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"ANALISIS NEUROFARMACOLOGICO DE LA ACTIVIDAD GABAERGICA DURANTE LA HIPEREXCITABILIDAD POR LA ABSTINENCIA AL GABA"

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El presente trabajo de investigación se realizó en los departamentos de neurociencias y biofísica del Instituto de Fisiología Celular de la UNAM, bajo la dirección inicial del Dr. Simón Brailowsky K. y bajo la dirección del Dr. José Bargas Díaz, durante el desarrollo posterior y conclusión del trabajo.

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Quienes con sus comentarios y sugerencias enriquecieron la calidad y contenido de la tesis.

A la memoria de Simón Brailowsky Klipstein, gran investigador y humano.

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RESUMEN

La interrupción súbita de la administración intracortical de ácido γ-aminobutírico (GABA), el neurotransmisor inhibitorio preponderante en el SNC, resulta en una hiperexcitabilidad neuronal y cambios conductuales. A este fenómeno se le denomina síndrome de abstinencia al GABA. La hiperexcitabilidad neuronal es totalmente reproducible *in vitro*, en rebanadas de cerebro. En el presente estudio se realizó un análisis cuantitativo de la hiperexcitabilidad neuronal inducida por la abstinencia al GABA y adicionalmente, se discute la posible relación entre este fenómeno y los mecanismos celulares de la epilepsia y de la abstinencia a drogas.

Los objetivos de la presente tesis son: a) Caracterizar electrofisiologicamente la hiperexcitabilidad hipocampal inducida por la abstinencia al GABA tanto *in vivo* como *in vitro*. b) Evaluar farmacológica y electrofisiologicamente la actividad GABAérgica durante la hiperexcitabilidad en esta región y c) analizar cuantitativamente los mecanismos postsinápticos que le subyacen al fenómeno.

Para la elaboración de este estudio se utilizaron ratas macho de la cepa Wistar. Se utilizaron las técnicas de electroencefalografía, registro extracelular poblacional, inmunocitoquímica y de radiomarcado por pegado específico (binding).

Los resultados obtenidos en la presente tesis muestran que la abstinencia al GABA produce actividad epileptógena tanto *in vivo* como *in vitro*. En la preparación de rebanadas de hipocampo con hiperexcitabilidad inducida por abstinencia al GABA, el análisis de curvas de entrada/salida (Input/Output) demuestra que la excitabilidad incrementa en mas del 100% con respecto a los controles y que radica preponderantemente en la postsinapsis. Durante la hiperexcitabilidad se observa un bloqueo de la inhibición recurrente en la región CA1 del hipocampo y la disminución en el umbral de inducción de la potenciación a largo plazo (LTP). Curvas dosis respuesta con el agonista del receptor $GABA_A$, muscimol, y el antagonista, bicuculina, demuestran una disminución en la eficacia de ambos ligandos, confirmando que la inhibición GABAérgica se encuentra alterada y sugiriendo una disminución en el número de receptores. El análisis de Schild demuestra que la afinidad del receptor $GABA_A$ no se modifica durante la hiperexcitabilidad. Experimentos con la técnica de saturación de la unión (binding) con el agonista $GABA_A$, muscimol, confirman un decremento en el pegado del [^{3}H]-muscimol después de la abstinencia al GABA, mostrando una alta correlación con el desarrollo temporal de la hiperexcitabilidad.

Tanto los análisis electrofisiológicos como los farmacológicos concuerdan en que la hiperexcitabilidad por abstinencia al GABA es causada principalmente por la disminución en la densidad del receptor $GABA_A$.

SUMMARY

The sudden interruption of the intracortical instillation of exogenous γ-aminobutyric acid (GABA) generates an epileptic focus in mammals. Seizures elicited by GABA withdrawal (GW) presents paroxysmal activity and behavioral changes that last for weeks. Withdrawal-induced hyperexcitability is also produced *in vitro*; in hippocampal slices. This thesis reports a quantitative analysis of GW-induced hyperexcitability produced in the hippocampus of rats.

GW produces polyspikes, spike and wave followed by hypersynchronic discharges *in vivo* and paroxysmal field potentials *in vitro*; in hippocampal slices. GW produced a left-ward displacement of the input/output (I/O) function in hippocampal slices, suggesting that the postsynaptic component is predominant to explain the hyperexcitability. Recurrent inhibition tested with the paired pulse paradigm shows that GABAergic inhibition is blocked, under this condition the threshold for long-term potentiation (LTP) was decreased. A decrease in the inhibitory efficacy of the $GABA_A$ receptor agonist, muscimol, and in the effect of the $GABA_A$ receptor antagonist; bicuculline, confirmed that inhibition was impaired. Binding saturation experiments demonstrated a decrease in [^{3}H]-muscimol binding after GABA withdrawal showing a close correlation with the development of hyperexcitability. All these modifications coursed without changes in receptor affinity (K_D) for muscimol or bicuculline as demonstrated by both binding studies and Schild analysis. It is concluded that, in the CA1 region of the hippocampus, it is the number of functional $GABA_A$ receptors, and not the affinity of the receptor, what is decreased during GW-induced hyperexcitability.

LISTA DE ABREVIATURAS

BZD	Benzodiacepinas
CA1	Cuerno de Ammon región 1
EC_{50}	Concentración efectiva requerida para el 50% del efecto máximo.
EEG	Electroencefalograma
Emáx	Concentración para el efecto máximo.
fPSPE	Potencial postsináptico excitatorio poblacional o de campo
GABA	Acido γ-aminobutírico
$GABA_A$	Receptor al ácido gama-aminobutírico tipo A
GAD	Glutamato descarboxilasa
GABA-T	GABA transaminasa
GFAP	Proteína acídica glial fibrilar
K_A	Constante de afinidad
K_D	Constante de disociación
LTP	Potenciación a Largo Plazo
PS	Espiga poblacional
RNAm	Acido Ribonucleico mensajero
SAG	Síndrome de abstinencia al GABA
SN	Sistema Nervioso
SNC	Sistema Nervioso Central

ESTRUCTURA Y ORGANIZACIÓN DE LA TESIS

La presente tesis esta organizada en 6 secciones: introducción, justificación, objetivos, resultados, discusión general y conclusiones.

Se presenta inicialmente una introducción, en donde se refieren algunos antecedentes históricos y bases fisiológicas que permiten contextualizar el problema central de la tesis dentro del marco de la neurofisiología y la transmisión sináptica. Se realiza una descripción de la neurotransmisión inhibitoria, centrándose en el papel del ácido γ-aminobutírico (GABA) como principal neurotransmisor inhibitorio en el sistema nervioso central, se describe su síntesis y mecanismos de acción, señalando a los receptores GABAérgicos, su estructura y modulación. Se profundiza en la descripción del síndrome de abstinencia al GABA (SAG) como un fenómeno plástico de incremento en la excitabilidad neuronal y como problema medular del presente estudio.

En la justificación se describe la relación de la neurotransmisión GABAérgica en la generación de trastornos neurológicos como la epilepsia y los síndromes de abstinencia a algunas drogas, así como la relevancia de su estudio. Se sitúa a la abstinencia al GABA como un modelo de hiperexcitabilidad para el estudio de mecanismos celulares involucrados en las alteraciones antes señaladas.

En la sección de objetivos se plantean los problemas centrales del presente estudio,

En la sección de resultados, que incluye la metodología empleada, se presentan los productos de las diferentes fases experimentales de la presente tesis. Se incluyen 3 publicaciones. En la primera se presenta evidencia del fenómeno, su caracterización como un fenómeno de hiperexcitabilidad neuronal y su relación con la epilepsia. En la segunda, se caracterizan cuantitativamente los mecanismos neurofarmacológicos involucrados y se relaciona al fenómeno con la abstinencia a drogas. En la tercera publicación, se ahonda en la hiperexcitabilidad del fenómeno y su relación con la plasticidad neuronal.

En la discusión general se integran los resultados de las diferentes fases experimentales, se discuten los resultados dentro de un marco más amplio y se proponen algunas perspectivas.

En las conclusiones se enuncian los aportes específicos del presente estudio.

INTRODUCCIÓN

El sistema nervioso (SN) está constituido por entidades individuales que fueron reconocidas a finales del siglo XIX como células nerviosas (Ramón y Cajal, 1894). A estas células se les considera la unidad estructural y funcional del SN y son denominadas neuronas. El nombre de neurona fue dado a la célula nerviosa por Waldeyer aunque su descripción inicial se le acredita a Ehrenberg, quien las describió en la corteza cerebral y en los ganglios espinales (Clarke y O'Malley, 1968).

Las neuronas son células especializadas responsables del funcionamiento y facultades del propio SN. Una de las propiedades más importantes de estas células, y que es la esencia del funcionamiento del SN, es su capacidad de señalización o transferencia de información. Esta característica permite que las neuronas se relacionen funcionalmente, recibiendo, procesando y transmitiendo señales entre sí o con otros tipos celulares. El sitio en donde se lleva a cabo esta comunicación neuronal se conoce por el nombre de sinapsis, del griego *synapto*, que significa broche, unión, conexión (Feldman et al., 1997).

El término sinapsis fue acuñado por el fisiólogo inglés Charles Sherrington en 1897 (Sherrington, 1906) y se refiere a la unidad anatómica neuronal en donde se transmite, modula y recibe la información. Las sinapsis pueden ser de naturaleza eléctrica o quimica; en estas últimas la comunicación entre ambas células está mediada por una sustancia o molécula denominada neurotransmisor.

Un neurotransmisor es una sustancia que en la mayoría de las veces se derivada del grupo de los aminoácidos o de su metabolismo intermediario, es sintetizado por reacciones enzimáticas en la neurona, acumulado en la misma, liberado por la terminal o botón sináptico y conlleva un papel fisiológico distintivo (Voet y Voet, 1995; Feldman et al., 1997; Deutch y Roth, 1999). El SN de los mamíferos utiliza varias decenas de sustancias como neurotransmisores. Algunas de estas sustancias son aminoácidos, como el glutamato o el ácido aspártico cuyo efecto es excitatorio, mientras que el aminoácido inhibitorio de mayor importancia en el sistema nervioso central (SNC) es el ácido γ-amino butírico (GABA) (Deutch y Roth, 1999; Kandel et al., 1991).

Acido γ-amino butírico (GABA)

El ácido γ-aminobutírico (GABA) es el neurotransmisor inhibitorio preponderante en el SNC de los vertebrados. Dependiendo de la región específica del cerebro, se estima que el GABA está presente en aproximadamente 20-50% de las sinapsis de la corteza cerebral (Bloom y Iversen, 1971).

El GABA fue identificado en los inicios de los años 50s como un componente del SNC (Roberts, 1974) y en los años 60s se estableció que el GABA tiene una función principal como neurotransmisor inhibitorio en el SNC (Curtis y Waltkins, 1965; Krnjevic y Schwartz, 1966).

El GABA es un aminoácido que es sintetizado en las terminales nerviosas GABAérgicas a partir del ácido glutámico, esta reacción es catalizada por la enzima descaboxilasa del ácido glutámico o glutamato descarboxilasa (GAD). Existen dos isoformas de esta enzima, la GAD65 y la GAD67 (Martin y Rimvall, 1993). La GAD actúa sobre el glutamato removiendo el grupo gama-carboxilo como CO_2 para producir al GABA. La presencia de la enzima GAD es considerada para la identificación positiva de neuronas GABAérgicas. Después de liberarse, el GABA es recapturado por células gliales y mitocondrias de la neurona, donde es metabolizado principalmente por transaminación. La GABA transaminasa (GABA-T) es la enzima que cataliza la transaminación, transfiriendo el grupo amino del GABA al alfa-cetoglutarato. Los productos de la reacción son glutamato y semialdehído succínico, este último es a su vez oxidado a ácido succínico o succinato en el ciclo del ácido cítrico en la células gliales. El fosfato de piridoxal, un derivado de la piridoxina (vitamina B_6), es un cofactor para GAD y GABA-T. Sin embargo, la descarboxilación, a diferencia de la transaminación, es esencialmente irreversible (Tapia, 1971; Ganong, 1990; Feldman et al., 1997). En consecuencia, el contenido de GABA en el cerebro está reducido cuando hay deficiencia de piridoxina. Esta deficiencia se asocia con signos de hiperexcitabilidad nerviosa y convulsiones, aunque el tratamiento con piridoxina no es útil en la mayor parte de los casos clínicos de epilepsia (Salazar y Tapia, 2001).

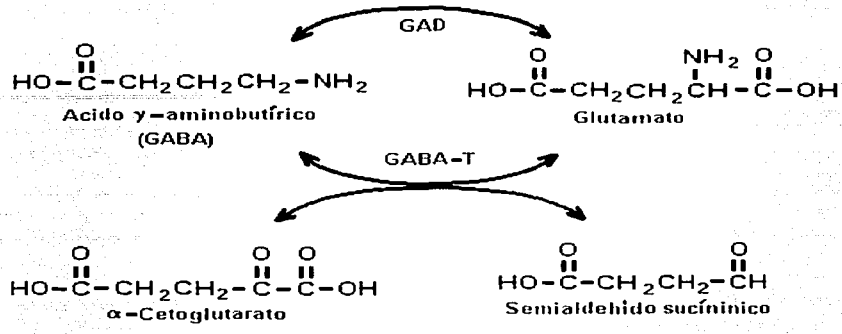

Fig. 1: Metabolismo del GABA (modificado de Siegel et al., 1995).

El GABA una vez sintetizado y liberado por la terminal sináptica es capaz de unirse y activar a tres diferentes tipos de receptores, denominados $GABA_A$ $GABA_B$ y $GABA_C$; cada uno con diferentes características con base en sus perfiles farmacológicos y fisiológicos (ver Tabla. 1).

	Receptor $GABA_A$	Receptor $GABA_B$	Receptor $GABA_C$
Categoría	Receptor asociado a canal iónico	Receptor asociado a proteína G	Receptor asociado a canal iónico
Subunidades	α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π	GBR1, GBR2	ρ_{1-3}
Agonistas	Muscimol, THIP, isoguvacina	Baclofen, CGP-97541	
Antagonistas	Bicuculina, Picrotoxina	Faclofen, CGP-35348	TPMPA, Picrotoxina
Desensibilización	Sí	?	No
Moduladores	Benzodiacepinas Barbitúricos		Zinc
Acción	(+) gCl^-	(-) gK+, (-) gCa2+, (-) AMPc	(+) gCl^-

Tabla 1. Características de los Receptores de GABA (Basado en: Mody et al., 1994; Qian y Dowling, 1995; Hevers y Lüddens, 1998; Mehta y Ticku, 1999).

Receptor $GABA_A$

El primero de estos receptores, el $GABA_A$, es un receptor ionotrópico o asociado a un canal iónico activado por ligando (e.i. Ligand-gated ion channels). Los receptores ionotrópicos combinan un sitio de unión a ligando con un canal iónico, o ionóforo, en su estructura. En el caso del receptor $GABA_A$ este canal es un cloróforo o canal de cloro (Harris y Allan, 1985; Hevers y Lüddens, 1998; Mehta y Ticku, 1999). El efecto inhibitorio del GABA esta mediado por los receptores presentes en la membrana neuronal y resulta en una reducción de la excitabilidad de las neuronas. Después de que el GABA fue reconocido por el receptor, es liberado y recapturado por las neuronas y células gliales circundantes, a través de transportadores de alta afinidad dependientes de sodio (Amara y Kuhar, 1993; Feldman et al., 1997). Las neuronas y células gliales reciclan al neurotransmisor para su uso posterior (Zimmermann, 1979). Los receptores $GABA_A$ son también el blanco de muchos compuestos, entre ellos fármacos psicoactivos y de acción terapéutica como las benzodiacepinas, los anestésicos generales, drogas sedantes, así como neuroesteroides y alcohol (Doble y Martin, 1996; Hevers y Lüddens, 1998; Mehta y Ticku, 1999).

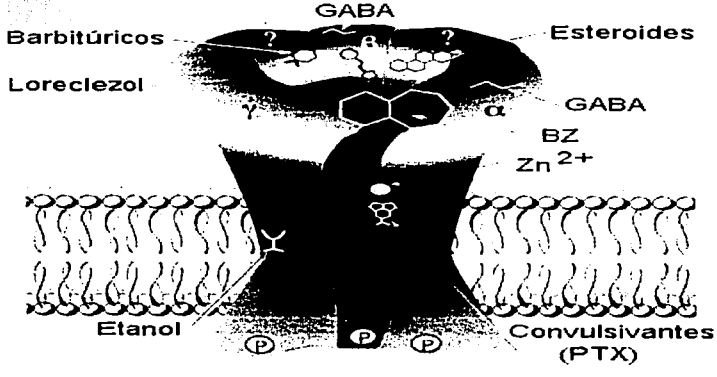

Figura 2: Receptor $GABA_A$ (basado en Hevers y Lüddens, 1998)

El receptor $GABA_A$ presenta un ensamble heteropentamérico derivado de la combinación de 5 subunidades proteícas (Hevers y Lüddens, 1998; Mehta y Ticku, 1999). Cada subunidad cruza transversalmente la membrana postsináptica generalmente de una manera simétrica y comprende un largo dominio N-terminal extracelular, cuatro dominios transmembranales hidrofóbicos (TM1-TM4) y un pequeño dominio C-terminal extracelular. En el cerebro de mamíferos se han clonado varias clases de subunidades, así como variantes o isoformas para cada subunidad, las cuales han sido clasificadas de acuerdo al grado de identidad en la secuencia de aminoácidos como: α1-α6, β1-β3, γ1-γ3, δ, ε, π y ρ1-ρ3 (MacDonald y Olsen, 1994; Hevers y Lüddens, 1998; Mehta y Ticku, 1999).

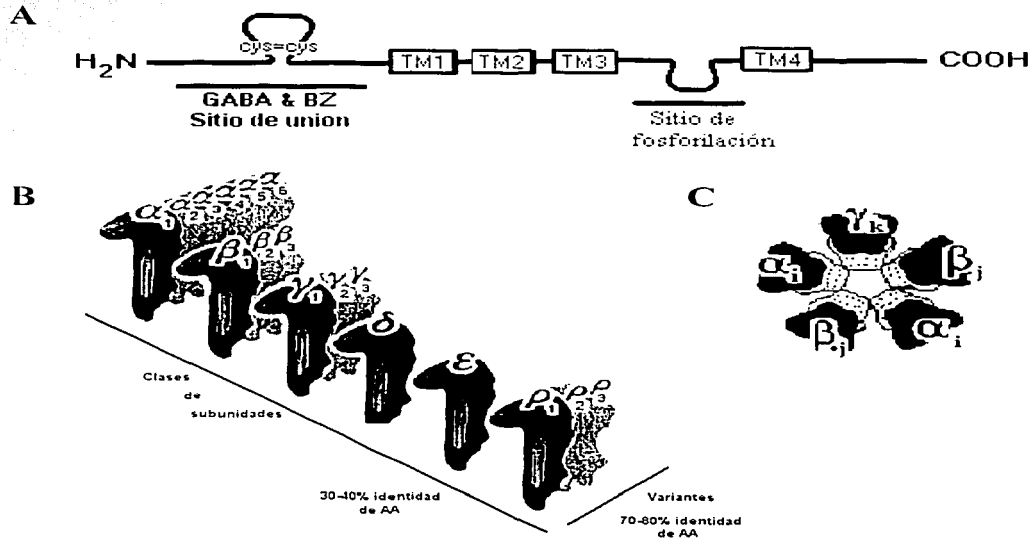

Figura 3: Receptor $GABA_A$. (A) Representación esquemática de la estructura primaria de una subunidad (B). Agrupamiento de las diferentes clases de subunidades de acuerdo a la identidad en la secuencia de sus aminoácidos. (C) Ensamble heteropentamérico del receptor (modificado de Hevers y Lüddens, 1998).

Se sugiere que varias de las subunidades ρ1-ρ3 ensamblan al receptor $GABA_C$, o bien a un receptor $GABA_A$ especializado dado que estos receptores son insensibles a bicuculina o baclofen (Mehta y Ticku, 1999).

La combinación de 5 de estas subunidades permite un número grande de posibles receptores, aunque no todos funcionales o presentes en el SN. La diversidad de combinaciones con acción fisiológica explica que el efecto de un número alto de drogas o psicofármacos que modulan al receptor difiera dramáticamente (Enna y Karbon, 1996; Hevers y Lüddens, 1998).

Las 5 subunidades que constituyen al receptor están arregladas para formar un poro central. Se ha propuesto que el dominio transmembranal TM2 de cada subunidad constituye la pared del canal iónico (Xu y Akabas, 1996). El receptor $GABA_A$ tiene un sitio específico al cual se unen las moléculas de GABA, se presume que el dominio N-terminal extracelular es el que putativamente incluye el sitio de unión al ligando y a la interacción del receptor con las benzodiacepinas (Hevers y Lüddens, 1998). La interacción entre el neurotransmisor y el receptor resulta en cambios conformacionales del arreglo protéico que, como consecuencia, abren el poro central. La apertura de este poro permite una corriente entrante de iones de cloro (Harris y Allan, 1985; Hevers y Lüddens, 1998; Mehta y Ticku, 1999) y conduce a una rápida hiperpolarización de la célula postsináptica, ya que el valor del potencial de equilibrio del cloro es generalmente más negativo que el valor del potencial de membrana en reposo. Lo cual explica el efecto inhibitorio del GABA a través de este receptor.

Receptor $GABA_A$ e hiperexcitabilidad neuronal

La alteración o interrupción de la activación del receptor $GABA_A$ tiene como consecuencia la disminución en la neurotransmisión inhibitoria (desinhibición). La desinhibición GABAérgica ha sido ampliamente referida como uno de los mecanismos sinápticos de los cuales depende, por lo menos en parte, la hiperexcitabilidad neuronal

(Kostopoulos y Psarropoulou, 1992; Feldman et al., 1997; Bernard et al., 1998; Ikeda-Douglas et al., 1998). La documentación sobre hiperexcitabilidad neuronal se basa principalmente en el número exagerado de potenciales de acción durante las descargas epileptiformes registradas durante las crisis convulsivas (Kostopoulos y Psarropoulou, 1992; Brailowsky, 1999). La relación de esta actividad con el receptor $GABA_A$ proviene de diferentes líneas de evidencia.

A) El efecto anticonvulsivo de los agentes GABAérgicos

Gran parte de la evidencia sobre la participación del receptor $GABA_A$ en la epilepsia proviene del efecto anticonvulsivo que presentan los agentes que directa o indirectamente aumentan la inhibición mediada por este receptor. Los mecanismos a través de los cuales actúan estos agentes son: reduciendo el catabolismo del GABA (vigabatrin), facilitando la liberación de GABA de las terminales presinápticas (gabapentin), a través de la inhibición de la recaptura de GABA (tiagabina), o incrementando el influjo de iones de cloro directamente en el receptor (benzodiacepinas y los barbitúricos) (Granger et al., 1995; Feldman et al., 1997; Ikeda-Douglas et al., 1998; Julien, 2001). Todos estos agentes incrementan la actividad GABAérgica y ejercen un efecto antiepiléptico.

B) El bloqueo del receptor GABAérgico

En sentido opuesto, la administración, en concentraciones lo suficientemente altas, de compuestos con la capacidad de bloquear al receptor $GABA_A$, produce descargas epileptiformes. Tanto la administración tópica en el SNC o sistémica de antagonistas del receptor $GABA_A$, como la penicilina, la bicuculina o la picrotoxina, producen hiperexcitabilidad persistente, depolarizaciones paroxísticas y actividad epileptiforme (Schwartzkroin y Prince, 1977; Wong y Traub, 1983; Connors y Gutnick, 1984; Karnup y Stelzer, 2001; Mackenzie et al., 2002). Asimismo, se ha referido la pérdida de la neurotransmisión inhibitoria durante crisis epilépticas como la producida por el

"kindling", o la producida en animales por diversos modelos, ya sea químicos o genéticos, de actividad epiletógena (Gibbs et al., 1997; Fueta et al., 1998; Ikeda-Douglas et al., 1998). No obstante, el incremento en la actividad GABAérgica también se ha reportado durante crisis epilépticas como el "kindling" (Gutiérrez y Heinemann, 2001), probablemente como un mecanismo emergente en repuesta a la sobreexcitación.

C) La abstinencia a fármacos agonistas GABAérgicos

La hiperexcitabilidad neuronal y las crisis epilépticas también se producen cuando se retira de manera abrupta la administración prolongada de drogas que incrementan la inhibición mediada por el receptor $GABA_A$. Tal es el caso de las benzodiacepinas, los barbitúricos y el alcohol (Ticku et al., 1983; Sandoval y Palermo, 1986; Carlen et al., 1990; Olsen y Avoli, 1997). Estas drogas causan tolerancia, dependencia y síndromes de abstinencia. El síndrome de abstinencia que resulta de la suspensión súbita en el consumo de estos compuestos se caracteriza por irritabilidad, ansiedad, temblor, cambios sensoperceptuales, alucinaciones y una gran variedad de crisis o patrones convulsivos, usualmente generalizados (Owen y Tyrer, 1983; Marciani et al., 1985; Davies et al., 1987; Lader, 1994). El análisis, *in vitro*, de rebanadas de cerebro obtenidas de animales sujetos a la exposición crónica de benzodiacepinas, barbitúricos o alcohol muestra que también bajo estas condiciones el tejido aislado presenta descargas epileptiformes y habitualmente tiene una alteración en la inhibición sináptica (Carlen et al., 1990; Saunders y Ho, 1990; Zeng et al., 1995).

La manipulación del sistema GABAérgico puede influenciar la excitabilidad cerebral y propiciar crisis epilépticas tanto en animales como en humanos. No sólo los agentes GABAérgicos o GABAmiméticos son capaces de modificar la excitabilidad cerebral; el retiro súbito de la administración del propio ligando endógeno, el GABA, resulta en un incremento de la excitabilidad neuronal.

Síndrome de abstinencia al GABA (SAG).

La interrupción súbita de la infusión intracortical de GABA conduce a un incremento en la excitabilidad neuronal en monos (Papio papio) y ratas. Este fenómeno llamado "Síndrome de Abstinencia al GABA" (SAG), se caracteriza por una actividad epileptógena de larga duración en el área que rodea al sitio de infusión; se acompaña de mioclonias en el área corporal correspondiente, cambios conductuales y puede durar de horas a días, dependiendo de la dosis y del tiempo de infusión (Brailowsky et al., 1987; 1988; 1990; 1999). Registros electroencefalográficos del fenómeno muestran la presencia de focos con descargas paroxísticas (poliespigas y actividad en forma de espiga-onda) en el área circunscrita a la infusión (Brailowsky et al., 1989; 1990). Se ha constatado la presencia de focos paroxísticos después de la infusión crónica de GABA en la corteza cerebral (Brailowsky et al., 1988) y el sistema límbico, particularmente en amígdala e hipocampo (Le Gal la Salle et al., 1988).

Un correlato del SAG *in vitro* se ha estudiado en rebanadas de neocorteza (Silva-Barrat et al., 1991; 1992) y en rebanadas de hipocampo de rata (García-Ugalde et al., 1992). El registro intracelular, en rebanadas neocorticales con SAG, muestra la presencia de un gran porcentaje de neuronas con descargas paroxísticas despolarizantes (rafagas o "burst") intrínsecas después de la inyección intracelular de corriente depolarizante o la estimulación sináptica. El registro de potenciales poblacionales, en rebanadas de hipocampo con SAG, presenta un incremento sustancial en el número y amplitud de las espigas poblacionales con respecto a sus valores control (García-Ugalde et al., 1992; Calixto et al., 2000).

Entre los mecanismos celulares asociados con la hiperexcitabilidad de las neuronas durante el SAG, se ha señalado cierta forma de tolerancia. La administración sistemática en dosis altas de drogas anticonvulsivas que aumentan la transmisión GABAérgica, tales como las benzodiacepinas, los barbitúricos y el valproato, no tuvieron efecto sobre las descargas paroxísticas focales en ratas con SAG (Silva-Barrat et al., 1989) y sólo la administración de dosis altas de GABA puede inhibir esta actividad (Brailowsky et al., 1989). Asimismo, registros intracelulares en rebanadas de

neocorteza con SAG, muestran niveles bajos en la conductancia neuronal tras la aplicación del agonista específico $GABA_A$, isoguvacina (Silva-Barrat et al., 1989; 1991).

Salazar et al. (1994), han reportado un decremento del 27-48% en la actividad de la enzima glutamato descarboxilasa (GAD) durante el SAG, así como Menini et al. (1991) un incremento del metabolismo energético en el sitio de infusión y en los núcleos talámicos de proyección.

