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MODULACIÓN POR BDNF Y NGF DE LA POTENCIACIÓN DE LARGO PLAZO EN EL GANGLIO CERVICAL SUPERIOR: ¿EFECTO DIFERENCIAL DE LOS RECEPTORES A NEUROTROFINAS p75NTR Y TrkA?

TESIS

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LISTA DE ABREVIATURAS

NGF: Factor de crecimiento neuronal.

BDNF: Factor de crecimiento derivado de cerebro.

NT-3: Neurotrofina 3.

SCG: Ganglio cervical superior.

CAP: Potencial de acción compuesto.

LTP: Potenciación de largo plazo.

TrkA: Receptor tirosina cinasa A.

TrkB: Receptor tirosina cinasa B.

p75NTR: Receptor de neurotrofinas p75NTR.

PA: Potencial de acción.

KCNQ: Canal de potasio Kv7.1-Kv7.5.

XE991: Bloqueador selectivo de canales KCNQ.

K252a: Inhibidor de la actividad enzimática de tirosin cinasa.

TrkA-IgG: Proteína de fusión que impide la interacción entre NGF y el receptor TrkA-IgG.

PFA: Paraformaldehído.

PBS-Tx: Buffer de fosfatos con tritón.

DMSO: Dimetil sulfóxido.

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RESUMEN

El sistema nervioso simpático es una de las vías eferentes del sistema nervioso autónomo que se encarga de regular la actividad de los tejidos somáticos en la periferia. La regulación de la actividad del sistema nervioso simpático es sumamente importante ya que controla la contracción del músculo liso y cardiaco, así como la secreción glandular, lo que lo convierte en un sistema clave para el mantenimiento de la homeostasis. Se sabe que la transmisión sináptica en el sistema simpático está regulada por una gran variedad de sustancias neuroactivas, como las neurotrofinas, por lo que se planteó como objetivo de este estudio determinar el efecto agudo del factor de crecimiento derivado de cerebro (BDNF) y del factor de crecimiento neuronal (NGF) sobre la eficacia de la transmisión sináptica del ganglio cervical superior (SCG) de la rata. Estudiamos el efecto de la aplicación aguda de BDNF y NGF sobre la transmisión sináptica ganglionar en la preparación del SCG en aislamiento registrando el potencial de acción compuesto (CAP) del nervio carotídeo interno en condiciones basales y durante la potenciación de largo plazo (LTP). En nuestro modelo la aplicación de neurotrofinas no modificó la transmisión basal, sin embargo, en presencia de BDNF 200 ng/ml ($BDNF_{200}$) o NGF 500 ng/ml (NGF_{500}) la LTP aumentó significativamente con respecto al control. Por otra parte, la aplicación de NGF 200 ng/ml (NGF_{200}) atenuó la LTP en un 40%. Caracterizamos la expresión de los receptores a neurotrofinas en neuronas ganglionares y encontramos que 83% de las neuronas expresan al receptor receptor tirosina cinasa A (TrkA), 52% expresan al receptor p75 (p75NTR) y 18% expresan al receptor receptor tirosina cinasa B (TrkB). Determinamos las contribuciones relativas de los receptores p75NTR y TrkA para el efecto de $BDNF_{200}$ y NGF_{200} sobre la LTP y encontramos que al mimetizar la activación de p75NTR con el compuesto c_2-ceramida la LTP se incrementó dramáticamente y que la activación del receptor TrkA es requisito para que se presente la atenuación de la LTP por NGF_{200} ya que al bloquear la activación del receptor utilizando inhibidores como tirofostina o un anticuerpo dirigido contra el dominio extracelular que impide la activación por NGF, el efecto de NGF_{200} sobre la LTP se bloquea. Caracterizamos la contribución de los canales KCNQ para el efecto de $BDNF_{200}$ y NGF_{200} sobre la LTP ganglionar utilizando XE991 y flupirtina, los cuales bloquean y activan a los canales respectivamente. Encontramos que en presencia de XE991 la LTP se atenuó significativamente con relación al control, mientras que al activar los canales KCNQ con flupirtina la LTP aumentó significativamente. Para determinar si los canales KCNQ contribuyen para los efectos de BDNF y NGF sobre la LTP, incubamos ganglios en presencia de XE991 y posteriormente los tratamos con neurotrofinas. En esta condición el tratamiento subsecuente con $BDNF_{200}$ o NGF_{500} no incrementó la LTP. En conclusión, nuestros datos muestran que BDNF y NGF regulan de manera bidireccional la LTP del SCG actuando a través de los receptores p75NTR y TrkA. Mientras que la activación de p75NTR por $BDNF_{200}$ y NGF_{500} aumenta la LTP, la activación de TrkA por NGF_{200} la atenúa. Proponemos que p75NTR y TrkA regulan de manera diferencial la actividad de los canales KCNQ y de esa manera modulan la excitabilidad de las neuronas ganglionares y por ende la forma en la que responden a los estímulos sinápticos, aumentando o disminuyendo su capacidad de expresar LTP.

