesi de DA.

Així doncs podem concloure que, aquest mètode ens ha permès determinar la síntesi i l'alliberament de DA en miniprismes de manera sel·lectiva, acurada, senzilla i ràpida. La nostra tècnica ens proporciona una sèrie d'avantatges respecte d'altres tècniques que hi ha a la literatura; com la rapidesa per obtenir resultats, la seva especificitat a l'hora de mesurar la DA, l'elevada precisió de la quantificació deguda a l'ús de patró intern i el seu baix cost econòmic. Tots aquests resultats contrastats amb la literatura citada donen validesa al nostre mètode.

Un cop ens vem assegurar que comptàvem amb un mètode de quantificació acurat, ens vem centrar en estudiar l'efecte de l'agonista H_3 imetit sobre la síntesi de DA en el miniprismes.

Tal i com s'ha descrit anteriorment, l'activació dels receptors histaminèrgics H_3 inhibeixen la síntesi de DA en estriat de rata (Molina-Hernandez, Nunez et al. 2000). En aquest article, l'agonista H_3 utilitzat va ser l'immepip a una concentració de 100 nM, obtenint una disminució de la síntesi de DA al voltant d'un 60-70%. Nosaltres vem voler reproduir aquest resultat amb el nostre mètode i amb un altre agonista H_3. Vem incubar els miniprismes d'estriat a diferents concentracions (1 pM a 1 µM) amb l'agonista H_3 imetit, i vem observar que a concentracions de 0.1 µM i 1 µM es produïa una inhibició màxima de la síntesi de DA del quasi el 60% **(capítol 2, figura 7).** Aquests resultats concorden amb l'afinitat que té l'agonista pel receptor H_3 IC_{50}=20 nM.

Degut a l'important paper de la DA en l'addicció, el resultat obtingut ens va fer pensar que seria una bon fàrmac per provar-lo en un model de desig d'autoadministració de cocaïna, d'autoadministració en fase d'extinció, recaiguda i de raó progressiva en rates que busquen el reforç.

Davant la dificultat i els problemes ètics que comporta treballar en humans, molt investigadors utilitzem models d'animals drogodependents (Deroche-Gamonet, Belin et al. 2004). Els més interessants i que presenten major validesa a l'hora d'obtenir resultats extrapolables als consumidors humans, són aquells que impliquen un consum voluntari, entenent per voluntari el fet que

l'animal pugui decidir si vol consumir cocaïna i la dosi que vol consumir (autoadministració). En general, els models animals drogodependents haurien de generar un consum que produeixi dependència, "craving" i motivació per la cerca a la substància (DSM-IV 2000; Deroche-Gamonet, Belin et al. 2004). Vem decidir utilitzar un model d'autoadministració fent servir la cocaïna com a reforçador, ja que la validesa d'aquest mètode per aquesta substància està àmpliament descrita a la literatura (Self, Genova et al. 1998). El procediment d'autoadministració de cocaïna (sense extinció) dut a terme en aquest treball va ser estandaritzat pel Dr. David Self, que compta amb 20 anys d'experiència en aquesta tècnica, amb qui hem establert una col·laboració, i replicat amb exactitud el seu procediment. Aquest protocol ens ha proporcionat uns patrons de consum estables duradors, idèntics als patrons que s'obtenen al laboratori del Dr. Self. Val a dir que el nostre treball ha estat pioner a la nostra universitat en l'utilització d'aquesta complexe tècnica conductual, amb l'excepció dels experiments duts a terme per Yolanda Peña al final de la seva tesi.

Un cop els subjectes arribaven a un consum estable de cocaïna, se'ls sotmetia a una sessió d'extinció on es testava l'efecte de l'imetit, amb l'objectiu d'estudiar si aquest fàrmac pot arribar a alterar la conducta de recerca del reforç.

Per fer-ho, ens vem fixar en dos paràmetres com a referència: el temps que tardaven els subjectes en iniciar la recerca de droga (latència de la primera resposta a la palanca) i la persistència en la recerca (número de respostes fetes durant la sessió, dividida en

blocs temporals). El primer experiment consistia en administrar l'imetit 3mg/kg s.c en una sessió d'extinció de dues hores. En aquestes sessions d'extinció no hi van haver canvis significatius respecte al grup control. Després d' aquest experiment les rates van tornar al protocol d' autoadministració diari a FR5 **(capítol 2 figura 1).**

En un segon experiment es va administrar l'imetit 3mg/kg endovenós i 10mg/kg endovenós en una sessió d'extinció d'una hora immediatament posterior a una sessió d'autoadministració. L'imetit 3mg/kg no va produir cap efecte significatiu sobre la conducta respecte el grup control. En canvi la dosi d'imetit 10 mg/kg, va augmentar la latència en l'emissió de la primera resposta, i va disminuir el número de respostes a la palanca durant els 20 min posteriors a l'administració del fàrmac **(capítol 2 figura 4B).** Des d'aquest moment fins al final de la sessió, el patró de resposta dels dos grups va ser idèntic. En el grup control, les respostes dels primers 20 minuts són superiors a les habituals, probablement degut a l'inesperada absència de recompensa, que porta a l'animal a apretar repetidament la palanca. La dosi d'imetit 10 mg/kg va contrarrestar per complert aquesta resposta.

Una altre experiment portat a terme, un cop les rates tenien un consum estable, va ser sotmetre-les a sessions de raó progressiva. Encara que els resultats no mostren una manifestació conductual del fàrmac de manera significativa, si que es pot apreciar que a l'inici de la sessió els subjectes tractats amb l'agonista H_3 a una dosi de 10mg/kg premïen menys la palanca, fet que podria ser

provocat per l'acció del fàrmac: un retard en el desitg per consumir cocaïna. Observant la gràfica es podria afirmar és que l'agonista H_3 no afecta la conducta motora de l'animal ja que no deixa de prémer la palanca respecta al grup control **(capítol 2 figura 6).**

Per comprovar la selectivitat conductual de l'efecte produït, vem pensar que seria interessant fer uns experiments d'autoadministració de cocaïna (sense fase d'extinció) administrant l'agonista H_3 a la mateixa concentració que la utilitzada anteriorment. Les rates es van administrar la mateixa quantitat de cocaïna que les rates control en la sessió d'autoadministració. Encara que no hi van haver diferències significatives entre els dos grups, els animals es van administrar menys quantitat de la droga en els dos primers blocs de 20 minuts i també hi va haver un augment en la latència. Aquest fet es podria interpretar com que el fàrmac no té efectes significatius sobre la conducta motora i sobre el comportament operant, tot i que permeten corroborar el retard en la latència observat en els experiments anteriors **(capítol 2 resultats complementaris figura 1B).**

Un altre experiment que vem voler portar a terme per reforçar els resultats anteriors sobre la selectivitat farmacològica va ser realitzar un experiment d'autoadministració de cocaïna administrant l'antagonista H_3 tioperamida juntament amb l'agonista imetit a les mateixes concentracions que les utilitzades anteriorment, per observar si l'antagonista impedia els efectes de l'agonista.

Els resultats no han estat significatius pel que es refereix a l'antagonisme de la latència en la primera resposta. Podria ser que la coadministració dels dos fàrmacs a dosis similars i amb dos minuts de diferència sigui insuficient per observar aquest antagonisme. En canvi, pensem que es podria interpretar que l'administració de l'antagonista incrementa el valor reforçant de la cocaïna, produïnt una disminució en el numero de palancades que es manté als tres blocs de 20 minuts **(capítol 2, resultats complementaris figura 1A).** És sap que dosis elevades de cocaïna produeixen una taxa de resposta menor que dosis més baixes. Per tant una menor taxa d'autoadministració és compatible amb un major valor reforçant de la combinació cocaïna + tioperamida, tot i que aquesta hipòtesi hauria de ser validada a altres concentracions de la droga.

Encara que no es tenen dades sobre la vida mitja de l'agonista imetit, hi ha estudis que indiquen que l'administració via oral de imetit 5mg/kg en ratolins inhibeix la formació de la 3-(tele)-metil-histamina un metabòlit inactiu de la histamina unes 3 hores després de l'administració d'aquesta molècula (Garbarg, Arrang et al. 1992). Aquest fet es podria interpretar com que l'administració via oral de l'imetit triga molt en arribar al cervell. Per tant, és difícil aventurar quina ocupació ha fet l'imetit sobre els receptors H_3 cerebrals, i quina ratio de concentracions imetit/tioperamida s'ha assolit durant l'administració endovenosa.

Com ja s'ha descrit anteriorment a la introducció, els receptors NMDA de glutamat estan localitzats presinapticament a les neurones dopaminèrgiques de l'estriat i la seva activació estimula l'activitat TH (Arias-Montano, Martinez-Fong et al. 1992a). El grup de Hansen *et al.,* ha descrit recentment que tant l'agonista H_3 l'imetit a 52 ± 100 µM com l'antagonista H_3 tioperamida a >100 µM tenen afinitat per receptors NMDA de glutamat (Hansen, Mullasseril et al. 2010). Encara que en aquestes concentracions es dificil poder-les asolir en experiments *in vivo,* aquests resultats es podrien interpretar com que la unió als receptors NMDA per part de l'agonista H_3 imetit podria contribuir a la inhibició de la formació de DA.

Està descrit que els receptors D_2 i D_3 regulen la recaptació de DA per part de DAT (Bolan, Kivell et al. 2007; Zapata, Kivell et al. 2007), però no coneixem referències que impliquin el DAT en la regulació dels receptors D_2, D_3 i encara menys H_3, mitjançant interaccions moleculars intracel·lulars. Tanmateix, la gran concentració de DA extracel·lular assolida en bloquejar el DAT podria sobresaturar el receptor D_2 i inhibir l'acció de l'agonista H_3 sobre aquest receptor, ja que canònicament utilitza la mateixa via de transducció. Això podria ajudar a explicar la manca d'efecte de l'imetit en presència de concentracions reforçants de cocaïna.

Esta descrit que el receptor H_4 té una organització similar al receptor H_3 i una seqüència d'aminoàcids molt similar (35%). Encara que aquest receptor té una baixa presència al còrtex, hipocamp i tàlem (Connelly, Shenton et al. 2009) és troba

àmpliament al sistema immunitari en cèl·lules T, monòcits, mastòcits, neutròfils i eosinòfils (Liu, Ma et al. 2001) i participa en processos inflamatoris (Zampeli and Tiligada 2009) i alèrgics (Dunford, O'Donnell et al. 2006). També està descrit que l'agonista H_3 imetit té afinitat pel receptor H_4, (Liu, Ma et al. 2001). No es pot descartar que la disminució en el numero de palancades també fos mediat parcialment pel receptor H_4.

Tots aquests resultats són totalment novedosos a la bibliografia, i poden ser importants de cara a buscar agents terapèutics per tractar l'addicció a la cocaïna (Ortiz and Self 2011).

Sabem que el nucli accumbens té una alta densitat de receptors H_1 i H_2 d'histamina, i concretament la zona de la shell és molt rica en receptors H_3 i en el seu mRNA (Pillot, Heron et al. 2002).

La hipòtesi que ens plantegem, és que l'imetit activaria els receptors H_3 histaminèrgics de la via mesolímbica (VTA-Nac), la qual cosa inhibiria la síntesi de DA al nucli accumbens. Aquesta inhibició comportaria que la rata deixés de tenir interès i poca motivació en la cerca del plaer. D'altre banda podríem pensar que al inhibir amb imetit la síntesi de DA a l'estriat, que també és molt rica en receptors H_3 (Lovenberg, Roland et al. 1999) provocaria certa hipoactivitat locomotora.

Està descrit (Chiavegatto, Nasello et al. 1998) que l'administració de histidina (5.4 i 54.3 nmol), l'aminoàcid precursor de la histamina, en rates produeix efectes bifàsics, amb un inici transitori d'hipoactivitat i després d'hiperactivitat locomotora. Aquesta disminució de l'activitat motora va ser inhibida per

l'antagonista H_3 tioperamida i induïda després per l'agonista histaminèrgic N-alfa-metilhistamina.

Els autors suggereixen que la hipoactivitat inicial provocada per la histidina es deu a l'activació d'heteroreceptors histaminèrgics H_3, reduint així l'activitat del sistema dopaminèrgic estriatal. Aquest efecte pot sobreposar-se amb la hiperactivitat induïda per activació pels antagonistes dels receptors histaminèrgics H_1, que tindria lloc més tard (Chiavegatto, Nasello et al. 1998). Val a dir que l'imetit també pot produir una disminució de l'alliberament d'histamina neuronal, que impediria l'acció hiperlocomotora de la histamina sobre receptors H_1. Tot i que pot provocar un efecte hipolocomotor, no sembla que l'acció hipolocomotora de l'imetit sigui molt evident, o si més no suficient per impedir una conducta operant.

Encara calen més estudis per comprovar si aquest fàrmac és un possible agent terapèutic, emprant altres models o patrons d'extinció i examinant l'efecte sobre el restabliment de la conducta (induït per agents externs com una dosi de "record" de cocaïna no contingent, estímuls visuals o estrés). En aquest sentit, resultats del nostre grup en col·laboració amb el grup del Dr. Self suggereixen que l'imetit podria disminuir el restabliment de la conducta de recerca de droga desencadenat per una dosi de record de cocaïna **(capítol 2 figura 3A).** En aquest estudi s'ha observat que rates en període d'extinció quan se'ls administra una dosi "record" de cocaïna 0.5mg/kg via endovenosa deixen de tenir interès en pressionar la palanca degut al pretractament d'imetit i

histidina, en canvi quan les dosis son a 2mg/kg el tractament amb l'agonista histaminèrgic L-histidina es inefectiu.

Un altre estudi que el nostre grup vol portar a terme, és realitzar en animals que prèviament hagin complert els criteris per assolir FR5, realitzar sessions d'autoadministració amb l'agonista H_3 imetit per comprovar si aquest fàrmac podria actuar com a reforç. De moment els resultats no son concloents i no és pot ni afirmar ni descartar aquesta hipòtesi.

Esta descrit que l'activació dels receptors H_3 al SNC produeix hipoactivitat locomotora en rates i ratolins i un augment del son en gats (McLeod, Aslanian et al. 1998). Aquest efecte sedant seria diferent al d'una benzodiazepina clasica ja que es tracta d'una classe farmacològica amb una diana totalment diferent.

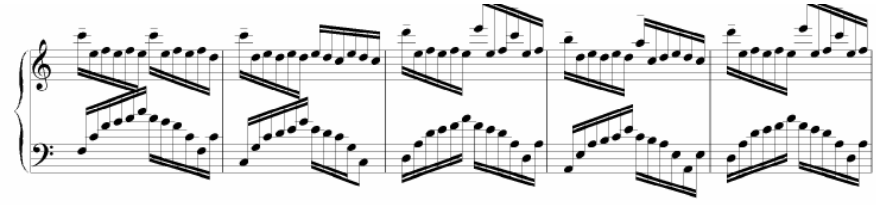
Per altra banda, altres estudis indiquen que l'administració d'agonistes H_3 com l'imepip i el R-α-methylhistamine no produieixen efectes sedants en el *open arms test* (Yokoyama, Yamauchi et al. 2009).