Durante la hiperexcitabilidad neuronal posterior a la interrupción de GABA se presenta un decremento en el tono GABAérgico que conduce a las células a un estado de excitabilidad anormal y a la aparición de la actividad epileptógena (Silva-Barrat et al., 1989; 1991; García-Ugalde et al., 1992; Calixto et al., 2000). Sin embargo, estos resultados no especifican si la alteración en la neurotransmisión GABAérgica responde a cambios mayoritariamente presinápticos, postsinápticos o ambos. Tampoco se ha analizado suficientemente las características y los mecanismos sinápticos de este fenómeno en el hipocampo. Considerando que el hipocampo es una estructura cerebral altamente epileptogénica y en ocasiones asociada a crisis refractarias.

La mayoría de los trabajos previos se llevaron a cabo induciendo la hiperexcitabilidad en el animal íntegro y estudiando sus características *in vivo* o bien, en muestras de tejido postmortem (*ex vivo*).

Con el presente trabajo de tesis se realizó un análisis neurofarmacológico de estas interrogantes, principalmente en la preparación de rebanadas de hipocampo. Lo que permite una mejor manipulación de las variables farmacológicas y un análisis del fenómeno a través de su curso temporal.

JUSTIFICACIÓN Y PLANTEAMIENTO DEL PROBLEMA.

Una de las premisas fundamentales sobre el funcionamiento del SN es que existe un balance entre la excitación y la inhibición neuronal. Anormalidades como el incremento en la excitación de las células principales, el decremento en los circuitos de inhibición (i.e. desinhibición) o ambas, se consideran como la base de la enfermedad neurológica más antigua que conoce el hombre y la de mayor incidencia: la epilepsia. De acuerdo con la Organización Mundial de la Salud (OMS), la epilepsia se define como una afección crónica, de etiología diversa, caracterizada por la repetición de crisis resultantes de la descarga excesiva de neuronas cerebrales (hiperexcitabilidad cerebral), independientemente de los síntomas clínicos o paraclínicos eventualmente asociados.

Más del 5% de la población mundial ha padecido al menos una crisis epiléptica en su vida, aunque, el diagnóstico de epilepsia se reserva sólo cuando se han padecido crisis repetidas. En estas condiciones (crisis repetidas o con necesidad de tratamiento) la prevalencia mundial de la epilepsia reportada por la OMS en 1997 fue de 7 por cada 1000 habitantes (0.72%). Esto es, si la población mundial actual es de seis mil millones de habitantes, entonces 43,200,000 de estas personas padecen epilepsia de algún tipo. La prevalencia es mayor en países en vías de desarrollo, como México, en los cuales la tasa de crecimiento anual de personas que padecen esta enfermedad es de 100 por cada 100 000 habitantes, en tanto que en países desarrollados es de 50 por cada 100 000 habitantes (OMS, 1997). Para otros autores el estimado se eleva a aproximadamente el 1% de la población, incluso en países desarrollados como los Estados Unidos (McNarama, 1994).

Adicionalmente, existen crisis epileptógenas como resultado del tratamiento con fármacos, el abuso de drogas, o bien por la interrupción precipitada de éstos. Este último es el caso de las drogas con efectos sedantes sobre el SNC. Los sedantes, que son principalmente agonistas del receptor GABAérgico, son las drogas más frecuentemente prescritas, consumidas y son también las de mayor abuso (OMS 1999; Carlen et al., 1990). Drogas tales como los barbitúricos, las benzodiacepinas y el

alcohol producen tolerancia funcional, dependencia y adicción. La supresión precipitada y/o súbita de la administración de estas sustancias resulta en síndromes de abstinencia que incluyen una gran variedad de patrones electroencefalográficos o crisis convulsivas, usualmente generalizadas (Owen y Tyrer, 1983; Marciani et al., 1985; Davies et al., 1987; Lader, 1994; Doble y Martin, 1996).

La actividad epileptógena no ha sido lo suficientemente analizada y en cuanto a los mecanismos básicos de la epilepsia humana ha sido extremadamente difícil dilucidar sus principios ya que existen demasiados tipos de crisis clínicas y eléctricas, a las cuales les subyacen etiologías ampliamente diversificadas y pobremente entendidas. Los registros electrofisiológicos unitarios, *in-situ*, en el cerebro humano resultan poco prácticos y los registros intracelulares en estas condiciones son prácticamente imposibles. Así, los modelos experimentales de epilepsia, y particularmente los modelos *in vitro*, se presentan como excelentes condiciones para registrar la actividad neuronal, controlar las condiciones ambientales del tejido y evaluar el efecto de fármacos con potencialidad terapéutica (Traub y Jefferrys, 1994). La preparación de rebanadas de hipocampo ha mostrado ser una técnica invaluable para investigar mecanismos de tolerancia a fármacos sedantes (Xie y Tietz, 1992).

Las descargas paroxísticas y espontáneas en el tejido cerebral durante la abstinencia al GABA se consideran un modelo de epileptogénesis (Brailowsky et al., 1987; 1988; 1989) y pueden representar un correlato de la actividad paroxística y la hiperexcitabilidad que se observa en los síndromes de abstinencia a sedantes o agonistas $GABA_A$, como las benzodiacepinas (Brailowsky et al., 1989; Calixto et al., 2000). De hecho, se han reportado casos humanos con síndrome de abstinencia similar al GABA después de la suspensión de la administración oral de γ-hidroxibutirato (GHB, también llamado éxtasis líquido), un depresor del SNC y agonista $GABA_A$ (e.g., Craig et al., 2000). El estudio de la hiperexcitabilidad por abstinencia al GABA permite el análisis experimental desde el inicio de la hiperexcitabilidad, distinguiendo entre los cambios propios del desarrollo de la hiperexcitabilidad de los cambios resultantes de la actividad eléctrica anormal, lo cual abre grandes perspectivas para entender las

condicionantes fisiológicas de estados de hiperexcitabilidad, como la epilepsia o la abstinencia a fármacos agonistas del receptor $GABA_A$. En ese sentido la tesis general del presente estudio se centra en el análisis neurofarmacólogico de la participación postsináptica en la generación de la hiperexcitabilidad provocada por la privación de GABA en el hipocampo. Específicamente, se realizó una evaluación cuantitativa de la hiperexcitabilidad y de los cambios en las propiedades y densidad de los receptores $GABA_A$, que le subyacen al fenómeno.

OBJETIVO GENERAL:

El análisis cuantitativo de la actividad GABAérgica durante la hiperexcitabilidad por abstinencia al GABA.

OBJETIVOS PARTICULARES:

1) Caracterizar electrofisiológicamente la hiperexcitabilidad hipocampal, tanto *in vivo* como *in vitro*, inducida por la abstinencia al ácido γ-aminobutírico (GABA)
2) Evaluar la actividad GABAérgica durante la hiperexcitabilidad por abstinencia al GABA, a través de la inhibición por pulsos pareados, la evaluación del efecto de agonistas y antagonistas $GABA_A$ sobre la actividad epileptogénica y a través de la inducción de la LTP.
3) Evaluar las propiedades farmacológicas (eficacia, potencia, constante de disociación, afinidad) del receptor $GABA_A$ que subyacen a la hiperexcitabilidad por abstinencia al GABA. Lo anterior con la ayuda del análisis de curvas dosis respuesta y del análisis de Schild.
4) Determinar, a partir del efecto sobre la respuesta electrofisiológica y del marcado en el pegado (binding) de ligandos específicos del receptor $GABA_A$, si la

hiperexcitabilidad por abstinencia al GABA es causada por cambios en la densidad del receptor $GABA_A$.

RESULTADOS

Los resultados de la presente tesis, así como la metodología y procedimientos empleados se describen con detalle en los 3 trabajos siguientes. Los resultados también son descritos e integrados durante el desarrollo de la discusión. De manera previa se ofrece a continuación una síntesis de los resultados en los artículos, los experimentos realizados y su relación con los objetivos planteados en la presente tesis.

El objetivo 1, la caracterización hipocampal de la hiperexcitabilidad por abstinencia la GABA, se aborda en el trabajo número 1 con los experimentos de electroencefalografía, inmunihistoquímica y con los registros y análisis de la respuesta extracelular de campo. En el trabajo número 2, con el registro del curso temporal del fenómeno y con las curvas de excitabilidad (Input/Ouput), éstas últimas para determinar si el fenómeno radica en la pre o en la postsinapsis.

El objetivo 2 y 3, la evaluación de la actividad GABAérgica y de las propiedades farmacológicas del receptor $GABA_A$, se lleva a cabo en el trabajo número 1 con el experimento de inducción de la LTP y la curva dosis/respuesta con el antagonista bicuculina. En el trabajo número 2, con la curva dosis/respuesta con el agonista específico muscimol, con el experimento de inhibición por pulsos pareados y con el análisis de Schild. En el trabajo número 3, con el experimento de inducción de la LTP en corteza y con la evaluación de diversos agentes farmacológicos y su efecto sobre la actividad epileptógena.

El objetivo 4, determinar si la hiperexcitabilidad por abstinencia al GABA es causada por cambios en la densidad del receptor $GABA_A$, se resuelve en el trabajo número 2 con el análisis de los parámetros farmacológicos en las curvas dosis/respuesta, el análisis de Schild y en el mismo trabajo con el análisis de la unión específica (binding).

Los trabajos se presentan a continuación bajo los siguientes títulos y en el orden respectivo:

Trabajo 1: Hyperexcitability induced by GABA withdrawal facilitates hippocampal Long-term potentiation.

Trabajo 2: Hippocampal hyperexcitabiity induced by GABA withdrawal is due to down-regulation of $GABA_A$ receptors.

Trabajo 3: Long-Lasting Effects of GABA Infusion into the Cerebral Cortex of the Rat.

El primero de ellos es un manuscrito terminado y listo para enviarse a publicación, el segundo se encuentra publicado en Epilepsy Research (2001) 47: 257-271 y el tercer artículo ha sido publicado en Neural Plasticity (2000) 7(1-2): 1-8.

DISCUSIÓN.

Sin duda la alteración en la relación entre inhibición y excitación neuronal es uno de los fenómenos neuronales de mayor interés científico, en gran medida por la alta frecuencia con la que ocurre y por el impacto que representa en la salud del ser humano. En la presente tesis se analizó, a través de técnicas electrofisiológicas, neurofarmacológicas y de inmunocitoquímica, la hiperexcitabilidad neuronal que resulta de la interrupción súbita de la administración de GABA en el hipocampo de ratas.

En el trabajo número 1, se reprodujo la actividad epiléptica como consecuencia de la abstinencia a GABA en el hipocampo de ratas, esta actividad se había reportado principalmente en corteza sensoriomotora (Montiel et al., 2000; Brailowsky et al., 1987; 1988; 1990; Fukuda et al., 1987; Le Gal La Salle et al., 1988; Silva-Barrat et al., 1989, 1991, 2000). Electroencefalográficamente se describe durante la abstinencia al GABA la presencia de poliespigas, espigas-onda y espigas seguidas de actividad hipersincrónica en el área circunscrita a la infusión. El análisis electrofisiológico, a través del registro extracelular de la actividad de campo, muestra que la actividad paroxística persiste en condiciones *ex vivo*, en rebanadas de hipocampo provenientes de estas ratas, dado que la estimulación única con pulsos de voltaje produjo espigas poblacionales múltiples en estas rebanadas. Adicionalmente, como se muestra en los trabajos 1 y 3, el tejido procesado para inmunomarcado de la proteína acídica glial fibrilar (GFAP) muestra una gliosis astrocítica intensa en el trayecto de la cánula y una moderada como reacción asociada a la hiperexcitabilidad. Este incremento de la actividad astrocítica ha sido reportada en la abstinencia al GABA (Le Gal la Salle et al., 1988) así como en otros modelos de epilepsia focal (Franke y Kittner, 2001).

En la presente tesis, logramos producir la hiperexcitabilidad por abstinencia al GABA totalmente *in vitro*, como se muestra en los trabajos 1 y 2. En este caso las rebanadas de hipocampo de rata fueron incubadas en presencia de GABA durante un periodo de 120 min. y como consecuencia del retiro súbito de este neurotransmisor se observó una potenciación de la espiga poblacional que sobrepasó la respuesta control y se produjeron descargas epileptiformes. Los cambios en estas condiciones fueron

significativos y coinciden en sus valores con estudios previos (García-Ugalde et al., 1992; Calixto et al., 2000). La concentración de GABA (5 mM) usada para inducir la abstinencia y la hiperexcitabilidad se aproxima a la concentración de GABA que se presenta de manera transitoria en el espacio sináptico (Cherubini y Conti, 2001).

La hiperexcitabilidad de la respuesta bajo esta condición se cuantificó a través de la función de "Entrada/Salida" sináptica o curvas I/O (Input/Output) (Kostopoulos y Psarropoulou, 1992). La función I/O es una medida de la descarga postsináptica como función de la actividad sináptica. En el caso de las rebanadas con abstinencia al GABA se determinó en el trabajo número 2 que la excitabilidad se incrementa en más del 100% con respecto a los controles y que esta hiperexcitabilidad radica esencialmente en la postsinapsis.

Posteriormente se analizó la hipótesis de una disminución en la inhibición GABAérgica que converge sobre las células piramidales de la región CA1 del hipocampo como un mecanismo sináptico subyacente a la hiperexcitabilidad (Bernard et al, 1998; Ikeda-Douglas et al, 1998). En el trabajo número 2 se confirmó, a través del análisis de pulsos pareados, que durante la hiperexcitabilidad se presenta una disminución significativa en la inhibición recurrente, i.e., la inhibición perisomática de las células piramidales. La disminución de la inhibición recurrente aparece reportada en una gran variedad de modelos de hiperexcitabilidad neuronal (Davies et al., 1990; Sloviter, 1991; Xie y Tietz, 1991; García-Ugalde et al., 1992; Olsen y Avoli, 1997; Ikeda-Douglas et al., 1998; Calixto et al., 2000). La inhibición dendrítica también se presenta alterada durante la hiperexcitabilidad por abstinencia al GABA, esto se demuestra en el trabajo número 1 en donde una estimulación aferente débil, que en condiciones control sólo produce una potenciación a corto plazo, durante la hiperexcitabilidad por abstinencia al GABA fue capaz de inducir una potenciación a largo plazo (LTP). Esta facilitación en la inducción de la LTP se corroboró tanto en condiciones *in vitro* como *ex vivo*. Esta última en rebanadas de hipocampo provenientes de ratas con abstinencia al GABA. Asimismo se corroboró en rebanadas de corteza sensoriomotora, como se muestra en el trabajo número 3. La evidencia

experimental proveniente de varios estudios señala que cambios en la excitabilidad de los circuitos neuronales, resultado de la hiperexcitabilidad y de las crisis epilépticas, inducen un incremento de larga duración en la eficacia sináptica del hipocampo (Schneiderman, 1997) y facilitan la inducción de la potenciación sináptica persistente en varias estructuras cerebrales (Kapur y Haberly, 1998), incluyendo el hipocampo (Becker et al., 1997., Ishizuka y Hayashi, 1998). Habitualmente esta relación entre hiperexcitabilidad y eficacia sináptica se asocia a una reducción en la transmisión sináptica GABAérgica (Schultz, 1997).

Estos resultados sugirieron que las modificaciones en la postsinapsis que conducen a la hiperexcitabilidad pueden ser ejercidas a través del receptor $GABA_A$. El decremento en la inhibición mediada por el receptor $GABA_A$ fue confirmado en los trabajos número 1 y 2 con la realización de curvas dosis-respuesta con el agonista específico para este receptor, el muscimol y con el antagonista competitivo bicuculina. En ambos casos se evaluó la eficacia y potencia de los fármacos sobre la amplitud de la espiga poblacional. El análisis de dobles reciprocas, Lineweaver-Burk, mostró una reducción significativa en la respuesta máxima (eficacia) del receptor sin cambios significativos en la potencia (EC_{50}) para ambos compuestos. Estos resultados señalan un cambio en el número de receptores sin una modificación significativa en su afinidad. No obstante, se realizó un análisis de Schild para asegurar la ausencia de cambios en la afinidad del receptor. Como se muestra en el trabajo número 2, se llevaron a cabo curvas dosis-respuesta con el agonista muscimol y se evaluó el desplazamiento progresivo de estas curvas ante diferentes dosis del antagonista competitivo bicuculina. Como resultado de este análisis no hubo diferencias significativas en la constante de disociación del antagonista durante la abstinencia al GABA. Los valores obtenidos en el análisis de Schild corresponden estrechamente a los valores reportados por otros grupos para el mismo antagonista (Krishek et al., 1996; Simmonds, 1982); usando la misma técnica de registro en la misma región del hipocampo (Kemp et al., 1986). De tal forma que el análisis de Schild, el análisis de las curvas dosis-repuesta y el análisis de Lineweaver-Burk, indicaron que cualquier

modificación que el receptor $GABA_A$ presente después de la abstinencia al GABA, no incluye un cambio en su afinidad ni para el agonista ni para el antagonista.

Una reducción en el número de receptores $GABA_A$ ha sido reportada como resultado de la administración crónica del mismo GABA o de agonistas GABAérgicos (Miller et al, 1988; Mehta y Ticku, 1992; Mhatre y Ticku, 1994; Calkin y Barnes, 1994). Para confirmar estructuralmente que la reducción en la transmisión mediada por el receptor $GABA_A$ durante la abstinencia al GABA se debe a una reducción en el número de lo receptores $GABA_A$ en la membrana postináptica, se realizaron experimentos de radiomarcado por pegado específico (binding) del agonista [^{3}H]-muscimol. En el trabajo número 2 se muestran los resultados de estos estudios, los cuales señalan una reducción en el número del receptor (eficacia) sin cambios en la afinidad o EC_{50} (potencia). Adicionalmente, la medición del curso temporal del fenómeno con la misma técnica, muestra que el decremento en el número de receptores se correlaciona con la evolución temporal de la hiperexcitabilidad.

Tanto los análisis electrofisiológicos como los farmacológicos coinciden en que la hiperexcitabilidad por abstinencia al GABA es causada principalmente por una disminución en la densidad del receptor $GABA_A$.

Hiperexcitabilidad, desinhibición y abstinencia al GABA

La reducción en el umbral de disparo en las neuronas (Lossin et al., 2002) y el número exagerado de potenciales de acción durante las descargas epileptiformes registradas durante las crisis convulsivas (Kostopoulos y Psarropoulou, 1992) caracterizan a la hiperexcitabilidad neuronal como la propiedad inherente más representativa del tejido epileptógeno. Sin embargo, la hiperexcitabilidad neuronal surge de mecanismos celulares y sinápticos en gran parte desconocidos (Brailowsky, 1999). Como se mencionó anteriormente, la desinhibición neuronal es uno de los

mecanismos sinápticos que más se han asociado al incremento en la excitabilidad neuronal.

La actividad electroencefalográfica epileptiforme y la actividad paroxística extracelular durante la hiperexcitabilidad por abstinencia al GABA, podría ser el resultado de respuestas oscilatorias y sincrónicas de las neuronas bajo el estado de desinhibición que produce la abstinencia al GABA. Se ha reportado que el incremento en la amplitud de la espiga poblacional y las espigas poblacionales múltiples epileptiformes se asocian con la pérdida de la inhibición GABAérgica (Ikeda-Douglas et al, 1998; Sloviter, 1991), o bien a través del bloqueo del receptor $GABA_A$ con penicilina, lo cual conduce a la aparición espontánea de trenes de descarga (rafagas o burst) sincrónicos y potenciales de campo oscilatorios persistentes en la red neuronal hipocampal (Schneiderman, 1997). De hecho los registros intracelulares de neuronas corticales durante la abstinencia al GABA (Silva-Barrat et al., 1989; 1991; 2000) muestran despolarizaciones paroxísticas en prácticamente cada neurona que rodea al área perfundida con GABA. La mayoría de estas neuronas presentan trenes de descarga (bursts) intrínsecos, además de un decremento correlacionado en la conductancia del receptor $GABA_A$ bajo la exposición al GABA o del agonista isoguvacina (Champagnat et al., 1990; Silva-Barrat et al., 1989; 1991; 1992; 2000). De tal forma que las neuronas con trenes de descarga pueden explicar los potenciales de campo oscilatorios y el EEG paroxístico (Olsen y Avoli, 1997) durante la hiperexcitabilidad por abstinencia al GABA.

La desinhibición asociada al decremento en la respuesta del receptor $GABA_A$ durante la abstinencia al GABA que se demuestra en esta tesis, coincide con resultados reportados en diferentes condiciones epileptógenas, especialmente en modelos farmacológicos, genéticos o eléctricos de epilepsia en el hipocampo. Por ejemplo, en el modelo de epilepsia inducida por pilocarpina, Gibbs et al. (1997) reportaron cambios en la eficacia y potencia del GABA endógeno sobre la respuesta del receptor $GABA_A$, Ikeda-Douglas et al. (1998) refieren la falla de la inhibición GABAérgica posterior a la aplicación de ácido kaínico en el hipocampo, Fueta et al.

(1998) señalan el decremento intrínseco de la inhibición mediada por el receptor $GABA_A$ en la región CA1 de ratones (E1) genéticamente epilépticos, así como Rossler et al. (2000) la refieren durante el desarrollo del "kindling". Asimismo hay modelos *in vitro*, e.g., la preparación de rebanadas de hipocampo, en donde la desinhibición conduce al tejido a trenes de descargas sincrónicos (Wong y Traub, 1983) y actividad epileptógena (Karnup y Stelzer, 2001).

La desinhibición por la alteración en la transmisión GABAérgica o en los influjos de cloro mediados por GABA también parece ser una constante después de exposiciones prolongadas a sedantes del SN. El tratamiento crónico con benzodiacepinas produce un decremento en la eficacia (Hu y Ticku, 1994) y una reducción en la potencia (Xie y Tietz, 1992) del receptor $GABA_A$ ante agonistas selectivos para este receptor. El mismo tratamiento crónico con GABA o benzodicepinas conduce a un bloqueo de la inhibición recurrente mediada por el receptor $GABA_A$ (Xie y Tietz, 1991; García-Ugalde et al., 1992; Zeng et al., 1994; Calixto et al., 2000) y a una reducción del 60% en la amplitud de los potenciales postsinápticos inhibitorios tempranos mediados por el receptor $GABA_A$ (Zeng et al., 1995). Esto resulta en una pérdida de las corrientes inhibitorias postsinápticas miniatura (Poisbeau et al., 1997), así como al decremento en el número de receptores $GABA_A$ (Mehta y Ticku, 1992; Barnes, 1996). En resumen, los tratamientos crónicos con benzodiacepinas conducen al desarrollo de tolerancia, incluyendo una subsensibilidad intrínseca al GABA, así como la reducción en la actividad de los circuitos GABAérgicos y a la modulación de las propiedades intrínsecas de la neurona (Wilson, 1996).

Estos datos sugieren que la hiperexcitabilidad por abstinencia al GABA puede ser relacionada con estados convulsivos observados después de la interrupción de la medicación crónica con anticonvulsivantes GABAérgicos, tales como los barbitúricos, fenitoína o benzodiacepinas (Owen y Tyrer, 1983; Marciani et al., 1985; Davies et al., 1987; Lader, 1994; Doble y Martin, 1996). La comparación de la hiperexcitabilidad por abstinencia al GABA con la abstinencia a agentes GABAérgicos, especialmente las benzodiacepinas, merece algunas consideraciones.

Comparación de los estados de hiperexcitabilidad por abstinencia a benzodiacepinas y por abstinencia al GABA.

La comparación directa entre estados de hiperexcitabilidad por abstinencia a benzodiacepinas y la hiperexcitabilidad derivada de la suspensión de GABA, aunque razonable desde su convergencia en el mismo receptor, no resulta sencilla. Las dificultades pueden considerarse desde la aproximación experimental con la que habitualmente se estudian los fenómenos de tolerancia y abstinencia a agonistas del receptor $GABA_A$. En estos estudios normalmente se observa la conducta, el EEG y posteriormente los cambios *in vitro* (*ex vivo*), ya sean electrofisiológicos, bioquímicos o farmacológicos, los cuales son analizados en diferentes momentos después de la administración crónica de estos compuestos o durante su abstinencia (Silva-Barrat et al., 1989; 2000; Brailowsky et al., 1990; Carlen et al., 1990; Kang y Miller, 1991; Zeng et al., 1994; 1995; Barnes, 1996; Wilson, 1996; Toki et al., 1996; Tietz et al., 1999). No obstante, las variaciones en los procedimientos hace difícil las comparaciones (Löscher et al., 1996; Fahey et al., 1999), las diferencias regionales en los cambios del receptor (Wilson, 1996) y los diferentes tiempos de observación después de la suspensión del tratamiento producen resultados a veces contrastantes (Silva-Barrat et al., 1989; 2000; Brailowsky et al., 1990; Carlen et al., 1990; Kang y Miller, 1991; Zeng et al., 1994; 1995; Barnes, 1996; Wilson, 1996; Toki et al., 1996; Tietz et al., 1999).

Por otro lado estudiar los mecanismos a partir de los cuales se genera la hiperexcitabilidad desde su inicio presenta algunos problemas. La administración prolongada de los agonistas $GABA_A$ o el retiro súbito de estas drogas, puede conducir a cambios electrofisiológicos, incluyendo crisis epileptiformes, antes de realizar las preparaciones y los registros mismos (Davies et al., 1987; Brailowsky et al., 1988; Silva -Barrat et al., 1989; Gibbs et al., 1997; Poisbeau et al., 1997). Este es el caso para las crisis epilépticas inducidas por cualquier medio, por ejemplo las crisis inducidas por pilocarpina, ácido kaínico o 4-aminopiridina, todas conducen a cambios profundos en la neurotransmisión para el GABA, el glutamato y para el N-metil-D-aspartato (NMDA) (Carlen et al., 1990; Traub y Jefferys, 1994; Gibbs et al., 1997; Sperk et al., 1998). El

registro electrofisiológico de neuronas una vez que se ha presentado una crisis muestra diferentes cambios debidos a la crisis misma, tales como el nivel de despolarización de las células, la aparición de potenciales de meseta y descargas en trenes o ráfagas, junto con los cambios en el influjo de calcio que acompañan a estos eventos (Carlen et al., 1990; Traub y Jefferys, 1994; Silva-Barrat et al., 2000). En cuanto al receptor $GABA_A$, se ha reportado su desensibilización, la regulación hacia arriba (up-regulation) del número de receptores, así como de las múltiples subunidades que conforman su estructura heteromérica y la disminución en el pegado de diferentes agonistas $GABA_A$ (Galpern et al., 1991; Kang y Miller, 1991; Hu y Ticku , 1994; Mhatre y Ticku, 1994; Barnes, 1996; Toki et al., 1996; Holt et al., 1997; Tietz et al., 1999; Lyons et al., 2000). El momento en el que se suceden todos estos cambios, así como su especificidad, no se conoce. Por su parte la total inducción *in vitro* de la hiperexcitabilidad por abstinencia al GABA, como se presenta en esta tesis, tiene la ventaja de que las rebanadas de cerebro pueden ser registradas a cualquier tiempo entre el retiro del agonista y la estabilización del nuevo estado de hiperexcitabilidad, lo cual permite separar entre dos condiciones diferentes, es decir, los cambios atribuibles a la abstinencia o tolerancia propiamente, de los cambios producto de la actividad epileptógena y las crisis convulsivas consecuentes. Aunque la tolerancia seguida de abstinencia conduce a la hiperexcitabilidad (Barnes, 1996), su separación es necesaria para comprender cómo es que ésta ocurre.

A pesar de las consideraciones anteriores, es notable la cantidad de resultados coincidentes entre la hiperexcitabilidad por abstinencia a las benzodiacepinas y por abstinencia al GABA en el hipocampo. Después de la administración crónica de GABA o de agonistas para el receptor $GABA_A$, se encuentra tolerancia, dependencia e hiperexcitabilidad inducida por la abstinencia (Brailowsky, 1988; Barnes, 1996; Silva-Barrat et al., 1989; 2000). El mismo fenómeno se observa claramente después de la administración crónica de benzodiacepinas (Davies et al., 1988; Miller et al., 1988; Carlen et al., 1990; Barnes, 1996; Toki et al., 1996). En ambos casos, existe un decremento en la sensibilidad del receptor $GABA_A$ ante sus agonistas (Miller et al.,

1988; Silva-Barrat et al., 1989; Xie y Tietz, 1992; Wilson, 1996; Cash et al., 1997). La hiperexcitabilidad inducida por la abstinencia ha sido total y agudamente inducida *in vitro* en la preparación de rebanadas de hipocampo, tanto para las benzodiacepinas como para el GABA (Davies et al., 1987; García-Ugalde, 1992), lo cual no se ha realizado para otros agonistas alostéricos. Por lo menos en la corteza, el curso temporal es muy similar para la abstinencia al GABA y a las benzodiacepinas (Calixto et al., 2000). La transmisión GABAérgica, la inhibición y los influjos de cloro mediados por GABA, como se señaló anteriormente, se encuentran alterados posterior a la exposición tanto de GABA como de las benzodiacepinas (Xie y Tietz, 1991; Zeng et al., 1994; 1995; Wilson, 1996; Barnes, 1996; Poisbeau et al., 1997; García-Ugalde et al., 1992; Mehta y Ticku, 1992; Hu y Ticku, 1994; Calixto et al., 2000). Finalmente, como se demuestra en la presente tesis, el decremento en el número de receptores $GABA_A$ también se observa después de la exposición crónica de GABA como de benzodiacepinas.