ABSTRACT

The aim of this study was to test if brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) can modulate cholinergic synaptic transmission in the superior cervical ganglion of the rat (SCG). The SCG is an important structure of the sympathetic nervous system for regulating the activity of target organs, such as the heart. A recent study has shown that cholinergic transmission between sympathetic neurons in culture can be modulated by acute application of BDNF or NGF via the neurotrophin receptor p75 (p75NTR) suggesting that neurotrophins may play a role in the regulation of synaptic transmission in the intact sympathetic ganglia. Here we characterized the effect of the acute application of BDNF or NGF on the population response (CAP) of the superior cervical ganglion (SCG) of the rat, recorded from the internal carotid nerve. We evaluated synaptic responses during basal conditions and during ganglionic long-term potentiation (LTP) and found that neurotrophins did not affect basal transmission, however, they affected LTP. In our experimental system BDNF 200 ng/ml ($BDNF_{200}$) increased LTP compared to control, in contrast, NGF showed a dual effect: at 200 ng/ml (NGF_{200}) it reduced LTP to 40% of control value, whereas it significantly increased LTP when applied at 500 ng/ml (NGF_{500}). To interpret our electrophysiological results, we characterized the expression of neurotrophin receptors in ganglionic sympathetic neurons of the intact SCG by immunofluorescence and found that 83% of ganglionic sympathetic neurons are tropomyosin receptor kinase A (TrkA) positive, whereas 52% are p75NTR positive and 18% are tropomyosin receptor kinase B (TrkB) positive. Based on this evidence we determined the relative contributions of p75NTR and TrkA to de modulation of LTP mediated by BDNF and NGF. First we induced LTP in presence of the compound c_2-ceramide to mimic p75NTR activation and we found that it produced a dramatic increase in LTP. On the other hand, we use the tyrosine kinase inhibitor tyrphostin AG879 and an anti-TrkA antibody to determine if the attenuating effect of NGF_{200} on LTP was due to TrkA activation. We found that both treatments blocked the effect of NGF_{200} on LTP, returning it to control values. Finally, we asked whether potassium KCNQ channels could play a role as effectors of the BDNF and NGF triggered signaling pathways involved in the regulation of LTP. To determine the involvement of KCNQ channels in the phenomenon we induced LTP in presence of the KCNQ channel blocker XE991 and the channel opener flupirtine respectively. Our experiments showed that blockade of KCNQ channels attenuated LTP in a similar fashion as NGF_{200} did. Conversely, the KCNQ channel activator flupirtine enhanced the magnitude of LTP in a similar way as BDNF did. Additionally, the stimulatory effect of $BDNF_{200}$ and NGF_{500} on LTP was abolished by pretreatment of sympathetic ganglia with XE991. In conclusion, BDNF and NGF acting on p75NTR enhance LTP, whereas NGF acting preferentially through TrkA receptors attenuate LTP. We propose that p75NTR and TrkA receptors differentially regulate KCNQ channel activity by an unknown mechanism and in that way they control the way in which synaptic stimuli are integrated in ganglionic neurons, leading to a differential regulation of synaptic plasticity.

INTRODUCCIÓN

La transmisión sináptica es el proceso que permite la propagación de las señales eléctricas de una neurona a otra a través del sistema nervioso. Para que el proceso pueda llevarse a cabo se requiere de estructuras especializadas denominadas sinapsis las cuales pueden ser de dos tipos: eléctricas o químicas. Las sinapsis eléctricas están formadas por proteínas llamadas conexinas que al ensamblarse crean poros en la membrana plasmática que permiten el flujo bidireccional de iones entre las dos neuronas conectadas sinápticamente; por su parte, las sinapsis químicas cuentan con una maquinaria especializada que acopla la llegada del impulso nervioso o potencial de acción (PA) a la terminal presináptica con la fusión de vesículas y la secreción de neurotransmisores hacia el espacio sináptico (Fig. 1A). En esta modalidad de neurotransmisión los transmisores difunden a través del espacio sináptico hasta la membrana postsináptica en donde se unen a sus receptores, los cuales al activarse generan una cambio en el potencial eléctrico de la membrana de la neurona postsináptica (Fig. 1A y 1B)