Està descrit que l'ansietat es un símptoma clau que es presenta davant l'abstinència a una substància en persones addictes i està considerat el factor que precipita a la recaiguda (Sarnyai, Biro et al. 1995). En el cas que l'imetit tingués un efecte ansiolític, potser seria una eina per tractar aquest aspecte de l'abstinència a la cocaïna. No obstant, el paper que juga el receptor H_3 en models animals amb ansietat necesita aclariment.

Tanmateix com a proves conductuals per comprendre millor si l'imetit produeix efectes motrius o sedants, es podria realitzar un test per mesurar l'activitat motora, com el rotarod o bé realitzar una prova de camp obert.

Encara que de moment no existeix cap fàrmac efectiu per a poder combatre la dependència a la cocaïna, l'objectiu de la recerca presentada en aquesta tesi ha estat entendre una mica més les addiccions per poder donar alguna eina per a fer-les front.

CONCLUSIONS GENERALS



CONCLUSIONS GENERALS

- El nostre protocol permet quantificar la síntesi i l'alliberament de DA en un mateix experiment amb l'automatització i l'eficàcia pròpia de l'HPLC millorant tècniques anteriors.
- Molècules addictives com la cocaïna i la nicotina faciliten l'alliberament de DA en estriat de rata.
- L'agonista H_3 imetit disminueix la síntesi de DA en estriat de rata.
- El tractament amb l'agonista histaminèrgic H_3 imetit a una dosi de 10mg/kg, fa augmentar la latència a la primera palancada. Això es posa de manifest en una sessió d'extinció, d'autoadministració i de raó progressiva.
- El tractament amb l'agonista histaminèrgic H_3 imetit a una dosi de 10mg/kg, no produeix canvis en la cerca per la droga en una sessió d'autoadministració.
- El pretractament amb l'antagonista histaminèrgic H_3 tioperamida 6mg/kg no disminueix de forma estadisticament significativa l'increment de la latència a la primera palancada provocada per l'agonista H_3 imetit 10mg/kg en una sessió d'autoadministració de cocaïna.
- El tractament amb l'agonista histaminèrgic H_3 a una concentració de 10mg/kg, no provoca canvis en un programa de raó progressiva sota reforç.

AGRAÏMENTS

Amb els suport de les subvencions del Govern Espanyol SAF2006-08.240 i SAF2009-12.510 i de la Xarxa de Trastorns Addictius RD06/0001/0015.

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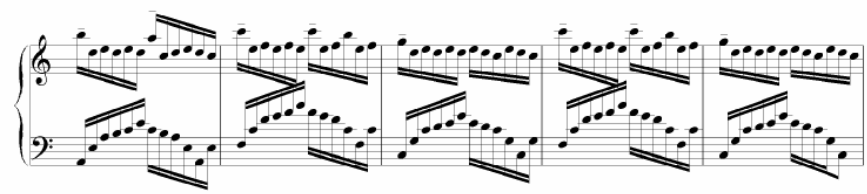

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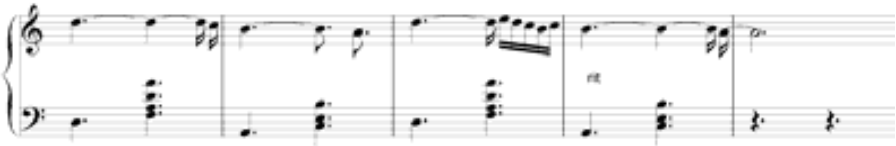
ANNEX



DUAL REGULATION OF BRAIN STRIATAL DOPAMINE SYNTHESIS BY ENZYMATIC END-PRODUCT FEEDBACK INHIBITION.

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ABSTRACT

Dopaminergic neurons play an important role in Parkinson's disease, psychosis, and addiction. Dopamine biosynthesis is rate-limited by tyrosine hydroxylase activity, which can be modified by phosphorylation and/or by dopamine in a process called end-product feedback inhibition. *In vitro* studies have shown that tyrosine hydroxylase has two binding sites for dopamine inhibitory effects: a high affinity site (regulated by phosphorylation) and a

low affinity site. When studying dopamine biosynthesis in rat brain striatal miniprisms we observe that: a) tyrosine hydroxylase is strongly inhibited by newly formed dopamine during preincubation, b) increasing extracellular or intracellular dopamine inhibits dopamine synthesis in a clear dose-dependent manner to an almost complete stop, c) tyrosine hydroxylase activation by phosphorylation appears to be reduced at high dopamine concentrations, suggesting a dual action of dopamine at high- and low affinity sites, and d) as expected the high affinity site appears to be regulated by dopamine and by phosphorylation through changes in the affinity for the tetrahydrobiopterin cofactor. Activation by phosphorylation would relieve dopamine from the high-affinity site and facilitate cofactor binding unless this equilibrium is altered by high dopamine concentrations. In addition, physiological concentrations of endogenous dopamine can inhibit tyrosine hydroxylase independently of its phosphorylation state.

INTRODUCTION

Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase; E.C. 1.14.16.2) is the first and rate-limiting enzyme in dopamine biosynthesis. Regulation of its activity is thought to be crucial to maintain dopamine levels, so permanent changes may turn in physiopathological phenotypes [1, 2]. TH activity is modulated by a long-term regulation of gene expression as well as by short-term

regulation of enzyme activity such as end-product feedback inhibition or phosphorylation [3]. Changes in TH phosphorylation state are usually considered as critically involved in the regulation of dopamine synthesis [4]. In particular, Ser^{40} phosphorylation induces TH activity, thereby stimulating synthesis of neurotransmitter [5, 6]. Modulation of TH activity by phosphorylation has been extensively investigated *in vitro*. However it is less clear what regulatory mechanisms could be more important *in vivo*, where other factors including end-product feedback inhibition should be present.

Previous works with recombinant TH have suggested the existence of at least two dopamine binding sites in TH [7]: a high affinity site (K_d 4nM), from which dopamine could only be dissociated by Ser40 phosphorylation [8, 9], and a low affinity binding site (K_d 90nM) from which dopamine is readily dissociable. Dissociation of dopamine from the low-affinity binding site increases TH activity in both the non-phosphorylated and $pSer^{40}$ forms of the enzyme. Moreover, the main alteration in TH upon Ser40 phosphorylation is a change in K_d value for catecholamines, leading to a lower affinity for dopamine [10]. Thus, end-product feedback inhibition might be the main regulatory mechanism unifying signaling inputs leading to phosphorylation with a dopamine level sensor. However, feedback inhibition in brain tissue has not been well characterized as compared to phosphorylation. After some initial classic studies [11, 12, 3], only some computational analysis pointed out the potential importance of feedback inhibition as the first principle to control

cytosolic dopamine levels in basal and depolarized neurons [13,14]. The study of end-product feedback inhibition in tissue is complex because of the unavoidable presence of endogenous dopamine bound to the enzyme at unknown amounts, which difficults to characterize the conditions of inhibition. Moreover, common methods to estimate tyrosine hydroxylase activity in brain typically prevent dopamine formation by inhibiting L-DOPA-decarboxylase to measure L-DOPA accumulation in tissue. This impairs to estimate actual feedback by dopamine. To overcome this limitation we improved radioisotopic methodology to determine [^{3}H]-dopamine synthesis by HPLC purification.

During set up of a study of [^{3}H]-dopamine synthesis in rat brain striatal miniprisms we observed a consistent and spontaneous decay of basal velocity in control tissue. We suspected end-product feedback inhibition by newly formed dopamine as causative of this effect.

To test this hypothesis we compared our radioisotopic methodology for [^{3}H]-dopamine synthesis and the prototypical estimation based on decarboxylase inhibition and L-DOPA accumulation. The effects of phosphorylating conditions, presence of dopamine and cofactor availability were also tested.

MATERIAL AND METHODS

Chemicals

Opti-Phase HiSafe-3 liquid scintillation cocktail was supplied by PerkinElmer Wallac (Turku, Findland). [3,5-^{3}H]L-tyrosine (^{3}H-Tyr, 50 Ci/mmol), from the same supplier, was purified by high-performance liquid chromatography (HPLC) before use as described (Purification of [^{3}H]-Tyrosine standards). db-cAMP was obtained from Biolog Life Science Institute (Bremen, Germany). Okadaic acid was purchased from Merck Biosciences (Darmstadt, Germany). NSD-1015, EDTA, HPLC standards, and other reagents were purchased from Sigma/RBI (Steinheim, Germany).

Purification of [^{3}H]-Tyrosine Standards

Ring-labeled [3,5-^{3}H]-L-tyrosine (40–60 Ci/mmol) shows a decomposition rate of at least 1–3% per month, which generates unwanted by-products that must be separated before its incubation with tissue. The main goal of this purification is to maintain a high degree of purity and control specific activity of [^{3}H]-tyrosine.

The system used for HPLC purification consisted of a reverse-phase C18 column (Tracer Extrasil ODS2, 5-μm particle size, 25 x 0.46 cm; Teknokroma, Spain) and a mobile phase with the following composition 100 mM sodium phosphate buffer, 1mM EDTA, 0.75 mM octanosulfonic acid and 1% (v/v) methanol (pH 3.4). The flow rate was 1 ml/min. Under these conditions, tyrosine eluted at 9–10 min. In each purification, 0.4mCi of [3,5-^{3}H]L-tyrosine were

injected into the HPLC and the whole tyrosine fraction (0.5–1 ml) was collected. The amount of [^{3}H]-tyrosine was quantified against an external standard calibration curve of nonradiolabeled tyrosine detected by UV absorbance at 285 nm. An aliquot of the purified fraction was subjected to liquid scintillation counting to obtain specific activity of the purified product, as total dpm in the purified fraction divided by the amount of tyrosine UV detected.

Preparation and preincubation of striatal miniprisms

Protocols for animal handling were previously approved by the Ethics Committee for Human and Animal Research (Universitat Autònoma de Barcelona) in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Male Sprague-Dawley rats weighing 200-250 g (Animal Service, Universitat Autònoma de Barcelona, Barcelona, Spain) were sacrificed by decapitation. Brains were chilled immediately in modified Krebs-Ringer-bicarbonate medium with the following composition: 120 mM NaCl, 0.8 mM KCl, 2.6 mM $CaCl_2$, 0.67 mM $MgSO_4$, 1.2 mM KH_2PO_4, 27.5 mM $NaHCO_3$, and 10 mM glucose, pH 7.4. In a 4ºC room, dorsal striata from both hemispheres were dissected and sliced using a McIlwain tissue chopper obtaining miniprisms of 0.3 x 0.3 mm/side. The miniprisms were suspended in ice-cold Krebs Ringer bicarbonate medium and washed by centrifugation and resuspension in order to remove debris of damaged cells. Striatal tissue from a single rat allowed obtaining up to 28 aliquots (25 µl each) of the settled slice

suspension corresponding to 24 incubations and 4 blank samples. Blank tubes were kept on ice and the rest were distributed into 2-ml polypropylene tubes and incubated at 37 ºC in an Eppendorf Thermomixer (5 Prime, Inc., Boulder, CO) under 95% O_2/5% CO_2 atmosphere. Samples were preincubated from 0 to 4 hours depending on the experiment. Preincubation was initially intended to reactivate metabolism of the different samples before quantification.

[^{3}H]-Dopamine synthesis

Purified [^{3}H]-tyrosine was added to all samples at the end of preincubation time to a final concentration of 0.12 μM and they were incubated for 10 min to synthesize [^{3}H]-dopamine. In experiments where drugs were needed, they were added during preincubation period. [^{3}H]-dopamine synthesis was stopped by the addition of a deproteinizing mixture containing trichloroacetic acid (1%) and 25nmol dopamine and 1mM ascorbic acid as internal standard. Samples were homogenized in a Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA). An aliquot was taken for protein quantification by the Lowry method to take into account the variability of tissue amounts inside each tube. Tissue homogenates were then centrifuged (12,000 g, 10 min, 4 ºC), and all supernatants were processed for [^{3}H]-dopamine purification by HPLC-UV (as described below). Dpm obtained in HPLC-purified [^{3}H]-dopamine fractions were corrected by dopamine internal standard recovery and dpm in blank samples. Velocity of [^{3}H]-

dopamine synthesis was estimated as the ratio of corrected dpm divided by protein content in each incubate and the incubation time in the presence of [^{3}H]-tyrosine. Results were expressed as percentage with respect to control samples run in each experiment.

[^{3}H]-Dopamine Purification by HPLC-UV

[^{3}H]-Dopamine formed during the incubation reaction was separated from [^{3}H]-tyrosine and purified by HPLC. The chromatography system consisted of a reverse-phase C18 column (Tracer Extrasil ODS2, 5-mm particle size, 25 x 0.46 cm; Teknokroma, Spain) and an ion-pair mobile phase, made up of 100mM sodium phosphate buffer, 1mM EDTA, 0,75 mM octanesulfonic acid plus 12% (v/v) methanol (pH 5). The flow rate was 1 ml/min. This HPLC system completely separates standards of tyrosine and dopamine detected by UV 285 nm (ring absorbance). Samples contain extremely low levels of radiolabeled tyrosine and dopamine that were undetectable by UV absorbance. Similarly, endogenous tyrosine and dopamine were negligible as compared to the amounts of internal standard dopamine used. The recovery of the internal standard in each sample (internal/external standard peak area) was quantified from dopamine HPLC-UV peak areas. Dopamine fractions were recovered in scintillation vials, mixed with Optiphase HiSafe III cocktail, and [^{3}H]-dopamine was quantified in a liquid scintillation counter.

Tyrosine Hydroxylase kinetic curves in homogenates

Miniprism samples were treated as described above in *Preparation and preincubation of striatal miniprisms.* Tubes were placed on ice, centrifuged at 1000 g in order to settle miniprisms and Krebs-Ringer buffer was removed. Cold phosphate buffer 10mM pH 7.4 was added and samples were homogenized using a glass Potter homogenizer. 200μl of homogenates were distributed in incubation tubes and 100μM NSD-1015, 0.25μM [^{3}H]-tyrosine, 20μM tyrosine and tetrahidrobioptherin (BH_4, at concentrations from 0 to 250μM) were added. After 30 min of incubation at 37ºC samples were placed in an ice block and deproteinizing mixture (containing trichloroacetic acid and 25nmol L-DOPA as internal standard) was added. L-DOPA was purified by HPLC-UV, recovered in a scintillation vial and mixed with Optiphase HiSafe III cocktail (Wallac). Coeluted [^{3}H]-L-DOPA was quantified using a scintillation counter. Dpm obtained in HPLC-purified [^{3}H]-L-DOPA fractions were corrected by L-DOPA internal standard recovery and dpm in blank samples. Velocity of [^{3}H]-L-DOPA synthesis was estimated as the ratio of corrected dpm divided by protein content in each incubate and incubation time in the presence of [^{3}H]-tyrosine. Results were expressed as percentage with respect to control samples in each experiment.