Los síndromes de abstinencia asociados con agonistas alostéricos del receptor $GABA_A$ son clínicamente tratados con otros agonistas alostéricos o directos (e.g. el síndrome de abstinencia al alcohol se trata con benzodicepinas o GABA). Una consideración pertinente ante este procedimiento es el riesgo de desarrollar una abstinencia de segundo orden y por tanto a la pregunta ¿cuál de las abstinencias llegará a convertirse en la menor o la peor?

Disminución en el número de receptores $GABA_A$

Después de los diferentes análisis electrofisiológicos y farmacológicos del presente estudio se concluye que la hiperexcitabilidad por abstinencia al GABA es causada principalmente por una disminución en la densidad del receptor $GABA_A$. La reducción en el número de receptores $GABA_A$ en la membrana sináptica ha sido reportada por otros autores como resultado de la administración crónica de agonistas GABAérgicos (Miller et al, 1988; Roca et al., 1990; Calkin y Barnes, 1994) con la consecuente

alteración de la neurotransmisión GABAérgica pero sin cambios en la K_D o EC_{50} (potencia) en la farmacodinamia del receptor $GABA_A$ (Hu y Ticku, 1994) tal como ocurre en el presente estudio. La disminución o regulación hacia abajo del receptor $GABA_A$ por el mismo GABA también ha sido reportada pero sólo en cultivos de neuronas corticales (Maloteaux, et al., 1987; Mehta y Ticku, 1992; Mhatre y Ticku, 1994; Calkin y Barnes, 1994; Barnes, 1996).

El decremento en el número de receptores $GABA_A$ con la alteración consecuente en la inhibición GABAérgica también se ha reportado en modelos de epilepsia inducidos con ácido kainico (Friedman et al., 1994), en el modelo de epilepsia del lóbulo temporal (Rice, et al., 1996) y durante el "kindling" (Kampuis et al., 1995). El decremento en el número de receptores $GABA_A$ funcionales y el estado de desinhibición GABAérgica hipocampal producto de la inhibición selectiva en la biosíntesis de la subunidad $\gamma 2$ del receptor $GABA_A$, a través de oligonucleóticos antisentido, conduce al desarrollo de estatus epilépticus límbico y a cambios neurodegenerativos severos en el hipocampo después del tratamiento prolongado con el antisentido (Karle, 2002). Asimismo, el análisis inmunohistoquímico de las subunidades que conforman al receptor en tejido proveniente del modelo de ratón con malformación cortical inducida e hiperexcitabilidad intrinseca, muestra una disminución en diversas regiones cerebrales de las subunidades $\alpha 1$, 2, 3 y 5 y de la subunidad $\gamma 2$. (Redecker et al., 2000). Aunque en algunos otros estudios la evidencia sobre la disminución en el número de receptores no presenta resultados uniformes o bien estos son contradictorios (Duncan, 1999; Zilles et al., 1999).

La medición de las subunidades que comúnmente conforman al receptor $GABA_A$ nativo del SNC ha sido explorada después del tratamiento crónico con GABA o agonistas GABAérgicos. Se ha reportado la regulación hacia abajo y el recambio del RNAm de las subunidades $\alpha 1$, $\beta 2S$, y $\gamma 1$ después de la incubación con GABA por un período de 48 hrs. en cultivos de neuronas (Lyons et al., 2000). El tratamiento crónico con benzodiacepinas, como el flurazepam, disminuye el RNAm para las subunidades

$\alpha 1$ y $\beta 2$ y 3 (Tietz et al., 1999) o con lorazepam durante 14 días disminuye la concentración del RNAm de las subunidades $\alpha 1$ y $\gamma 2$ en aproximadamente un 50% (Kang y Miller, 1991). En el mismo periodo de tiempo, el tratamiento con diazepam (valium, 15mg/kg) decrementa el nivel de trascripción del gen para la subunidad gama2 (Holt et al., 1997). Lo cual apunta no sólo a la degradación del RNAm sino también a la disminución en la tasa de trascripción de las subunidades como mecanismos asociados. La subunidad $\alpha 4$ también parece jugar un papel importante en la abstinencia a neuroesteroides, dado que su supresión previene las características de la abstinencia a esteroides endógenos (Smith et al., 1998). En estos casos habitualmente la alteración en los niveles de RNAm ocurre de manera previa a la disminución del número de receptores y los cambios significativos aparecen después de varios días. En el caso de la hiperexcitabilidad por abstinencia al GABA, la disminución en el número de receptores ocurre en periodos mucho más cortos como se demuestra en el trabajo 2, lo cual apunta a que previo a una disminución en la síntesis del receptor existe un mecanismo de degradación más rápido asociado con la desensibilización y la hiperexcitabilidad.

La disminución o regulación hacia abajo del receptor $GABA_A$ después de tratamientos con GABA o agonistas GABAérgicos ha sido asociada con endocitosis. Se ha reportado el secuestro de receptores $GABA_A$ en vesículas cubiertas de clatrina después de la exposición prolongada de GABA o benzodiacepinas (Calkin y Barnes, 1994; Tehrani y Barnes, 1997; Barnes, 2000). No obstante, los mecanismos celulares de secuestro del receptor $GABA_A$ en vesículas cubiertas de clatrina no queda claro, pero la estabilidad del receptor en la superficie de la célula parece depender de la actividad de proteínas-cinasas, como la PKC y de la composición de las subunidades (Connolly et al., 1999; Krishek et al., 1994). La endocitosis del receptor $GABA_A$ pareciera ser el mecanismo más probable para explicar la desensibilización durante la abstinencia al GABA. Los resultados obtenidos en el presente estudio concuerdan entonces con estudios previos (Lyons et al., 2000). Sin embargo, la exploración sobre diferentes tiempos de exposición al agonista es necesaria para comprender en qué

momento se desarrollan los cambios. Así también, es recomendable estudiar los mecanismos de señalización intracelular que preceden al fenómeno.

En conclusión, encontramos que los diferentes mecanismos asociados a la abstinencia al GABA así como sus características electrofisiológicas y farmacológicas presentan interesantes analogías con la epilepsia y la abstinencia a fármacos sedantes. Para ambos casos, la pregunta se sostiene; ¿son los mismos mecanismos celulares los que le subyacen a todos estos estados de hiperexcitabilidad?. Si bien la epilepsia presenta una gran variedad de clasificaciones cada una con su respectiva sintomatología, así como una etiología ampliamente diversificada, sería difícil que un solo modelo experimental incluyera todas sus dimensiones. La abstinencia al GABA presenta una ventaja sobre otros modelos farmacológicos y es que la hiperexcitabilidad se genera a partir del propio agonista endógeno, el GABA, circunstancia que provee al fenómeno de implicaciones fisiológicas relevantes. En cuanto a la abstinencia al GABA como un modelo de abstinencia a fármacos sedantes como las benzodicepinas, los barbitúricos o el alcohol, también presenta notables virtudes, entre otras su rápida inducción y su reproductibilidad *in vitro*. Independientemente de la validación de fenómenos de hiperexcitabilidad como modelos experimentales, el adecuado funcionamiento del SNC requiere del balance entre excitación e inhibición en cada uno de sus circuitos. Bajo condiciones normales, los circuitos neuronales pueden desplegar un amplio rango de respuestas y cambios en la actividad dependiente de uso (plasticidad). Sin embargo, la alteración en el balance de los niveles de excitación-inhibición en el SNC resulta habitualmente en disfunciones de los circuitos neuronales involucrados, por lo que dichas alteraciones merecen del interés científico.

CONCLUSIONES.

La hiperexcitabilidad por abstinencia al GABA presenta:

A) EEG paroxístico (poliespigas, espigas-onda y espigas seguidas de actividad hipersincrónica) en el área circunscrita a la infusión.

B) Gliosis astrocítica moderada como reacción asociada a la hiperexcitabilidad.

C) Un incremento en la amplitud de la espiga poblacional y la aparición de espigas poblacionales múltiples epileptiformes (*in vitro* y *ex vivo*).

D) Un incremento de más del 100% en la excitabilidad neuronal, la cual radica esencialmente en la postsinápsis.

E) Una disminución significativa en la inhibición recurrente

F) Una facilitación en la inducción de la LTP.

G) Una reducción significativa en la respuesta máxima (eficacia) del receptor $GABA_A$ sin cambios significativos en la potencia (EC_{50}) ante la presencia de agentes GABAérgicos.

H) No se presentan cambios significativos en la afinidad del receptor (K_D) para agentes GABAérgicos.

I) Una disminución en el número del receptores $GABA_A$

J) Una correlación estrecha entre el decremento en el número de receptores y la evolución temporal de la hiperexcitabilidad.

REFERENCIAS GENERALES.

Amara, S.G. and Kuhar, M.J. (1993) Neurotransmitter transporters: recent progress. Annu. Rev. Naurosci. 16: 73-93.

Barnes E.M. Jr. (1996) Use-dependent regulation of $GABA_A$ receptors. Int. Rev. Neurobiol. 39:53-76

Barnes, E.M. Jr. (2000) Intracellular trafficking of $GABA_A$ receptors. Life Sci 66, 1063-1070

Becker, A., Krug, M. and Schroder, H. (1997) Strain differences in pentylenetetrazol-kindling development and subsequent potentiation effects. Brain Res. 763, 87-92.

Bernard, C., Esclapez, M., Hirsch, J.C. and Ben-Ari, Y. (1998) Interneurones are not so dormant in temporal lobe epilepsy: a critical reappraisal of the dormant basket cell hypothesis. Epilepsy Research 32: 93-103

Bloom, F.E. and Iversen, L.L. (1971) Localizing 3H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. Nature, 229: 628-630.

Brailowsky, S., Menini, C., Silva-Barrat, C. and Naquet, R. (1987) Epileptogenic gamma-aminobutiric acid-withdrawal syndrome after chronic, intracortical infusion in baboons. Neurosciences Letters. 74: 75-80.

Brailowsky, S., Kinimoto, M., Silva-Barrat, C., Riche, D. and Naquet, R. (1988) The GABA-Withdrawal syndrome: a new model of focal epileptogenesis. Brain Res. 442: 175-179.

Brailowsky, S., Silva-Barrat, C., Menini, C., Riche, D. and Naquet, R. (1989) Effects of localized chronic GABA infusion into different cortical areas of the photosensitive baboon Papio papio. Electroenceph. Clin. Neurophysiol. 72: 147-156.

Brailowsky, S., Kunimoto, M., Silva-Barrat, S., Menini, C. and Naquet, R. (1990) Electroencephalografhic study of the GABA-Withdrawal syndrome in rats. Epilepsia. 31(4): 369-377.

Brailowsky, S. Epilepsia. Enfermedad sagrada del cerebro. Fondo de Cultura Económica, México, D.F. 1999.

Calkin, P. and Barnes, E. Jr. (1994). Gamma-aminobutyric acid-A (GABAA) agonist down-regulate GABAA/benzodiazepine receptor polypeptides from the surface of chick cortical neurons. J. Biol. Chem. Jan 14; 269(2): 1548-1553.

Calixto, E., López-Colomé, A.M., Casasola C., Montiel, T., Bargas, J. and Brailowsky, S. (2000) Neocortical hyperexcitability after GABA withdrawal in vitro. Epilepsy Res. 39, 13-26.

Carlen, P.L., Davies, M.F., Rougier-Naquet, I., Reynolds, J.N. and Spigelman, I., 1990, Sedative drugs withdrawal seizures: cellular electrophysiological mechanisms. In: Avoli, M., Gloor, P., Kostopoulos, G., Naquet, R. (Eds.). Generalized Epilepsy: Neurobiological Approaches. Birkhauser. Boston. pp. 460-470.

Cash, D.J., Serfözö, P. and Allan, A.M. (1997) Desensitization of a gamma-aminobutyric acid type A receptor in rat is increased by chronic treatment with chlordiazepoxide: a molecular mechanism of dependence. J Pharmacol Exp Ther. 283: 704-71.

Champagnat, J., Silva-Barrat, C., Brailowsky, S. and Menini, Ch., 1990. Possible epileptogenic consequences of misused GABAergic relationships. In: Avoli, M., Gloor, P., Kostopoulos, G.,

Naquet, R. (Eds.). Generalized Epilepsy: Neurobiological Approaches. Birkhauser. Boston. pp. 118-125.

Cherubini, E. and Conti, F., 2001. Generating diversity at GABAergic synapses. Trends Neurosci. 24, 155-162

Clarke, E. and O'Malley, C. D. The human brain and spinal cord. University of California Press. Los Angeles, USA, 1968.

Connolly, C.N., Kittler, J.T., Thomas, P., Uren, J.M., Brandon, N.J., Smart, T. G. and Moss, S.J. (1999) Cell Surface Stability of -Aminobutyric Acid Type A Receptors. Dependence on protein kinase C activity and subunit composition. J. Biol. Chem. 274: 36565-36572.

Connors, B.W. and Gutnick. M.J., 1984. Cellular mechanisms of neocortical epileptogenesis in a acute experimental model. In P. Schwartzroin and H. Wheal (Eds.). Electrophysiology of epilepsy. Academic, London. pp. 79-105.

Craig, K., Gomez, H.F., McManus, J.L. and Bania, T.C. (2000) Severe gamma-hydroxybutyrate withdrawal: a case report and literature review. J. Emerg. Med. 18: 65-70

Curtis, D.R. and Watkins, J.C. (1965) The pharmacology of amino acids related to gamma-aminobutyric acid. Pharmacol. Rev. 17: 347-391.

Davies, C., Davies, S. and Collinridge, G. (1990) Paired pulse depression on monosynaptic GABA mediated inhibitory postsynaptic response in the rat hippocampus. J. Physiol. (Lond). 424, 513-531.

Davies. M.F., Sasaki, S.E. and Carlen, P.L. (1987) Benzodiazepine-induced epileptiform activity in vitro. Brain Res. 437, 239-244.

Davies, M.F., Sasaki, S.E. and Carlen, P.L. (1988) Hyperexcitability of hippocampal CA1 neurons in brain slices of rats chronically administered clonazepam. J. Pharmachol. Exp. Ther. 247: 737-744.

Deutch. A. and Roth, R. Neurotransmitters. En Zigmond, M., Bloom, F, Landis, S., Roberts, J. y Squire , L. Fundamental Neurosciences. Cap. 8. Academic Press. California, USA, 1999.

Doble, A. and Martin, I. (1996) The GABAA /benzodiazepine receptor as a target for psychoactive drugs. Neuroscience Intelligence Unit, Chapman and Hall, New York.

Duncan. J.S. (1999) Positron emission tomography receptor studies. Adv Neurol. 79: 893-899.

Enna, S.J. and Karbon, E.W., (1986) GABA receptors: an overview. En Olsen, R.W. and Ventes, J.C. (Eds.) Benzodiacepine/GABA receptors and chloride channels: structural and functional properties. pp: 41-56.

Fahey, J.M., Pritchard, G.A., Grassi, J.M., Pratt, J.S., Shader, R.I. and Greenblatt, D.J. (1999) In situ hybridization histochemistry as a method to assess GABA(A) receptor subunit mRNA expression following chronic alprazolam administration. J Psychopharmacol. 13(3): 211-218.

Feldman, R.S., Meyer, J.S. and Quenzer, L.F. Principles of neuropsychopharmacology. Sinauer Associates, Inc. Sunderland, Massachusetts, USA, 1997.

Franke. H. and Kittner, H. (2001) Morphological alterations of neurons and astrocytes and changes in emotional behavior in pentylenetetrazol-kindled rats. Pharmacol Biochem Behav. Oct-Nov; 70(2-3): 291-303.

Friedman, L.K., Pellegrini-Giampietro, D.E., Sperber E.F., Bennett, M.V.L. and Zukin, R. S. (1994) Kainate-induced status epilepticus alters glutamate and GABAA receptor gene expression in adult rat hippocampus: an in situ hybridization study. J. Neurosci. 14: 2697-2707.

Fueta. Y., Kawano, H., Ono, T., Mita, T., Fukata, K. and Ohno, K. (1998) Regional differences in hippocampal excitability manifested by paired-pulse stimulation of genetically epileptic El mice. Brain Res. 1;779(1-2): 324-328.

Fukuda, H., Brailowsky, S., Menini, C., Silva-Barrat, C., Riche, D. and Naquet, R. (1987) Anticonvulsant effect of intracortical, chronic infision of GABA in kindled rats: Focal seizures upon withdrawal. Exp. Neurol. 98, 120-129.

Galpern, W.R., Lumpkin, M., Greenblatt, D.J., Shader, R.I. and Miller, L.G. (1991) Chronic benzodiazepine administration. VII. Behavioral tolerance and withdrawal and receptor alterations associated with clonazepam administration. Psychopharmacology. 104: 225-230.

Ganong, W.F. Fisiología Médica. Ed. El manual moderno, S.A. de C.V. México, 1990. pp 86-88

García-Ugalde, G., Galarraga, E., Bargas, J. and Brailowsky, S. (1992) Hyperexcitability of hipocampal CA1 region in brain slices after GABA withdrawal. Neurosciences letters. 142: 229-232.

Gibbs III, J.W., Shumate, M.D. and Coulter, D.A. (1997) Differential epilepsy-associated alterations in postsynaptic $GABA_A$ receptor function in dentate granule and CA1 neurons. J. Neurophysiol. 77: 1924-1938.

Granger, P., Biton, B., Faure, C., Vige, X. Depoortere, H., Graham, D., Langer, S.Z., Scatton, B. and Avonet P. (1995) Modulation of the Gamma-aminobutyric acid type A receptor by the antiepileptic drug carbamazepine an phenytoin. Molecular Pharmacology 47: 1189-1196.

Gutiérrez, R. and Heinemann, U. (2001) Kindling induces transient fast inhibition in the dentate gyrus--CA3 projection. Eur J Neurosci. Apr; 13(7): 1371-1379.

Harris, R.A. and Allan, A.M. (1985) Functional coupling of gamma-aminobutyric acid receptors to chloride channels in brain membranes. Science. 228: 1108-1110.

Hevers, W. and Lüddens, H. (1998) The diversity of $GABA_A$ receptors. Molecular Neurobiology. 18: 35-86.

Hu, X. and Ticku, M. (1994) Chronic flurazepam treatment produces decreased efficacy of the benzodiazepine ligands and pentobarbital with g -aminobutyric acid A receptor subtypes. J. Pharmacol. Exp. Ther. 270: 485-490.

Holt, R.A., Martin, I.L. and Bateson, A.N. (1997) Chronic diazepam exposure decreases transcription of the rat $GABA_A$ receptor γ2-subunit gene. Mol Brain Res. 48: 164-166.

Ikeda-Douglas, C. J., Head, E., Holsinger, R. M. D., Tremblay, L., Racine, R. and Milgram, N. W. (1998) Selective loss of early suppression in the dentate gyrus precedes kainic acid induced electrographic seizures. Epilepsy Res. 31, 143-152.

Ishizuka, S. and Hayashi, H. (1998) Spontaneous epileptiform bursts and long-term potentiation in rat CA3 hippocampal slices induced by chaotic stimulation of mossy fibers. Brain Res. 790, 108-114.

Julien, R.M. A primer of drug action: a concise, nontechnical guide to the actions, uses and side effects of psychoactive drugs. Ninth Edition, Worth Publishers, NY, USA, 2001.

Kampuis, W., DeRijk, T.C. and daSilva, F.H.L. (1995) Expression of GABAA receptor subunit mRNAs in hippocampal pyramidal and granular neurons in the kindling model of epileptogenesis: an in situ hybridization study. Brain Res. 31: 33-47.

Kandel, E., Schwartz, J. y Jessell, T. (Eds.) Principles of Neural Science. Third Edition. Ed. Elsevier Science Publishing Co., Inc. New York. 1991.

Kang, I. and Miller, L.G. (1991) Decreased $GABA_A$ receptor subunit mRNA concentration following chronic lorazepam administration. Br. J. Pharmacol. 103: 1285-1287.

Kapur, A. and Haberly, L.B. (1998) Duration of NMDA-dependent synaptic potentiation in piriform cortex in vivo is increased after epileptiform bursting. J. Neurophysiol. 80, 1623-1629.

Karle, J. (2002) Antisense studies of brain GABAA receptors. Dan Med Bull.49(2):130-44.

Karnup, S. and Stelzer, A. (2001) Seizure-like activity in the disinhibited CA1 minislice of adult guinea-pigs. J. Physiol. 532(Pt 3): 713-730.

Kemp, J. A., Marshall, G. R. and Woodruff, G. N. (1986) Quantitative evaluation of the potencies of GABA-receptor agonists and antagonists using the rat hippocampal slice preparation. British. Journal of Pharmacology. 87: 677-884.

Kostopoulos, G.K. and Psarropoulou, C.T., 1992. Possible mechanisms underlying hyperexcitability in the epileptic mutant mouse tottering. J. Neural Transm. [Suppl] 35, 109-124.

Krishek, B. J., Xie X., Blackstone, C., Huganir, R.L., Moss, S.J. and Smart, T.G. (1994) Regulation of $GABA_A$ receptors function by protein kinase C phosphorylation. Neuron. 12: 1081-1095.

Krishek, B. J., Moss, S. J. and Smart, T. G. (1996) A functional comparison of the antagonists bicuculline and picrotoxin at recombinant $GABA_A$ receptor. Neuropharmacol. 35: 1289-1298.

Krnjevic, K. and Schwartz, S. (1966) Is gamma-aminobutyric acid an inhibitory transmitter? Nature. Sep 24;211(56): 1372-4

Lader, M. (1994) Biological processes in benzodiazepine dependence. Addiction. 89: 1413-1418.

Le Gal la Salle, G., Brailowsky, S., Menini, C. and Naquet, R. (1988) Local asymptomatic status epilepticus induced by withdrawal of GABA infusion into limbic structures. Exp. Neurol. 101: 411.

Loscher, W., Rundfeldt, C., Hönack, D. and Ebert, U. (1996) Long-term studies on anticonvulsant tolerance and withdrawal characteristics of benzodiazepine receptor ligands in different seizure models in mice. I. Comparison of diazepam, clonazepam, clobazam and abecarnil. J Pharmacol Exp Ther. 279: 561-572.

Lossin, C., Wang, D.W., Rhodes, T.H., Vanoye, C.G. and George, A.L. (2002) Molecular basis of an inherited epilepsy. Neuron. Jun 13;34(6):877-84.

Lyons, H.R., Gibbs, T.T. and Farb, D.H. (2000) Turnover and down-regulation of GABA(A) receptor $\alpha 1$, $\beta 2S$, and $\gamma 1$ subunit mRNAs by neurons in culture. J Neurochem. 74: 1041-1048.

MacDonald, R.L. and Olsen, R.W. (1994) $GABA_A$ receptor channels. Annu. Rev. Neurosci. 17: 569-602.

Mackenzie, L., Medvedev, A., Hiscock, J.J., Pope, K.J. and Willoughby, J.O. (2002) Picrotoxin-induced generalised convulsive seizure in rat: changes in regional distribution and frequency of the power of electroencephalogram rhythms. Clin. Neurophysiol. Apr;113(4): 586-596.

Maloteaux, J.-M., OctaveJ.-N., Gossuin, A., Laterre, C. and Trouet, A. (1987) GABA induces down-regulation of the benzodiazepine-GABA receptor complex in the rat cultured neurons. Eur. J. Pharmacol. 144: 173-183.

Marciani, M.G., J. Gotman, F. Anderman and A. Oliver. (1985) Patterns of seizure activation after withdrawal of antiepileptic medication. Neurology. 35: 1537-1543.

Martin, DL and Rimvall K. (1993) Regulation of gamma-aminobutyric acid syntesis in the brain. J Neurochem. 60: 395-407.

McNarama, J.O. (1994) Cellular and Molecular basis of epilepsy. J. Neurosciences. 14: 3413-3425.

McNaughton, B. L. and Barnes, C. A. (1990) From cooperative synaptic anhancement to associative memory: brinding the abyss. Semin. Neurosci. 2:403-416.

Mehta, A.K. and Ticku, M.K. (1992) Chronic GABA exposure down-regulates GABA-benzodiazepine receptor-ionophore complex in cultured cerebral cortical neurons. Mol Brain Res. 16, 29-36.

Mehta, A.K. and Ticku, M.K. (1999) An update of $GABA_A$ receptors. Brain Research Reviews. 29: 196-217

Menini, CH., Mraovitch, S., Calando, Y., De la Sayette, V., Silva-Barrat, C., Brailowsky, S. and Seylaz, J. (1991) Metabolic anatomy of the focal epilepsy produced by cessation of chronic intracortical GABA infusión in the rat. Neuroscience, 41(2/3): 607-615.

Mhatre, M.C. and Ticku, M.K. (1994) Chronic GABA treatment downregulates the GABAA receptor alpha 2 and alpha 3 subunit mRNAS as well as polypeptide expression in primary cultured cerebral cortical neurons. Mol. Brain Res. 24: 159-165.

Miller, L.G., Greenblatt, D.J., Barnhill, J.G. and Shader, R.I. (1988) Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric $acid_A$ receptor function. J. Pharmacol. Exp. Ther. 246: 170-176.

Mody, Y., DeKoninck, Y., Otis, T.S. and Sotesz, I. (1994) Bridging the cleft at GABA synapses in the Brain. TiNS. 17: 517-525.

Montiel, T., Almeida, D., Arango, I., Calixto, E., Casasola, C. and Brailowsky, S. (2000) Long-Lasting Effects of GABA Infusion into the Cerebral Cortex of the Rat. Neural Plasticity. 7, 1-8.

Olsen, W. R. and Avoli, M., (1997) GABA and Epileptogenesis. Epilepsia. 38: 399-407.

OMS. World Healt Organzation (1997). http://www.who.int/inf-fs/en/fact165.html

Owen, R.T. and Tyrer, P. (1983) Benzodiazepine dependence. A review of the evidence. Drugs. 25: 385-398.

Poisbeau, P., Williams, S.R. and Mody, I. (1997) Silent $GABA_A$ synapses during flurazepam withdrawal are region-specific in the hippocampal formation. J Neurosci. 17, 3467-3475.

Qian, H. and Dowling, J.E. (1995) $GABA_A$ and $GABA_C$ receptors on hybrid bass retinal bipolar cells. J. Neurophysiol. 74: 1920-1928.

Ramón y Cajal, S. (1894) Croonian Lecture. La fine structure des centres nerveux. Proc R. Soc. (London). 55: 444-468.

Redecker, C,. Luhmann, H.J., Hagemann, G., Fritschy, J.M. and Witte, O.W. (2000) Differential downregulation of GABAA receptor subunits in widespread brain regions in the freeze-lesion model of focal cortical malformations. J. Neurosci. 1;20(13): 5045-53.

Rice, A., Rafiq, A., Shapiro, S.M., Jakoi, E.R., Coulter, D.A. and DeLorenzo R. J. (1996) Long-lasting reduction of inhibitory function and g-aminobutyric acid type A receptor subunit mRNA expression in a model of temporal lobe epilepsy. Proc. Natl Acad. Sci. 93,:9665-9669.

Roberts, E. (1974) Gamma-aminobutyric acid and nervous system function – a perspective. Biochem. Pharmacol. 23: 2637-2649.

Roca, D.J., Rozenberg, I., Farrant, M. and Farb, D.H. (1990) Chronic agonist exposure induces down-regulation and allosteric uncoupling of the γ-aminobutyric acid/benzodiazepine receptor complex. Mol. Pharmacol. 37: 37-43.

Rossler, A.S., Launay, J.M., Venault, P., Dodd, R.H., Chapouthier, G. (2000) Changes in benzodiazepine binding in a subkindling situation. Epilepsia. Jun;41(6): 651-4.

Salazar, P., Montiel, T., Brailowsky, S. and Tapia, R. (1994) Decrease of glutamate decarboxylase activity after *in vivo* cortical infusion of γ-aminobutyric acid. Neurochem. Int. 24 (4): 363-368.

Salazar, P. and Tapia, R. (2001) Seizures induced by intracerebral administration of pyridoxal-5'- phosphate: effect of GABAergic drugs and glutamate receptor antagonists. Neuropharmacology, 41: 546-553.

Sandoval, M.R. and Palermo-Neto, J. (1986) GABAergic influences on barbital withdrawal induced convulsions. Gen. Pharmacol. 17: 431-435.