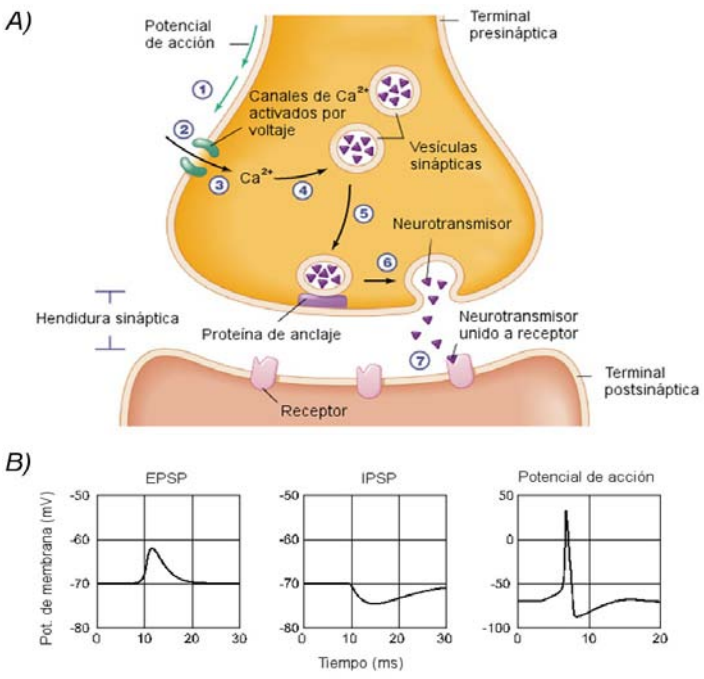

Figura 1: La transmisión sináptica química. *A)* Esquema de una sinapsis química y secuencia de eventos importantes ocurridos durante la transmisión del impulso nervioso: 1) Llegada del potencial de acción y despolarización de la terminal presináptica; 2) Apertura de canales de Ca^{2+} sensibles a voltaje; 3) Incremento en la concentración de Ca^{2+} intracelular; 4) Movilización de vesículas con neurotransmisores hacia la membrana plasmática; 5) Fusión de vesículas y secreción de neurotransmisores; 6) Unión de los neurotransmisores a sus receptores post-sinápticos. *B)* La unión de los neurotransmisores a sus receptores postsinápticos produce un cambio en el potencial de membrana de la neurona postsináptica que puede ser inhibidor o excitador, e incluso generar un potencial de acción (EPSP: Potencial post-sináptico excitador; IPSP: Potencial post-sináptico inhibidor). Imagen tomada de www.studyblue.com. Copyright 2006, Pearson Education.

A la capacidad que tiene una terminal presináptica para generar un cambio en el potencial eléctrico de una terminal postsináptica se le conoce como eficacia sináptica (López 2002), dicha capacidad esta regulada por diversos factores exógenos y endógenos tales como el nivel de actividad de la sinapsis y la acción de diversas sustancias neuroactivas como las neurotrofinas, de las cuales hablaremos más adelante.

Las modificaciones de la eficacia sináptica producidas por patrones específicos de actividad presináptica se conocen como fenómenos de plasticidad. Una forma bien caracterizada de plasticidad sináptica es la LTP, la cual fue descubierta en 1973 por Bliss y Lømo mientras realizaban estudios electrofisiológicos en el hipocampo de conejo (Bliss & Lømo 1973). Al inicio la LTP se consideró un fenómeno exclusivo de las sinapsis del hipocampo pues tras su descubrimiento se le relacionó inmediatamente con los procesos de aprendizaje y memoria, sin embargo, más adelante otros estudios demostraron que la LTP se expresa también en otras regiones del sistema nervioso, por lo que se aceptó que se trata de una propiedad general de las sinapsis. En 1982 Brown y McAfee publicaron un artículo en la revista *Science* donde describieron por primera vez un fenómeno de plasticidad sináptica semejante a la LTP del hipocampo en el SCG de la rata al cual llamaron LTP ganglionar (Brown & McAfee 1982).

La LTP ganglionar se expresa como un aumento en la amplitud de la respuesta postsináptica del SCG en respuesta a la estimulación tetánica de las terminales preganglionares, dicho aumento decae con una cinética bi-exponencial y tiene una duración promedio de 60 minutos (Vargas et al. 2007). Un modelo propone que el mecanismo de la LTP es trans-sináptico y participan activamente la pre- y la post-sinapsis. La despolarización repetida de las terminales preganglionares genera la liberación masiva de acetilcolina por acumulación de Ca^{2+}, lo que corresponde a la primera exponencial(Briggs et al. 1985), sin embargo, se ha propuesto que hay un segundo mecanismo que participa en el mantenimiento de la LTP ganglionar (Morales et al. 1994).

El modelo trans-sináptico propone que la fase de mantenimiento de la LTP se debe a un aumento en la sensibilidad de los receptores nicotínicos producido por la acción de uno o varios inductores que se liberan desde las terminales presinápticas como resultado de la estimulación de alta frecuencia (Cifuentes et al. 2013). Se sabe que algunos factores neurotróficos como las familia neurotrofinas regulan la eficacia de la transmisión colinérgica entre neuronas simpáticas del SCG en cultivo (Fig. 2A), por lo cual, podrían ser candidatos para funcionar como inductores de la LTP.

En un trabajo reciente se reportó que la aplicación aguda de 100 ng/ml de BDNF o NGF, así como de otros agonistas del receptor p75NTR (e. g. c_2-ceramida) es suficiente para aumentar la sensibilidad de la post-sinapsis a la acción de la acetilcolina liberada de forma espontánea desde la presinapsis. En este sistema el tratamiento con neurotrofinas genera un aumento observable en la amplitud de las corrientes postsinápticas excitadoras mediado por la activación del receptor p75NTR (Fig. 2B) (Luther et al. 2013).