Estimation of tyrosine hydroxylase activity *in situ* by L-DOPA accumulation

Tyrosine hydroxylase activity in miniprisms of rat striatum was estimated using the classical method described by Lindgren et al, 2000 with slight modifications. Miniprisms were treated as *Preparation and preincubation of striatal miniprisms* and then treated with NSD-1015 (100 μM) to inhibit L-aminoacid aromatic decarboxylase. After 30 min of incubation the accumulation of L-DOPA was quantified by HPLC and coulometric detection. The stationary phase consisted of a reverse-phase C18 column (2.5μm particle Fortis C18, 100 x 4.6, Sugelabor, Spain) and an ion-pair mobile phase, made up of 100mM sodium phosphate buffer, 1mM EDTA, 5 mM octanesulfonic acid plus 1% (v/v) methanol (pH 2.5). The flow rate was 1 ml/min. This HPLC system completely separated standards of tyrosine, L-DOPA and dopamine that were detected by a coulometric detector (Coulochem II; ESA) with a detection limit of 0,2nmol for L-DOPA. Standards of L-DOPA at different concentrations (4-40nmol) were injected in every experiment to quantify L-DOPA in miniprisms by the external standard method. Fmols of L-DOPA in samples were corrected by fmols present in blank samples and protein content in each incubate. Results were expressed as percentage versus control samples in each experiment.

Western blot

Each sample was added 100 µL of ice-cold lysis buffer made of 1 mM orthovanadate, 50 mM Tris-HCl (pH 7.5), 25 mM sodium pyrophosphate, 50 mM NaCl, 1% Triton X100, 50 mM sodium fluoride, 5 µM zinc chloride, 2 mM DTT, phosphatase inhibitor cocktail 1 (Sigma) and protease inhibitor cocktail 1 (Sigma). Samples were sonicated on ice for 15 s and centrifuged (13000 g, 20 min, 4 ºC). Supernatant protein concentration was determined by a modified Lowry's method where 30 µL of 4% SDS was added 5 min prior to folin reagent to avoid interference with Triton X100 [15]. Proteins were dissolved in denaturating loading buffer and boiled at 99 ºC for 5 min. Equal amounts of protein (10 µg) were separated by 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride membrane (Millipore), washed in phosphate buffered saline– 0.05%Tween 20 (PBS-T) or Trizma buffered saline-0.05% Tween 20 (TBS-T), followed by blocking in 3% BSA dissolved in TBS-T. Primary antibodies were prepared in 1% BSA dissolved in TBS-T and incubated over night at 4 ºC with gentle agitation. Horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated at room temperature for 1 h. Antibody binding was detected by enhanced chemiluminecence (Millipore).

Immunoblotting was carried out using primary antibodies against: tyrosine hydroxylase (1:5000, AB1542, Chemicon) and tyrosine hydroxylase phosphoSer40 (1:4000, AB5935, Chemicon).

Horseradish peroxidase-coupled secondary antibodies used were goat anti-rabbit (1:1000, #7074 Cell Signaling) and donkey anti-sheep (1:3000, AP147P, Chemicon). A CCD camera (Gene Gnome Syngene Bio Imaging) was used to reveal chemiluminescence. Semi-quantitative analysis was performed by ImageJ (NIH image). Results were expressed in arbitrary units of optical density.

Statistical analysis

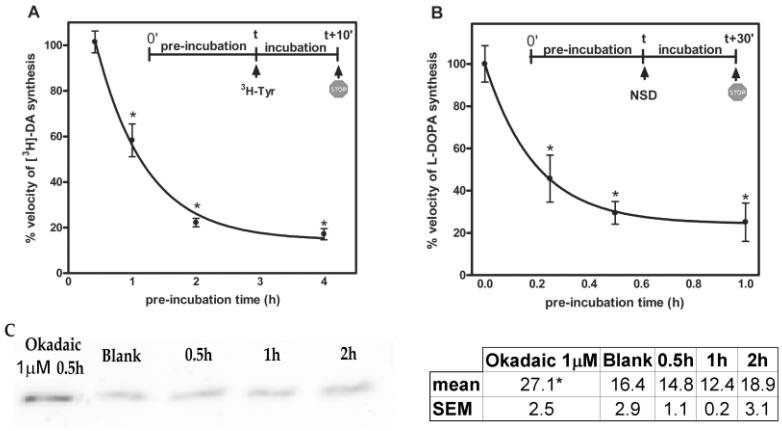
Statistical significance of differences between groups was assessed by analysis of variance (ANOVA) followed by Bonferroni post hoc tests. Statistical significance was set at $p<0.05$.

RESULTS

Effect of preincubation or incubation time in L-DOPA and dopamine syntheses.

Tissue preincubation is frequently used to recover and to stabilize metabolism after sample processing in ice-cold buffer. We expected TH activity to increase during preincubation. However using preincubation times above 25 min we observed a significant reduction of [^{3}H]-dopamine synthesis velocity **(Fig 1A).** This reduction of dopamine synthesis was consistently observed across experiments. To reaffirm this result we measured TH activity by L-DOPA accumulation after decarboxylase inhibition with NSD-1015, obtaining a parallel decrease with the same characteristics **(Fig 1B).** The latter result also discarded the possibility that degradation of newly formed [^{3}H]-dopamine was the cause of the observed

decrease. Nevertheless, we confirmed that monoamino oxidase inhibition did not suppress the observed decrease of the velocity of [^{3}H]-dopamine synthesis (data not shown). Moreover, no modification of TH phosphorylation at Ser40 was detected **(Fig 1C).**



	Okadaic 1μM	Blank	0.5h	1h	2h
mean	27.1*	16.4	14.8	12.4	18.9
SEM	2.5	2.9	1.1	0.2	3.1

Fig. 1 Velocity of dopamine and L-DOPA syntheses decrease in a time-dependent manner in rat brain striatal miniprisms. [^{3}H]-dopamine (DA, A) or L-DOPA (B) synthesis was measured in rat striatal slices using different preincubation times. See Methods for details on [^{3}H]-dopamine synthesis (A) and TH activity estimation *in situ* by L-DOPA accumulation (B). [^{3}H]-Tyrosine or NSD1015 were only present during the last 10 or 30 minutes respectively. In both cases synthesis velocity decreased significantly to a 20% although for L-DOPA synthesis this descent is much faster. C) In spite of this velocity decrease, TH phosphorylation at Ser^{40} does not change with preincubation time. Optical density was measured in arbitrary units. Okadaic acid stimulation is shown as a positive control. Blank samples were not incubated. Experimental design is shown as a time bar. Data represent the means ±SEM of N equal to A) 28-20; B) 3-5 and C) 3 replicates. *p<0.05 vs. respective control, oneway-ANOVA plus Bonferroni's test.

Furthermore, the presence of D_2-like autoreceptor blockers such as haloperidol or sulpiride did not impair this decrease (data not shown). According to previous *in vitro* reports [16, 7] we suspected that TH activity might be decreasing due to the accumulation of

newly formed dopamine which would exert end-product feedback inhibition on the TH enzyme. Reports of such effect have been hardly obtained in brain tissue [11, 12], although it is well characterized in recombinant TH and other cells [3, 10]. In order to observe if feedback inhibition could be responsible of such decrease, we incubated samples with [^{3}H]-Tyr or NSD-1015 for different times without preincubation. In the presence of NSD-1015 no new dopamine should be formed. In the absence of NSD-1015 the velocity of [^{3}H]-dopamine synthesis decayed exactly in the same way as did in previous preincubation experiments **(Fig 2, continuous line).** However in the presence of NSD-1015 the velocity of L-DOPA synthesis remained constant versus incubation time **(Fig 2, discontinuous line).** These results strongly suggest that *de novo* dopamine synthesis, impaired when NSD-1015 was present from the beginning, is resposible of end-product inhibition of TH.

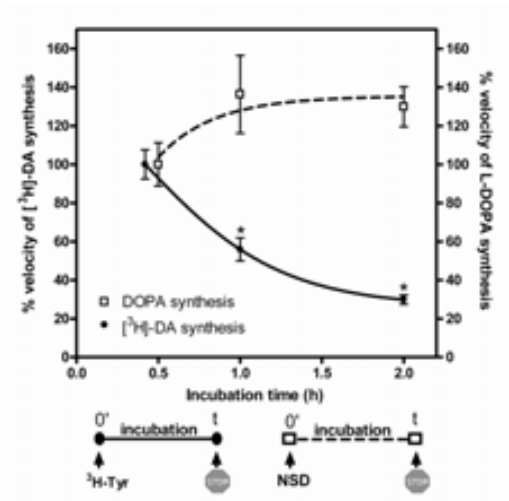


Fig. 2 Incubation with NSD1015 from the beginning impairs the time-dependent decrease in the velocity of L-DOPA synthesis. [^{3}H]-dopamine (DA) or L-DOPA synthesis velocities were measured in rat striatal slices incubated different times without preincubation. Thus, [^{3}H]-Tyrosine or NSD1015 were present from the beginning. Although the significant decrease in [^{3}H]-dopamine synthesis from [^{3}H]-Tyrosine is maintained as compared to Fig. 1, L-DOPA synthesis does not decrease as NSD1015 is present throughout. Experimental design is shown as a time bar. Data represent the means ±SEM of N equal to 8-4 (dopamine) and 6 (L-DOPA) replicates, *$p<0.05$ vs. respective control, oneway-ANOVA plus Bonferroni's test.

Exogenous and endogenous dopamine inhibit [^{3}H]-dopamine and L-DOPA syntheses.

Next, we increased the concentrations of dopamine. The addition of exogenous dopamine during incubation of striatal miniprisms decreased [^{3}H]-dopamine synthesis in a clear concentration dependent manner **(Fig 3A, continuous line).** This effect was caused by an impairment of TH activity, as confirmed by measuring L-DOPA accumulation. Similarly, the inhibitor of the vesicular monoamine transporter tetrabenazine (TBZ) also inhibited both [^{3}H]-dopamine synthesis **(Fig 3B, continuous line)** and TH activity. Since TBZ impairs dopamine storage in vesicles, the effect of TBZ should be due to endogenous dopamine accumulation in the cytoplasm.

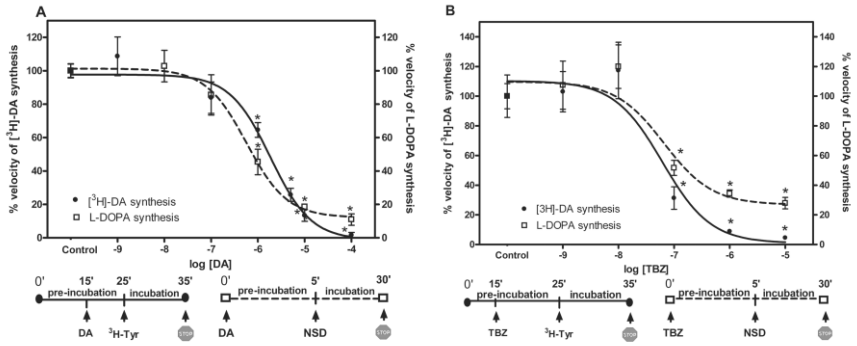


Fig. 3 Exogenous or endogenous dopamine decrease velocity of dopamine synthesis by inhibiting TH. DA (A) and the inhibitor of the vesicular monoamine transporter tetrabenazine (TBZ; B) decrease both [^{3}H]-dopamine (DA) and L-DOPA synthesis velocities in a concentration-dependent manner. Similar IC_{50} were obtained for [^{3}H]-dopamine and L-DOPA syntheses ($1.8e^{-6}$ M vs $5.7e^{-7}$ M for DA and $2.9e^{-8}$ M vs $6.6e^{-8}$ M for TBZ, respectively). Experimental design is shown in each graph as a time bar. Data represent the means ±SEM of N equal to A) 26-4 (DA), 4 (L-DOPA) and B) 8-6 (DA), 4-3 (L-DOPA) replicates, *p<0.05 vs. respective control, oneway-ANOVA plus Bonferroni's test.

Modulation of end-product inhibition by stimulation of TH phosphorilation.

It is well known that PKA activators as well as inhibitors of protein phosphatases induce TH activity by phosphorylation in Ser^{40} [4]. Phosphorylation also increases TH affinity for BH_4 and decreases dopamine occupation of the high affinity dopamine binding site. Thus, we wondered if the previously observed decay on dopamine synthesis would be affected by TH phosphorylation.

We observed that the time-dependent decay of dopamine synthesis attributed to end-product feedback inhibition during preincubation was similar in all conditions assayed (control, db-cAMP, and okadaic acid) **(Fig 4).** As expected, PKA activation and okadaic acid (an inhibitor of Ser/Thr protein phosphatases)

increased initial TH activity. However the time-dependent decay observed had the same extent than control samples.

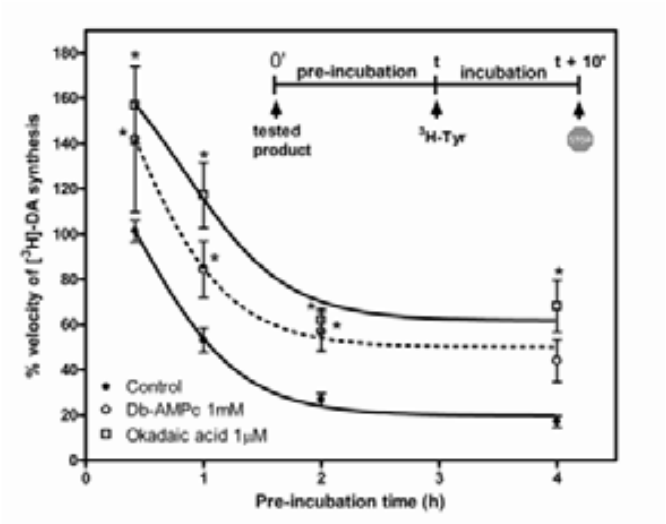


Fig. 4 Time-dependent decrease of dopamine síntesis does not depend on TH phosphorylation. The PKA activator db-AMPc and the phosphatase inhibitor okadaic acid applied during preincubation increase [^{3}H]-dopamine (DA) synthesis velocity, but the pattern of time-dependent inhibition is maintained. Experimental design is shown as a time bar. Data represent the means ±SEM of N equal to 32-20 (control), 8-4 (okadaic) and 8-3 (db-AMPc) replicates. Every data group was statistically significant vs. its 25 minute value; ***** $p<0.05$ vs. control curve, oneway-ANOVA plus Bonferroni's test.