Saunders, P.A. and Ho, I.K. (1990) Barbiturates and the $GABA_A$ receptor complex. Prog Drug Res. 34: 261-286

Schneiderman, J. H. (1997) The role of Long-term Potentiation in persistent epileptiform burst-induced hyperexcitability following $GABA_A$ receptor blockade. Neuroscience. 81: 1111-1122.

Schultz, L.M. (1997) GABAergic inhibitory processes and hippocampal long-term potentiation. Neuroscientist. 3, 226-236

Schwartzkroin, P.A. and Prince, D.A. (1977) Penicillin-induced epileptiform activity in the hippocampal in vitro preparation. Ann. Neurol. 1: 463-469.

Sherrington, C. S. (1906) The integrative action of the nervous system. Scribner, NY (2nd Ed., New Haven CT: Yale University Press, 1947)

Siegel, G., Agranoff, B., Albers, W. and Molinoff, P. Basic Neurochemistry. Raven Press, N. Y. 1994.

Silva-Barrat, C., Champagnat, J., Brailowsky, S., Menini, C. and Naquet, R. (1989) Relationship between tolerance to $GABA_A$ agonist and bursting properties in neocortical neurons during GABA-withdrawal syndrome. Brain Research, 498: 289-298.

Silva-Barrat, C., Champagnat, J., Brailowsky, S., Menini, C., and Naquet, R. (1991) In vivo and in vitro studies of the epileptic focus induced by tha interruption of a cortical GABA infusion. Experimental Brain Research. Series 20: 107-110.

Silva-Barrat, C., Araneda, S., Menini, CH., Champagnat, J. and Naquet, R. (1992) Burst generation in neocortical neurons after GABA withdrawal in the rat. Journal of Neurophysiology. 67(3): 715-727.

Silva-Barrat, C., Champagnat, J. and Menini, C. (2000) The GABA-withdrawal syndrome: a model of local status epilepticus. Neural Plasticity. 7: 9-18.

Simmonds, M. A. (1982) Classification of some GABA antagonists with regard to site of action and potency in slices of rat cuneate nucleus. Eur. J. Pharmac. 80: 347-358.

Sloviter, R.S. (1991) Feedforward and feddback inhibition of hippocampal principal cell activity evoked by perforant path stimulation: GABA-mediated mechanisms that regulate excitability. Hippocampus 1, 31-40.

Smith, S.S., Gong, Q.H., Hsu, F.C., Markowitz, R.S., ffrench-Mullen, J.M. and Li, X. (1998) $GABA_A$ receptor $\alpha 4$ suppression prevents withdrawal properties of an endogenous steroid. Nature. 392: 926-930.

Sperk, G., Schwarzer, C., Tunashima, K. and Kandlhofer, S. (1998) Expression of $GABA_A$ receptor subunits in the hippocampus of the rat after kainic acid-induced seizures. Epilepsy Res. 32: 129-139.

Tapia, R. Biochemical pharmachology of GABA in CNS. In:. Iversen, L.L., Iversen, S.D., Snyder, S.H. (Eds), Handbook of Psychopharmacology, vol. 4. New York, Plenum Press. 1971.

Tehrani, M.H. and Barnes, E.M. Jr. (1997) Sequestration of gamma-aminobutyric acidA receptors on clathrin-coated vesicles during chronic benzodiazepine administration in vivo. J Pharmacol. Exp. Ther. 283: 384-90.

Ticku, M.K., Burch, T.P. and Davis, W.C. (1983) The interactions of ethanol with the benzodiazepine-GABA receptor-ionophore complex. Pharmacol. Biochem Behav. 18 (Suppl 1): 15-18.

Tietz, E.I., Huang, X., Chen, S. and Ferencak, W.F. (1999) Temporal and regional regulation of $\alpha 1$, $\beta 2$ and $\beta 3$, but not $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 1$ or $\gamma 2$ $GABA_A$ receptor subunit messenger RNAs following one-week oral flurazepam administration. Neuroscience. 91: 327-41.

Toki, S., Saito, T., Nabeshima, A., Hatta, S., Watanabe, M. and Takahata, N. (1996) Changes in $GABA_A$ receptor function and cross-tolerance to ethanol in diazepam-dependent rats. Alcohol Clin Exp Res. 20: 40A-44A

Traub, D. R. y Jefferys G. R. J. (1994) Are there unifying principles underlying the generation of epileptic afterdischarges in vitro? Progress in Brain Research, 102(chap. 26): 383-394

Voet, D. and Voet, J.G. Biochemistry. Second Edition. Jhon Wiley & Sons, Inc. NY, USA, 1995.

Wilson, M.A. (1996) GABA physiology: modulation by benzodiazepines and hormones. Crit. Rev. Neurobiol. 10, 1-37

Wong, R.K.S. and Traub, R. D. (1983) Syncronized burst discharges in disinhibited hippocampal slices. I. Initiation in CA2-CA3 region. J. Neurophysiol. 49: 442-458.

Xie, X.H. and Tietz, E.I. (1991) Chronic benzodiazepine treatment of rats induces reduction of paired-pulse inhibition in CA1 region of in vitro hippocampus. Brain Res. 561, 69-76.

Xie, X.H. and Tietz, E.I. (1992) Reduction in potency of selective gamma-aminobutyric acidA agonists and diazepam in CA1 region of in vitro hippocampal slices from chronic flurazepam-treated rats. J Pharmacol. Exp. Ther. Jul, 262(1): 204-211.

Xu, M. and Akabas, M.H. (1996) Identification of channel-lining residues in the M2 membrane-spanning segment of the $GABA_A$ receptor alfa1 subunit. J. Gen. Physiol. 107: 195-205.

Zeng, X., Xie, X.-H. and Tietz, E.I. (1994) Impairment of feedforward inhibition in CA1 region of hippocampus after chronic benzodiazepine treatment. Neurosci. Lett. 173, 40-44.

Zeng, X., Xie, X.-H. and Tietz, E.I. (1995) Reduction of GABA-mediated inhibitory postsynaptic potentials in hippocampal CA1 pyramidal neurons following oral flurazepam administration. Neuroscience. 66: 87-99.

Zilles, K., Qu, M.S., Kohling, R. and Speckmann, E.J. (1999) Ionotropic glutamate and GABA receptors in human epileptic neocortical tissue: quantitative in vitro receptor autoradiography. Neuroscience. 94(4):1051-61.

Zimmermann H. (1979) Vesicle recycling and transmitter release. Neuroscience 4(12): 1773-804.

PAGINACIÓN
DISCONTINUA

HYPEREXCITABILITY INDUCED BY GABA WITHDRAWAL FACILITATES HIPPOCAMPAL LONG-TERM POTENTIATION.

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[*] Posthumous work.

ABBREVIATIONS:

BFS	Brief frequency stimulation
BZD	Benzodiazepines
CA1	Ammon's horn region 1
CNS	Central nervous system
EC_{50}	Effective concentration required for half-maximal activation
EEG	Electroencephalographic
Emax	Effective concentration required for maximal activation
fEPSP	Field excitatory postsynaptic potential
GABA	Gamma-aminobutyric acid
$GABA_A$	Gamma-aminobutyric acid receptor type A
GFAP	Glial fibrillary acidic protein
GW	GABA withdrawal
K_A	Affinity constant
K_D	Dissociation constant
LTP	Long-term potentiation
PS	Population spike

Running title: GABA withdrawal facilitates hippocampal LTP

ABSTRACT

In some mammals, epileptic seizures have been induced in the cerebral cortex, hippocampus and other limbic structures after the sudden suppression of chronically infused γ-aminobutyric acid (GABA). This hyperexcitability state induced by the endogenous neurotransmitter resembles the withdrawal seizure-responses to other $GABA_A$ receptor agonists such as benzodiacepines, barbiturates and alcohol.

Hyperexcitability-induced by GABA withdrawal also persists in *ex-vivolin-vitro* conditions. Hippocampal slices, obtained from rats with seizures induced by GABA-withdrawal showed field potential oscillations and paroxysmal activity in the CA1 region. During GABA-withdrawal hyperexcitability the threshold of hippocampal Long-term Potentiation (LTP) decreased to a point in which a brief frequency stimulation that normally failed to produce long lasting changes in the synaptic strength, was now able to induce LTP. Facilitation of the LTP induction was associated with a decreased $GABA_A$-mediated inhibitory activity, because the effect of the $GABA_A$ receptor antagonist, bicuculline, was occluded during hyperexcitability and the dose-response curve for bicuculline showed a 50% efficacy reduction with a shift in the EC_{50} from 4.5 to 1.1 mM relative to controls. Nevertheless, the K_D of the antagonist did not change significantly.

Our results support the idea that changes in hippocampal plasticity under altered inhibitory neurotransmission states, like those induced by withdrawal syndromes to anxiolytic, sedative or anticonvulsant drugs may be engaged during seizures.

Key words: LTP, GABA withdrawal, Plasticity, Seizure, Hippocampus, $GABA_A$ receptor.

INTRODUCTION

The correct functioning of the central nervous system (CNS) requires the balance between excitation and inhibition in each of its circuits. Under normal conditions, neuronal circuits may display an ample range of responsiveness and activity-dependent (plastic) changes. However, impairments in the excitation-inhibition balance due to lesions in the CNS often result in misfunction of the circuits involved. If synaptic inhibition is impaired, the consequence is an increase in the excitability of neuronal cells (i.e. hyperexcitability). In the CNS the major inhibitory neurotransmitter is the γ-aminobutyric acid (GABA) whose inhibitory effect is mainly mediated by the $GABA_A$ receptor. The topical or systemic administration of compounds that block the $GABA_A$ receptor, such as penicillin, bicuculline or picrotoxin, produces hyperexcitability, paroxysmal depolarization shifts and epileptiform activity (Schwartzkroin and Prince, 1977; Wong and Traub, 1983; Connors and Gutnick, 1984; Schneiderman, 1997; Czlonkowska et al., 2000; Karnup and Stelzer, 2001).

Neural hyperexcitability and seizure activity can also be produced when drugs that increase $GABA_A$-mediated inhibition, like benzodiazepines, barbiturates and alcohol, are suddenly withdrawn (Ticku et al., 1983; San~~doval and Palermo,~~

1986; Carlen et al., 1990; Olsen and Avoli, 1997). These $GABA_A$ agonists are commonly prescribed as anxiolytic, sedative or anticonvulsant drugs and are among the most used and abused drugs. Their prolonged administration can result in tolerance and physical dependence. When these compounds are abruptly suspended withdrawal syndromes are produced, which are characterized by irritability, anxiety, tremors, changes in perception, as well as hallucinations and seizures (Owen and Tyrer, 1983; Davies et al., 1987; Lader, 1994; Doble and Martin, 1996). Brain slices obtained from animals subjected to chronic exposure to benzodiazepines (BZD), barbiturates or steroids display hyperexcitability and have impaired synaptic inhibition (Davies et al., 1987, 1988; Silva-Barrat et al., 1989; Zeng et al., 1995; Carlen et al., 1990; Saunders and Ho, 1990; Smith et al., 1998).

GABA withdrawal (GW) itself, also induces hyperexcitability and epileptic seizures. Sudden interruption of chronic intracortical infusion of GABA produces electroencephalographic (EEG) spikes, polyspikes and spikes-and-wave discharges in baboons and rats (Brailowsky et al., 1987; 1988; 1990; Le Gal La Salle et al., 1988; Silva-Barrat et al., 1989, 2000); this paroxysmal activity may be associated with myoclonic activity, wet-dog shakes, salivation, and changes in motor and exploratory behavior. A similar case of human GABA-withdrawal syndrome has been reported after discontinuation of orally administered gamma-hydroxybutyrate (Craig et al., 2000). Furthermore, hyperexcitability by GABA withdrawal is induced completely in the *in vitro* slice preparations after sudden interruption of superfused GABA in both hippocampal and cortical slices (Garcia-

Ugalde et al., 1992; Calixto et al., 2000). These spontaneous and paroxysmal discharges have been considered as an experimental model of local epileptogenesis (Brailowsky et al., 1988) and resemble the withdrawal responses to other $GABA_A$ receptor agonists, like benzodiazepines, barbiturates and alcohol (Casasola et al., 2001; Calixto et al., 2000; Brailowsky and Garcia, 1999; Carlen et al., 1990), but presently induced by the endogenous neurotransmitter itself.

Modification of the strength of synaptic connections is critical to many aspects of nervous system physiology. Long-term potentiation (LTP) is a plastic phenomenon originally described by Bliss and Lømo (1973) and defined as a long-lasting increase of the synaptic efficacy following brief afferent tetanic stimulation (Gustafsson and Wigström, 1988). LTP has become the dominant model of activity-dependent synaptic plasticity in the mammalian brain and has been related with learning and memory (Bliss and Collingridge, 1993; Malenka, 1991, 1995; Lynch et al., 1990). LTP depends mainly on glutamatergic transmission and calcium influx (Malenka, 1995; Bliss and Collingridge, 1993; Collingridge et al., 1983) but $GABA_A$-mediated inhibitory can play an important role in this phenomenon (Ishizuka and Hayashi, 1998; Schneiderman, 1997; Schultz, 1997; Wigström and Gustafsson, 1986, 1985).

Evidence suggests that changes in network excitability resulting from hyperexcitability and epileptic seizures induce a hippocampal long lasting increase in synaptic efficacy (Schneiderman, 1997) or have an enabling action on the

induction of persistent synaptic potentiation in piriform cortex (Kapur and Haberly, 1998), and in hippocampus after kindling (Becker et al., 1997). Inversely, LTP induction can be followed by epileptiform activity (Ishizuka and Hayashi, 1998) and by a reduction in the GABAergic recurrent and/or feedforward inhibitory synaptic transmission that may contribute to seizure hyperexcitability (for a review see Schultz, 1997).

Hippocampus is an interesting brain region to analyze how mismatches in the inhibition/excitation balance affects neuronal circuitry function, since is a classical plasticity-inductable brain region and also the generator of intractable epileptic seizures; both characteristics coexist but not enough is known of the interaction between them. To analyze this interaction, we first explored if hyperexcitability induced by GABA withdrawal affects LTP induction in rat hippocampal brain slices. In addition we explored the possible role of a decrease in GABAergic neurotransmission in this phenomenon.

EXPERIMENTAL PROCEDURES

Animal preparation and EEG recordings

Male wistar rats (U.N.A.M. breeding unit) weighing 200-250 g were prepared as already described (Brailowsky, 1988) and all efforts were made to minimize the number of animals used and their suffering. Under general anesthesia (Halotane

5.0% and gas mixture of 95% O_2 / 5% CO_2), rats were implanted with epidural screws for chronic EEG recordings. Additionally each rat was implanted bilaterally with stainless-steel cannula (0.6 mm o.d.) in the CA1 hippocampus region (AP: -3.6, L: +/- 2.5, V: 2.5mm from bregma and bone surface; see ref. Paxinos and Watson, 1986), cannulas were also used to record the intrahippocampal EEG, allowing drug infusion and electrical recording. Reference electrode was located over the frontal sinus. One week after surgery, in separate groups of animals (n=14), saline or GABA (50 μg/μl, delivery rate 3 μl/2h) was unilaterally applied into the hippocampus for a period of 120 min using a programmable pump (Harvard). EEG was recorded (P5 grass pre-amplifier) before, during and after GABA or saline infusion in the control group.

Immunocytochemistry.

Some of the rats with GABA or saline infusion (n=6) were used for histological analysis. After saline infusion or GABA withdrawal, rats were perfused intracardially with cold buffered saline followed by PLP (phosphate-lisine-paraformaldehyde) fixative for glial fibrillary acidic protein (GFAP) immunohistochemistry.

Coronal sections (40 μm) were sequentially incubated in the following solutions: 1) primary antibody against GFAP (Dako, Denmark), diluted 1:2000 in PBS 0.1 M, containing 0.3% Triton X-100 and 2% normal goat serum for 72 h at 4°C; 2) biotinylated secondary antibody, diluted 1:200 in the same buffer as in 1,

for 2 h; 3) avidin-biotin-peroxidase complex (ABC) (vector, Inc.), prepared according to manufacturer's instructions in PBS-GT (vector, Inc.); 4) 0.05% 3,3'-diaminobenzidine in trizma (Sigma), pH 7.2, with 0.03% hydrogen peroxide for 5-10 min. Three 10-min rinses were performed between steps. All incubations were performed at room temperature unless otherwise noted. GFAP-reactive areas were mapped based on the atlas of Paxinos and Watson (1986).

Slice preparation and extracellular recording.

Rats were anesthetized with ether and decapitated. The brain was rapidly removed and each hemisphere sectioned with a vibratome in sagital slices of 400 μm. Slices containing dorsal hippocampus were placed in cold Ringer-Krebs solution of the following composition (in mM): 125 NaCl, 3 KCl, 1.0 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, 11 glucose, pH was adjusted to 7.4 after saturation with 95% O_2 and 5% CO_2, 300 mOsM/L. The slices were left to stabilize 45 min in the same solution before any further procedure. All the experiments were performed with the slices totally submerged in the recording chamber with a Ringer-Krebs perfusion rate of 2-3 ml/min. (34 °C). Extracellular field recordings of synaptic and population spikes (PS) were obtained with glass microelectrodes (3-5 MΩ) filled with NaCl (0.9%) and placed in CA1 stratum pyramidale. Bipolar concentric electrodes were used for orthodromic stimulation of the Shaffer collateral-commissural fibres. Stimulus were square wave pulses (40-60 μs) delivered at 0.08 Hz for continuous recording. This stimulation frequency did not produce potentiation, depression or

multiple spikes in control slices. Excitability curves of the PS were obtained with stimulus values ranging from threshold to maximal response and measures were made from the first deflection of the field excitatory postsynaptic potential (fEPSP) to the peak negativity of the spike. For the remainder of the experiment the stimuli were adjusted to produce a response 50-60% of maximum. Recordings were amplified with an AC amplifier (Grass P5; filters = 0.1 Hz-3.0 KHz), displayed in a digital oscilloscope and stored for later analysis.

Hyperexcitability induced by GABA withdrawal and LTP parameters.

In-vitro hyperexcitability by GABA withdrawal was induced in hippocampal slices from a completely new group of rats as previously reported (García-Ugalde et al., 1992; Calixto et al., 2000). After a stabilization period of 45 min slices were incubated with GABA (5 mM) dissolved in Ringer-Krebs for 120 min at room temperature (21 °C). GABA was then washed off abruptly. Control slices were incubated for the same time in the Ringer-Krebs solution without GABA. Continuous recordings were taken and PS monitored. Stability of PS-amplitude normally takes place after 120-150 min. of GABA washout, at this moment both GABA withdrawal and control slices were stimulated with a brief frequency stimulation (BFS) that consists of 8 pulses at 40Hz (200mseg) every 5 sec., 2 times at low orthodromic stimulation strength. This BFS normally does not induce a LTP or if it does it is a very weak one.

Pharmacological analysis.

New control and GABA-withdrawal hyperexcitability slices were obtained and after stabilization of the PS amplitude, dose-response relationships (Fig. 6) were constructed for the action of the competitive $GABA_A$ receptor antagonist, bicuculline on the PS amplitude. Stimulus strength was adjusted to produce a response of about 50 to 60% of the maximal response (approximately 1.5 - 2 fold the threshold value) and 20 min. of base line was recorded, then either control and GABA-withdrawal hyperexcitability slices were exposed to the different doses of bicuculline, the drug was perfused in the bathing medium for 10 min. Hyperexcitability by GABA withdrawal was produced as described above.

Concentration-response plots were fitted with the Hill equation (Fig. 6), such that PS amplitude response, DR, at any bicuculline concentration, [bicuc], is given by:

$$DR = Emax / (1+(EC_{50}/[bicuc]))^{n} \quad (1)$$

where *Emax* corresponds to the maximal PS amplitude in the presence of bicuculline as a percentage of control, *[bicuc]* is the bicuculline concentration plotted in logarithmic scale, EC_{50} is the effective concentration of bicuculline that increases the PS amplitude to 50% of its maximal amplitude, and *n* is the Hill coefficient. To see the shift of the function along the concentration axis, the maximum effect (*Emax*) obtained from the fitted function data was normalized to 1

(Fig. 6B). Student's t-tests were used to predict the level of statistical significance (p value < 0.05) as appropriate. To assess the significance in the bicuculline dose-response shifts were determined from the fits of the Hill equation to the individual data sets.

Drugs.

GABA and bicuculline were purchased from Sigma (St. Louis, MO). They were applied from freshly prepared stock solutions to the superfusion saline.

RESULTS

Hippocampal EEG paroxysmal activity after GABA-withdrawal (GW).

One week after surgery, and before any other manipulation, basal EEG was recorded for confirmation of clinical EEG normality (Fig 1A). Subsequently GABA (50 μg/μl, 3 μl/120min) was unilaterally applied to the hippocampus. Approximately 90 min after cessation of GABA infusion paroxysmal discharges appear bilaterally (Fig. 1B) in 100% of the rats (n=14), as previously reported (Brailowsky et al., 1987, 1988, 1990; Le Gal La Salle et al., 1988; Silva-Barrat et al., 1992). As shown in Fig. 1C, seizures induced by GW were characterized by polyspikes and spike-and-wave discharges followed by hypersynchronic activity. Abnormal activity lasted for several days (Fig. 1D). Electroencephalic activity was associated with myoclonic activity, wet-dog shakes, salivation and behavior changes.

INSERT FIG. 1 HERE

Reactive gliosis after GW.

Immunocytochemical analysis was used to confirm the correct electrode location and hippocampal cellular response to the hyperexcitability induced by GABA withdrawal. In groups of rats from GABA or from saline infusion (n=6), antibodies against the glial fibrillary acidic protein (GFAP) were used to determine reactive gliosis. Tissue perfused with GABA or saline had positive reactions against astroglial protein in the area surrounding the cannula trajectory across the cortex and at the end of the cannula tract, corresponding to the CA1 hippocampal region (Fig. 2 A y B). Moderate increase in the reactive tissue was notorious in the area infused in the GABA treated rats (Fig. 2B; arrows) when compared with the saline infused group (Fig. 2A).

INSERT FIG. 2 HERE

Cellular electrophysiological correlates of GW seizures

To evaluate if hyperexcitability by GW persists in an *ex-vivo* fashion, slices were obtained from the hippocampus of rats that had been previously infused with GABA. Extracellular field recordings in the CA1 region of slices taken from animals that presented EEG paroxysmal activity, exhibited epileptiform field potential

responses to a single orthodromic stimulus (Fig. 3A). The field potentials recorded from the periphery of the GABA infused area had at least 3 or 4 population spikes (PS) at intermediate or at high stimulus intensities (Fig. 3B). Control slices from saline infused group only had one PS, even at high intensities of afferent stimulation ($n=8$, $p < 0.001$ T-Student). These results confirm that epileptiform activity after the GABA withdrawal is maintained both *in-vivo* as *in-vitro* conditions, in the hippocampus as in other structures (Champagnat et al., 1990; Silva-Barrat et al. 1991, 1992; Garcia-Ugalde et al., 1992).

INSERT FIG. 3 HERE

LTP was significantly facilitated by GW.

To test for a convergence point between classical hippocampus plasticity and epileptiform activity, we analyzed LTP induction in GW slices. Hyperexcitability by GABA-withdrawal was reproduced in hippocampal slices by superfusing and withdrawing GABA (Garcia-Ugalde et al. 1992; Calixto et al., 2000). Responses before and after GABA withdrawal were compared ($n=7$). Under normal conditions slices were stimulated with brief frequency stimulation (BFS; see methods), which only produced short lasting changes in the PS amplitude (Fig. 4A). Subsequently slices were exposed to GABA; the inhibitory effect of the neurotransmitter was rapidly observed (Fig. 4B). After GABA cessation hyperexcitability was progressively produced (Fig. 4C), under this condition the same BFS induced

Long-term potentiation (Fig. 4D). In other words, the threshold for LTP induction was reduced under hyperexcitability.

INSERT FIG. 4 HERE

To verify that the time since slices were obtained or the time in which slices were stimulated did not influence LTP induction, BFS was applied to control slices that had never been exposed to GABA but at exactly the same time that it was applied in the GABA-exposed and withdrawal slices. BFS applied to control slices only produced short-lasting effects but in GW slices BFS produced an enhancement of 127% ± 0.4 in the mean of the PS amplitudes (Fig. 5). Significant differences (t-Student, $p < 0.05$) were notorious in the recordings when both groups were compared (n=8 for control and n=7 for GW). It was also observed that when the slices were stimulated with a frequency sufficient to induce strong LTP in normal conditions, the PS amplitude in both GW and normal slices did not differ (data not shown), indicating that GW facilitates the LTP generation, but not its maximal amplitude. This may suggest that both phenomena share mechanisms or synaptic resources.

INSERT FIG. 5 HERE

Decreased $GABA_A$-mediated inhibitory action after GW.

To analyze the $GABA_A$ receptor activity, dose-response curves of the $GABA_A$ receptor antagonist bicuculline were performed in control and GW slices. Figure 6A shows that bicuculline increased PS amplitude in a dose-dependent manner in both control and GW slices. However, maximal enhancement produced by bicuculline was reduced in a 49.9% in GW slices. Emax in the PS amplitude was: 56.7 ± 12.3 % in control slices and 28.3 ± 3.1 % in GW slices ($n=6$, $p < 0.05$; Student's t). Fitting of the entire dose-response curves gave almost identical Hill coefficients (1:1 control slices and 1:2 for GW slices).

Dose-response curves were normalized to their maximal effect (Fig. 6B), despite a right-ward shift observed in GW slices, the decrease in the bicuculline effect was not associated with a significant alteration in the sensitivity to the $GABA_A$ antagonist. The bicuculline EC_{50} was 1.1 ± 0.6 μM for controls and 4.5 ± 1.1 μM in GW slices. This result suggests that the difference in bicuculline effect was not due to a change in receptor affinity. To further test this inference, a Lineweaver-Burk analysis (Fig. 6C) of dose-response curves was performed. This analysis confirmed that the change in efficacy was not accompanied by a significant change in the potency of bicuculline; in control and GW slices, the EC_{50} value was 0.8 μM.

INSERT FIG. 6 HERE

DISCUSSION

Withdrawal of GABAergic drugs, such as barbiturates, benzodiazepines or alcohol, leads to an increase in the hippocampal excitability and epileptiform activity (Olsen and Avoli, 1997; Carlen et al., 1990; Sellers, 1988; Davies et al., 1987, 1988; Theodore et al., 1987). This abnormal neural activity is frequently associated with a reduction of the GABA-mediated inhibitory action (Zeng et al., 1994, 1995; Xie and Tietz, 1992; Saunders et al., 1992). In this study we reproduced hippocampal epileptic seizures as a result of the withdrawal of GABA (Montiel et al., 2000; Brailowsky et al., 1987, 1988, 1990; Fukuda et al., 1987; Le Gal La Salle et al., 1988; Silva-Barrat et al., 1989, 1991, 2000). Extracellular recordings showed that hyperexcitability by GW is very consistent *in-vivo* as *in-vitro*. Paroxysmal activity was highly preserved in the hippocampal slices obtained from seizure GW-induced rats, in which multiple population spikes were elicited by a single stimulus. Additionally, tissue from rats with GW processed for glial-fibrillary acidic protein immunoreactivity showed astrocytic reactive gliosis in the hippocampal infused site as reaction associated with hyperexcitability.

Epileptic EEG activity and the correlated extracellular paroxysmal activity during GW may result from the oscillatory and synchronous responses of neurons under a decreased inhibitory activity. Intracellular recordings from cortical slices with GW (Silva-Barrat et al., 1989, 1991, 2000) showed paroxysmal depolarization shifts in virtually every neuron surrounding the GABA infused site. Most of these neurons presented intrinsic bursting properties and correlated decrease in the conductance of the $GABA_A$ receptor under the exposure to GABA or the agonist

isoguvacine (Champagnat et al., 1990; Silva-Barrat et al., 1989, 1991, 1992, 2000). Bursting neurons may explain oscillatory field potentials and paroxysmal EEG (Olsen and Avoli, 1997) during GW. The decrease in the inhibitory activity during GW is clearly observed in the bicuculline dose-response relation described in this study, in which efficacy of bicuculline was reduced to the 50% after GW without significant changes in the K_A. These results are consistent with the $GABA_A$ subsensitivity to the GABA itself or to a diversity of other $GABA_A$ receptor agonists, including benzodiazepines (Mehta and Ticku, 1992) during withdrawal states and also with the reduction in the GABA-mediated recurrent inhibition during paired pulse test in slices with GW (Casasola et al., 2001; Calixto et al., 2000; Garcia-Ugalde et al., 1992).