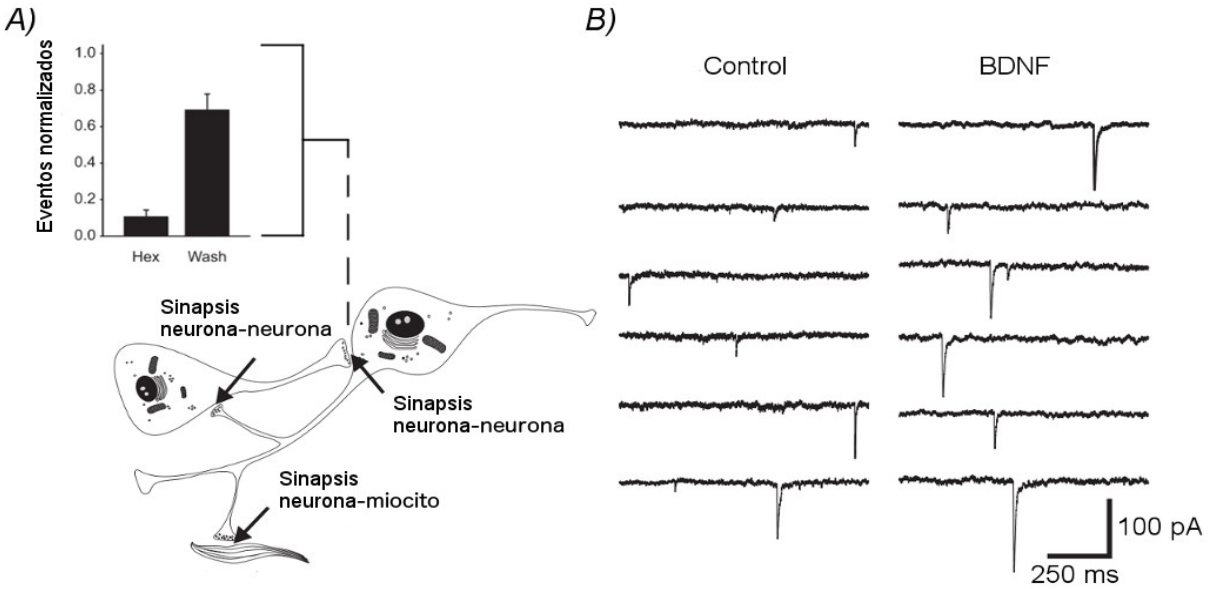

Figura 2: BDNF modula la transmisión colinérgica entre neuronas simpáticas en co-cultivo con miocitos cardiacos. *A)* Esquema de las sinapsis que se forman en el modelo de co-cultivo celular de neuronas simpáticas con miocitos cardiacos. Se muestra como la transmisión sináptica entre neuronas simpáticas es sensible a hexametonio, lo que indica su naturaleza colinérgica. *B)* BDNF aumenta la amplitud de las corrientes sinápticas excitadoras. Experimento de voltage clamp registrado a V_h=-60 mV donde se observa que la amplitud de las corrientes unitarias espontáneas se incrementa tras 15 minutos de la aplicación de BDNF (Tomado de Luther et al., 2013).

El trabajo de Luther y colaboradores sugiere que las neurotrofinas BDNF y NGF podrían modular la transmisión ganglionar colinérgica del sistema simpático *in vivo*, probablemente acoplando la excitabilidad de la postsinapsis con el nivel de actividad requerido por la presinapsis o viceversa.

Las neurotrofinas también modulan la eficacia de otras sinapsis. El grupo del Dr. Mu Ming Poo estudió los cambios agudos en la actividad sináptica generados por el tratamiento con neurotrofinas utilizando como modelo la sinapsis neuromuscular de *Xenopus* en cultivo (Fig. 3A). En ese sistema registraron las corrientes sinápticas en los miocitos con la técnica de *patch clamp.* En un experimento clásico, Poo y colaboradores pudieron observar que el tratamiento con 50 ng/ml de NT-3 o BDNF incrementó la actividad sináptica espontánea de la sinapsis neuromuscular de *Xenopus* (Fig. 3B y C), por otra parte, NGF no tuvo efecto sobre la actividad de esta sinapsis (Lohof et al. 1993).