Okadaic acid effects were also assayed in presence of increasing concentrations of dopamine. Although, as expected, okadaic acid initially enhanced [^{3}H]-dopamine synthesis in control samples, this effect was progressively lost when increasing concentrations of exogenous dopamine were added, leading to convergence of control and okadaic curves **(Fig 5).** Similar results were obtained using TBZ to increase cytoplasmatic dopamine concentrations (data not shown). These results suggest that high dopamine

concentrations compete with phosphorylation effects, which can facilitate binding pf BH_4 cofactor [10].

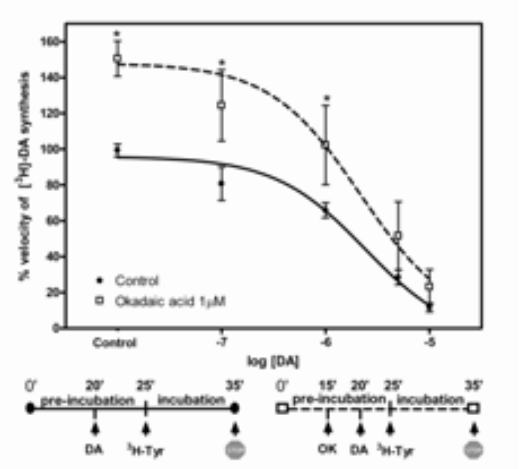


Fig. 5 High dopamine concentrations reduce the effects of okadaic acid on [^{3}H]-dopamine synthesis. Although okadaic acid (Ok) enhances velocity of [^{3}H]-dopamine (DA) synthesis as expected, its absolute effect is reduced from 51 to 11 % as added dopamine concentration increases leading to convergence of both curve fits. Note that the relative effects of okadaic acid appear preserved at low and high added dopamine concentrations, suggesting that phosphorylation still activates TH molecules. These results are compatible with phosphorylation increasing TH apparent Km for dopamine, which could be compensated by increasing dopamine concentrations. Experimental design is shown as a time bar. Data represent the means ±SEM of N equal to 29-16 (control) and 15-6 (okadaic) replicates. *p<0.05 vs. control curve, oneway-ANOVA, Bonferroni's test.

TH kinetics vs. BH_4 is affected by dopamine and okadaic acid.

To assess whether our previous results could be due to a change in the kinetics of TH related to BH_4, after incubation of tissue miniprisms in the presence of dopamine or okadaic acid we homogenized them and reincubated the homogenates with increased BH_4 concentrations (see *Tyrosine Hydroxylase kinetic*

curves in homogenates). We performed this assay by measuring [^{3}H]-L-DOPA accumulation in the presence of NSD-1015 to specifically determine TH activity in homogenates. As expected, with no BH_4 added, TH activity in homogenates was increased by previous addition of okadaic acid, and decreased by previous addition of dopamine to tissue miniprisms. In all cases, addition of BH_4 resulted in increased TH activity in homogenates. Notably, when dopamine had been added, a higher Km of TH for BH_4 was observed (control EC_{50} $1.88x10^{-5}$ M vs. dopamine EC_{50} $1.31x10^{-4}$ M). Conversely, when TH was phosphorylated due to previous addition of okadaic acid, Km for BH_4 decreased (control EC_{50} $1.88x10^{-5}$ vs. okadaic acid EC_{50} $5.42x10^{-6}$) **(Fig 6).** These results would agree with dopamine- and phosphorylation effects being mediated by changes in affinity for the BH4 cofactor. Schematic representation of the occupation of the TH regulatory sites **(Fig 7).**

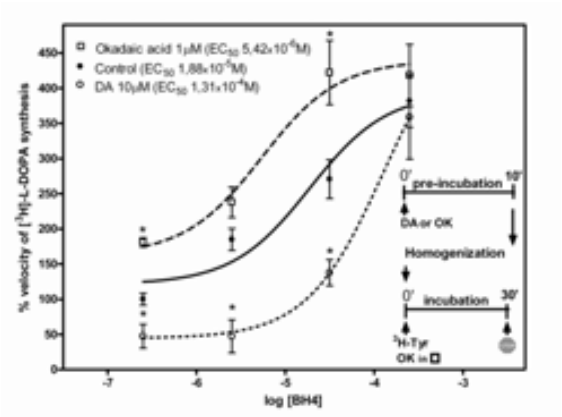


Fig. 6 Dopamine feedback inhibition on TH activity is impaired by BH_4 cofactor in a concentration-dependent manner. Slices were preincubated in the presence of dopamine or okadaic acid, then homogenized, and the homogenates were incubated with [^{3}H]-tyrosine, NSD1015 and BH_4 as explained in *Methods, Tyrosine hydroxylase kinetic curves in homogenates*. Experimental

design is shown as a time bar. The cofactor BH_4 increased TH activity as expected. However, when BH_4 concentration was increased to 250μM, dopamine feedback inhibition on TH activity was reduced or completely abolished. This is in agreement with a dopamine effect based on a decrease of TH apparent affinity for BH_4. Conversely, TH affinity for BH_4 is increased due to okadaic acid-mediated high phosphorylation of the enzyme as expected. Data represent the means ±SEM of N equal to 15-10 (control), 3 (okadaic) and 6-2 (dopamine, DA) replicates. *p<0.05 vs. control curve, oneway-ANOVA Bonferroni's test.

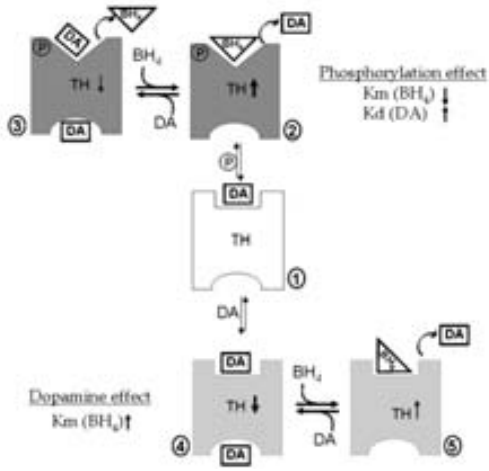


Fig. 7 Schematic representation of the occupation of the TH regulatory sites. Clear/dark figures represent TH in different phosphorylation conditions. (1) dopamine is bound to the high affinity site in control conditions. (2) Phosphorylation increases affinity for BH_4 and decreases affinity for dopamine, which dissociates the catecholamine from the high affinity site. (3) Nevertheless, high dopamine concentrations compete with BH_4 at the high affinity site. (4) Moderate dopamine concentrations can inhibit TH through binding to the low-affinity site. (5) Higher BH_4 concentrations compete with dopamine, at least at the high affinity site.

DISCUSSION

We unexpectedly observed a time-dependent decay of the velocity of basal dopamine synthesis that was independent of phosphorylation. This effect facilitated to characterize end-product feedback inhibition, which had been hard to study in brain striatal

tissue using classical techniques. We show that end-product feedback inhibition plays a central role on the regulation of tyrosine hydroxylase activity by phosphorylation and by physiological dopamine concentrations, using a double mechanism.

Two types of end-product feedback inhibition has been well described *in vitro* using recombinant TH [7, 17, 18]. Its importance in brain has been hypothesized, but proofs of its generic relevance are limited to classic papers [11] or results obtained in other tissues and cell types easier to work with [3, 12]. In brain this is the first report of a phosphorylation-independent end-product feedback inhibition. Our results show that TH is strongly regulated by feedback inhibition in striatal tissue under conditions as close as possible to physiological, with tissue manipulation restricted to chopping and washing in Krebs buffer. We have exploited the methodological advantage of our highly sensitive radioisotopic technique which determines [^{3}H]-dopamine synthesized during a relatively short period of time. Nevertheless, equivalent results have been obtained with the classical L-DOPA accumulation assay performed in parallel as described by Lindgren et al. [6] in striatal slices. Using both techniques we observed a decrease of the velocity of synthesis when preincubation times increased (Fig. 1). However no such decrease was observed when we inhibited L-DOPA decarboxylation from the start to completely block endogenous dopamine formation, pointing out to the inhibitory effect of newly synthesized dopamine. Such effect was not altered

by phosphorylation (Fig. 4) and did not alter phosphorylation: In a previous study in PC12 cells, Gordon et al. [17] observed that TH phosphorylation in Ser^{40} remained constant despite feedback inhibition. Using brain striatal tissue here we describe that TH phosphorylation in Ser^{40} remains constant versus time (Fig. 1C), validating previous data suggesting a phosphorylation-independent inhibition.

Computational analyses predict that cytosolic dopamine concentration in dopaminergic terminals is extremely regulated by dopamine inhibitory control on TH as well as by the velocity of transport into vesicles [14]. Once dopamine is stored into vesicles, it can be released to the synaptic cleft and subsequently reuptaken by the dopamine transporter into cytosol, participating again in TH regulation by end-product feedback inhibition. This mechanism might potentially limit secondary waves of synthesis and release independently of D_2-like autoreceptors. In agreement, inhibition of the vesicular transporter elicits similar curves of end-product feedback inhibition than extracellularly added dopamine. Feedback inhibition could be the key regulator of dopamine synthesis, facilitating crosstalk with release mechanisms and phosphorylation regulated by D_2-like autoreceptors.

TH can be phosphorylated at several serine residues by diverse protein kinases such as PKA, ERK or CaMKII, and it can be dephosphorylated by $PP2_A$ and $PP2_C$ protein phosphatases. TH phosphorylation by PKA in Ser^{40} or inhibition of protein phosphatases increases enzymatic velocity [4]. In agreement we

show an increased TH activity after PKA stimulation with dbcAMP or phosphatase inhibition with okadaic acid. Nevertheless, end-product feedback inhibition still occurs independently of the higher steady-state level of dopamine synthesis reached (Fig. 4). This suggests that, in phosphorylated state, TH kinetic properties allowing a higher dopamine synthesis can still be finely tuned by independent feedback mechanisms. The low affinity dopamine binding site appears responsible of such effect. TH phosphorylation in Ser^{40} alters dopamine binding but only to the high affinity site. Despite phosphorylation, dopamine is still able to inhibit TH activity by binding to the low affinity site, which has been proposed as the physiological dopamine sensor of TH [7, 17]. Our results in striatal tissue completely agree and validate these *in vitro* data as long as we observe independent effects of phosphorylation (probably at the high-affinity site) and newly formed dopamine (at the low-affinity site; Fig. 4). This agrees with the view that phosphorylation in Ser^{40} increases TH activity through relief from already bound dopamine inhibiting TH at the high-affinity site.

In incubations of brain tissue it is difficult to know which dopamine binding sites are occupied in each assayed condition because of the presence of endogenous dopamine. However, taking into account physiological levels of dopamine lower than 100nM [19], Gordon et al. [7] suggested that the high affinity binding site might be completely occupied by dopamine, while the low affinity binding site would be able to respond to an increase of cytosolic dopamine concentrations (Fig. 7, step 1). Our results are in

agreement with this suggestion. First, dopamine synthesis quickly decreases after a cytosolic dopamine increase (Fig. 7, step 4), and inhibition is maintained as long as cytosolic dopamine remains increased. Physiological dopamine levels must be similar to those obtained during our preincubation, as no new tyrosine was added (except for 0.12 μM [^{3}H]-tyrosine in radioisotopic experiments). Maximum inhibition appears at 2 hours, and then dopamine synthesis is stabilized (Fig. 1) perhaps due to limited availability of endogenous tyrosine. Second, TH phosphorylation by PKA activators or induced by okadaic acid initially increase dopamine synthesis but they do not change the time-dependent pattern of end-product feedback inhibition. This suggests that the observed time-dependent pattern is caused by new binding of endogenous dopamine to the low affinity binding site of TH, while phosphorylation reliefs dopamine binding at the high affinity site facilitating BH_4 binding (Fig. 7, step 2).

It is thought that the effect of phosphorylation on TH activity can only be reversed by dephosphorylation by protein phosphatases. However, our results suggest that high dopamine concentrations are able to decrease phosphorylation effects. In Fig 5, the absolute okadaic acid effect on [^{3}H]-dopamine synthesis is reduced at high dopamine concentrations (5-10μM), leading to convergence of both curves in samples having equivalent TH protein levels. This could be explained by the high amount of added dopamine preventing BH_4 binding (Fig. 7, step 3). However, even at such high dopamine concentrations the relative okadaic acid effect appears

preserved (in Fig. 5, the low [^{3}H]-dopamine synthesis at 5-10 μM dopamine appears nearly doubled from 12 to 23 % of control samples), suggesting that phosphorylation does activate TH molecules but excess dopamine difficults its expulsion from the high-affinity site to allow BH_4 binding. Conversely, dopamine might be displaced out of the high-affinity site by increasing concentrations of BH_4 (Fig. 6 and Fig. 7, step 5). Even though micromolar levels of cytosolic dopamine might not be physiologically reached, this result agrees with previous suggestions that the main change in phoshorylated TH is the decrease of the affinity for dopamine binding to a site overlapping with the BH_4 site [10]. Provided that BH_4 and dopamine compete for overlapping sites, TH activity depends on the K_d for BH_4 and dopamine, but also on cytosolic BH_4 and dopamine concentrations. Even though pSer^{40}-TH has a lower K_d for BH_4, a high amount of dopamine or BH_4 might displace the equilibrium, making changes in K_d almost irrelevant. Studies in PC12 cells have also demonstrated that dopamine end-product inhibition impairs TH activity even when TH phosphorylation is activated by forskolin [17].

Dopamine binding to the high affinity site should inhibit TH by increasing the apparent Km for BH_4 and decreasing Vmax, whereas dopamine binding to the low affinity binding site should only increase Km for BH_4. In agreement, TH phosphorylation stimulated by okadaic acid decreased Km for BH_4 while the opposite was observed by adding dopamine (Fig. 6). No changes in Vmax were

searched for as tyrosine concentrations were not changed. Although it has been established that phosphorylation can expel dopamine from the high affinity binding site the relevance of its effects on Vmax can only be speculated [10].

To summarize, in physiological conditions the high affinity binding site is probably occupied by dopamine and TH activity should be low (Fig. 7, step 1). In this situation, cytosolic dopamine (exogenous, synthesised or reuptaken) should exert its inhibitory effect through the low-affinity binding site (Fig. 7, step 4). Phosphorylation would increase TH activity by dissociating dopamine from the high-affinity site and facilitating BH_4 binding (Fig. 7, step 2). If dopamine levels reached μM concentrations, even phosphorylated TH could be inhibited by dopamine competition with BH_4 for the high-affinity site (Fig. 7, step 3).