The cellular decrease in the sensitivity to GABAergic agonist after chronic exposures to GABA also appear after benzodiazepines, barbiturates and alcohol withdrawal (Xie and Tietz, 1992; Cash et al., 1997; Olsen and Avoli, 1997), suggesting that the $GABA_A$ receptor had gone through affinity changes, number of receptors, subunit composition or coupling between $GABA_A$ receptors and BZD receptors (Olsen and Avoli, 1997). Recently we have demonstrated that down regulation of $GABA_A$ receptor is temporally and functionally involved in the hippocampal hyperexcitability induced by GW (Casasola et al., 2001). Environmentally induced epilepsies may result from "aberrant plasticity" (Olsen et al., 1997; Olsen and Avoli, 1997) in which $GABA_A$ receptor may be persistently impaired under conditions such as discontinuation of chronic GABAergic drugs,

stress trauma or kindling. Aberrant plasticity may involve mechanisms such as a subunit switch (e.g., a new receptor isoform that is less functional might become relatively more abundant), a change in a regulatory system (e.g. steroids, zinc, or phosphorylation, including calcium regulated kinases or phosphatases) or a change in the number of receptors (e.g. intracellular trafficking of GABA receptors; for a review see Barnes, 2000).

Strong and persistent GABAergic inhibitory inputs to the hippocampal principal cells may prevent changes in synaptic strength by bursts of afferent activity during injury, but when desinhibition occurs (i.e. hyperexcitability and bursting neurons in GW), synaptic strength may be altered and epileptogenic activity may engage synaptic plasticity mechanisms. It must be considered that the hippocampus, apart from its wide range of plastic changes, is a region especially susceptible to intractable epileptic seizures. Mesial temporal lobe epilepsy is characterized by recurrent complex partial seizures and hippocampal damage often associated with Ammon's horn sclerosis (Kardos, 1999; Olsen and Avoli, 1997) and is accompanied by severe gliosis and sprouting in this area (Babb et al., 1989).

The whole cellular and synaptic bases of hippocampal epileptic syndromes remain to be determined. However, robust evidence suggests that long lasting changes in hippocampal synaptic efficacy take place during network hyperexcitability, resulting from seizure (Schneiderman, 1997; Chavez-Noriega et

al., 1989; Kapur and Haberly, 1998; Becker et al., 1997). Studies using epileptic animal models reported significantly greater LTP under hyperexcitability (Wieraszko and Seyfried, 1993) or additive effects between electrical and chemical kindling, indicating the existence of mechanisms in common (Bradford, 1995). Hyperexcitability may take place spontaneously after plasticity; LTP induction can be followed by epileptiform activity (Ishizuka and Hayashi, 1998; Schultz, 1997). On the other hand drugs usually employed in psychiatric therapeutics, with the property of enhanced GABA-mediated inhibition, can suppress LTP induction with corresponding impaired spatial learning (McNarama et al., 1993).

An essential requirement for generating LTP is presynaptic activity during sufficient postsynaptic membrane depolarization (Wingström and Gustafsson, 1986; Gustafsson and Wigström, 1988; Bliss and Collingridge, 1993; Malenka, 1991, 1995). Several studies had shown that LTP induction can be facilitated by factors that increase postsynaptic depolarization (Kelso et al., 1986; Wingström and Gustafsson, 1986; Gustafsson y Wigström, 1990; McNaughton and Barnes, 1990; Nicoll and Malenka, 1995), among them, the use of $GABA_A$ antagonists like bicuculline, penicillin and picrotoxin (Wigström and Gustafsson, 1985). In this study the facilitated LTP induction may be related with the increase in the postsynaptic depolarization in response to the minor GABAergic activity during seizures.

The marked reduction of $GABA_A$-inhibitory action during GW could engage and take advantage of synaptic plasticity resources in the hippocampal network, or

at least of a kind of "aberrant plasticity". In this sense epileptogenic activity and neural plasticity may in some circumstances share cellular and synaptic mechanisms and thus promote a cooperation effect between them. This cooperative relation may contribute to hyperexcitability getting stronger and rejecting classical therapeutics or pharmachological approaches in some kinds of epileptic seizures.

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REFERENCES

Babb, T.L., Pretorius, J.K., Kupfer, W.R. and Crandall, P.H. (1989) Glutamate decarboxylase-immunoreactive neurons are preserved in human epileptic hippocampus. J. Neurosci. **9**, 2562-2574.

Barnes, E.M. Jr. (2000) Intracellular trafficking of $GABA_A$ receptors. Life Sci. **66**, 1063-1070.

Becker, A., Krug, M. and Schroder, H. (1997) Strain differences in pentylenetetrazol-kindling development and subsequent potentiation effects. Brain Res. **763**, 87-92.

Bliss, T.V.P. and Lømo, T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anesthetized rabbit following stimulation of the perforant path. J. Physiol. (London). **232**, 331-356.

Bliss, T.V.P. and Collingridge, G. L. (1993) A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature*. **361**, 31-39.

Bradford, H.F. (1995) Glutamate, GABA and epilepsy. Prog. Neurobiol. **47**, 477-511.

Brailowsky, S., Menini, C., Silva-Barrat, C. and Naquet, R. (1987) Epileptogenic gamma-aminobutiric acid-withdrawal syndrome after chronic, intracortical infusion in baboons. Neurosci. Lett. **74**, 75-80.

Brailowsky, S., Kinimoto, M., Silva-Barrat, C., Riche, D. and Naquet, R. (1988) The GABA-Withdrawal syndrome: a new model of focal epileptogenesis. Brain Res. **442**, 175-179.

Brailowsky, S., Kunimoto, M., Silva-Barrat, S., Menini, C. and Naquet, R. (1990) Electroencephalografhic study of the GABA-Withdrawal syndrome in rats. Epilepsia. **31**, 369-377.

Brailowsky Simón. and Garcia Octavio. (1999) Ethanol, GABA and Epilepsy. Arch. Med. *Res.* **30**, 3-9.

Calixto, E., López-Colomé, A.M., Casasola C., Montiel, T., Bargas, J. and Brailowsky, S. (2000) Neocortical hyperexcitability after GABA withdrawal in vitro. Epilepsy Res. **39**, 13-26.

Carlen, P.L., Davies, M.F., Rougier-Naquet, I., Reynolds, J.N. and Spigelman, I., 1990, Sedative drugs withdrawal seizures: cellular electrophysiological mechanisms. In: Avoli, M., Gloor, P., Kostopoulos, G., Naquet, R. (Eds.). Generalized Epilepsy: Neurobiological Approaches. Birkhauser. Boston. pp. 460-470.

Casasola, C., Bargas, J., Arias-Montaño, J.A., Calixto, E., Montiel, T., Galarraga E., and Brailowsky S. (2001) Hippocampal hyperexcitability induced by GABA withdrawal is due to down-regulation of $GABA_A$ receptors. Epilepsy Res. **47**(3), 257-271.

Cash, D.J., Serfözö, P., Allan, A.M. (1997) Desensitization of a gamma-aminobutyric acid type A receptor in rat is increased by chronic treatment with chlordiazepoxide: a molecular mechanism of dependence. J Pharmacol Exp Ther 283, 704-71

Champagnat, J., Silva-Barrat, C., Brailowsky, S. and Menini, Ch., 1990. Possible epileptogenic consequences of misused GABAergic relationships. In: Avoli,

M., Gloor, P., Kostopoulos, G., Naquet, R. (Eds.). Generalized Epilepsy: Neurobiological Approaches. Birkhauser. Boston. pp. 118-125.

Chavez-Noriega, L.E., Bliss, T.V.P. and Halliwell, J.V. (1989) The EPSP-spike (E-S) component of long-term potentiation in the rat hippocampal slice is modulated by GABAergic but not cholinergic mechanisms. Neurosci. Lett. **104**, 58-64.

Collingridge, G., Kehl, S. and McLennan, H. (1983) The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones in vitro. J. Physiol. (London). **334**, 33-46.

Connors, B.W. and Gutnick. M.J., 1984. Cellular mechanisms of neocortical epileptogenesis in a acute experimental model. In P. Schwartzroin and H. Wheal (Eds.). Electrophysiology of epilepsy. Academic, London. pp. 79-105.

Craig, K., Gomez, H.F., McManus, J.L. and Bania, T.C. (2000) Severe gamma-hydroxybutyrate withdrawal: a case report and literature review. J. Emerg. Med. **18**, 65-70.

Czlonkowska, A.I., Krzascik, P., Sienkiewicz-Jarosz, H., Siemiatkowski, M., Szyndler, J., Bidzinski, A., and Plaznik. A. (2000) The effects of neurosteroids on picrotoxin-, bicuculline- and NMDA-induced seizures, and a hypnotic effect of ethanol. Pharmacol. Biochem. Behav. **67**, 345-53.

Davies, M.F., Sasaki, S.E. and Carlen, P.L. (1987) Benzodiazepine-induced epileptiform activity in vitro. Brain Res. **437**, 239-244.

Davies, M.F., Sasaki, S.E. and Carlen, P.L. (1988) Hyperexcitability of hippocampal CA1 neurons in brain slices of rats chronically administered clonazepam. J. Pharmachol. Exp. Ther. **247**, 737-744.

Doble, A. and Martin, I. (1996) The GABAA /benzodiazepine receptor as a target for psychoactive drugs. Neuroscience Intelligence Unit, Chapman and Hall, New York.

Fukuda, H., Brailowsky, S., Menini, C., Silva-Barrat, C., Riche, D. and Naquet, R. (1987) Anticonvulsant effect of intracortical, chronic infision of GABA in kindled rats: Focal seizures upon withdrawal. Exp. Neurol. **98**, 120-129.

Garcia-Ugalde, G., Galarraga, E., Bargas, J. and Brailowsky, S. (1992) Hyperexcitability of hippocampal CA1 region in brain slices after GABA withdrawal. Neurosci. lett. **142**, 229-232.

Gustafsson, B. and Wigström, H. (1988) Physiological mecanisms underlying long-term potentiation. Trens in Neurosci. **11**, 156-162.

Gustafsson, B. and Wingström, H. (1990) Long-term potentiation in the hipoccampal CA1 region: its induction and early temporal development. Prog. Brain Res. **83**, 223-232.

Ishizuka, S. and Hayashi, H. (1998) Spontaneous epileptiform bursts and long-term potentiation in rat CA3 hippocampal slices induced by chaotic stimulation of mossy fibers. Brain Res. **790**, 108-114.

Kardos, J. (1999) Recent advances in GABA research. Neurochem. Int. **34**, 353-358.

Kapur, A. and Haberly, L.B. (1998) Duration of NMDA-dependent synaptic potentiation in piriform cortex in vivo is increased after epileptiform bursting. J. Neurophysiol. **80**, 1623-1629.

Karnup, S. and Stelzer, A. (2001) Seizure-like activity in the disinhibited CA1 minislice of adult guinea-pigs. J. Physiol. **532**(Pt 3), 713-730.

Kelso, S. R., Ganong, A. H. and Brown, T. H. (1986) Hebbian synapses in hippocampus. Proc. natn. Acad. Sci. USA **83**, 5326-5330.

Lader, M. (1994) Biological processes in benzodiazepine dependence. Addiction **89**, 1413-1418.

Le Gal la Salle, G., Brailowsky, S., Menini, C. and Naquet, R. (1988) Local asymptomatic status epilepticus induced by withdrawal of GABA infusion into limbic structures. Exp. Neurol. **101**, 411-417.

Lynch, G., Kessler, M., Arrai, A. and Larson, J. (1990) The nature and causes of hippocampal long-term potentiation. Prog. Brain Res. **83**, 233-250.

Mehta, A.K. and Ticku, M.K. (1992) Chronic GABA exposure down-regulates GABA-benzodiazepine receptor-ionophore complex in cultured cerebral cortical neurons. Mol. Brain Res. **16**, 29-36.

Malenka R.C. (1991) Pstsynaptic factors control the duration of synaptic enhancement in area CA1 of the hippocampus. Neuron, **6**, 53-60.

Malenka, R. (1995) LTP and LTD: Dynamic and interactive processes of synaptic plasticity. Neuroscientist, **1**, 35-42.

McNamara. R., DePape, G. and Skelton, R. (1993) Differential effects of benzodiazepine receptor agonists on hippocampal long-term potentiation and espatial learning in the Morris water maze. Brain Res. **629**, 63-70.

McNaughton, B. L. and Barnes, C. A. (1990) From cooperative synaptic anhancement to associative memory: brinding the abyss. Semin. Neurosci. **2**, 403-416.

Montiel, T., Almeida, D., Arango, I., Calixto, E., Casasola, C. and Brailowsky, S. (2000) Long-Lasting Effects of GABA Infusion into the Cerebral Cortex of the Rat. Neural Plasticity. **7**, 1-8.

Nicoll, R. A. and Malenka, R. C. (1995) Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature. **377**, 115-118.

Olsen, W. R. and Avoli, M., (1997) GABA and Epileptogenesis. Epilepsia **38**, 399-407.

Olsen, R.W., DeLorey, T.M., Gordey, M. and Kang, M.H., 1997. GABA receptor function and epilepsy. In: Delgado-Escueta, A.V., Wilson, W.A., Olsen, R.W. and Porter, R.M. (Eds.) Basic mechanisms of the epilepsies. New York: Lippincott-Raven.

Owen, R.T. and Tyrer, P. (1983) Benzodiazepine dependence. A review of the evidence. Drugs. **25**, 385-398.

Paxinos, G and Watson, C. (1986) The Rat Brain in estereotaxic coodinates. Second Edition. Academic Press INC. USA.

Saunders, P.A., Kimura, T., Miyaoka, T. and Ho, IK. (1992) Effects of pentobarbital tolerance and dependence on convulsant and $GABA_A$ receptor antagonist binding. Life Sci. **50**, 1701-1709.

Sandoval, M.R. and Palermo-Neto, J. (1986) GABAergic influences on barbital withdrawal induced convulsions. Gen. Pharmacol. **17**, 431-435.

Schneiderman, J. H. (1997) The role of Long-term Potentiation in persistent epileptiform burst-induced hyperexcitability following $GABA_A$ receptor blockade. Neuroscience. **81**, 1111-1122.

Schwartzkroin, P.A. and Prince, D.A. (1977) Penicillin-induced epileptiform activity in the hippocampal in vitro preparation. Ann. Neurol. **1**, 463-469.

Sellers, E.M. (1988) Alcohol, barbiturate and benzodiazepine withdrawal syndromes: clinical management. CMAJ. **139**, 113-120.

Silva-Barrat, C., Champagnat, J., Brailowsky, S., Menini, C. and Naquet, R. (1989) Relationship between tolerance to GABAa agonist and bursting properties in neocortical neurons during GABA-withdrawal syndrome. Brain Res. **498**, 289-298.

Silva-Barrat, C., Champagnat, J., Brailowsky, S., Menini, C., and Naquet, R. (1991) In Vivo and In Vitro Studies of the epileptic focus induced by the interruption of cortical GABA infution. Exp. Brain Res. **20**, 107-110.

Silva-Barrat, C., Araneda, S., Menini, Ch., Champagnat, J. and Naquet, R. (1992) Burst generation in neocortical neurons after GABA withdrawal in the rat. J. Neurophysiol. **67**, 715-727.

Silva-Barrat, C., Champagnat, J. and Menini, C. (2000) The GABA-withdrawal syndrome: a model of local status epilepticus. Neural Plasticity. **7**, 9-18.

Smith, S.S., Gong, Q.H., Hsu, F.C., Markowitz, R.S., ffrench-Mullen, J.M. and Li, X. (1998) $GABA_A$ receptor $\alpha 4$ suppression prevents withdrawal properties of an endogenous steroid. Nature **392**, 926-930.

Schultz, L.M. (1997) GABAergic inhibitory processes and hippocampal long-term potentiation. Neuroscientist. **3**, 226-236.

Theodore, W.H., Porter, R.J. and Raubertas, R.F. (1987) Seizures during barbiturate withdrawal: relation to blood level. Ann. Neurol. **22**, 644-647.

Ticku, M.K., Burch, T.P. and Davis, W.C. (1983) The interactions of ethanol with the benzodiazepine-GABA receptor-ionophore complex. Pharmacol. Biochem Behav. **18** (Suppl 1), 15-18.

Wieraszko, A. and Seyfried, T.N. (1993) Influence of audiogenic seizures on synaptic facilitation in mouse hippocampal slices is mediated by N-methyl-D-aspartate receptor. Epilepsia. **34**, 979-984.

Wingström, H. and Gustafsson, B. (1985) Facilitation of hippocampal long-lasting potentiation by GABA antagonists. Acta Physiol. Scand. **125**, 159-172.

Wingström, H. and Gustafsson, B. (1986) Postsynaptic control of hippocampal long-term potentiation. J. Physiol. *(Paris)*, **81**, 228-236.

Wong, R.K.S. and Traub, R. D. (1983) Syncronized burst discharges in disinhibited hippocampal slices. I. Initiation in CA2-CA3 region. J. Neurophysiol. **49**, 442-458.

Xie, X.-H., Tietz E.I. (1992) Reduction in potency of selective gamma-aminobutiric acidA agonist and diazepam in CA1 region of in Vitro hippocampal slices from chronic flurazepam-treated rats. J. Pharmacol. Exp. Ther. **262**, 204-211.

Zeng, X., Xie, X.H. and Tietz, E.I. (1994) Impairment of feedforward inhibition in CA1 region of hippocampus after chronic benzodiazepine treatment. Neurosci. Lett. **173**, 40-44.

Zeng, X., Xie, X.H. and Tietz, E.I. (1995) Reduction of GABA-mediated inhibitory postsynaptic potentials in hippocampal CA1 pyramidal neurons following oral flurazepam administration. Neuroscience. **66**, 87-99.

FIGURE LEGENDS

Figure 1. Representative intrahippocampal EEG recordings before and after GABA-withdrawal (GW). **A.** Control EEG recordings taken from the left (L) and the right (R) hippocampus before GABA infusion. **B.** Paroxysmal activity began spontaneously after 90 min of cessation of GABA infusion in the left hippocampus. Note strong propagation to the right hippocampus. **C.** Spike-and-waves followed by hypersynchronic discharges are present bilaterally 120 min after GW. **D.** Seizures due to GW persisted for several days.

Figure 2. Moderate reactive gliosis after hippocampal GW. Micrograph of coronal sections of rats' brains processed for immunocytochemical GFAP analysis after hippocampal saline infusion (A) or hippocampal GW (B). Note the reactive tissue along the cannula trajectory, in the end of the cannula tract corresponding to the hippocampal CA1 region and a moderate major reaction in the side infused with GABA (arrows in B) as compared with the control slice (A). The same cannula served as electrode for intrahippocampal EEG recording.

Figure 3. Epileptiform discharges are evoked in slices from animals with GW. **A.** Extracellular population spikes (PS) in the CA1 pyramidal cell layer in normal slices (left) and in slices taken from GABA-withdrawal animals (right). Paroxysmal discharges in the periphery of the GABA infusion area are preserved *ex vivo*. Responses are evoked at different stimulus strength. **B.** The box plot summarizes statistical significance in the number of PS evoked after GW as compared with the controls (n=8, $p < 0,001$, Student's *t* test). Stimulus strength was 3 X threshold.

Figure 4. GABA-withdrawal facilitates Long-term potentiation. Circles represent PS amplitude evoked by a single stimulus. **A.** In normal conditions brief frequency stimulation (BFS) produced short lasting changes in PS amplitude. **B.** The addition of 5 mM GABA (2 hrs) to the saline bath inhibited the PS. **C.** After a sudden GABA washout, the tissue develops hyperexcitability as seen by a progressive increase in PS amplitude. **D.** 120-150 min after GW, the amplitude and

PS number had stabilized. Now, the same BFS is capable of producing LTP (n=7, $p < 0.05$, Student's *t* test).

Figure 5. Time course of PS amplitude after a train of stimuli. Time courses are compared in control (white circles, n = 8) and GW (dark circles, n = 7) slices after they were stimulated with a couple of brief frequency trains (BFS = 200mS at 40Hz two times at 5Hz). This stimuli fails to induce long-lasting changes (LTP) in control slices but successful induces it when the slices are in a hyperexcitability state by the GABA-withdrawal. Statistical differences ($p < 0.05$) are marked with asterisks. In both conditions the slices have exactly the same time since they were obtained from the corresponding rats' brains.

Figure 6. A decrease in sensitivity to bicuculline was found in GABA-withdrawal slices. A. The $GABA_A$ receptor antagonist bicuculline enhances PS amplitude in a dose-dependent way. Dose-response plots for bicuculline are shown for controls (white circles) and for slices after GW (dark circles). Note After GW, bicuculline has lost efficacy. PS was increased by 28% after GW and 57% in the controls (n=6). **B.** Normalized dose-response plots show a rightward shift in EC_{50} for bicuculline: 1.1 μM in the control vs. 4.5 μM after GW. **C.** Lineweaver-Burk analysis shows that this shift does not represent a change in receptor affinity. Thus, bicuculline efficacy may be due to a decrease in the number of $GABA_A$ receptor (Casasola et al., 2001).