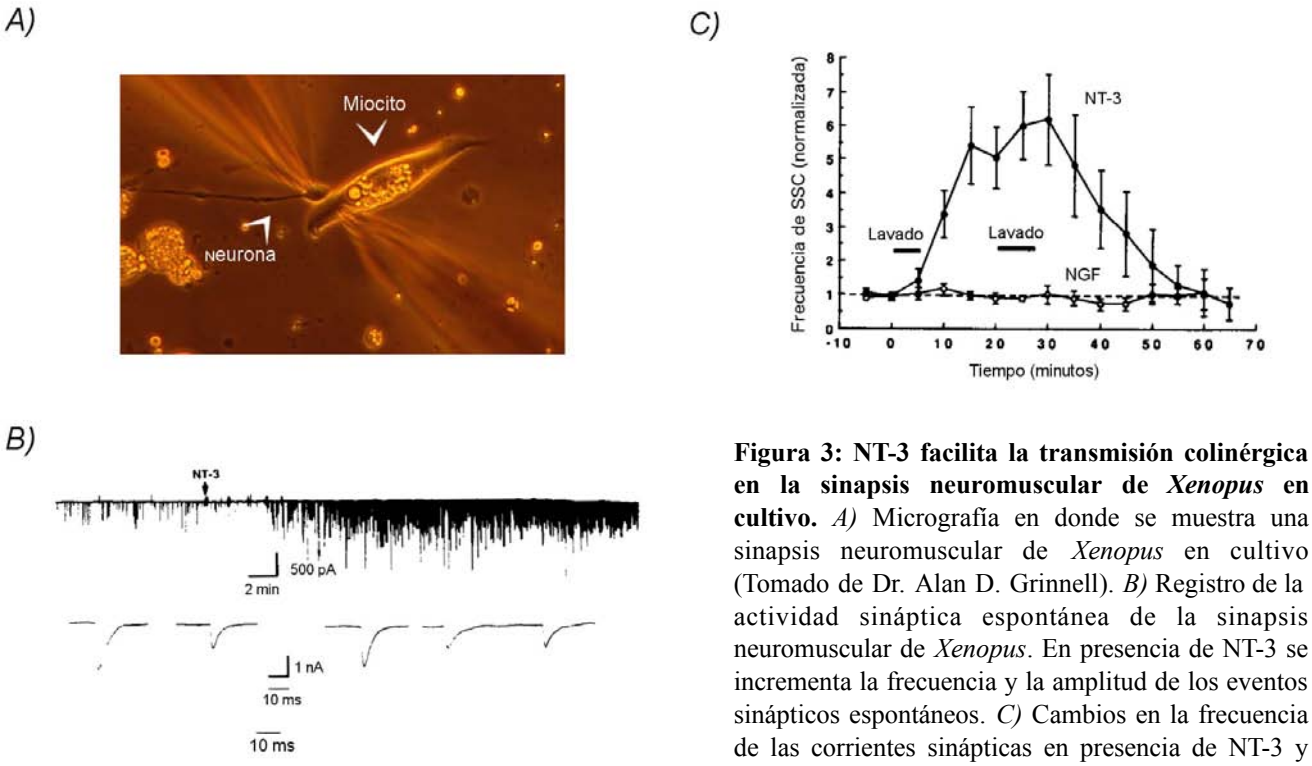

Figura 3: NT-3 facilita la transmisión colinérgica en la sinapsis neuromuscular de *Xenopus* en cultivo. *A)* Micrografía en donde se muestra una sinapsis neuromuscular de *Xenopus* en cultivo (Tomado de Dr. Alan D. Grinnell). *B)* Registro de la actividad sináptica espontánea de la sinapsis neuromuscular de *Xenopus*. En presencia de NT-3 se incrementa la frecuencia y la amplitud de los eventos sinápticos espontáneos. *C)* Cambios en la frecuencia de las corrientes sinápticas en presencia de NT-3 y NGF, normalizado con relación al control (Tomado de Lohof et al., 1993).

Con otro experimento demostraron que la activación del receptor TrkB en la presinapsis es necesaria para que se produzca la facilitación sináptica mediada por BDNF y NT-3 (Lohof et al. 1993). Dado que el tratamiento con NGF no modifica la transmisión sináptica y el inhibidor de tirosin cinasa K252a bloquea el efecto de BDNF y de NT-3 (Fig. 3C), los autores concluyeron que el efecto facilitador de BDNF sobre la transmisión sináptica en su modelo se debía a un aumento en la probabilidad de liberación de acetilcolina mediado por el receptor TrkB (Lohof et al. 1993).

Tras el trabajo de Lohof y colaboradores, otros grupos de investigación se interesaron en estudiar el efecto agudo de las neurotrofinas sobre la eficacia sináptica en otros sistemas, entre ellos el grupo del Dr. Rodolfo Llinás. El grupo del Dr. Llinás reportó por primera vez que la aplicación exógena de NGF tiene un efecto atenuador sobre la transmisión sináptica, utilizando la preparación del ganglio estelar del manto de calamar (Fig. 4A). Al registrar el potencial postsináptico con un microelectrodo insertado en el sitio preciso de la sinapsis y estimular en la presinapsis observaron que la aplicación de 100 ng/ml de NGF disminuye paulatinamente la amplitud del potencial de acción post-sináptico a partir de los 10 minutos de la aplicación y hasta que la amplitud del potencial se abate casi por completo a los 50 minutos después del tratamiento (Moreno et al. 1998) (Fig. 4B).

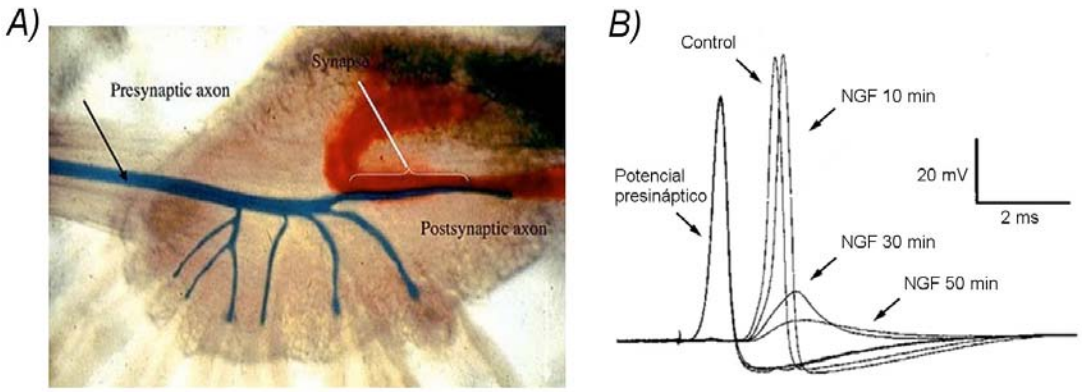

Figura 4: NGF atenúa la transmisión sináptica en el ganglio estelar de calamar. *A)* Micrografía en donde se puede observar la estructura macroscópica de la sinapsis del manto del calamar *Loligo,* se observa la zona activa de la sinapsis y los axones pre- y post-sinápticos. *B)* Potenciales de acción pre- y post-sinápticos registrados en la sinapsis de *Loligo* con microelectrodos de vidrío antes y después de la aplicación extracelular de NGF 100 ng/ml. Se observa que la respuesta post-sináptica se encuentra abatida casi en su totalidad a los 50 minutos de la aplicación de NGF (Tomado de Moreno et al., 1998).