Disregulation of inhibitory mechanisms on TH might lead to an increase of cytosolic dopamine levels, storage in vesicles, and higher dopamine release. It is known that different behavioral profiles such as impulsivity, novelty-seeking or emotional reactivity are associated (by cause or consequence) to differences in dopamine levels in particular brain areas [20-22]. Understanding inhibition of dopamine synthesis could lead to new treatment strategies to modulate behavior in dopamine-related disorders, particularly psychoses and addiction. TH end-product feedback inhibition is a central regulatory mechanism integrating different modulatory inputs: receptors, phosphorylation, dopamine storage into vesicles, reuptake and dopamine release. A highly regulated

tyrosine hydroxylase activity is of key importance for normal function of dopamine neurotransmission, and a real possibility for pharmacological intervention.

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CELLULAR DISTRIBUTION OF THE HISTAMINE H_3 RECEPTOR IN THE BASAL GANGLIA: FUNCTIONAL MODULATION OF DOPAMINE AND GLUTAMATE NEUROTRANSMISSION

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ABSTRACT

Histamine H_3 receptors (H_3R) are widely expressed in rat brain where they participate in sleep-wake cycle and cognitive functions among others. Despite their high expression in some regions of the basal ganglia, their functional role in this forebrain neural network remains unclear. The present findings provide in situ hybridization and

immunohistochemical evidence for H_3R expression in several basal ganglia neuronal populations but not in astrocytes (glial fibrillary acidic protein immunoreactive cells). We demonstrate the presence of H_3R mRNA and protein in dopaminergic neurons (tyrosine hydroxylase positive) of the ventral tegmental area and substantia nigra. In addition we found H_3R in cholinergic neurons (choline acetyltransferase immunoreactive) and GABAergic neurons (substance P, proenkephalin or dopamine D_1 receptor positive) as well as in corticostriatal terminals (VGLUT1-immunoreactive) of the dorsal and ventral (nucleus accumbens) striatal complex. Double-labelling experiments in the medial prefrontal cortex showed that H_3R is expressed in D_1R-positive interneurons and VGLUT1-positive corticostriatal output neurons. Functional experiments confirmed that H_3R ligands modulated dopamine synthesis and the probability of glutamate release in the striatum. The presence of H_3R in such different neuronal populations and its functional involvement in the control of striatal dopaminergic and glutamatergic transmission credits a complex role to H_3R in the functional basal ganglia neural network.

Abbreviations

Acb: nucleus Accumbens; D1R: dopamine receptor 1; H3R: histamine receptor 3; mPFC: medial prefrontal cortex; PE: proenkephalin; SNc: substance nigra pars compacta; SNr: substance nigra pars reticulata; SP: substance P; SSC: sodium saline citrate; TBS: 50mM tris buffer saline pH 7.6; TSA: tyramide signal amplification; VGLUT1: vesicular glutamate transporter 1 ; VTA: ventral tegmental área.

INTRODUCTION

Histamine is involved in a variety of brain functions such as the sleep-wake cycle, attention, learning, memory and locomotion control (Yanai and Tachiro, 2007) through its interaction with four identified G-protein coupled receptors. Three of them (H_1 to H_3) are widely distributed in the central nervous system while the H_4 receptor is expressed mostly in bone marrow and leukocytes, displaying very low levels in brain (Oda et al., 2000; Connelly et al., 2009).

The histamine H_3 receptor (H_3R) was initially characterized as an autoreceptor controlling histamine release and synthesis in histaminergic terminals of the central nervous system (Arrang et al. 1983, 1987). Later, its heteroreceptor role was demonstrated as it modulated release of other cerebral neurotransmitters including serotonin (Schlicker et al. 1988), norepinephrine (Schlicker et al. 1989), dopamine (Molina-Hernandez et al. 2000), glutamate (Molina-Hernandez et al. 2001) and GABA (Arias-Montaño et al. 2001) in brain samples. Six H_3 mRNA isoforms have been described in the rat, four of which are functionally active: $H_{3(445)}$, $H_{3(413)}$, $H_{3(410)}$, $H_{3(397)}$ and two inactive: $H_{3(nf1)}$ $H_{3(nf2)}$. Isoforms are generated by differential splicing of the three exons and two introns of the gene. All of them have a variable expression level in cerebral structures (Morisset et al. 2001) being more expressed in cortex, thalamus and caudate-putamen of humans (Lovenberg et al. 1999). This regional localisation matches the relative distribution of histaminergic projections arising from the tuberomammillary nucleus. In addition, radioligand binding studies in rodents show high H_3 receptor levels in olfactory nucleus, cortex, substantia nigra pars reticulata (SNr), amygdala, thalamus and hypothalamus (specially in tuberomammillary

nucleus) with the highest density in dorsal striatum and nucleus accumbens (Acb) (Pillot et al. 2002a).

The rat striatum is the main input structure of the basal ganglia involved in sensory-motor behavioural aspects. It can be divided in two big regions: 1) the dorsal striatum (caudate-putamen) that has been implicated in the initiation and development of a voluntary motor behaviour and 2) the ventral striatum (nucleus accumbens) which plays a central role in motivated and goal directed behaviours because it integrates the motor and limbic systems (Morgane et al. 2005). In spite of the different afferences and efferences of the dorsal and ventral striatum, both present the same neuronal phenotypes. Thus 95% of striatal neurons are GABAergic spiny projection neurons, which can be divided in two populations: striato-nigral pathway neurons which show dynorphin, substance P (SP) and D_1-like dopamine receptors and striato-pallidal pathway neurons that coexpress enkephalin and the D_2-like dopamine receptor (Le Moine et al. 1995, Sonomura et al. 2007). Nevertheless, discrimination between these two populations is not absolutely clear because all of these neurons can express low levels of characteristic receptors of the other neuronal type (Aizman et al. 2000; see also Valjent et al. 2009). The remaining 5% of striatal neurons is composed by interneurons of two subtypes: cholinergic and GABAergic (Kawaguchi et al. 1997).

Striatal neurons are a major target of mesencephalic dopaminergic neurons, which are implicated in complex neurological and psychiatric disorders such as Parkinson, Huntington, schizophrenia and addiction. The role of H_3R in normal striatal functions such as locomotion is not clearly elucidated (Chiavegatto et al. 1998, Toyota et al. 2002), and even less in pathological conditions. However, it has been proposed that

antagonism of H_3R has therapeutic potential in sleep-wake disorders, dementia, epilepsy and schizophrenia (Sander et al. 2008). Conversely, stimulation of H_3R decreases L-dopa-induced chorea and turning behaviour in animal models of Parkinson's disease (Huotari et al., 2000; Gomez-Ramirez et al. 2006). In the present work functional histamine H_3R expression in basal ganglia neuronal populations is studied to evaluate the possibility that H_3 receptors constitute new targets for treatments of these disorders.

EXPERIMENTAL PROCEDURES

Tissue preparation for histochemistry

Experiments performed in the present study conformed to the Ethics Committee for Human and Animal Research (Universidad Autónoma de Barcelona) in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Brains were obtained from male Sprague-Dawley rats weighing 200-250 g (Animal Service, Universidad Autónoma de Barcelona, Barcelona, Spain). Animals were sacrificed by decapitation, their brain was removed rapidly, immediately frozen (-40 ºC) by immersion in isopenthane and stored at -20 ºC. Brain sections (10µm) were prepared on a cryostat and thaw-mounted onto Superfrost slides. Slices were fixed for 40min at 4 ºC in freshly prepared 4% formaldehyde made up in 0.1M phosphate buffer pH 7.4, rinsed three times (5min each) in 0.1M phosphate buffered saline, pH 7.4, dehydrated through graded ethanol and dried. All the sections were stored at -20ºC until use.

Fluorescent in situ hybridization histochemistry

Brain sections were incubated at 37ºC for 10 min with proteinase K (5µg/ml), acetylated for 10min (in 0.1M triethanolamine, pH 8 and 0.25% acetic anhydride) at room temperature and dehydrated in graded ethanol up to 100%. Hybridization was performed overnight at 55ºC in the presence of 10-20 ng of biotin/digoxigenine labelled antisense or sense probes in hybridization buffer (50% formamide, 10% dextran sulfate, standard saline citrate 2x (SSC), 1% Denhart's solution, 50mM Tris-HCl buffer, 0.1% NaPPi, 0.2mg/ml tRNA, 1mM EDTA). Subsequently, sections were rinsed with: 50% formamide in SSC2x (30min at 55ºC), SSC2x (5min at 55ºC, 2 times 10min at room temperature) and incubated for 40min at 37ºC with ribonuclease. Then, sections were washed in graded SSC (4x and 2x, 15min each, 0.1x 30min at 60ºC and 0.1x 10min room temperature), Tris buffered saline 50mM pH 7.6 (TBS) –Tween20 0.05% three times (5min each) and blocked with 1% blocking reagent (Roche Applied Science) in TBS for 1h at 37ºC. They were then incubated for 2h at room temperature in a humid chamber with the primary antibodies sheep anti-digoxigenine–peroxidase (Roche Applied Science, 1/100) and mouse anti-Biotin (Jackson Immunoresearch, 1/200) diluted in buffer (TBS, 0.1% acetylated bovine serum albumin, 1% goat serum, 0.1% Tween20). After three rinses with TBS-Tween20 0.05% (5min each) sections were incubated with the green secondary antibody anti-mouse-Alexa488 (Invitrogen, 1/200) diluted in TBS-Tween20 0.1% for 2h at room temperature. Two more washes followed by one in TBS without Tween20 (5min) were performed before the incubation with the red fluorophore, which is conjugated to tyramide signal amplification (TSA)-Cy3 activated by peroxidase (Perkin Elmer, 1/500). Finally, sections were washed and mounted with Mowiol.

The H_3R probe corresponded to nucleotides 636-1243 of the rat H_3R sequence. It was previously shown to hybridize to the various H_3R mRNA isoforms expressed in the brain or peripheral tissues (Morisset et al., 2001; Pillot et al., 2002). The tyrosine hydroxylase probe used to detect dopaminergic neurons was complementary to rat tyrosine hydroxylase mRNA sequence from nucleotide 25 till the end. cDNAs for proenkephalin (PE) and SP were obtained by polymerase chain reaction and corresponded to nucleotides 335-641 (GenBank accession n° AH002996) and nucleotides 80 - 227 (AH002233), respectively. They were subcloned into pGEM-4Z (Promega) plasmids. Antisense and sense cRNA riboprobes were prepared by in vitro transcription.

Fluorescent immunohistochemistry

Brain sections were thawed at 4ºC, washed in TBS for 5min, then in sodium borohydrate 1% (diluted in disodium hydrogenphosphate 0.1M pH 7.8) for 20min and rinsed in phosphate buffer saline 50mM pH 7.4 and TBS (3min each). Blocking was made with 7% donkey serum in TBS for 1h at 37ºC. Primary antibodies (listed in **Table 1**) were diluted in buffer (TBS, acetylated bovine serum albumin 1%, Tween20 0.1%, 7% donkey serum) and incubated overnight at 4ºC.

A

Antigen	Abbreviation	Dilution	Reference	Host
Histamine H_3 receptor	anti-H_3Rab	1/300	Abcam (ab-13014)	Rabbit (P)
Histamine H_3 receptor	anti-H_3Rc	1/200	Chemicon (ab5660)	Rabbit (P)
Histamine H_3 receptor	anti-H_3Ral	1/200	Alfa Diagnostics (H3R31-A)	Rabbit (P)
Tyrosine Hydroxylase	anti-TH	1/500	Millipore (AB1542)	Sheep (P)
Dopamine D1 receptor	anti-D1	1/300	Frontier Institute (D1rgpaf501)	G. Pig (P)
Choline Acetyl Transferase	anti-ChAT	1/100	Chemicon (ab144P)	Goat (P)
Vesicular Glutamate Transporter 1	anti-VGLUT1	1/300	Synaptic Systems (135 511)	Mouse (M)
Glial fibrillary Acidic Protein	anti-GFAP	1/500	Sigma Aldrich (G3893)	Mouse (M)

P, polyclonal; M, monoclonal

B

Detection system	Dilution	Source	Host
Anti Digoxigenin -peroxidase	1/100	Roche (11207733910)	Sheep
Anti-rabbit-peroxidase	1/200	Cell signalling (7074)	Goat
TSA-Cy3 (amplification system)	1/100-500	Perkin Elmer (NEL 744)	-
Anti biotine	1/200	Jackson Immun. (200-002-211)	Mouse
Anti mouse -Alexa 488	1/200	Invitrogen (A11029)	Goat
Anti sheep -FITC	1/40	Sigma Aldrich (F7634)	Donkey
Anti goat -Alexa 488	1/200	Invitrogen (A11055)	Donkey
Anti guinea pig -Alexa 488	1/200	Invitrogen (A11073)	Goat
Anti rabbit –Alexa594	1/200	Invitrogen (A21442)	Chicken

Table 1. List of antibodies (A) and fluorescent detection systems (B) used for immunofluorescence and fluorescent in situ hybridization studies. Histamine H_3 receptor protein was detected using the Abcam 13014 antibody in dopaminergic and cholinergic neurons and the Alfa Diagnostics H3R31A antibody in the rest of experiments.

Histamine H_3 receptor expression in dopaminergic and cholinergic neurons was detected using the Abcam 13014 antibody while the Alfa Diagnostics H3R31A antibody was employed for the other experiments. After three washes in TBS-Tween20 0.05%, sections were incubated with the secondary antibodies diluted in the same buffer as the primary. Three more washes with TBS-Tween20 0.05% and TBS were done prior the incubation of 5min with Hoechst33258 diluted 1/1000 in TBS. Final washes in TBS were made before the mounting with Mowiol.

Sections were analyzed with a Nikon Eclipse 90i microscope equipped with conventional fluorescence: (1) DAPI filters (340-380nm excitation, 435-485nm emission), (2) FITC filters (465-495nm excitation, 515-555nm emission) and (3) G2-A filters (510-560nm excitation, 590 emission). Fluorescent images were captured with a high resolution (1280x1024 pixels) Nikon digital DXM1200F camera interfaced with the ACT-1 Nikon software. Image signal levels were adjusted using Adobe Photoshop software for a better visualization. Original images have been kept. Confocal images are a Z-stack of 5 photos (1µm/slice) made with an Olympus FluoView FV1000 (Olympus) microscope equipped with a UPLSAPO 60x NA: 1.35 objective. The following lasers were used: a 488nm laser (488nm excitation, 520nm emission) and a 559nm laser (559nm excitation, 618nm emission). Since the intensity of immunolabelling for the antibodies used could be due to many variables that cannot be individually quantified, this study does not attempt to quantify the relative amounts of labelled antigens. Colocalization of both antigens were measured with the FV10-ASW 1.7 software (Olympus) and shown as average overlap ± standard deviation of seven different regions of interest for each image. When the overlap value is above 0.5 we consider both antigens colocalized.