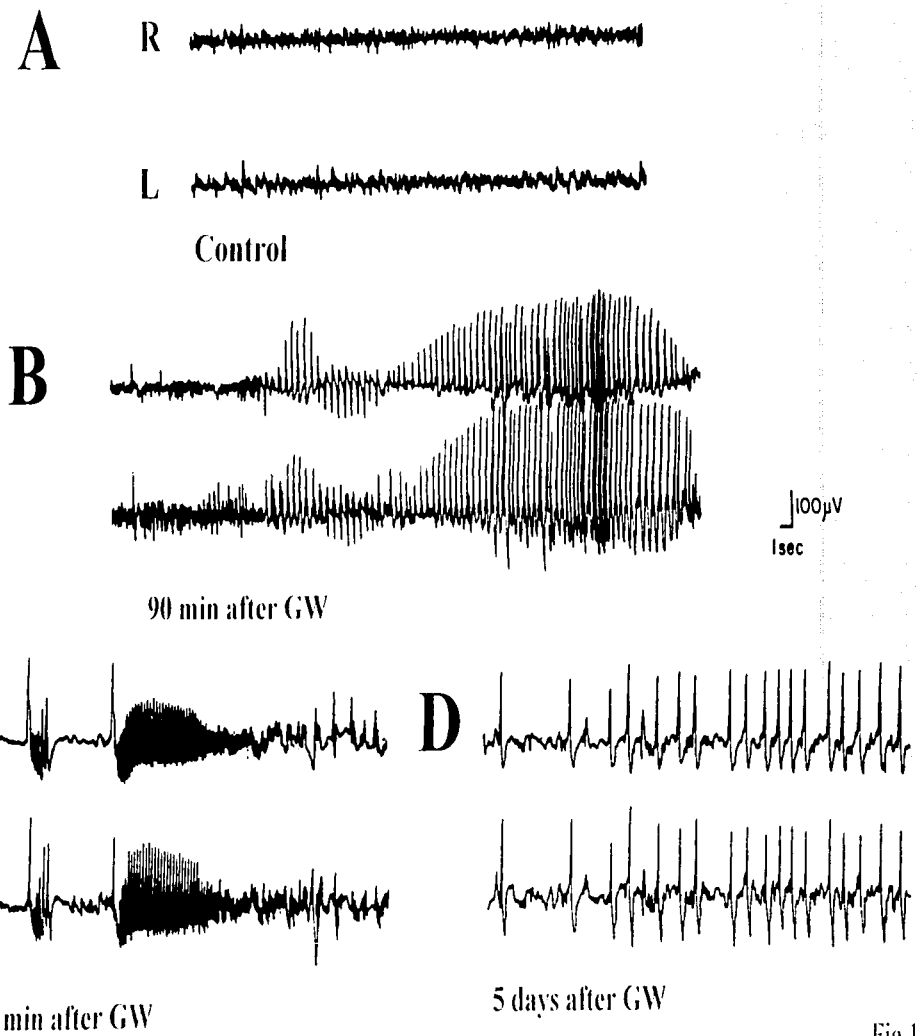

Fig 1

Fig 2

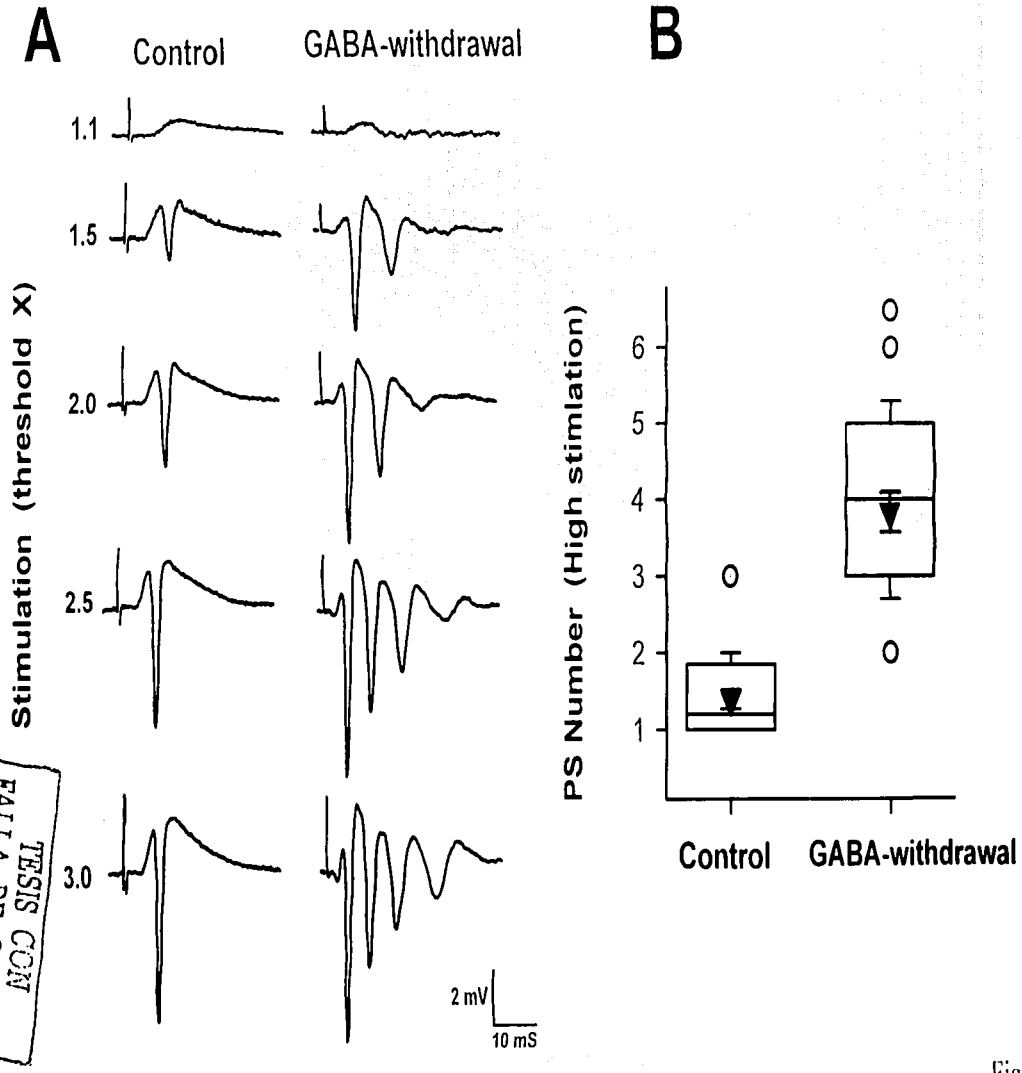

Fig 3

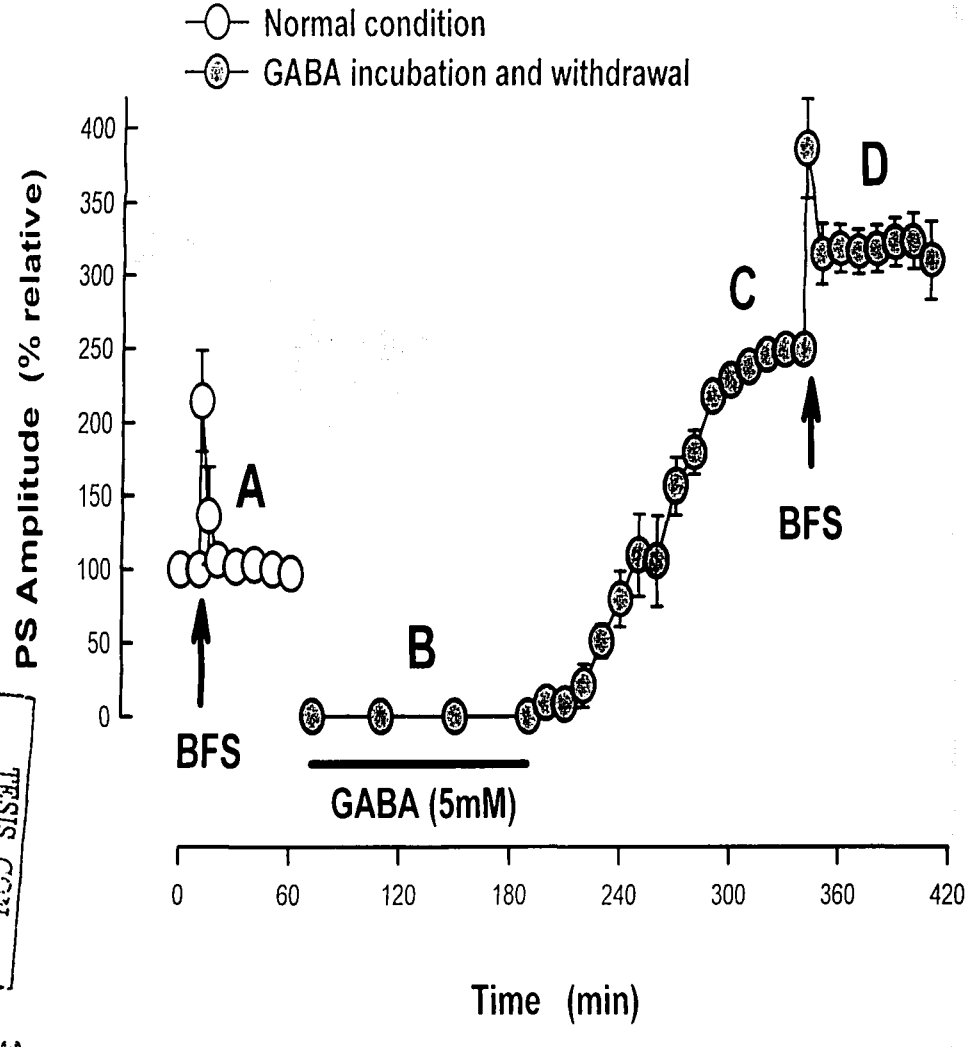

Fig 4

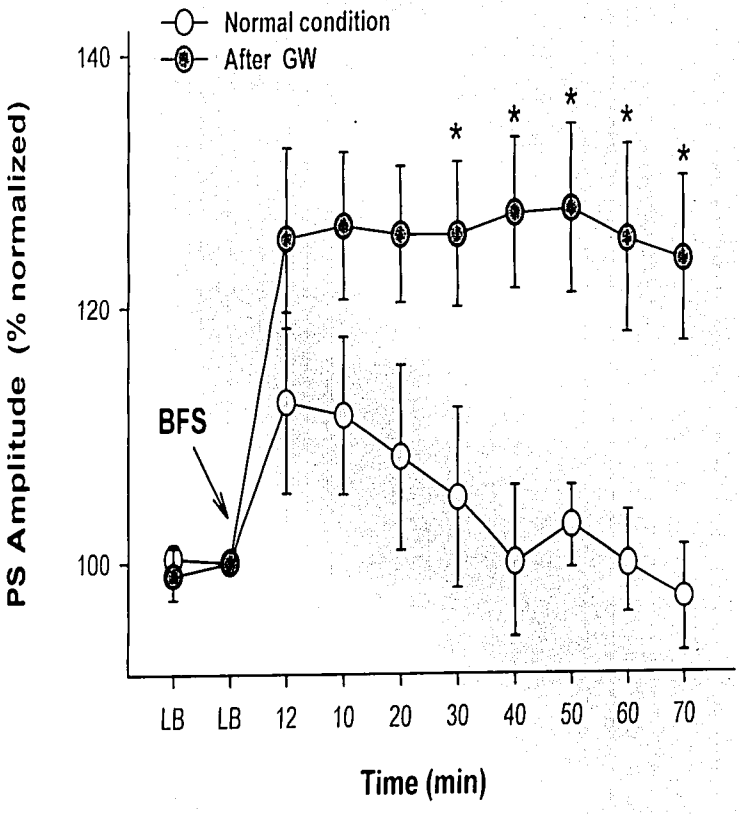

Fig 5

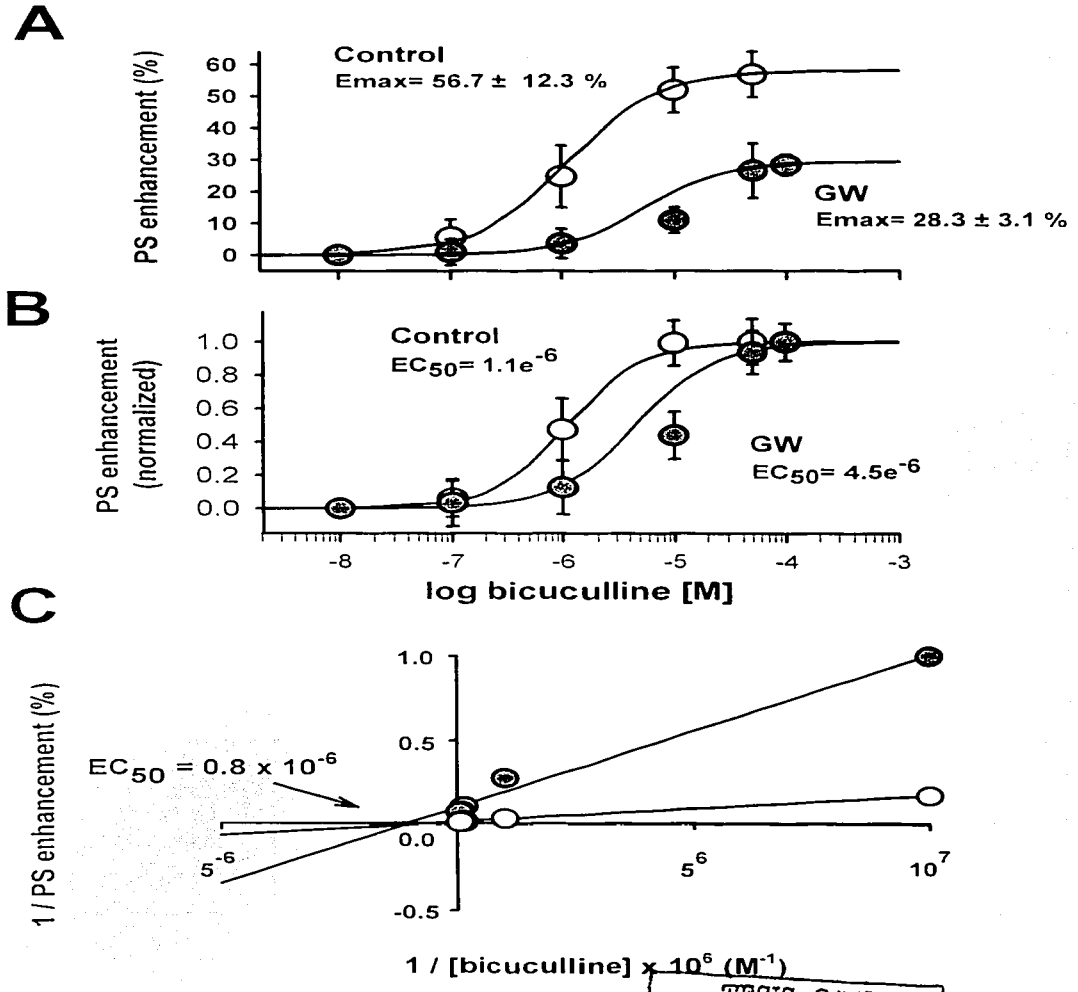

Fig 6

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Hippocampal hyperexcitability induced by GABA withdrawal is due to down-regulation of $GABA_A$ receptors

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Abstract

The sudden interruption of an intracortical instillation of exogenous γ-aminobutyric acid (GABA) generates an epileptic focus in mammals. Seizures elicited by GABA withdrawal (GW) last for weeks. A similar withdrawal-induced hyperexcitability is also produced by several $GABA_A$ receptor agonists. This work reports a quantitative analysis of GW-induced hyperexcitability produced in the hippocampus in vitro. GW produced a left-ward displacement of the input output (I/O) function, suggesting that the postsynaptic component is predominant to explain the hyperexcitability. A decrease in the inhibitory efficacy of the $GABA_A$ receptor agonist, muscimol, confirmed that inhibition was impaired. Binding saturation experiments demonstrated a decrease in [^{3}H]-muscimol binding after GABA withdrawal showing a close correlation with the development of hyperexcitability. All these modifications coursed without changes in receptor affinity (K_D) for muscimol or bicuculline as demonstrated by both binding studies and Schild analysis. It is concluded that, in the CA1 region of the hippocampus, it is the number of functional $GABA_A$ receptors, and not the affinity of the receptor, what is decreased during GW-induced hyperexcitability. © 2001 Published by Elsevier Science B.V.

Keywords: GABA; $GABA_A$ receptors; Epilepsy; GABA withdrawal syndrome; Withdrawal syndromes; Hippocampus; Benzodiazepine withdrawal

1. Introduction

The prolonged use of drugs that increase GABAergic activity such as barbiturates, benzodiazepines and alcohol causes functional tolerance and physical dependence. The abrupt suppression of these drugs may cause a withdrawal syndrome characterized by irritability, anxiety, tremor, changes in sensation and perception, as well as hallucinations and seizures (Carlen et al., 1990; Lader, 1994; Doble and Martin, 1996). Moreover, the withdrawal of GABA itself after a brief, but sustained administration, results in cortical hyper-

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excitability (Brailowsky et al., 1987; Silva-Barrat et al., 2000). GW-induced hyperexcitability is characterized by polyspikes, spike-and-wave discharges associated with myoclonic activity of the corresponding body areas, salivation, wet-dog shakes, and changes in motor and exploratory behavior (Le Gal la Salle et al., 1988; Brailowsky et al., 1987, 1988, 1990; Silva-Barrat et al., 1989, 2000).

Brain slices obtained from animals subject to a chronic exposure to GABA, benzodiazepines (BZD), barbiturates or steroids are hyperexcitable and or have impaired synaptic inhibition (Silva-Barrat et al., 1989; Zeng et al., 1995; Carlen et al., 1990; Saunders and Ho, 1990; Smith et al., 1998). Furthermore, GW-induced hyperexcitability, i.e. potentiation of evoked population spikes (PS) accompanied by epileptiform discharges, can be produced completely in the in vitro slice preparation after sudden withdrawal of superfused GABA in both hippocampal and cortical slices (Garcia-Ugalde et al., 1992; Calixto et al., 2000). Similarly, the interruption of benzodiazepines superfusion may produce hyperexcitability in vitro (Davies et al., 1987). A decrease in the sensitivity to GABA, isoguvacine and benzodiazepines (i.e. tolerance) appears during GW (Silva-Barrat et al., 1989, 2000; Mehta and Ticku, 1992) and also after benzodiazepine withdrawal (BZD-W) (Xie and Tietz, 1992; Cash et al., 1997; Olsen and Avoli, 1997), suggesting that the $GABA_A$ receptor had gone through changes in affinity, number, subunit composition or coupling between $GABA_A$ receptors and BZD receptors (Olsen and Avoli, 1997).

The in vitro slice preparation allows to record the time course of excitability enhancement before the advent of fully developed epileptiform discharges (Traub and Jefferys, 1994; Calixto et al., 2000). Therefore, the present work used the in vitro slice preparation to perform a quantitative pharmacological analysis of the cellular correlates of GW in the CA1 region of the hippocampus. Evidence is presented that major changes occur in number but not receptor affinity. Part of these results have been presented in the abstract form (Casasola et al., 2000).

2. Methods

2.1. Recordings

The experiments were performed on rat hippocampal slices maintained in vitro. Male Wistar rats (100–120 g) were anesthetized with ether and decapitated. The brain was rapidly removed and placed into cold saline solution containing (in mM): 125 NaCl; 3 KCl, 1.0 $MgCl_2$; 2.0 $CaCl_2$; 25 $NaHCO_3$, 11 glucose, pH = 7.4 after saturation with 95% O_2 and 5% CO_2, 290 mOsM L. Vibratome-cut sagittal slices of 400 μm thick were left to stabilize 45 min in the same solution. Recordings were performed in a submerged recording chamber and superfused with the same saline (34 °C; 2–3 ml/min). Bipolar concentric electrodes (50 μm diameter) were used to stimulate the Schaffer's collateral commissural fibers. Stimuli were square wave pulses (40–60 μs) delivered at 0.08–0.1 Hz. Stimulation frequency was chosen to avoid producing potentiation or depression with time (see control in Fig. 1). Extracellular field recordings of synaptic and population spikes (PS) were obtained with glass microelectrodes (3–4 MΩ) filled with NaCl (9%) and placed in the CA1 stratum pyramidale (Fig. 2A). Recordings were amplified with an AC amplifier, displayed in a digital oscilloscope and stored for later analysis.

2.2. Hyperexcitability induced by GABA withdrawal

Slices were either superfused or incubated with GABA (5 mM) dissolved in saline for 120 min (34 °C). GABA was then washed off abruptly (Fig. 1), and PS monitored for several minutes. As previously reported (Garcia-Ugalde et al., 1992; Calixto et al., 2000), a gradual potentiation of the recorded PS was obtained after GABA withdrawal (Fig. 1), that reached a maximum amplitude at about 120 min. Potentiated PS were accompanied by epileptiform discharges (Fig. 1 inset) that never appeared in control slices, not exposed to GABA, and recorded in parallel (Fig. 1). For most pharmacological experiments (Section 2.3), stimulus strength was adjusted to produce a response of about 50–60% of the maximal

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response (approximately 1.5–2-fold the threshold value; see Fig. 2D). Excitability functions (Fig. 2) were obtained: stimulus strength was varied to obtain population postsynaptic potentials and population spikes (PS) from threshold to maximal response (saturation) (see Fig. 2D). The slope of the field excitatory postsynaptic potential (fEPSP-slope; V/s) was measured in the 20–80% portion of its ascending phase (Fig. 2A) and plotted against stimulus strength normalized to threshold. It represents a relative measure of evoked synaptic activity (input) (Fig. 2C). The postsynaptic response (output) is represented by the summed amplitude of all spikes in the evoked discharge (mV). It was also plotted against stimulus strength (Fig. 2D). Summed PS amplitudes (Fig. 2B) were preferred to integration or spike count but all these methods gave similar results. Summed PS amplitude was then plotted against fEPSP-slope to get the 'input–output function' (I/O plot) (Kostopoulos and Psarropoulou, 1992) (Fig. 2E). This is a measure of the postsynaptic discharge as a function of synaptic activity. An epileptiform discharge (Fig. 2B) was never observed in the control condition.

Although an increase in excitability could be obvious by simple inspection in treated slices (cf. Fig. 2A and B), the hyperexcitability was quantified to see if the enhanced discharge is the product of a rise in synaptic activity or, on the contrary, if the same synaptic activity (input) is able to produce an enhanced discharge (Kosto-

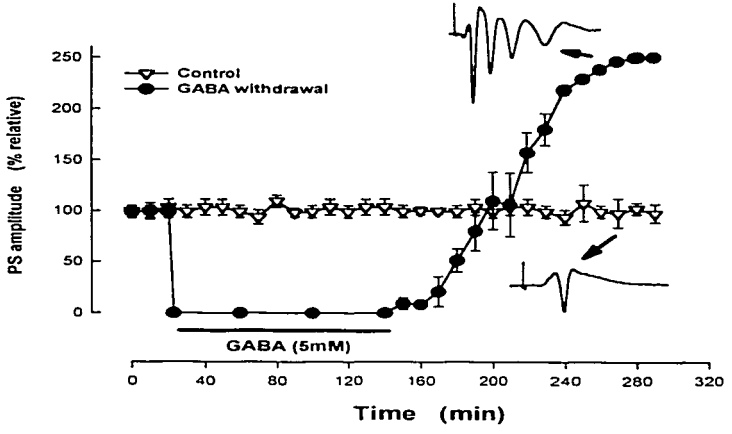

Fig. 1. GABA withdrawal-induced hyperexcitability. Population spikes (PS) were recorded in the CA1 region of the hippocampus. PS recorded in control slices (triangles) had a stable amplitude for several hours ($n = 16$ slices). Slices superfused with saline containing 5 mM GABA, for 120 min (circles), were recorded in parallel ($n = 25$ slices). GABA superfusion is marked by the horizontal bar. Note that the PS was strongly inhibited during GABA superfusion. After GABA washout, PS amplitude recovered and then reached an amplitude 150% larger than the control amplitude in about 120–150 min. Epileptiform discharges appear in the potentiated PS (top inset). Epileptiform discharges never appear in the control recordings (bottom inset). PS were evoked by stimulating the Schaffer collaterals with bipolar electrodes. Stimulus strength was adjusted to that required to elicit a response equal to half the maximal response. Stimulus strength was maintained during the whole experiment.

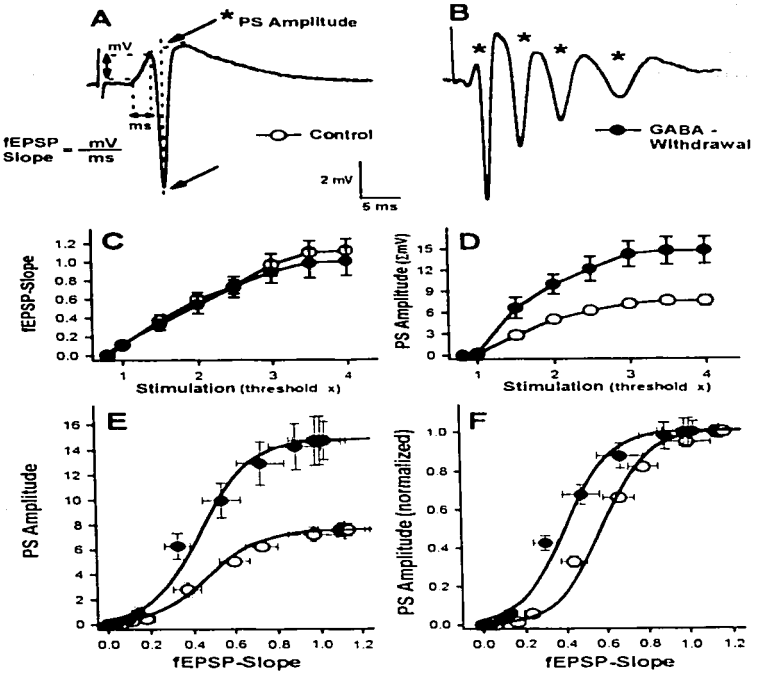

Fig. 2. Input–Output (I–O) function. (A) PS evoked by a single stimulus in a control slice. Dashed lines indicate both the method to measure the slope of the field Excitatory Postsynaptic Potential (fEPSP-slope in mV/ms) and the method to measure the amplitude of the PS. Linear regression was used to determine the slope of the fEPSP. (B) After GABA withdrawal, an epileptiform discharge can be evoked by a single stimulus. This is never seen in control slices (cf. A and B). (C) The magnitude of the fEPSP-slope as a function of stimulus strength is depicted for both control (empty circles) and GABA withdrawal (GW) slices (filled circles) ($n = 8$). (D) The summed amplitude of all PS after a single stimulus is also plotted as a function of stimulus strength in both conditions. (E) I–O function: since the range of the stimulus strength is the same for C and D, PS amplitude (output) was plotted as a function of synaptic activity as represented by the fEPSP-slope (input). Note that the same synaptic activity produces a larger postsynaptic response in the GW slices. Epileptiform discharges are produced by the same synaptic activity (input) that produces single PS in control slices. (F) If the I–O functions are normalized to 1.0, the function corresponding to GW slices is shifted to the left suggesting that the causes for hyperexcitability are postsynaptically located.

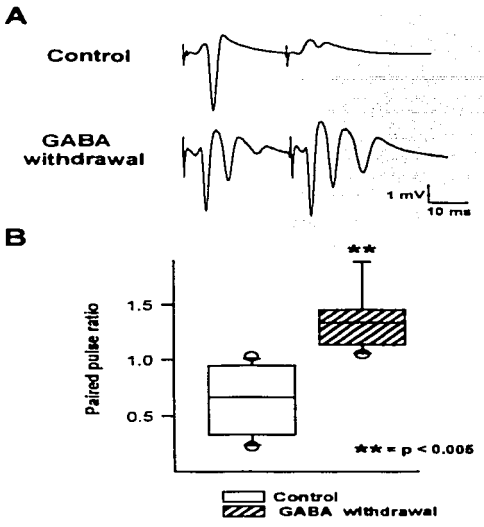

Fig. 3. Recurrent inhibition tested with the paired pulse paradigm. (A) A pair of stimuli was delivered to the Schaffer's collaterals with an interstimulus interval (ISI) equal to 20 ms. Control responses exhibit a typical reduction in the second response; indicative of GABAergic inhibition. In GW slices, the second response (with similar stimulus strength) was not inhibited but facilitated. (C) Box plots summarize nine experiments. Three horizontal lines in the box indicate quartiles 25, 50 (median) and 75 of the sample. Extremes of the bars indicate quartiles 1 and 99. Open circles outside the bars are outliers.

poulos and Psarropoulou, 1992). Thus, in order to quantify the excitability, I–O data were fitted by a sigmoid function of the form:

$$\text{amp} = \text{maxamp}\,(1 + (\quad k(\text{fEPSPslope} - \text{syn50})))\qquad(1)$$

where amp is the summed PS amplitude; maxamp is the maximal postsynaptic response; fEPSPslope corresponds to the slope of the field EPSP and syn50 is the synaptic activity necessary to obtain a discharge equal to 50% of the maximal (Fig. 2E).

2.3. Pharmacological analysis

Concentration–response plots were constructed for the inhibitory action of the $GABA_A$ receptor agonist, muscimol, on the PS amplitude. They were fitted by the equation:

$$DR = (i \quad i\text{max})\,(1 + ([m]/IC_{50})) \quad i\text{max}\qquad(2)$$

where i is the PS amplitude in the presence of muscimol as a percentage of control, $i$max corresponds to the maximal muscimol inhibition, $[m]$ is the muscimol concentration (plotted in logarithmic scale); and IC_{50} is the concentration of muscimol that reduces the PS amplitude to 50% of its control amplitude (Fig. 4A). To see the shift of the function along the concentration axis, the maximal block ($i$max) was normalized to 1 and a simpler equation was fitted:

$$DR = 1/(1 + (IC_{50}\,[m]))\qquad(3)$$

Thereafter, a Lineweaver–Burk analysis of the concentration–response plot was performed: the reciprocal of muscimol concentration was plotted against the reciprocal of the inhibitory effect (Fig. 4C).

Using muscimol inhibition as in Fig. 4A, a Schild analysis was performed by testing different concentrations of the $GABA_A$ receptor antagonist bicuculline (Fig. 5). Families of DR plots were performed for the control and the GW situations. Data were fitted with an antagonist model of the form:

$$\log\,[A'/(A' - 1)] = \log\,[B] - \log K_B\qquad(4)$$

where A is the concentration of the agonist (muscimol) that induces 50% of PS inhibition (IC_{50}) and A' represents similar IC_{50} values obtained in the presence of different concentrations of bicuculline. $[B]$ is the concentration of the antagonist, and $-\log K_B$ is the inverse log of the dissociation constant (Tallarida et al., 1979; Kenakin, 1982).

For the characterization of [^{3}H]-muscimol binding to hippocampal membranes, slices from rat hippocampus were homogenized in 10 mM Tris–HCl buffer, pH 7.4, containing 5 mM EGTA,

Homogenates were centrifuged at 20 000 × g for 30 min and the pellets thus formed were washed by three cycles of resuspension in 50 mM Tris–HCl buffer and centrifugation (20 000 × g for 30 min).

For time-course experiments the analysis was carried out in 1 ml 50 mM Tris–HCl buffer containing 10 nM [^{3}H]-muscimol and ~0.2 mg membrane protein (Lowry et al., 1951). Equilibration was for 60 min at 4 °C and was terminated by filtration through Whatman GF/B glass fiber paper, presoaked in 0.3% polyethylenimine for 2 h, using a Brandel (Gaithersburg, MD, USA) cell harvester. The filters were washed three times with ice-cold buffer and then transferred to vials containing 10 ml scintillation solution and allowed to stand at room temperature for at least 2 h before determination of the tritium content. Non-specific binding was determined as that insensitive to inhibition by 100 μM GABA.

Saturation analysis was carried out in 1 ml 50 mM Tris–HCl buffer containing increasing concentrations (1–40 nM) of [^{3}H]-muscimol and ~2.0 mg membrane protein in the absence or presence of 100 μM GABA. After 60 min at 4 °C incubations were terminated and above data were fit by the one-site equation:

$$B = (B_{max}[m]) / (K_D + [m]) \quad (5)$$

where B is the bound [^{3}H]-muscimol, B_{max} is the maximal [^{3}H]-muscimol bound, $[m]$ is the [^{3}H]-muscimol concentration and K_D is the dissociation constant. Eqs. (2), (3) and (5) are variations of the same equation.

2.4. Drugs

GABA and bicuculline were acquired from Sigma (St. Louis, MO) and muscimol was acquired from Research Biochemical International (Natick, MA). They were applied from freshly prepared stock solutions to the superfusion saline. [^{3}H]-muscimol for binding essays was acquired from Amersham Pharmacia Biotech (Accesolab; México City).

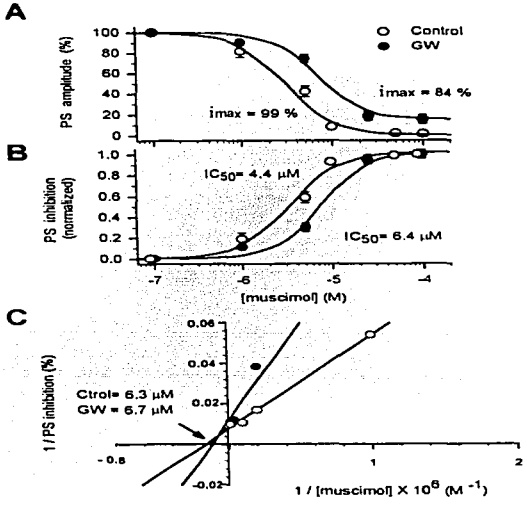

Fig. 4. Muscimol inhibition is less efficient in GW slices. (A) The inhibition of PS by muscimol is concentration-dependent. However, maximal inhibition is lower after GW. (B) Normalization of concentration–response plots to 1.0 shows that IC_{50} tends to be shifted to the right in GW slices; however, this shift is not statistically significant. (C) Lineweaver–Burk analysis of concentration–response plots above.

3. Results

3.1. GW-induced hyperexcitability

GABA withdrawal-induced hyperexcitability (Fig. 1) was elicited as previously reported (Garcia-Ugalde et al., 1992; Calixto et al., 2000). In contrast to control slices that do not exhibit PS potentiation or epileptiform discharges, during several hours of continuous recording (triangles in Fig. 1; inset at the bottom is a representative control recording), slices superfused or incubated with 5 mM GABA exhibit a gradual PS potentiation after GABA was retired from the bath (filled

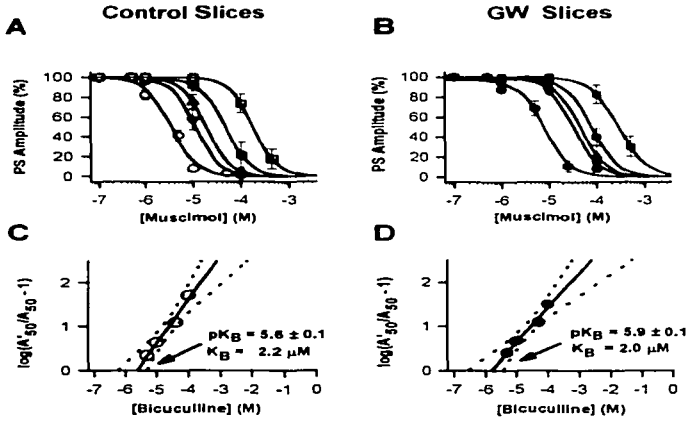

Fig. 5. Schild analysis shows that receptor affinity is unchanged after GW. (A) Concentration–response plots for muscimol in the presence of increasing bicuculline concentrations: 0, 5, 10, 50 and 100 μM (from left to right: empty circles, downward triangles, upward triangles, hexagons, and squares; respectively). (B) Similar concentration–response plots for GW slices. The same bicuculline concentrations were used. Filled symbols are arranged in the same order. Error bars represent standard errors of at least four experiments and slices with the same concentration of muscimol and bicuculline. Absence of error bars indicates small error. (C) IC_{50} values obtained from plots in A were used to build a Schild plot for control slices (see Section 2 and Eq. (4)). (D) A Schild relation was also built from GW slices, plots in B. When linear regression of Schild plots was constrained to slope = 1.0, pK'_B estimates were the same for both samples, 5.7. When they were not constrained, neither slope was significantly different than 1.0 and pK'_B values were very similar. Dashed lines are 95% confidence intervals.

circles in Fig. 1; horizontal bar indicates time of GABA superfusion and inset at the top is a representative recording). The same stimulus strength was used throughout the experiment, but epileptiform discharges were only observed in GW slices 120–150 min after GABA wash out (García-Ugalde et al., 1992). PS amplitude was virtually zero during GABA superfusion (Calixto et al., 2000).