Al microinyectar de forma intracelular inhibidores de tirosin cinasa en la postsinapsis, como K252a o genisteina, observaron que ambos bloquearon el efecto de NGF, por lo cual, concluyeron que la activación del receptor TrkA por NGF disminuye la eficacia de la transmisión sináptica y que el sitio en donde se lleva a cabo dicha modulación es en la post-sinapsis.

Con respecto a la modulación de la LTP por neurotrofinas se sabe que tanto BDNF como NGF modulan la LTP en estructuras del sistema nervioso central como el hipocampo y la corteza visual de la rata. Por un lado, el tratamiento con BDNF (2 nM) promueve la inducción de la LTP en la sinapsis que se establecen entre las fibras de Schaffer y las neuronas de la región CA1 del hipocampo (Fig. 5A). En este modelo se observó que en animales en etapa post-natal donde la LTP no se expresa, la aplicación de BDNF permite la expresión de LTP de manera robusta en respuesta a la estimulación tetánica (Fig. 5B) (Figurov et al. 1996). La expresión de BDNF parece ser fundamental para la capacidad de las sinapsis de expresar LTP en el hipocampo ya que en animales BDNF *knock-out* no hay expresión de LTP (Korte et al. 1998).

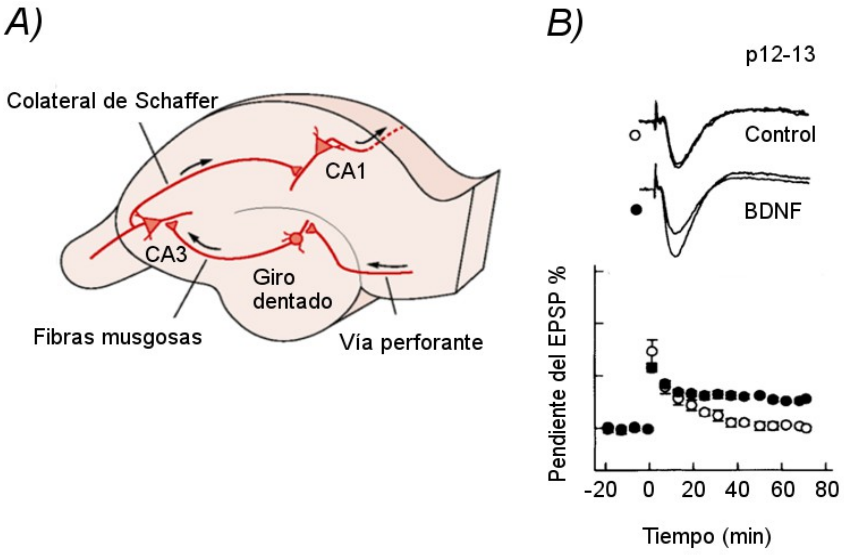

Figura 5: BDNF promueve la expresión de LTP en el hipocampo. *A)* Esquema del hipocampo en donde se puede observar la sinapsis que se forma entre las colaterales de Schaffer y las neuronas de CA1 (Tomado de studyblue.com). *B)* Potenciación de la eficacia sináptica por estimulación a alta frecuencia en la sinapsis de CA1. Arriba, se muestra el registro de el EPSP poblacional antes y después de la aplicación del tren de alta frecuencia en condiciones control y en presencia de BDNF; abajo, cinética de la potenciación sináptica. Estímulo tetánico 3 trenes de 30 pulsos a 100 Hz por 3 segundos (Tomado de Figurov et al., 1996).

En el caso de NGF, la aplicación de 100 ng/ml en las capas II/III de la corteza visual bloquea la LTP durante el periodo crítico para la deprivación monocular en ratas (p16 – 18) (Fig. 6A y B) (Fagiolini et al. 1994), que es la etapa en que la expresión de LTP se encuentra normalmente en su punto máximo (Kirkwood & Bear 1995). De manera interesante, en el mismo modelo el tratamiento con TrkA-IgG en los días p30 – 35, edad en la que el periodo crítico de la deprivación monocular está casi extinto y la LTP se encuentra regulada a la baja, restablece la expresión de LTP (Pesavento et al. 2000). Este trabajo muestra que NGF es un regulador negativo de la LTP y que en la corteza visual limita la plasticidad de las sinapsis una vez que las conexiones necesarias han sido formadas a través de la señalización mediada por el receptor TrkA.