Electrophysioligy

The protocol used was adapted from Lante et al. (2006) and was as follows: Brains from male Sprague-Dawley rats were rapidly extracted and placed in ice-cold Krebs buffer containing 124 mM NaCl, 3.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM glucose, 10 mM Hepes (pH 7.4) and oxygen saturated (95% O_2/5% CO_2). Sagital corticostriatal slices (350 µm) were prepared from tissue blocks of

the brain with a Vibratome (Leica, VT1000S) and maintained at least 2 h at room temperature in the Krebs buffer, containing 2 mM $CaCl_2$. This medium was used for further recordings. A single slice was transferred to a recording chamber of an upright microscope (DMLFS, Leica). The slice was positioned under a nylon mesh and continuously superfused with oxygen saturated Krebs solution (flow rate 2 ml/min) maintained at 30-32 ºC. Extracellular recordings were made using a glass micropipette (flame polished borosilicate capillary, inside diameter 0.58 mm, outside diameter 1 mm, Warner instruments; resistance 4-7 MΩ) with a wire (silver, 250 µm, A-M Systems) filled with perfusion medium and placed in the dorsal striatum. Afferent fibers were stimulated by delivering monophasic voltage pulses at a frequency of 0.05 Hz to a bipolar electrode (nickel chrome wire, bare diameter 50 µm, A-M Systems) connected to a multi-channel external stimulator (STG4, Multi channels systems) controlled by the software MC Stim (Multi channels systems). The stimulating electrode was placed in the corpus callosum, or in the striatum close to the border of the corpus callosum. Stimulation intensity ranged from 100-800 µA and 80-600 ms. The recording electrode was connected to a patch-clamp amplifier (Axopatch 200 B, Axon Instruments). Signals were digitized (Digidata 1200 Interface, Axon Instruments) and filtered at 2 kHz. Paired-pulse ratio was obtained by applying two stimuli with a 50 ms interval and calculated by dividing the second field potential by the first field potential (P2/P1). Data were collected by Win LTP (Kind gift by Dr. W. Anderson, University of Bristol, UK). The amplitude of the field potential was used as a measure of synaptic transmission. After obtaining a stable baseline of at least 15 min, thioperamide was diluted in the Krebs solution and bath-applied. Field potential amplitudes were normalized to baseline amplitude. Each data

point used for statistics was the mean of 8 consecutive field potentials. Results were pooled in Sigmaplot (Sigmaplot version 9) and represented as means (± SEM) of n= 5 animals.

Dopamine synthesis

Fresh rat brains were chilled immediately in modified Krebs-Ringer-bicarbonate medium with the following composition: 120 mM NaCl, 0.8 mM KCl, 2.6 mM $CaCl_2$, 0.67 mM $MgSO_4$, 1.2 mM KH_2PO_4, 27.5 mM $NaHCO_3$, and 10 mM glucose, pH 7.4, saturated with 95% O_2/5% CO_2. In a 4ºC room, striata (including nucleus accumbens) were dissected and sliced using a McIlwain tissue chopper to obtain miniprisms of 0.3-0.3 mm/side. Miniprisms were suspended in the same medium and washed by centrifugation and resuspension to remove cell debris. Miniprisims were distributed into 2-ml polypropylene tubes and preincubated for 15 min at 37 ºC in an Eppendorf Thermomixer under 95% O_2/5% CO_2 atmosphere. Imetit (H_3R agonist) or vehicle was added and preincubation continued for 10 min. Then ring-labeled [3,5-^{3}H]-L-tyrosine (40–60 Ci/mmol) was added to all samples (final concentration of 0.12 µM) and incubation continued for 10 min to synthesize [^{3}H]-dopamine. Synthesis was stopped by the addition of a deproteinizing solution containing trichloroacetic acid and 100 nmol internal standard dopamine per tube. Blank tubes contained deproteinizing solution prior to [^{3}H]-tyrosine and were kept ice-cold throughout. All samples were homogenized in a Dynatech/Sonic Dismembrator (Dynatech Labs). An aliquot was taken for protein quantification by the Lowry method to take into account the variability of tissue amounts in each tube. Tissue homogenates were then centrifuged (12,000 g, 10 min, 4 ºC), and supernatants were recovered for [^{3}H]-dopamine purification by HPLC-UV.

The chromatography system consisted of a reverse-phase C18 column (Tracer Extrasil ODS2, 5-mm particle size, 25 x 0.46 cm; Teknokroma) and an ion-pair mobile phase, made up of 100mM sodium phosphate buffer, 1mM EDTA, 0,75mM octanesulfonic acid plus 12% (v/v) methanol (pH 5). Flow rate was 1 ml/min. Internal standards were detected by UV 285 nm. Radiolabelled and endogenous tyrosine and dopamine were undetectable by UV absorbance. Recovery of the internal standard was quantified in each sample (internal/external standard peak area). Dopamine fractions were collected in scintillation vials, mixed with Optiphase HiSafe III cocktail (Wallac), and [^{3}H]-dopamine was quantified in a liquid scintillation counter. Dpm obtained were corrected by dopamine internal standard recovery, dpm in blank samples, and protein content in each incubated tube. Results were expressed as percentage versus control samples in the same experiment.

RESULTS

H_3R mRNA and protein expression in mesencephalic dopaminergic neurons. Effects on dopamine synthesis.

In a previous study of H_3R mRNA distribution in several brain regions (Pillot et al. 2002a), no expression was apparent in the ventral tegmental area (VTA). In contrast in the present work we describe H_3R expression in the VTA using the same H_3R cRNA probe for in situ hybridization as Pillot et al. 2002a. TSA amplification permitted to visualize H_3R expression particularly in dopaminergic neurons of the VTA identified by tyrosine hydroxylase mRNA and protein (**Fig. 1A and B**). In agreement with Pillot et al (2002a) we confirm H_3R mRNA and protein presence in substantia nigra pars compacta (SNc, **Fig. 1C**), particularly in dopaminergic neurons, as well as in other neuronal populations of SNr (**Fig. 1A**). The Pillot et al (2002a) study also showed a lower but significant binding of the H_3R

ligand [^{125}I]-iodoproxyfan in the VTA as compared to the SNc. Our results would then agree with those of Pillot et al (2002a) after improvement of signal detection with TSA.

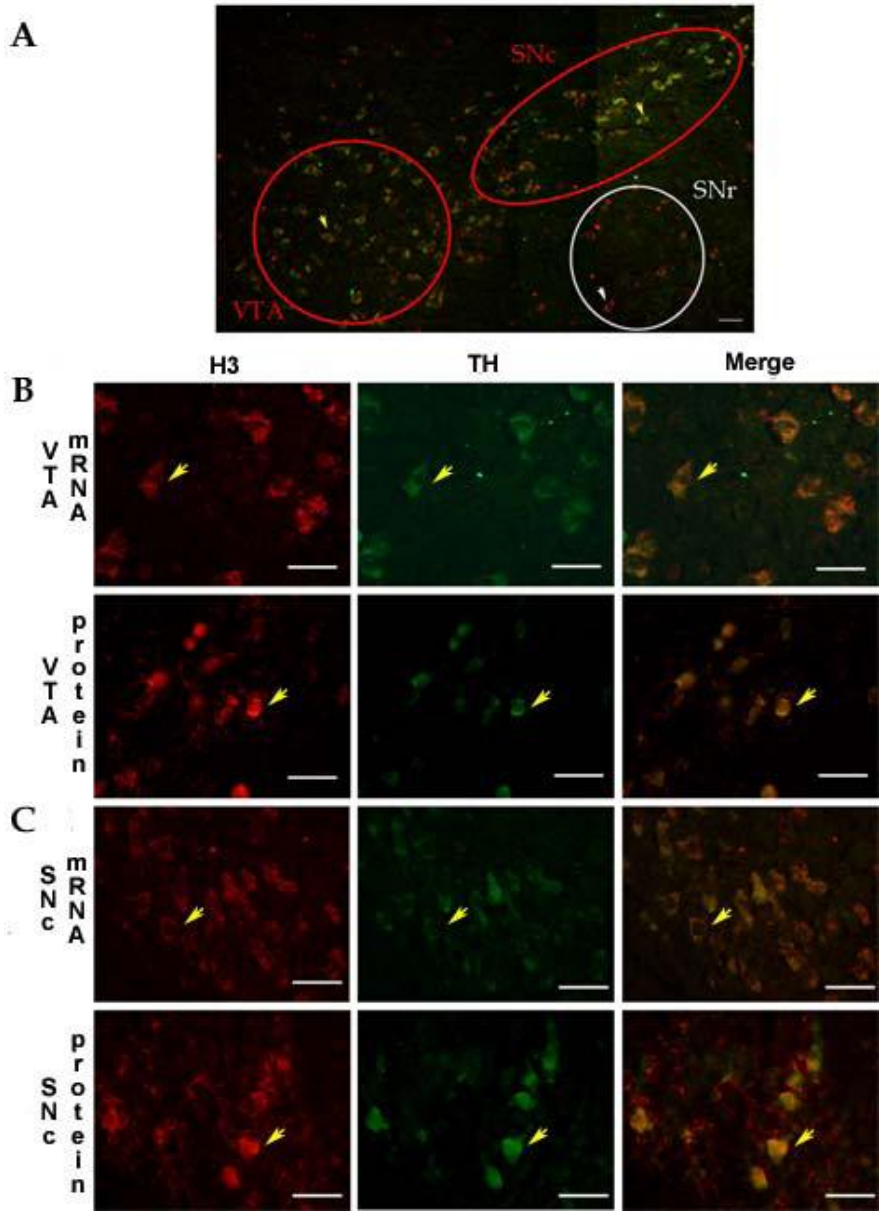

Figure 1. H_3R expression in mesencephalic dopamine neurons. A) Low magnification illustrating representative colocalization of H_3R and TH mRNAs in the SN-VTA region (assembly of 4 images, scale bar 50µm). B) Higher magnification of the colocalization of H_3R and TH mRNAs (upper images) and protein immunoreactivities (lower images) in the VTA (scale bar 40µm). C) Colocalization of H_3R and TH mRNAs (upper images) and protein immunoreactivities (lower images) in the SNc (scale bar, 40µm). H_3R protein immunoreactivity is detected with a rabbit anti-H_3R antibody and an anti-rabbit secondary coupled to DIG. H_3R mRNA is detected with a H_3R-specific cRNA-DIG probe. In both protocols, an anti-DIG-peroxidase antibody is used followed by TSA-Cy3 signal amplification, which gives a red labelling to H_3R expressing cells. TH protein is detected with a sheep anti-TH antibody and an anti-sheep FITC-coupled secondary antibody. TH mRNA is detected with a cRNA-biotin probe, followed by a mouse anti-biotin antibody and an anti-mouse Alexa 488-coupled antibody. In both cases a green label shows TH-expressing cells. Yellow arrows show neurons with positive colocalization. A white arrow shows a neuron with H_3R mRNA but no TH mRNA.

Nevertheless, we controlled the specificity of the H_3 antisense probe used for *in situ* hybridization with the sense probe. In both our study and Pillot et al (2002a) a very low non-specific signal was detected in the VTA with the sense probe (**Fig. 2**), but it was almost undetectable when compared with the specific signal of the antisense probe in the same conditions. We also controlled the specificity of the H_3R antbodies used.

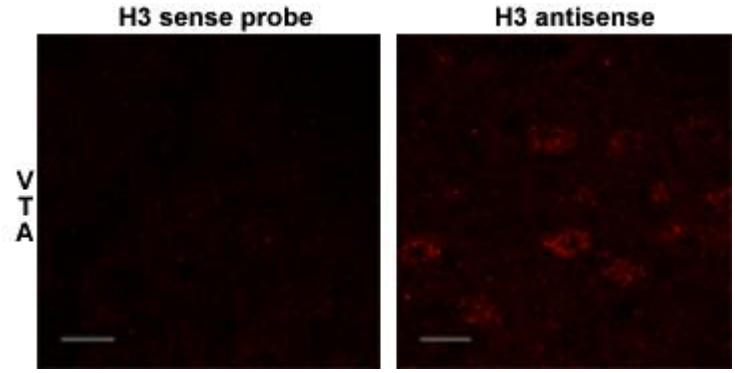

Figure 2. Comparison between sense and antisense H_3R cRNA probes. A faint signal due to low non-specific binding of the sense H_3R cRNA probe can be found in the VTA. Scale bar 35µm.

Three different commercial antibodies (listed in **Table 1**) gave the same signal pattern. Moreover, the signal of the Alfa Diagnostics anti-H3R (anti-H3Ral) antibody was completely abolished by preincubating it with its blocking peptide (**Fig. 3**). All these antibodies lacked non-specific signal in negative controls made in the absence of primary antibodies or probe (data not shown) validating the results obtained.

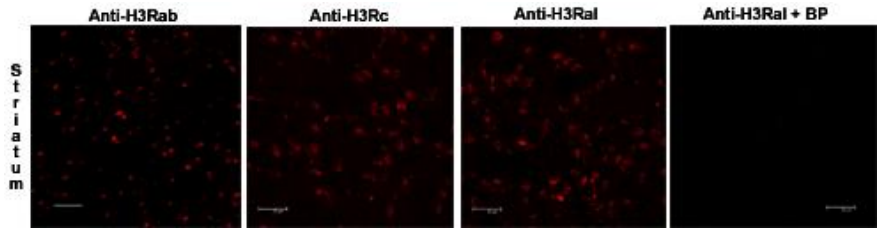

Figure 3. Comparison among diverse H_3R commercial antibodies. Similar H_3R immunostaining pattern was obtained by using three different commercial antibodies. Alfa Diagnostics anti-H3R (anti-H3Ral) signal was completely abolished by preincubation with its blocking peptide. See Table 1 for antibody details. Scale bar 35µm.

Striatal dopamine synthesis takes place in dopaminergic terminals arising from VTA and SNc. To search for H_3R function in dopaminergic neurons we chose to determine dopamine synthesis in miniprism preparations of freshly dissected striatum. In the presence of the H_3R agonist imetit (100 nM), ^{3}H-dopamine synthesis from ^{3}H-tyrosine was decreased by 50 % vs. controls ($p<0.05$ Student's t-test; **Fig. 4**). This result confirms a previous report with a different H_3R agonist (Molina-Hernandez et al., 2000) suggesting that H_3R are functional inhibitory heteroreceptors in dopaminergic neurons.