Since the amplitude and number of components in the response became stable 150 min after GABA withdrawal, we compared the excitability of control and GW slices at this time. Excitability was quantified with I–O functions (see Section 2 and Fig. 2). Panel A of Fig. 2 shows that synaptic activity was estimated as the slope of the field postsynaptic potential (fEPSP-slope), whereas postsynaptic activity (synchronous firing or discharge) was measured with the amplitude of the field population spike (PS). Panel C of Fig. 2 shows that similar stimulus strength results in similar synaptic activity in both control ($n = 7$) and GW slices ($n = 8$). In contrast, Fig. 2B and D show that synchronous firing (summed PS amplitude) is enhanced in GW slices as compared with control slices (Fig. 2A and B) for the same stimulus strength. When firing is plotted as a function of synaptic input (Kostopoulos and Psarropoulou, 1992) (Fig. 2E), it is evident that more synchronous discharge is produced in GW slices for

the same synaptic activity with maximal excitability 7.6 ± 0.5 mV ($n = 7$; median = 7.4) in controls vs. 14.7 ± 2.1 mV ($n = 8$; median = 16.5) in GW slices ($P < 0.03$; Mann–Whitney's U-test). By normalizing I/O functions (Fig. 2F) a left-ward shift was observed in the plot for GW slices suggesting that less synaptic activity was necessary to get a half maximal response; in spite of the larger amplitude of the response (Fig. 2E). Synaptic activity required to elicit half the maximal discharge (syn50 in Eq. (1)) was: 0.58 ± 0.05 (V/s; median = 0.55) in controls vs. 0.34 ± 0.06 (V/s; median = 0.34) in GW slices ($P < 0.01$; Mann–Whitney's U-test). Thus, similar synaptic activation elicits about 100% more synchronous firing in GW slices than in control slices. Since less synaptic activity is necessary to produce a larger discharge, GW slices appear to be hyperexcitable after GABA withdrawal. The analysis also points out that hyperexcitability is mainly due to postsynaptic, and not presynaptic, modifications.

3.2. Impairment of synaptic transmission as a result of GW

To gain insight into which postsynaptic modifications might be present (Traub and Jefferys, 1994; Bernard et al., 1998; Ikeda-Douglas et al., 1998) we tested the hypothesis of a reduction in GABAergic transmission. Hippocampal PS is regulated by feed-back interneurons whose action can be manifested by the inhibition of the second response evoked by a pair of stimuli (Buszaki, 1984; Newberry and Nicoll, 1984; Xie and Tietz, 1991; Zeng et al., 1994; Olsen and Avoli, 1997). Paired stimuli were delivered with a 20 ms interstimulus interval. Fig. 3A shows that inhibition of the second response is present in control slices but absent in GW slices, where the second response is actually facilitated. The box plot at the bottom (Fig. 3) summarizes these effects. The paired pulse ratio between the second and the first response was (mean ± S.E.M.) 0.64 ± 0.1 in controls ($n = 9$; median = 0.67) and 1.4 ± 0.11 in GW slices ($n = 8$; median = 1.3; $P < 0.005$, Mann–Whitney's U-test).

These results suggest that postsynaptic modifications leading to hyperexcitability may be exerted at $GABA_A$ receptors. Therefore, a pharmacological analysis of the $GABA_A$ receptor function was performed.

3.3. Sensitivity of PS to muscimol

Concentration–response plots for the $GABA_A$ receptor agonist, muscimol, were performed to compare control and GW slices. Fig. 4A shows that muscimol inhibits, in a concentration-dependent manner, PS amplitude in both control and GW slices. However, maximal inhibition produced by muscimol was reduced in GW slices. Maximal inhibition was: 99 ± 0.5% ($n = 6$; median = 99%) in control slices and, 84 ± 3.2% ($n = 6$; median = 83%) in GW slices, respectively ($P < 0.004$; Mann–Whitney's U-test). Concentration–response plots were normalized to maximal inhibition set at 1.0 (Eq. (3)), and a right-ward shift was observed in GW slices. However, this shift was not statistically significant: $IC_{50} = 4.4 \pm 0.64$ μM ($n = 6$; median = 3.95 μM) for controls and $IC_{50} = 6.4 \pm 1.11$ μM ($n = 6$; median = 7.04 μM) in GW slices ($P > 0.3$, Mann–Whitney's U-test, and $P \geq 0.16$ Student's t-test). This result suggests that the difference in muscimol inhibition was not due to a change in receptor affinity. To further test this inference, a Lineweaver–Burk analysis (Fig. 4C) of concentration–response plots was performed. This analysis confirmed that a major change in maximal inhibition was not accompanied by a significant change in the IC_{50} values for muscimol, which were 6.3 ± 0.2 and 6.7 ± 0.3 μM for control and muscimol-treated slices, respectively. Both these constants are similar to those reported previously (Kemp et al., 1986).

Nevertheless, a Schild analysis with the help of bicuculline, a competitive antagonist at $GABA_A$ receptors, was performed to ensure this conclusion (Fig. 5A and B). Thus, several concentration–response plots for muscimol were performed in the presence of different concentrations of bicuculline (5, 10, 50 and 100 μM), that resulted in progressive right-ward displacements of the plots in a concentration-dependent manner for both control and GW slices. Each concentration was used in at least four different slices. Noticeably,

these displacements were parallel to the control concentration–response plot. IC_{50} ratios in the presence and the absence of the antagonist were used to build Schild plots (Eq. (4)). Fig. 5C and D illustrate the intercepts of these plots in the concentration axis. They were not significantly different: suggesting that receptor's dissociation constants for bicuculline did not vary after GABA withdrawal. pK_B values (Eq. (4)) were (log concentration ± estimated error): 5.6 ± 0.11 and 5.9 ± 0.1 in control and GW slices, respectively (NS); corresponding to dissociation constants of 2.2 and 2.0 μM, for controls and GW slices, respectively. These measurements fully agree with those reported previously (Simmonds, 1982; Kemp et al., 1986; Krishek et al., 1996). Moreover, when Shild plots were constrained to have a slope identical to 1.0, both control and GW slices yielded the same pK_B value: 5.7.

Therefore, both Schild and Lineweaver–Burk analyses indicated that any modifications that $GABA_A$ receptors had suffered after GABA withdrawal did not include a change in agonist or antagonist affinities. Therefore, the impairment of GABA transmission is mainly attributed to a reduction in the number of $GABA_A$ receptors in the postsynaptic membrane.

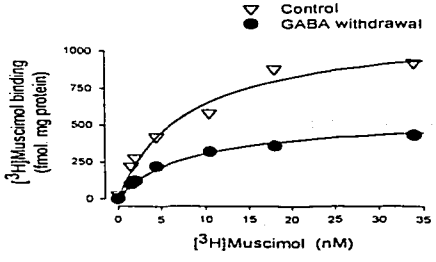

Fig. 6. [^{3}H]-muscimol specific binding to CA_1 membranes. This representative experiment illustrates that specific muscimol binding is lower in membranes obtained from GW slices. Bmax = 1097 ± 129 fmol mg prot. for controls and 556 ± 17 fmol mg prot. for GW slices ($n = 3$). K_D = 8.9 ± 0.5 nM for controls and 8.9 ± 0.6 nM for GW slices.

In order to test this hypothesis, the equilibrium binding of the $GABA_A$ receptor agonist, [^{3}H]-muscimol, was characterized in membranes from slices subject to the same manipulations described above (Fig. 1). Fig. 6 shows that the density (B_{max}) of [^{3}H]-muscimol specific binding sites was (mean ± S.E.M.) 1097 ± 129 fmol mg prot. for controls and 556 ± 17 fmol mg prot. for GW slices ($n = 3$). This represents a 48 ± 5% reduction in total binding, suggesting that the $GABA_A$ receptor is down-regulated after GABA withdrawal. Dissociation constants obtained with equilibrium binding experiments confirmed that receptor affinity was not changed since K_D values were (mean ± S.E.M.) 8.9 ± 0.5 and 8.9 ± 0.6 nM for control and GW slices, respectively. These results support the notion that hyperexcitability caused by GABA withdrawal is mainly due to a decrease

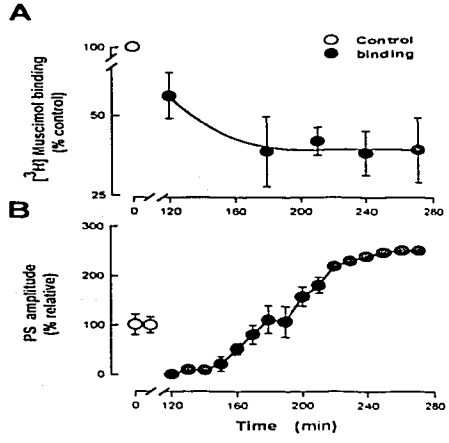

Fig. 7. The number of $GABA_A$ receptors falls continuously after GW. (A) Specific [^{3}H]-muscimol binding was measured at different times after GW. Note that the number of receptors decays progressively with time. (B) The enhancement of excitability coincides with receptor decay, after GW.

in the number of $GABA_A$ receptors. Therefore, the time course of the decrease in receptor number was compared to the development of hyperexcitability. Fig. 7 shows that specific [^{3}H]-muscimol binding decays progressively as PS amplitude increases, after GABA withdrawal.

4. Discussion

4.1. GABA withdrawal induces down-regulation of $GABA_A$ receptors

The results of the present experiments show that the first steps of the use-dependent down-regulation of $GABA_A$ receptors (Barnes, 1996) can be triggered acutely, in an in vitro, previously unexposed, slice preparation. The sudden interruption of GABA superfusion initiates an enhancement of excitability that surpasses control responses and becomes stable after 120–150 min (García-Ugalde et al., 1992; Calixto et al., 2000). This excitability enhancement is characterized by a potentiation of the population spike and the appearance of evoked epileptiform discharges that could be measured by means of I/O functions (Kostopoulos and Psarropoulou, 1992). I/O functions indicated a postsynaptic locus for the hyperexcitability. A deficit in GABA neurotransmission was shown with the paired pulse paradigm (Davies et al., 1990; Sloviter, 1991; Nie and Tietz, 1991; García-Ugalde et al., 1992; Olsen and Avoli, 1997; Ikeda-Douglas et al., 1998; Calixto et al., 2000). A decrease in the inhibitory action of the $GABA_A$ receptor agonist, muscimol, was confirmed on the basis of concentration response analysis. Schild analysis and equilibrium binding showed that a receptor reduction (efficacy) occurred without changes in affinity or IC_{50} (potency). Moreover, the time course of receptors decrease was closely correlated with the time course of the enhancement in excitability.

Altogether, these results make a case for the down-regulation of $GABA_A$ receptors as the cause of the hyperexcitability found as a result of sudden GABA withdrawal. Probably, a diminished de novo synthesis cannot replenish down-regulated receptors in these conditions.

4.2. Separating withdrawal from seizures

A commonly used experimental paradigm to investigate tolerance and withdrawal phenomena associated with the use of $GABA_A$ receptor agonists has been the chronic administration of the agonists, by several pathways from orally to intracortically, and thereafter observe behavioral, EEG, and in vitro (ex vivo) electrophysiological, biochemical or pharmacological changes at several times after the withdrawal (Silva-Barrat et al., 1989, 2000; Brailowsky et al., 1990; Carlen et al., 1990; Kang and Miller, 1991; Zeng et al., 1994, 1995; Barnes, 1996; Wilson, 1996; Toki et al., 1996; Tietz et al., 1999). There are two problems in comparing and synthesizing these results. First, a lack of paradigm standardization makes comparisons difficult (Löscher et al., 1996; Fahey et al., 1999). Regional differences on $GABA_A$ receptor changes (Wilson, 1996), and different observation times after withdrawal, yield contrasting results (Kang and Miller, 1991; Olsen and Avoli, 1997; Poisbeau et al., 1997; Holt et al., 1997; Tietz et al., 1999). Second, prolonged administration of agonists, or the withdrawal of drugs before making the in vitro preparations, may lead to electrophysiological changes, including seizures (Davies et al., 1987; Brailowsky et al., 1988; Silva-Barrat et al., 1989; Gibbs et al., 1997; Poisbeau et al., 1997), before any recording is done. Yet, once seizures have been elicited by any means (e.g. pilocarpine, kainic acid, 4-aminopyridine), they lead to profound changes in GABA, glutamate and N-methyl-D-aspartate (NMDA) transmission and receptors (Carlen et al., 1990; Traub and Jefferys, 1994; Gibbs et al., 1997; Sperk et al., 1998). Hence, any study on the cellular basis of withdrawal-induced hyperexcitability, relying on preparations that had undergone previous hyperexcitability or seizures, has the problem of sorting out which changes are attributable to tolerance or withdrawal and which are attributable to seizures themselves. An advantage of the preparation described here is that brain slices may be recorded at any time between agonist withdrawal and the stabilization of the new excitable state. Although tolerance followed by withdrawal leads to hyperexcitability (Barnes, 1996), the separation of the

different steps is necessary to elucidate how this happens. In contrast, once seizures have occurred, depolarizing shifts, plateau potentials and bursting are common, with an accompanying calcium influx that leads to numerous consequences (Carlen et al., 1990; Traub and Jefferys, 1994; Silva-Barrat et al., 2000). Not a major change is the modification of $GABA_A$ receptor subunit composition (Sperk et al., 1998). Thus, desensitization, up- and down-regulation of $GABA_A$- and BZD-receptors, uncoupling of BZD and $GABA_A$ receptors, and up- and down-regulation of the multiple subunits of the heteroligomeric protein that makes up the receptor, have been found after different tolerance and withdrawal paradigms (Galpern et al., 1991; Kang and Miller, 1991; Hu and Tieku, 1994; Mhatre and Tieku, 1994; Barnes, 1996; Toki et al., 1996; Holt et al., 1997; Tietz et al., 1999; Lyons et al., 2000) but the timing and specificity of all these changes has only begun to be elucidated. Because of the above reasons, a non-exposed, in vitro slice preparation, was used for the present quantitative work. It is shown the time course of the hyperexcitability enhancement and measurements were done when hyperexcitability had stabilized but before spontaneous epileptiform discharges had appeared (Traub and Jefferys, 1994). Future studies will concentrate on specific points of the time course. Slices were treated with 5 mM GABA since this concentration approximates the GABA concentration transiently present in the synaptic cleft (Cherubini and Conti, 2001). However, a concentration- and exposure time response relationship has not been done.

4.3. A comparison of hyperexcitable states induced by benzodiazepine and GABA withdrawal

In spite of the above, a contextual synthesis of previous, mostly coincident results obtained with a variety of techniques, can be attempted. The results in a extensively studied area, the CA1 region of the hippocampus, will be mostly preferred for this synthesis, although mention of cortical tissue is necessary. Thus, a point to point comparison between GABA withdrawal- (GW) and benzodiazepine withdrawal- (BZD-W) induced hyperexcitablity follows: After chronic administration of GABA or $GABA_A$ receptor agonists, tolerance, dependence and withdrawal-induced hyperexcitability are found (Brailowsky et al., 1988; Barnes, 1996; Silva-Barrat et al., 1989, 2000). The same phenomena are found after chronic BZD (Davies et al., 1988; Miller et al., 1988; Carlen et al., 1990; Barnes, 1996; Toki et al., 1996). In both cases, there is a decreased sensitivity of the $GABA_A$ receptor towards its agonists (Miller et al., 1988; Silva-Barrat et al., 1989; Nie and Tietz, 1992; Wilson, 1996; Cash et al., 1997; this work). Moreover, withdrawal-induced hyperexcitability has been totally and acutely induced in in-vitro slice preparations for both GABA and BZD (Davies et al., 1987; Garcia-Ugalde et al., 1992). This has not been done for other allosteric agonists. At least in the cortex, the time course is very similar for GW and BZD-W (Calixto et al., 2000). GABA transmission and inhibition, or GABA-induced chloride fluxes, are impaired after exposure to both GABA and BZD (Xie and Tietz, 1991; Zeng et al., 1994, 1995; Wilson, 1996; Barnes, 1996; Poisbeau et al., 1997; Garcia-Ugalde et al., 1992; Mehta and Tieku, 1992; Hu and Tieku, 1994; Calixto et al., 2000; this study). Impairment of GABA transmission has been associated with a decreased number of receptors (efficacy) in the synaptic membrane rather than with K_d or IC_{50} changes (potency) for both BZD and GABA (Hu and Tieku, 1994; this study). In both cases there have been reports of down-regulation of the $GABA_A$ receptor upon prolonged exposure (Mehta and Tieku, 1992; Mhatre and Tieku, 1994; Barnes, 2000; this study). Sequestration of $GABA_A$ and BZD receptors on clathrin-coated vesicles after exposure to both GABA and BZD has also been reported (Calkin and Barnes, 1994; Tehrani and Barnes, 1997; Barnes, 2000). At least, the turnover and down-regulation of some receptor subunits (γ1, γ1,2), suitable to assemble the $GABA_A$ receptor sensitive to BZD regulation, have been reported in both cases (Kang and Miller, 1991; Holt et al., 1997; Tietz et al., 1999; Barnes, 2000; Lyons et al., 2000). Other sub-units may up-regulate (e.g. γ4,5) as in the case of steroid withdrawal (Smith et al., 1998), and this coincides with a decreased sensitiv-

ity or 'uncoupling' between BZD and GABA (Xie and Tietz, 1992; Barnes, 1996; Cash et al., 1997; Smith et al., 1998; Tietz et al., 1999). Finally, a common gene-loci has been proposed to explain withdrawal-induced phenomena (Buck and Finn, 2001). Thus, in spite of the many discrepancies found in the literature, many due to experimental and regional differences (Wilson, 1996; Löscher et al., 1996; Fahey et al., 1999), there are important coincidences between GW and BZD-W phenomena (Barnes, 1996).

This coincidences logically rise a hypothetical question: are we looking at the same phenomenon? Many allosteric agonists produce a left-ward shift in the dose–response function of $GABA_A$-receptor agonists. Therefore, we can suppose that allosteric agonists make $GABA_A$ receptors 'to see' a higher GABA concentration, and for a longer period of time, than the actual concentration in the synaptic cleft. As a consequence, the prolonged use of a BZD may be equivalent to a prolonged, non-physiologically high GABA concentration. Then, non-physiological millimolar GABA-concentrations, as those used here, may pave the way to 'calibrate' the BZD potential to produce withdrawal-induced hyperexcitability. A complete dose and time study is necessary to approximate the doses of GABA that the receptor 'needs to see' in order to produce an equivalent withdrawal-induced hyperexcitability with a BZD. Of course, GABA is in high concentrations for only a few milliseconds in the synaptic cleft, however, what we would like to hypothesize is that in the presence of an allosteric modulator, the receptor protein may undergo changes leading to tolerance and withdrawal as though the GABA concentration were staying high and prolonged. That is, the changes that the receptor protein undergoes occur either with physiological GABA and a modulator or with a high GABA concentration. This interpretation forces an implicit hypothesis of physiopathological importance: that both GW and BZD-W are the same phenomenon. This hypothesis needs further experimentation to be proved or discarded.

Also, before reaching additional conclusions or to further extend these comparisons to other allosteric modulators associated with the $GABA_A$ receptor (neurosteroids, ethanol, barbiturates) (Barnes, 1996; Wilson, 1996), more results coming from a similar experimental paradigm as the one presented here are needed. This time-limited, simple, preparation, may contribute towards this end, since a few minutes superfusion triggers a dynamic process that continues for a long time after GW or BZD-W (Calixto et al., 2000). Correlations with chronic in vivo exposures, and the testing of a diverse array of agonists and cerebral regions, are also needed.

Regional differences need to be evidenced by making observations at the same time after withdrawal. Thus, in a previous study in the somatosensory cortex and using the same experimental paradigm, we found that muscimol binding first decreased and then increased again, although accompanied with a changed sensitivity to steroids (Calixto et al., 2000). However, in the CA1 hippocampal region, only a decrease was found after withdrawal; which correlates with previous findings (Lyons et al., 2000). However, the exploration needs to be done at longer and shorter times. If a subunit turnover is present in both the CA1 region of the hippocampus and the somatosensory cortex, it is in the CA1 region where the turnover is not enough to compensate for total receptor loss.

Finally, a note of caution may be necessary after looking at all experimental coincidences available today. Clinically, withdrawal syndromes associated with allosteric $GABA_A$ receptor agonists are treated with other allosteric or direct $GABA_A$ receptor agonists (e.g. alcohol withdrawal is being treated with BZDs). The question is, which withdrawal is going to be the worst?

5. Conclusions

A quantitative analysis of the GABA withdrawal-induced hyperexcitability was performed in the in vitro hippocampal slice preparation. Both electrophysiological and pharmacological methods agree in that this hyperexcitability is mainly caused by the down regulation of the $GABA_A$ receptors. This process continues several minutes after GABA withdrawal and is tempo-

rally correlated with the development of hyperexcitability. It occurs without noticeable changes in receptor affinity although future work is necessary to probe other receptor agonists and modulators.

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References

Barnes, E.M. Jr., 1996. Use-dependent regulation of $GABA_A$ receptors. Int. Rev. Neurobiol. 39, 53–76.

Barnes, E.M. Jr., 2000. Intracellular trafficking of $GABA_A$ receptors. Life Sci. 66, 1063–1070.

Bernard, C., Esclapez, M., Hirsch, J.C., Ben-Ari, Y., 1998. Interneurones are not so dormant in temporal lobe epilepsy: a critical reappraisal of the dormant basket cell hypothesis. Epilepsy Res. 32, 93–103.

Bralowsky, S., Menini, C., Silva-Barrat, C., Naquet, R., 1987. Epileptogenic gamma-aminobutyric acid-withdrawal syndrome after chronic intracortical infusion in baboons. Neurosci. Lett. 74, 75–80.

Bralowsky, S., Kinimoto, M., Menini, C., Silva-Barrat, C., Riche, D., Naquet, R., 1988. The GABA-withdrawal syndrome: a new model of focal epileptogenesis. Brain Res. 442, 175–179.

Bralowsky, S., Kinimoto, M., Silva-Barrat, S., Menini, C., Naquet, R., 1990. Electroencephalographic study of the GABA-withdrawal syndrome in rats. Epilepsia 31, 369–377.

Buck, K.J., Finn, D.A., 2001. Genetic factors in addiction: QTL mapping and candidate gene studies implicate GABAergic genes in alcohol and barbiturate withdrawal in mice. Addiction 96, 139–149.

Buszaki, G., 1984. Feed-forward inhibition in the hippocampal formation. Prog. Neurobiol. 22, 131–153.

Calkin, P., Barnes, E. Jr., 1994. Gamma-aminobutyric acid-A ($GABA_A$) agonist down-regulate $GABA_A$ benzodiazepine receptor polypeptides from the surface of chick cortical neurons. J. Biol. Chem. 269, 1548–1553.

Calixto, E., Lopez-Colome, A.M., Casasola, C., Montiel, T., Bargas, J., Bralowsky, S., 2000. Neocortical hyperexcitability after GABA withdrawal in vitro. Epilepsy Res. 39, 13–26.

Carlen, P.L., Davies, M.F., Rougier-Naquet, T., Reynolds, J.N., Spigelman, I., 1990. Sedative drugs withdrawal seizures: cellular electrophysiological mechanisms. In: Avoli, M., Gloor, P., Kostopoulos, G., Naquet, R. (Eds.), Generalized Epilepsy: Neurobiological Approaches. Birkhauser, Boston, pp. 460–470.

Casasola, C.C., Velazquez-Martinez, D.N., Arias-Montano, J.A., Calixto, E., Galarraga, E., Bargas, J., Bralowsky, S., 2000. Downregulation of $GABA_A$ receptors explains hyperexcitability produced by GABA withdrawal. Soc. Neurosci. Abs. 26, 1780.

Cash, D.J., Serfozo, P., Allan, A.M., 1997. Desensitization of a gamma-aminobutyric acid type A receptor in rat is increased by chronic treatment with chlordiazepoxide: a molecular mechanism of dependence. J. Pharmacol. Exp. Ther. 283, 704–771.

Cherubini, E., Conti, F., 2001. Generating diversity at GABAergic synapses. Trends Neurosci. 24, 155–162.

Davies, C., Davies, S., Collinridge, G., 1990. Paired pulse depression on monosynaptic GABA mediated inhibitory postsynaptic response in the rat hippocampus. J. Physiol. (Lond.) 424, 513–531.

Davies, M.F., Sasaki, S.E., Carlen, P.L., 1987. Benzodiazepine-induced epileptiform activity in vitro. Brain Res. 437, 239–244.

Davies, M.F., Sasaki, S.E., Carlen, P.L., 1988. Hyperexcitability of hippocampal CA1 neurons in brain slices of rats chronically administered clonazepam. J. Pharmacol. Exp. Ther. 247, 737–744.

Doble, A., Martin, I.L., 1996. The $GABA_A$ Benzodiazepine Receptor as a Target for Psychoactive Drugs. Neuroscience Intelligence Unit. Chapman and Hall, New York.

Fahey, J.M., Pritchard, G.A., Grassi, J.M., Pratt, J.S., Shader, R.I., Greenblatt, D.J., 1999. In situ hybridization histochemistry as a method to assess $GABA_A$ receptor subunit mRNA expression following chronic alprazolam administration. J. Psychopharmacol. 13, 211–218.

Galpern, W.R., Lumpkin, M., Greenblatt, D.J., Shader, R.I., Miller, L.G., 1991. Chronic benzodiazepine administration. VII. Behavioral tolerance and withdrawal and receptor alterations associated with clonazepam administration. Psychopharmacology 104, 225–230.

Garcia-Ugalde, G., Galarraga, E., Bargas, J., Bralowsky, S., 1992. Hyperexcitability of hippocampal CA1 region in brain slices after GABA withdrawal. Neurosci. Lett. 142, 229–232.

Gibbs III, J.W., Shumate, M.D., Coulter, D.A., 1997. Differential epilepsy-associated alterations in postsynaptic $GABA_A$ receptor function in dentate granule and CA1 neurons. J. Neurophysiol. 77, 1924–1938.

Hu, N., Tieku, M., 1994. Chronic flurazepam treatment produces decreased efficacy of the benzodiazepine ligands and pentobarbital with γ-aminobutyric acid A receptor subtypes. J. Pharmacol. Exp. Ther. 270, 485–490.

Holt, R.A., Martin, I.L., Bateson, A.N., 1997. Chronic diazepam exposure decreases transcription of the rat $GABA_A$ receptor γ2-subunit gene. Mol. Brain Res. 48, 164–166.

Ikeda-Douglas, C.J., Head, E., Holsinger, R.M.D., Tremblay, L., Racine, R., Milgram, N.W., 1998. Selective loss of early suppression in the dentate gyrus precedes kainic acid induced electrographic seizures. Epilepsy Res. 31, 143–152.

Kang, I., Miller, L.G., 1991. Decreased $GABA_A$ receptor subunit mRNA concentration following chronic lorazepam administration. Br. J. Pharmacol. 103, 1285–1287.

Kenakin, T.P., 1982. The Schild regression in the process of receptor classification. Can. J. Physiol. Pharmacol. 60, 249–265.

Kemp, J.A., Marshall, G.R., Woodruff, G.N., 1986. Quantitative evaluation of the potencies of GABA-receptor agonists and antagonists using the rat hippocampal slice preparation. Br. J. Pharmacol. 87, 677–684.

Kostopoulos, G.K., Psarropoulou, C.T., 1992. Possible mechanisms underlying hyperexcitability in the epileptic mutant mouse tottering. J. Neural Transm. [Suppl.] 35, 109–124.

Krishek, B.J., Moss, S.J., Smart, T.G., 1996. A functional comparison of the antagonists bicuculline and picrotoxin at recombinant $GABA_A$ receptors. Neuropharmacol. 35, 1289–1298.

Lader, M., 1994. Biological processes in benzodiazepine dependence. Addiction 89, 1413–1418.

Le Gal la Salle, G., Brailowsky, S., Menini, C., Naquet, R., 1988. Local asymptomatic status epilepticus induced by withdrawal of GABA infusion into limbic structures. Exp. Neurol. 101, 411–417.

Lowry, D.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Loscher, W., Rundfeldt, C., Honack, D., Ebert, U., 1996. Long-term studies on anticonvulsant tolerance and withdrawal characteristics of benzodiazepine receptor ligands in different seizure models in mice. I. Comparison of diazepam, clonazepam, clobazam and abecarnil. J. Pharmacol. Exp. Ther. 279, 561–572.