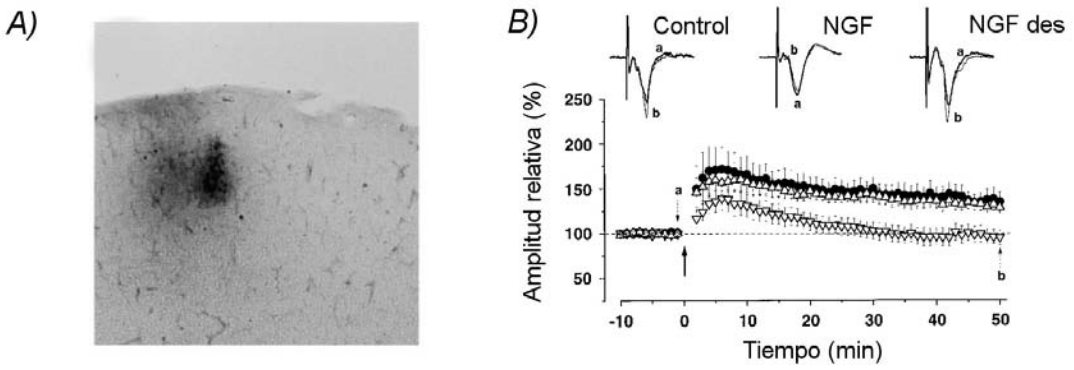

Figura 6: Efecto de NGF sobre la LTP en la corteza visual. *A)* Micrografía de una rebanada de cerebro donde se observa una región de neuronas marcadas con biocitina cercanas a la región de la corteza en donde se realizó el registro, capas II/III. *B)* Efecto de NGF sobre la LTP de la corteza visual. La aplicación de NGF 100 ng/ml (triángulo invertido) reduce la LTP de la corteza visual en relación al control (círculo) y al grupo al que se le aplicó NGF desnaturalizado por temperatura (triángulo). Se eplicó un estímulo tetánico de 3 trenes de 100 pulsos a 100 Hz por 10 segundos (Tomado de Pesavento et al., 2000).

Los mecanismos neuronales finos que permiten la modulación de la eficacia sináptica mediada por neurotrofinas no se conocen en detalle, sin embargo, hay varios estudios en los que se muestra que BDNF y NGF regulan la actividad de diversos canales iónicos a través de la activación de los receptores TrkA y p75NTR. Se sabe que uno de lo blancos de los receptores a neurotrofinas son los canales KCNQ (Kv7.2 y Kv7.3), los cuales acarrean la corriente de K^+ tipo M (I_M) (Wang 1998).

Esta corriente es fundamental para regular el patrón de disparo en las neuronas simpática ganglionares y en otros tipos neuronales del sistema nervioso central como las neuronas del giro dentado (Jia et al. 2008; Luther & Birren 2009; Nieto-Gonzalez & Jensen 2013).

I_M es una corriente que se activa a potenciales subumbrales (~60 mV) y no presenta inactivación por lo que genera una corriente saliente constante que contribuye para el mantenimiento del potencial de membrana en respuesta a corrientes despolarizantes, al tener activación lenta I_M no contribuye para la repolarización de los PA individuales pero tiene un profundo efecto sobre la excitabilidad neuronal y la regulación del patrón de disparo neuronal (Brown & Passmore 2009). Uno de los efectos más notables de bloquear los canales KCNQ con drogas como linopiridina o XE991 es el aumento en la frecuencia de disparo (Fig. 7A) (Brown & Passmore 2009).

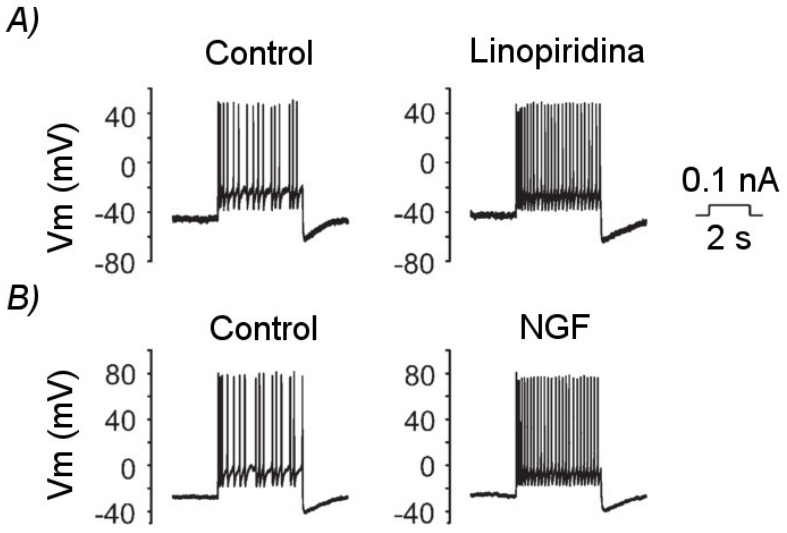

Figura 7: Aumento en la frecuencia de disparo de neuronas simpáticas en cultivo en respuesta al bloqueo de los canales KCNQ. *A)* La aplicación de linopiridina (30 µM, 1 min) o *B)* NGF (20 ng/ml, 2 min) en el cultivo de neuronas simpáticas produce un aumento en la frecuencia de disparo neuronal debido a la atenuación de la corriente M por el bloqueo de los canales KCNQ (Tomado de Jia et al., 2008).

Por otro lado, drogas como flupirtina y retigabina, que aumentan la actividad de los canales KCNQ, disminuyen la frecuencia de disparo neuronal y favorecen el patrón de disparo fásico (Fig. 8A) (Brown & Passmore 2009).