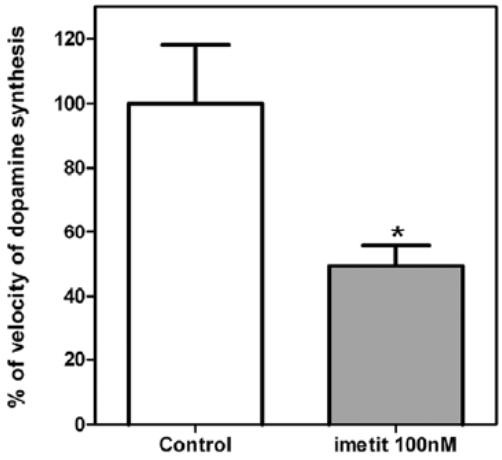

Figure 4. Inhibition of dopamine synthesis by a H_3R agonist. The H_3R agonist imetit (100 nM) decreased ^{3}H-dopamine synthesis in fresh striatal miniprisms incubated with ^{3}H-tyrosine. Results are expressed as % of dopamine synthesis in control samples in the same experiment. * $p<0.05$ vs controls, two-tailed Student's t-test.

H_3R protein and mRNA expression in striatal neurons

We studied the presence of H_3R mRNA (**Fig. 5**) and protein (**Fig. 6**) in striatal GABAergic projection neurons expressing either SP or PE. As seen in **Fig. 5**, approximately half of H_3R mRNA-positive neurons correspond to each population of GABAergic projection neurons. Almost all SP or PE mRNA positive neurons express H_3R mRNA.

Dopamine D_1 receptor immunoreactivity showed a general pattern of expression suggesting terminal labelling, although the signal was more intense in neuronal somas. We found H_3R-D_1R colocalization (**Fig. 6**) in the H_3R positive neurons of the mPFC, dorsal striatum and nucleus accumbens (overlap values 0.71±0.01, 0.67±0.05 and 0.71±0.03 respectively) and also in H_3R positive terminals of these areas (overlap values 0.70±0.02, 0.70±0.03 and 0.74±0.04 respectively).

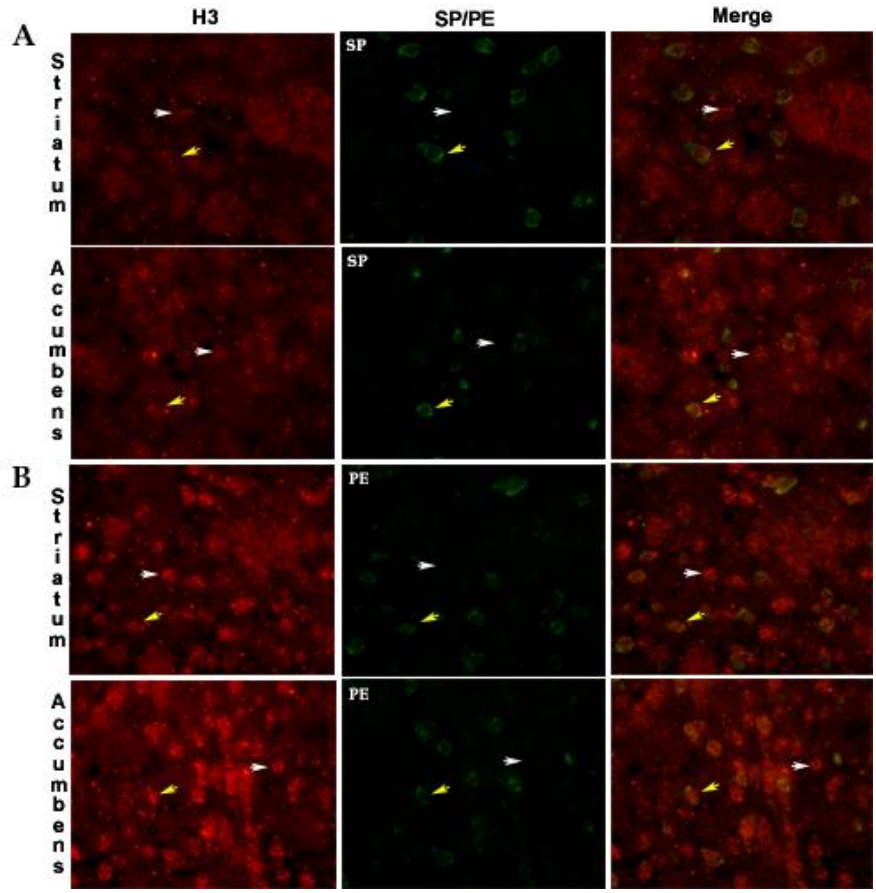

Figure 5. H_3R mRNA expression in GABAergic spiny projection neurons of the striatum and nucleus accumbens. A) H_3R mRNA colocalization with SP mRNA B) H_3R mRNA colocalization with PE mRNA. H_3R mRNA is detected as a red fluorescent signal obtained using a H_3R-specific cRNA-DIG probe recognized by an anti-DIG-peroxidase antibody and followed by a TSA-Cy3 signal amplification. PE or SP mRNAs are detected as green fluorescence signals obtained using PE or SP cRNA-Biotin probes recognized by a mouse Anti-biotin antibody and followed by a goat anti-mouse Alexa 488-coupled antibody. White arrows show neurons single-labelled with H_3R mRNA only. Yellow arrows show positive neurons for both probes. Scale bar 40μm.

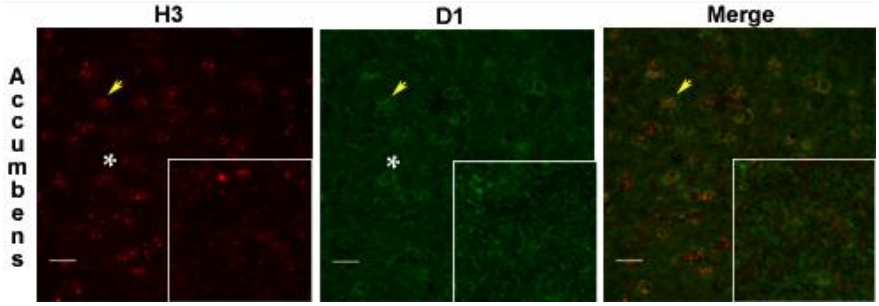

Figure 6. Colocalization of H_3R and D_1R in the nucleus accumbens neurons. H_3R and D_1R protein colocalize at neuronal bodies and axon terminals. No neurons were found with only H_3R immunoreactivity. H_3R protein is detected as red fluorescence obtained with a rabbit anti-H_3R antibody followed by an anti-rabbit-peroxidase and the TSA-Cy3 system. D_1 protein is detected as green fluorescence obtained with the guinea pig anti-D_1 antibody followed by the goat anti-guinea pig-Alexa488 antibody. Yellow arrows show positive neurons for both antibodies. Asterisks mark colocalization of immunoreactivity at nerve terminals. The insert show a zoom of the area marked by the asterisk. Scale bar 20μm.

Although H_3R activation modulates acetylcholine release (Clapham and Kilpatrick 1992; Arrang et al. 1995; Prast et al., 1999), the presence of H_3R on cholinergic neuron terminals had not yet been clearly established. We found H_3R immunoreactivity in virtually all the striatal cholinergic interneurons, as shown in **Fig. 7**.

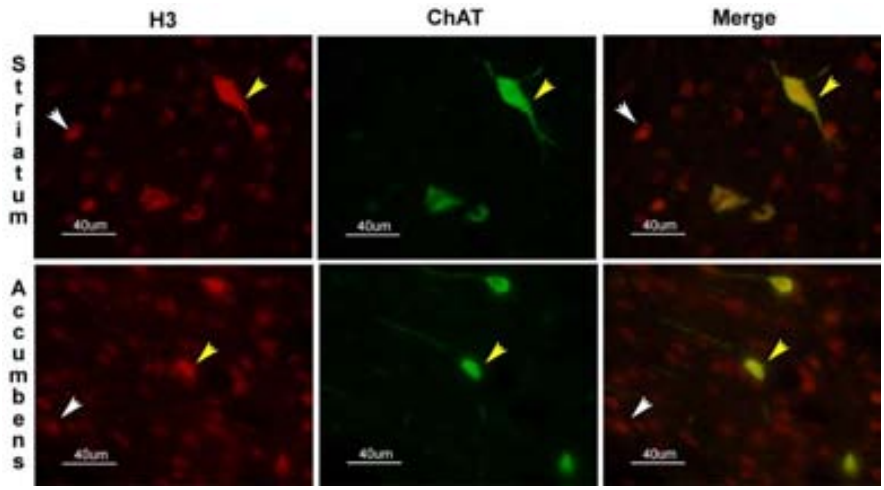

Figure 7. Presence of H_3R immunoreactivity in cholinergic (ChAT-immunoreactive) neurons in striatum and nucleus accumbens. ChAT immunoreactivity is found in big size H_3R-immunoreactive neurons. H_3R positive – ChAT negative neurons are also found. H_3R protein is detected as red fluorescence obtained with a rabbit anti-H_3R antibody developed with a chicken anti-rabbit-Alexa594 antibody. ChAT protein is detected as green fluorescence obtained by the goat anti-ChAT antibody developed with the donkey anti-goat-Alexa488 antibody. White arrows show neurons with H_3R protein immunoreactivity only. Yellow arrows show colocalization of both antibodies. Scale bar 40μm.

H_3R protein expression in glutamatergic endings. Effects on glutamate release.

The striatum receives a modulatory histaminergic input arising from the tuberomammillary nucleus of the hypothalamus (Panula et al. 1989) and three major synaptic inputs: the dopaminergic nigrostriatal pathway and the glutamatergic corticostriatal and thalamostriatal pathways (Smith et al. 2004). These two glutamatergic inputs to striatal neurons can be easily distinguished on the basis of the vesicular transporter used for glutamate storage. Thalamic neurons express the vesicular glutamate transporter 2 while cortical neurons innervating the striatum are

believed to use the vesicular glutamate transporter 1 (VGLUT1) (Kaneko and Fujiyama 2002). As **Fig. 8** shows, corticostriatal glutamatergic (VGLUT1 immunoreactive) endings are highly expressed in mPFC, striatum and nucleus accumbens, where they surround H_3R positive neurons (overlap values 0.68±0.08, 0.75±0.08 and 0.72±0.04 respectively). Although colocalization of VGLUT1 and H_3R is found at the neuronal body in these areas it is limited to the external border of some H_3R positive neurons (overlap values 0.54±0.03, 0.60±0.03 and 0.62±0.05 respectively), while central area of the neurons only shows H_3R immunoreactivity.

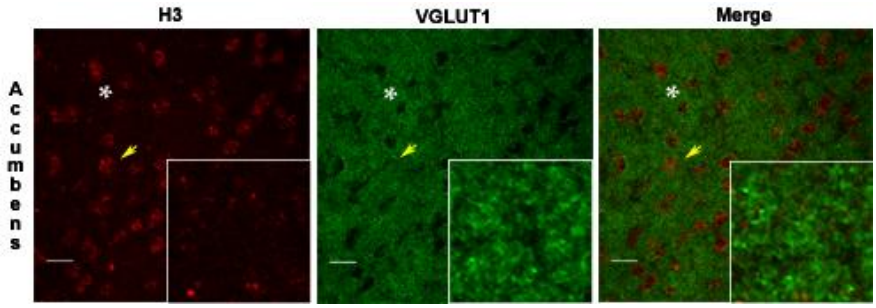

Figure 8. Presence of glutamatergic (VGLUT1-immunoreactive) terminals surrounding H_3R immunoreactive neurons in the nucleus accumbens. There is a clear terminal-axon VGLUT1 pattern of expression that spares most H_3R labelling. However colocalization between H_3R and VGLUT1 can be found only in the external border of some H_3R immunoreactive neurons, not inside cell bodies. H_3R protein is detected as red fluorescence obtained with a rabbit anti-H_3R antibody followed by an anti-rabbit-peroxidase and the TSA-Cy3 system. VGLUT1 protein is detected as green fluorescence by the mouse anti-VGLUT1 antibody developed with the goat anti-mouse-Alexa488 antibody. Yellow arrows show positive neurons for both antibodies while asterisks mark immunoreactive colocalization at terminals. The insert shows a zoom of the area marked by the asterisk. Scale bar 20μm.

H_3 receptors found in glutamatergic corticostriatal endings are functional as long as the application of H_3R inverse agonist thioperamide (100 nM) to fresh brain slices decreased the paired-pulse ratio of field potentials elicited by cortico-striatal stimulation (**Fig. 9**). This effect persisted during thioperamide application (30 min) and after washout ($p<0.05$ two-way ANOVA). A change in paired-pulse ratio is generally interpreted as a changed probability of neurotransmitter release reflecting a presynaptic action of the studied molecule.

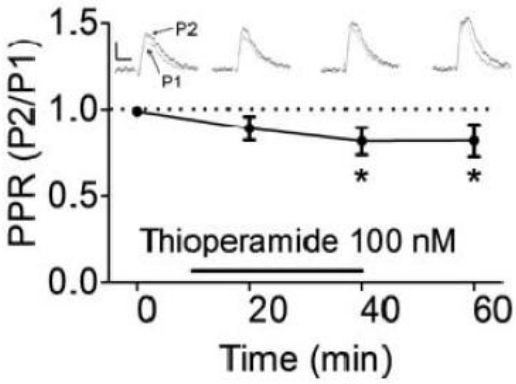

Figure 9. Paired-pulse depression elicited by a H_3R inverse agonist in fresh corticostriatal slices. Bath application of the H_3R inverse agonist thiopermide (100 nM, horizontal line) decreased paired-pulse ratio of field potentials elicited by stimulation of the corpus callosum, which suggests that presynaptic H_3R alter the probability of neurotransmitter release. Results are expressed as mean paired-pulse ratio (P2/P1) ± SEM obtained from N=5 animals, where each data point is the mean of 8 consecutive field potentials normalized to baseline. Representative electrophysiological traces are shown above each time point. * $p<0.05$ versus controls, two-way ANOVA.

H_3R protein is not expressed in GFAP-immunoreactive astrocytes.

Astrocytes detected with an antibody against its specific marker the glial fibrillary acidic protein (GFAP) are present in diverse brain areas including the neostriatum, the substantia nigra and the cortex (Savchenko et al. 2000). We analyzed the possible presence of H_3R in astrocytes and found no colocalization between H_3R and GFAP proteins in mPFC, striatum or nucleus accumbens (**Fig. 10**; overlap values 0.29±0.03, 0.26±0.07 and 0.27±0.07 respectively).