Lyons, H.R., Gibbs, T.T., Farb, D.H., 2000. Turnover and down-regulation of GABA(A) receptor γ1, β2S, and γ1 subunit mRNAs by neurons in culture. J. Neurochem. 74, 1041–1048.

Mehta, A.K., Ticku, M.K., 1992. Chronic GABA exposure down-regulates GABA-benzodiazepine receptor-ionophore complex in cultured cerebral cortical neurons. Mol. Brain Res. 16, 29–36.

Mhatre, M.C., Ticku, M.K., 1994. Chronic GABA treatment downregulates the $GABA_A$ receptor alpha 2 and alpha 3 subunit mRNAs as well as polypeptide expression in primary cultured cerebral cortical neurons. Mol. Brain Res. 24, 159–165.

Miller, L.G., Greenblatt, D.J., Barnhill, J.G., Shader, R.I., 1988. Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric acid A receptor function. J. Pharmacol. Exp. Ther. 246, 170–176.

Newberry, N.R., Nicoll, R.A., 1984. A bicuculline-resistant inhibitory postsynaptic potential in rat hippocampal pyramidal cells in vitro. J. Physiol. (Lond.) 348, 239–254.

Olsen, W.R., Avoli, M., 1997. GABA and epileptogenesis. Epilepsia 38, 399–407.

Poisbeau, P., Williams, S.R., Mody, I., 1997. Silent $GABA_A$ synapses during flurazepam withdrawal are region-specific in the hippocampal formation. J. Neurosci. 17, 3467–3475.

Saunders, P.A., Ho, I.K., 1990. Barbiturates and the $GABA_A$ receptor complex. Prog. Drug Res. 34, 261–286.

Silva-Barrat, C., Champagnat, J., Brailowsky, S., Menini, C., Naquet, R., 1989. Relationship between tolerance to $GABA_A$ agonist and bursting properties in neocortical neurons during GABA-withdrawal syndrome. Brain Res. 498, 289–298.

Silva-Barrat, C., Champagnat, J., Menini, C., 2000. The GABA-withdrawal syndrome: a model of local status epilepticus. Neural Plasticity 7, 9–18.

Simmonds, M.A., 1982. Classification of some GABA antagonists with regard to site of action and potency in slices of rat cuneate nucleus. Eur. J. Pharmac. 80, 347–358.

Slowiter, R.S., 1991. Feedforward and feedback inhibition of hippocampal principal cell activity evoked by perforant path stimulation: GABA-mediated mechanisms that regulate excitability. Hippocampus 1, 31–40.

Smith, S.S., Gong, Q.H., Hsu, F.C., Markowitz, R.S., ffrench-Mullen, J.M., Li, X., 1998. $GABA_A$ receptor γ3 suppression prevents withdrawal properties of an endogenous steroid. Nature 392, 926–930.

Sperk, G., Schwarzer, C., Tunashima, K., Kandlhofer, S., 1998. Expression of $GABA_A$ receptor subunits in the hippocampus of the rat after kainic acid-induced seizures. Epilepsy Res. 32, 129–139.

Tallarida, R.J., Cowan, A., Adler, M.W., 1979. pA2 and receptor differentiation: a statistical analysis of competitive antagonism. Life Sci. 25 (8), 637–654.

Tehrani, M.H., Barnes, E.M. Jr., 1997. Sequestration of gamma-aminobutyric acid A receptors on clathrin-coated vesicles during chronic benzodiazepine administration in vivo. J. Pharmacol. Exp. Ther. 283, 384–390.

Tietz, E.I., Huang, X., Chen, S., Ferencak, W.F., 1999. Temporal and regional regulation of γ1, β2 and β3, but not γ2, γ4, γ5, γ6, β1 or γ2 $GABA_A$ receptor subunit messenger RNAs following one-week oral flurazepam administration. Neuroscience 91, 327–341.

Toki, S., Saito, L., Nabeshima, A., Hatta, S., Watanabe, M., Takahata, N., 1996. Changes in $GABA_A$ receptor function and cross-tolerance to ethanol in diazepam-dependent rats. Alcohol Clin. Exp. Res. 20, 40A–44A.

Traub, D.R., Jefferys G.R.J., 1994. Are there unifying principles underlying the generation of epileptic afterdischarges in vitro? In: Van Pelt, J., Corner, M.A., Uylings, H.B.M., Lopez da Silva, (Eds.), Progress in Brain Res. 102, pp. 383–394.

Wilson, M.A., 1996. GABA physiology, modulation by benzodiazepines and hormones. Crit. Rev. Neurobiol. 10, 1–37.

Xie, X.-H., Tietz, E.I., 1992. Reduction in potency of selective γ-aminobutyric acid A agonist and diazepam in CA1 region of in vitro hippocampal slices from chronic flurazepam-treated rats. J. Pharmacol. Exp. Ther. 262, 204–211.

Xie, X.-H., Tietz, E.I., 1991. Chronic benzodiazepine treatment of rats induces reduction of paired-pulse inhibition in CA1 region of in vitro hippocampus. Brain Res. 561, 69–76.

Zeng, N., Nie, X.H., Tietz, E.I., 1995. Reduction of GABA-mediated inhibitory postsynaptic potentials in hippocampal CA1 pyramidal neurons following oral flurazepam administration. Neuroscience 66, 87–99.

Zeng, N., Nie, X.H., Tietz, E.I., 1994. Impairment of feedforward inhibition in CA1 region of hippocampus after chronic benzodiazepine treatment. Neurosci. Lett. 173, 40–44.

Long-Lasting Effects of GABA Infusion into the Cerebral Cortex of the Rat

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SUMMARY

In electrophysiological terms, experimental models of durable information storage in the brain include long-term potentiation (LTP), long-term depression, and kindling. Protein synthesis correlates with these enduring processes. We propose a fourth example of long-lasting information storage in the brain, which we call the GABA-withdrawal syndrome (GWS). In rats, withdrawal of a chronic intracortical infusion of GABA, a ubiquitous inhibitory neurotransmitter, induced epileptogenesis at the infusion site. This overt GWS lasted for days. Anisomycin, a protein synthesis inhibitor, prevented the appearance of GWS *in vivo*. Hippocampal and neocortical slices showed a similar post-GABA hyperexcitability *in vitro* and an enhanced susceptibility to LTP induction. One to four months after the epileptic behavior disappeared, systemic administration of a subconvulsant dose of pentylenetetrazol produced the reappearance of paroxysmal activity. The long-lasting effects of tonic $GABA_A$ receptor stimulation may be involved in long-term information storage processes at the cortical level, whereas the cessation of $GABA_A$ receptor stimulation may be involved in chronic pathological conditions, such as epilepsy. Furthermore, we propose that GWS may represent a common key factor in the addiction to GABAergic agents (for example, barbiturates, benzodiazepines, and ethanol). GWS represents a novel form of neurono-glial plasticity. The mechanisms of this phenomenon remain to be understood.

KEYWORDS

Focal epilepsy, GABA-withdrawal syndrome, GWS, memory, motor cortex, GFAP, plasticity

INTRODUCTION

Over the past few years, we have been involved in studies addressing GABA-mediated inhibition in several different models of epilepsy. In photo-sensitive baboons and in kindled rats, we have demonstrated that intracortically applied GABA has powerful anticonvulsant effects (Fukuda, 1987; Brailowsky, 1989). In all cases, we observed that cessation of GABA infusion was associated with the appearance of epileptogenic activity at the site of the GABA infusion (Brailowsky, 1987). Phenomenologically, we named this event the "GABA-withdrawal syndrome (GWS)".

Although originally described in the baboon, GWS was also induced in the rat somatomotor cortex (Brailowsky, 1988), hippocampus, and amygdala (Le Gal la Salle, 1988). We also showed, using a 100

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μg/μL/h dose, an inverse relation between GABA infusion time (from 6 h to 2 wk) and the latency and duration of epileptogenic activity (Brailowsky, 1988). With this procedure, a 3-h infusion of GABA was not effective in inducing GWS. We later found that hippocampal slices, perfused with GABA for 120 min, show an enhanced response to electrical stimulation and decreased paired-pulse during the washing period, GABA-mediated inhibition (Garcia-Ugalde, 1992).

Searching for neurotransmitter receptor specificity and for the minimum time of continuous GABA infusion that is required for inducing GWS, we produced a long-lasting GWS after a relatively short intracortical infusion of either GABA or $GABA_A$ agonist. The absence of an effect using antagonists of the excitatory neurotransmitter glutamate further suggests that the GWS phenomenon is mainly dependent, at least in the first stages, on changes in inhibitory mechanisms.

TABLE 1

Occurrence of epileptogenic EEG activity[1] consecutive to intracortical microinfusion of various agents.

Drug	Dose	Infusion time (min)[2]	% GWS (n)	Latency (min)	Duration (days)
GABA	50 mM	120	76.9 (13)	35.2±3.4	2.2±0.4
	100 mM	120	100 (11)	39.9±6.5	3.3±0.5
	500 mM	120	100 (9)	95.4±10.2	7.0±0.4
	"	60	84.6 (13)	115.0±7.1	3.27±0.9
	"	30	58.3 (12)	99.7±11.8	7.0±2.0
	"	15	0 (8)	—	—
Aniso -> GABA+Aniso.	75 mM ->+ 0.5 M	60 -> 120	0 (4)	—	—
Aniso + GABA	75 mM+ 0.5 M	120	0 (4)	—	—
Isoguvacine	61 mM	120*	100 (7)	127.8±39.9	11.42±0.6
	"	15*	100 (2)	158.5±	6.5±
	"	2*	100 (7)	232.5±13.3	5.0±0.6
THIP	10 mM	120*	100 (8)	98.6±7.7	1.5±0.5
	1 mM	120*	0 (5)	—	—
	100 μM	120	0 (2)	—	—
	10 mM	2*	0 (4)	—	—
	1 mM	2*	0 (4)	—	—
	100 μM	2	0 (2)	—	—
GABA -> APH	0.5 M -> 444 mM	120, 180	(5)	100±22.7	6.0±1.8
GABA -> CNQX	0.5 M -> 100 μM	120, 180	(8)	98.8±11.3	2.8±0.9
Glycine	0.5 M	7 days	0 (4)	—	—
		120	0 (4)	—	—

[1] percent of subjects

[2] All infusions, except the acute (0.4 μL in 2 min) infusion, were performed at a rate of 3 μL/h. Latency (in min) and duration (in days) of GWS are indicated (mean±s.e.m.). Aniso = anisomycin; The antibiotic was given either before and/or with GABA. * = doses that induced EEG slowing during drug infusion. Other abbreviations are given in the text.

EXPERIMENTAL METHODS

Experiments were performed in male Wistar rats (200 to 250g), prepared for chronic recording and intracortical infusion (Brailowsky, 1988). One week after surgery, saline or pharmacological agents (listed in Table 1) were unilaterally applied into the cortex for various periods of time. In separate groups of animals, immediately after GABA or saline disconnection, the respective NMDA and AMPA/quisqualate receptor antagonists, 444 mM APH (aminophosphonoheptanoate) and 100μM CNQX (6-cyano-7-nitrouinoxaline-2,3-dione), were administered locally at 1 μL/h for 180 min. All drug and saline solutions contained 0.2 mg/mL of direct blue to mark the infusion site and the diffusion area. The rats were sacrificed either 10 d after the electrical signs of GWS had disappeared or 30 to 120 d after overt GWS had disappeared, but with the administration of PTZ or its vehicle (saline) before the sacrifice (see below). The brains were processed for Nissl staining and for glial fibrillary acidic protein (GFAP) immunohistochemistry (Hsu, 1981).

RESULTS

In all animals in which GABA was successfully infused, except those of the 15-min infusion group, GWS was observed (Table 1). The $GABA_A$ agonists

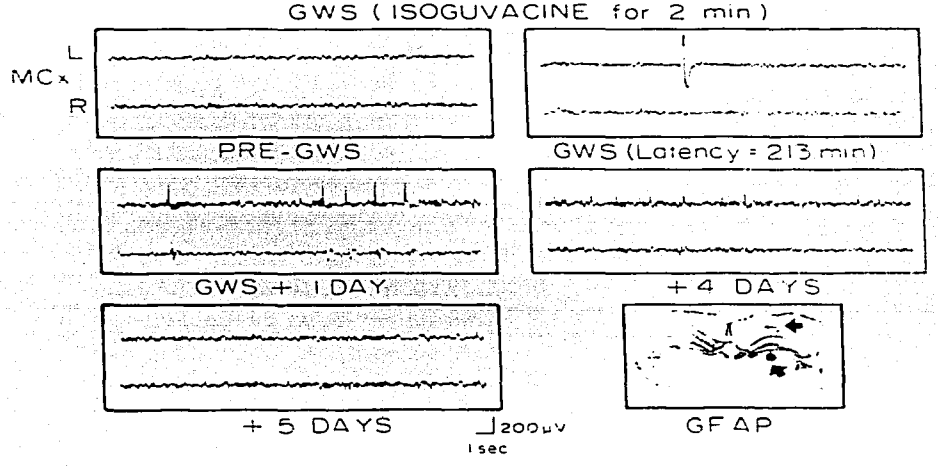

Fig. 1: An example of a GWS-like syndrome induced by acute, intracortically administered isoguvacine, a specific $GABA_A$ agonist, into the left somatomotor cortex. The latency for the appearance of the first epileptic spike was 213 min after injection. Insert: Micrograph of a coronal section of the rat brain processed for glial-fibrillary acidic protein (GFAP) immunoreactivity. Note the astrocytic reaction in the GABA-infused side, both at the cortical site and in ipsilateral thalamic structures (arrows).

used, isoguvacine and THIP, induced GWS with electrographic features similar to those induced by GABA (Table 1). It is noteworthy that acute micro-injections of isoguvacine (2 min) were effective in inducing paroxysmal activity for 5 d or more (see Table 1 and Fig. 1).

Histological analysis showed an area of gliosis at the cortical infusion site and in the thalamic projection site, in particular on the upper portion of the ventrolateral (VL) and posterior groups, and in the reticular nucleus (Fig. 1). GWS induction was not prevented by either APH or CNQX, which when given alone, had no effect.

Intracortical administration of glycine produced neither a behavioral nor an EEG abnormality in the 200-min follow-up period after drug infusion. Sixty to 120 days after the electrical signs of GWS disappeared, rats were injected systemically with a subconvulsant dose (20 to 25 mg/kg i.p.) of PTZ, a widely used epileptogenic agent (Dedeyn, 1992), or with the PTZ vehicle (saline). In 12 of 14 rats, PTZ induced the reappearance of localized, high-voltage paroxysmal activity at the GABA-infused site, similar to the previous GWS and lasting from 60 to 90 min (Fig. 2).

In search of a possible relation between the in vitro analog of GWS and in vitro long-term potentiation (LTP) (Bliss, 1993), we treated cortical slices with GABA, applying the same methods as

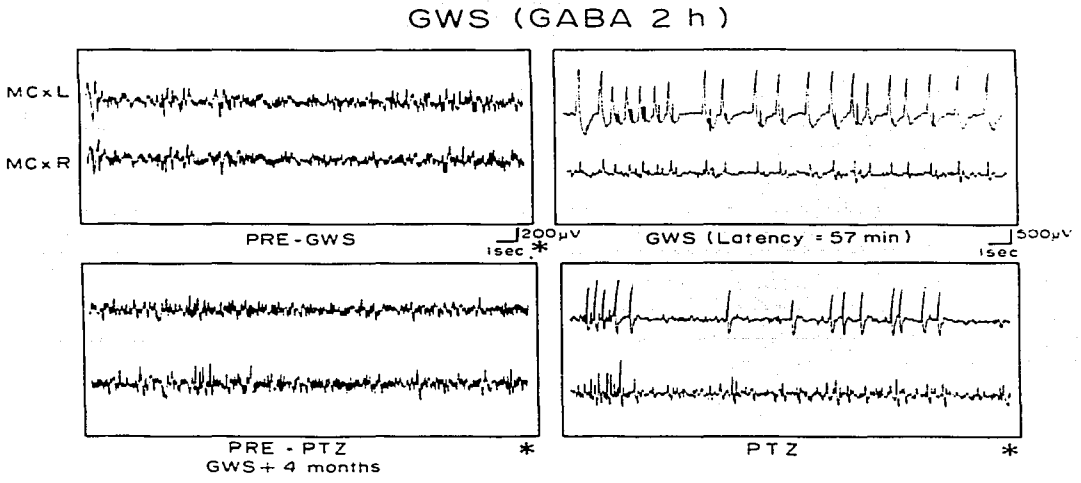

Fig. 2: An example of the electrographic changes typical of a GWS induced after 120 min of continuous intracortical GABA infusion. GABA was applied into the left somatomotor cortex (MCx). The effects of i.p. injection of pentylenetetrazol (20 mg/kg) 4 months after the disappearance of electrographic signs of GWS epileptogenesis are also shown. Note the reactivation at the cortical focus, with characteristics similar to those observed originally.

those used with hippocampal slices. We found that (a) cortical slices also show hyperexcitability after GABA incubation (Fig. 3), and (b) electrical stimulation that failed to produce LTP (40 Hz, 200 ms × 10, at 0.2 Hz) in control slices (n=3) elicited a further enhancement of the already facilitated response in GWS slices (n=4) and most notably, the appearance of additional components.

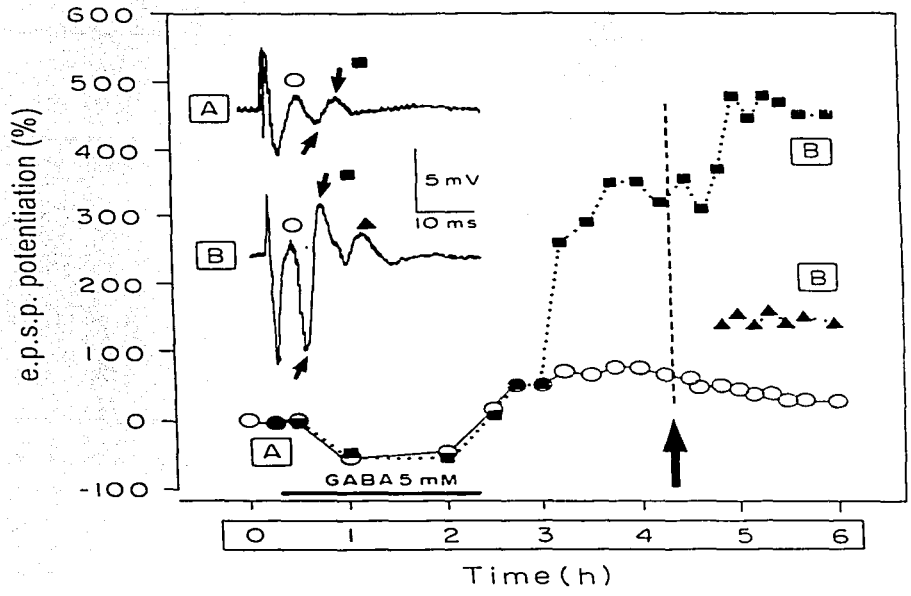

Fig. 3: *In vitro* induction of cortical GWS and the effects of electrical stimulation. Longitudinal brain slices (400 µm), obtained from anesthetized young adult (150 g) Wistar rats, were incubated for 60 min in Ringer-Krebs solution and then for 120 min in 5 mM GABA, followed by a wash. The graph plots the slope of the rising phase of the various components (indicated by symbols) of the population evoked response (e.p.s.p.), recorded from the superficial layers (I-II) of the cortex in response to test stimuli applied to the deep (V-VI) cortical layers. Each point represents the average of 10 responses. The first response is shown in empty circles, the second component, indicated by arrows, is illustrated with filled squares, and in triangles, a late component that appeared after GWS induction and electrical stimulation (40 Hz for 200 ms × 10, at 0.2 Hz). (A) control response; (B) response after GWS induction and electrical stimulation. Note that GABA incubation produced an inhibition of the e.p.s.p. response, followed by a facilitation of about 350%. Electrical stimulation, applied at the arrow, produced a further enhancement of the response of about 100% and the appearance of an additional component (filled triangles) in the response. Calibration, 5 mV, 10 ms.

DISCUSSION

Both the qualitative and quantitative features of the GWS found in this study are comparable to those previously observed (Brailowsky, 1988) in rats, in which a 6-h GABA infusion time was μL/h. It is thus possible that by increasing the infusion rate, we obtained a larger GABA diffusion and, therefore, a larger population of affected cells; alternatively, a saturation of GABA transport or turnover processes, or both may have occurred.

Among the $GABA_A$ agonists, the quantitative differences in GWS may be related to their pharmacokinetic profiles: isoguvacine remains longer in the extracellular space because it is not taken up, whereas THIP is known for its short duration of action (Krogsgaard-Larsen, 1994). This distinction may explain why an acute micro-injection of isoguvacine was as effective as a 30 min GABA infusion.

The specificity of the effects of GABA is supported by

a) the lack of abnormalities observed after the cessation of glycine infusion;
b) the effects of specific $GABA_A$ agonists (this study), and
c) the potentiation of GWS produced by the neurosteroid allopregnanolone, an allosteric modulator of the $GABA_A$ receptor (Calixto, 1995).

The participation of $GABA_B$ receptors in the induction of GWS can be excluded because baclofen does not produce withdrawal signs (on the contrary, the drug itself induces paroxysmal activity). In addition, the specific $GABA_B$ antagonists, phaclofen and CGP 35348, do not modify GWS features (Brailowsky, 1995). In addition, GWS was not prevented by the glutamate receptor antagonists APH or CNQX.

In the current study, we also confirmed the histological changes previously reported by Brailowsky (1988), both at the infusion site and in ipsilateral thalamic areas, adding immunohistochemical evidence (GFAP staining) of astrocytic participation in this reaction. The thalamic changes are similar to those reported with cortical epileptogenic foci that were induced with convulsant agents, such as bicuculline or penicillin, and shown to be excitotoxic in nature because they can be prevented by NMDA antagonists (Clifford, 1989). We propose that this event, gliosis, is but one manifestation of many important changes that are occurring in glial function. An explanation of these events will be critical to understanding the mechanisms involved in GWS.

Chronic GABA exposure may induce the creation of some form of "epileptic" GABA receptor or an enhanced vulnerability to seizures, or both. For example, in Angelman syndrome, a clinical condition in which epileptic seizures are frequent, a deletion of genes encoding the $GABA_A$ receptor subunits, alpha-5, beta-3, and gamma-3, in chromosome 15q11-13, has been reported (Mclean, 1995).

The $GABA_A$ receptor is a member of the superfamily of ionotropic receptors comprising several subunits, whose combination determines the particular pharmacology reported for diverse brain regions (Olsen, 1990; Macdonald, 1994). This property of allosteric modulation is relevant to widely used and clinically important drugs, such as the benzodiazepines, barbiturates, neurosteroids, and ethanol. After chronic administration, all these drugs, except the neurosteroids, can induce physical dependence and withdrawal signs. A common neurochemical mechanism involving GABA function has been suggested for such abstinence symptoms (Cowen, 1982). The premenstrual syndrome has been proposed to be a possible withdrawal syndrome to progesterone, a positive modulator of the $GABA_A$ receptor (Gallo, 1993). A progesterone metabolite, allopregnanolone, significantly potentiates GWS (Calixto, 1995). Despite the clinical differences in these withdrawal syndromes, an understanding of the pathophysiology of GWS may enable us to decipher the mechanisms responsible for sedative, anxiolytic, and hypnotic drug addiction, a

significant epidemiological problem in many countries. In fact, GWS may represent a common factor in all such addictions.

A fascinating result of this study was the long-lasting (months) consequence of a relatively short exposure to GABA or to $GABA_A$ agonists (but not to glycine), protracted effects that could be unmasked by the systemic administration of PTZ (but not saline), even 4 months after the apparent disappearance of epileptogenic activity. If we consider such enduring epileptogenic activity as a synaptic expression that is analogous to memory (an "epileptogenic" experience), then a role for GABA in memory processes (conceived as cellular information storage) can be postulated.

A further suggestion for a relation between GWS and information storage derives from our results with anisomycin, an inhibitor of protein synthesis. A wide variety of experimental models, from snails to humans, have demonstrated that when administered from 1 h before and up to 7 h after a training or sensitization procedure, antibiotics like anisomycin induce amnesia to the task (Barzilai, 1989; Oleary, 1995). Anisomycin blocks GWS induction and, interestingly, also interferes with drug dependence (Williams,1994).

The GWS is a remarkable example of synaptic plasticity: its basic mechanism, neuronal and glial, may be analyzed from the rich perspectives offered by studies in epilepsy, drug dependence, and information storage.

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REFERENCES

Barzilai A., Kennedy TE, Sweatt JD, Kandel ER. 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in *Aplysia* sensory neurons. Neuron 1989; 2: 1577–1586.

Bliss TVP, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 1993; 361: 31–39.

Brailowsky S, Kunimoto M, Menini C, Silva-Barrat C, Riche D, Naquet R. The GABA-withdrawal syndrome: a new model of focal epileptogenesis. Brain Res 1988; 442: 175–179.

Brailowsky S, Menini C, Silva-Barrat C, Naquet R. Epileptogenic γ-aminobutyric acid-withdrawal syndrome after chronic intracortical infusions in baboons. Neurosci Lett 1987; 74: 75–80.

Brailowsky S, Montiel T, Meneses S, Discala G. Effects of GABA(B) receptor antagonists on two models of focal epileptogenesis. Brain Res 1995; 702: 126–132.

Brailowsky S, Silva-Barrat C, Menini C, Riche D, Naquet R. Effects of localized chronic GABA infusions into different cortical areas of the photo-sensitive baboon *Papio papio*. Electroencephalogr Clin Neurophysiol 1989; 72: 147–56.

Calixto E, Montiel T, Lemini C, Brailowsky S. Allopregnanolone potentiates a GABA-withdrawal syndrome in the rat cerebral cortex. Neurosci Lett 1995; 195: 73–76.

Clifford DB, Zorumski CF, Olney JW. Ketamine and MK-801 prevent degeneration of thalamic neurons induced by focal cortical seizures. Exp Neurol 1989; 105: 272–279.

Cowen PJ, Nutt DJ. Abstinence symptoms after withdrawal of tranquilizing drugs: is there a common neurochemical mechanism? Lancet 1982; 360–362.

Dedeyn PP, Dhooge R, Marescau B, Pei YQ. Chemical models of epilepsy with some reference to their applicability in the development of anticonvulsants. Epilepsy Res 1992; 12: 87–110.

Fukuda H, Brailowsky S, Menini C, Silva-Barrat C, Riche D, Naquet R. Anticonvulsant effect of intra-cortical chronic infusions of GABA in kindled rats: focal seizures upon withdrawal. Exp Neurol 1987; 98: 120–129.

Gallo MA, Smith SS. Progesterone withdrawal decreases latency to and increases duration of electrified prod burial: a possible rat model of PMS anxiety. Pharmacol Biochem Behav 1993; 46: 897–904.
Garcia-Ugalde G, Galarraga E, Bargas J, Brailowsky S. Hyperexcitability of hippocampal CA1 region in brain slices after GABA withdrawal. Neurosci Lett 1992; 147: 229–232.
Hsu SM, Raine L, Fanger H. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577–580.
Krogsgaard-Larsen P, Frolund B, Jorgensen FS, Schousboe A. GABA-A receptor agonists partial agonists and antagonists. Design and therapeutic prospects. J Med Chem 1994; 37: 2489–2505.
Le Gal La Salle G, Brailowsky S, Menini C, Naquet R. Local asymptomatic status epilepticus induced by withdrawal of GABA infusion into limbic structures. Exp Neurol 1988; 101: 411–417.
Macdonald RL, Olsen RW. GABA(A) receptor channels. Ann Rev Neurosci 1994; 17 569–602.
Mclean PJ, Farb DH, Russek SJ. Mapping of the alpha(4) subunit gene (GABRA4) to human chromosome 4 defines an (alpha2)-(alpha4)-(beta1)-(gamma1) gene cluster: Further evidence that modern GABA(A) receptor gene clusters are derived from an ancestral cluster. Genomics 1995; 26: 580–586.
Oleary FA, Byrne JH, Cleary LJ. Long-term structural remodeling in *Aplysia* sensory neurons requires *de novo* protein synthesis during a critical time period. J Neurosci 1995; 15: 3519–3525.
Olsen RW, Tobin AJ. Molecular biology of $GABA_A$ receptors. FASEB J 1990; 4: 1469–1480.
Williams MB, Jope RS. Protein synthesis inhibitors attenuate seizures induced in rats by lithium plus pilocarpine. Exp Neurol 1994; 12: 169–173.