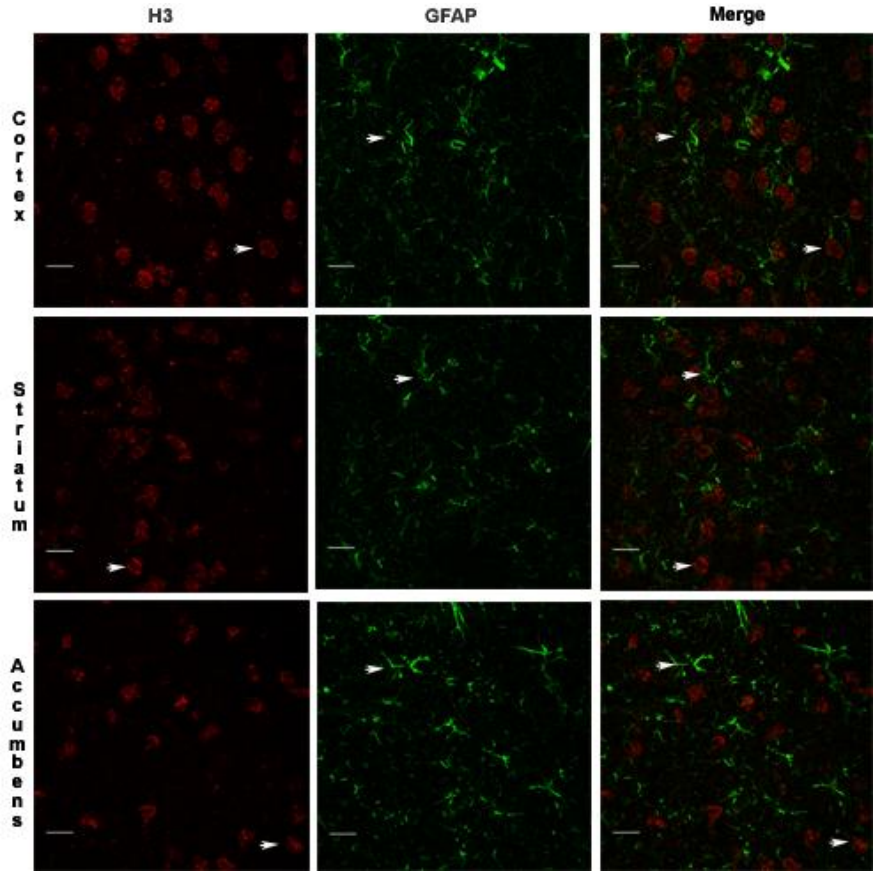

Figure 10. Absence of H_3R protein in astrocytes (GFAP-immunoreactive cells) in the rat forebrain. GFAP-positive astrocytes surround H_3R immunolabelled neurons in the mPFC, striatum and nucleus accumbens. H_3R protein is detected as red fluorescence obtained with a rabbit anti-H_3R antibody followed by an anti-rabbit-peroxidase and the TSA-Cy3 system. GFAP protein is detected as green fluorescence obtained with the mouse anti-GFAP antibody developed with the goat anti-mouse-Alexa488 antibody. White arrows show immunostained cells for only one of the antigens. Scale bar 20μm.

DISCUSSION

In the present work we describe the presence of histamine H_3 receptor expression in several neuronal types of rat brain: (1) dopaminergic neurons of the VTA and SNc; (2) striatonigral and striatopallidal GABAergic neurons; (3) striatal cholinergic interneurons and (4) glutamatergic terminals. In addition, we show that H_3 receptors seem to be functional in these cells.

H_3R mRNA distribution in several brain regions was previously shown (Pillot et al. 2002a). However in the Pillot et al. study H_3R mRNA was not detected in the VTA. The reason for this discrepancy could be due to methodological procedures of different sensitivity. In the Pillot work a ^{33}P radioactive probe was used for a one-week exposition time, a relatively short and probably insufficient period for low levels of mRNA expression. In our case we used the same probe as Pillot et al. but we labelled it with digoxigenin. Detection with the potent TSA amplification system revealed H_3R mRNA expression in the VTA. Pillot et al. 2002a also studied the brain distribution of H_3R protein by means of binding studies with the H_3R antagonist ^{125}I-iodoproxyfan, and found a minimum binding in the VTA, much lower than in the dorsal striatum, Acb and substantia nigra (noteworthy weaker in SNc than in SNr, suggesting high H_3R expression in striatonigral neurons). Using fluorescence we have confirmed H_3R

receptor presence in those areas (cortex, dorsal striatum, Acb, SNc and also VTA), but a comparison between regions was not attempted due to the difficulties of fluorescence quantification. Although H_3R expression can be lower in the VTA and SNc than in other regions, it is clearly present in dopaminergic neurons characterized both by tyrosine hydroxylase immunoreactivity and mRNA expression. Furthermore, H_3R stimulation decreased dopamine synthesis in striatal tissue (Figure 4 and Molina-Hernandez et al., 2000), as expected according to the typically inhibitory role of H_3R in other cells (Arrang et al., 1983, 1987). Dopamine release is also modulated by H_3R, although this effect has been more clearly observed in the prefrontal cortex than in the striatum (Ligneau et al., 2007; Schlicker et al 1993). In the Acb shell H_3R ligands modulate methamphetamine-stimulated dopamine release, but fail to modulate release when not stimulated (Munzar et al., 2004). Thus despite low expression levels, functional inhibitory H_3R are present in dopaminergic neurons projecting to the prefrontal cortex, dorsal striatum and Acb.

Colocalization of PE or SP mRNA with H_3R mRNA in the striatum and nucleus accumbens confirms H_3R expression in all GABAergic medium spiny projection neurons (Ryu et al., 1994; Moreno et al., 2011). The presence of functional H_3R in striatonigral neurons agrees with: 1) the fact that H_3R stimulation reduces dopamine D_1 receptor dependent GABA release (Arias-Montano et al. 2001); 2) H_3R-mediated inhibition of the D_1 dopamine receptor-stimulated cAMP accumulation (Sanchez-Lemus 2004), and 3) D_1R - H_3R receptor heteromers stimulating MAP kinase specifically in these cells (Moreno et al., 2011). On the other hand, H_3R presence in striatopallidal neurons agrees with 1) a previous report of H_3R mRNA presence in PE positive neurons (Pillot et al 2002b) and 2) D_2 - H_3 colocalization (Moreno et al., 2011). D_2 and H_3 receptors can interact

forming heteromers in living cells *in vitro*, and locomotor tests suggest they could also interact in vivo (Pillot et al 2002b; Ferrada et al. 2008), but it is still uncertain whether this interaction actually occurs in striatal neurons (Humbert-Claude et al., 2007).

The strict separation of dopamine D_1-like and D_2-like receptors between populations of striatonigral and striatopallidal projection neurons has been challenged by evidence showing that: (1) in the ventral striatum dopamine D_3 receptors can be co-expressed with D_1 or D_2 receptors (Le Moine and Bloch 1996); (2) D_4 and D_5 receptors can be colocalized in other striatal efferent neurons (Smith and Kieval 2000) and (3) neurons expressing D_1 receptors contain low levels of D_2 receptors and vice versa (Aizman et al., 2000). Currently, the existence of a population of medium spiny neurons which express both D_1 and D_2 receptors is accepted (Valjent et al. 2009, Perreault et al. 2010). Furthermore, two additional populations of D_1-expressing projection neurons can be found in striatum: one projects principally to SNc and co-express SP and PE (Wang 2007), and a second one projects to the substantia innominata and produces neurokinin B instead of SP or PE (Sonomura 2007). This implies that a low percentage of neurons can express both SP and PE or none, preventing a complete separation of the neuronal populations. In this paper we found that H_3R-mRNA positive neurons express SP or PE mRNA, and all H_3R-protein immunoreactive neurons show D_1 protein immunoreactivity in the areas studied. This would imply H_3R expression in neurons where D_1 and D_2 receptors are colocalized and in neurokinin B neurons. However as these populations represent only a small part of the medium spiny neurons, the specificity of the antibodies should be discussed. The specificity of the antibody against the dopamine D_1 receptor we used was described for the first time by Narushima et al.

(2006). In Narushima's paper, D_1 receptor immunoreactivity showed a partial colocalization with the D_2 receptor immunoreactivity, although this fact was not discussed by the authors. The specificity of the H_3R antibody used here has been tested (Fig. 3) but we can not discard the possibility that it could also recognize low levels of histamine H_4 receptors present in the striatum (Connelly et al. 2009).

GABAergic and cholinergic interneurons of the caudate/putamen express dopamine D_1R (David 2005). Thus the terminal pattern of colocalization of D_1R and H_3R immunoreactivity that we found should be due to H_3R presence in interneuron arborizations, as glutamatergic inputs express only dopamine D_2-like receptors (David 2005). Similarly, the D_1R - H_3R protein colocalization found in the mPFC could be due to the H_3R presence in the D_1R-expressing GABAergic interneurons of the cortex.

We found H_3R immunoreactivity in corticostriatal glutamatergic inputs to the striatum. It is well known that excitatory corticostriatal afferents to the striatum innervate both populations of medium-sized spiny GABAergic projection neurons (Lei et al. 2004). The colocalization of H_3R and VGLUT1 agrees with functional studies showing that 1) the H_3 ligand thioperamide elicits paired-pulse depression in corticostriatal synapses (Fig. 9), an index of altered probability of presynaptic neurotransmitter release (Thomson, 2000); 2) H_3R mediate histamine-induced synaptic depression in corticostriatal inputs (Doreulee 2001), and 3) H_3R activation in striatal synaptosomes inhibits glutamate release (Molina-Hernandez 2001).

Our results show that striatal cholinergic neurons present histamine H_3 receptor immunoreactivity. Due to the low number of striatal cholinergic interneurons, we were unable to show direct effects of H_3R ligands specifically in these cells, but there are numerous studies where H_3R modulation of acetylcholine release has been observed in diverse brain regions: (1) in the amygdala H_3R agonists increased acetylcholine release (Cangioli et. al 2002); (2) in the cortex H_3R agonists decreased acetylcholine release (Clapham & Kilpatrick, 1992; Arrang et al., 1995); (3) in the hippocampus H_3R antagonists increased acetylcholine release (Bacciottini et al. 2002); (4) in the ventral striatum of freely moving rats acetylcholine release was stimulated by both H_3R agonists and inverse agonists by acting at H_3 receptors putatively located in different populations of neurons, although no evidence was obtained of direct effects of H_3 ligands on cholinergic neurons (Prast et al. 1999). Thus, the possibility that H_3 receptors in striatal cholinergic interneurons are functional is still uncertain.

Although we did not find H_3R presence in GFAP-immunoreactive astrocytes in the mPFC, striatum or nucleus accumbens, this colocalization was recently shown at the caudal spinal trigeminal nucleus of non-human primates (Sekizawa 2010). Thus H_3R presence in astrocytes could be limited to some areas. In cultured astrocytes histamine seems to act through H_1 receptors to induce inositol phosphate accumulation (Kondou 1991) and calcium entry (Jung 2000) but we have found no literature evidence of H_3 receptor-mediated effects in astrocytes.

Due to the presence of functional H_3R in such different cellular types, what role should these receptors have in striatal function? Regarding locomotion, it has been shown that histamine action on H_3R elicits a fast

hypokinetic effect, and afterwards histamine would activate H_1 receptors to elicit hyperactivity (Chiavegatto et al., 1998). The hypokinetic effect could be explained by a transient decrease of dopamine neurotransmission provoked by H_3R stimulation. However H_3R KO mice exhibit lower locomotion than wild-type mice (Toyota et al., 2002), a finding that is hard to reconcile with the H_3-mediated hypokinetic effect. An interpretation given by these authors is that the absence of H_3 inhibitory autoreceptors in histamine neurons of KO mice could favor histamine release, which could decrease neuronal histamine levels, which in turn could decrease activation of H_1 receptors (Toyota et al., 2002). Expression of H_3R as auto- and heteroreceptors in such many different cellular types difficults to interpret the results obtained, most notably those of KO mice, wich experiment developmental adaptations.

Several H_3R agonists decrease L-dopa and apomorphine-induced turning behavior in 6-hydroxydopamine lesioned rats (Huotari et al., 2000; Liu et al., 2008) as well as L-dopa-induced chorea in MPTP-lesioned monkeys (Gomez-Ramirez et al. 2006). These effects could be due to H_3R-mediated decrease of GABA release in the SNr (García-Ramirez et al., 2004, but see Yanovsky et al., 2011). In reserpinized mice, H_3 receptor stimulation decreases locomotion induced by dopaminergic agonists (Ferrada et al., 2008). These effects should be independent of H_3R expression in dopaminergic neurons. In brains from Parkinson disease patients a strong H_3R binding was found in the SNr (Anichtchik et al., 2001). Thus, it is likely that H_3R in the GABAergic direct pathway could account for the majority of effects of H_3R agonists in models of Parkinson's disease. H_3R and D_1R in these neurons have opposite effects (Arias-Montaño et al, 2001; Moreno

et al., 2011) and dopaminergic lesions may sensitize them (Anichtchik et al., 2001; Sanchez-Lemus and Arias-Montaño, 2004).

The effects of H_3R antagonists / inverse agonists can be more difficult to interpret. H_3R inverse agonists would (1) stimulate histamine release, which may lead to H_1-mediated effects such as hyperlocomotion (Chiavegatto et al., 1998; Zhou et al., 2006), (2) facilitate glutamate release from corticostriatal terminals (Fig. 9), and (3) stimulate cortical dopamine release (Ligneau et al., 2007). In reserpinized mice, the H_3R antagonists / inverse agonist thioperamide potentiates locomotion elicited by dopaminergic agonists (Ferrada et al., 2008). However, antagonism of H_3R in striatonigral neurons could also block D_1-H_3 receptor heteromers (Moreno et al., 2011), whose involvement in locomotion is uncertain, but could hypothetically contribute to dopamine-mediated effects. D_1-H_3 receptor heteromers work as processors integrating dopaminergic and histaminergic signals that can be blocked by antagonists of either receptor partner (Moreno et al., 2011). According to H_3R expression in several neuronal types of the basal ganglia, further studies would help to determine whether H_3R ligands could be useful as therapeutic agents in Parkinson's disease or related movement disorders.

In conclusion, functional H_3 receptors are present in many different neuronal populations where they control striatal dopaminergic and glutamatergic transmission. These results could help to understand the role of H_3R in basal ganglia neural network.

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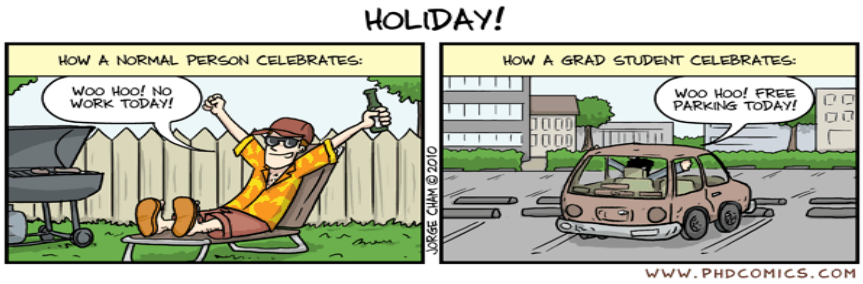

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