De manera interesante, BDNF y NGF han demostrado tener efectos sobre I_M y el patrón de dispar, los cuales dependen del tipo de receptor sobre el cual actúan. Por ejemplo, la aplicación de 20 ng/ml de NGF inhibe la amplitud de I_M en neuronas simpáticas disociadas del SCG de rata, produciendo un aumento en la frecuencia de disparo similar al observado en presencia de linopiridina (Fig. 7B) (Jia et al. 2008); mientras que el tratamiento de neuronas del giro dentado con retigabina (10 μM) favorece el disparo fásico al reducir el número de potenciales de acción (Fig. 8A), efecto que se mimetiza al aplicar 15 ng/ml de BDNF (Fig. 8B) (Nieto-Gonzalez & Jensen 2013).

Los efectos de BDNF y NGF sobre el patrón de disparo neuronal se han reportado en neuronas simpáticas en cultivo, donde la aplicación de BDNF favorece el disparo fásico por medio de la activación del receptor p75NTR e incrementa la amplitud de I_M, mientras que NGF favorece el disparo tónico al activar al receptor TrkA y atenúa la amplitud de I_M (Luther & Birren 2009).

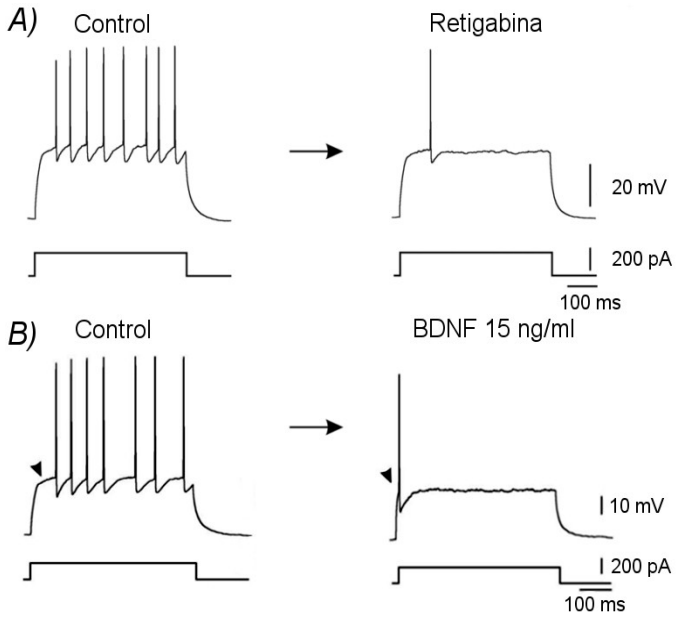

Figura 8: La activación farmacológica de los canales KCNQ por retigabina o BDNF favorece el modo de disparo fásico. *A)* Registro en fijación de corriente en una interneurona con inmunoreactividad para parvalbúmina que muestra el disparo antes y 15 minutos después de la aplicación de retigabina (*A*) o BDNF (15 ng/ml) (*B*). Se observa el cambio en el patrón de disparo de tónico a fásico producido por la activación de los canales KCNQ, la cabeza de flecha en *B)* indica una disminución en la primera latencia (Tomado de Nieto-Gonzales et al., 2013).

PLANTEAMIENTO DEL PROBLEMA CIENTÍFICO

Los estudios mencionados nos permiten plantear las siguientes preguntas: 1) ¿Las neurotrofinas modulan la transmisión sináptica y la LTP en el SCG de la rata? Y 2) ¿Los canales KCNQ contribuirán para la posible modulación de la transmisión ganglionar mediada por neurotrofinas?

Nuestras hipótesis son que por analogía a lo que se ha reportado en otros sistemas y a la evidencia obtenida del modelo de cultivo celular, las neurotrofinas modulan la eficacia sináptica de manera aguda en el SCG de la rata a través de la activación de los receptores p75NTR y/o TrkA, los cuales a su vez, modularían la actividad de diversos canales iónicos como los canales KCNQ. Pensamos que la regulación de la corriente M contribuye para la modulación de la eficacia de la transmisión sináptica mediada por neurotrofinas.

HIPÓTESIS

- BDNF y NGF modulan la transmisión sináptica y la plasticidad tipo LTP del SCG en aislamiento a través de la activación diferencial de los receptores p75NTR y TrkA.

- Los canales KCNQ contribuyen para la regulación de la LTP del SCG mediada por BDNF y NGF como efectores.

OBJETIVOS

- Caracterización de los efectos de BDNF y NGF sobre la transmisión basal y la LTP del SCG en aislamiento.

- Detección de la presencia de los receptores p75NTR, TrkA y TrkB en las neuronas ganglionares simpáticas del SCG de ratas adultas.

- Caracterización de la contribución de los canales KCNQ para la regulación de la LTP ganglionar mediada por BDNF y NGF.

MATERIALES Y MÉTODOS

Preparación biológica

Se utilizaron ratas *Wistar* macho de 250 – 300 g de peso, en conformidad con los lineamientos éticos para el uso y cuidado de animales de laboratorio aprobados por nuestro comité local (NOM-062-ZOO-1999). Así mismo, nos encargamos de reducir al máximo el número de animales utilizados y su nivel de estrés durante las manipulaciones experimentales. La disección de los ganglios se realizó quirúrgicamente con los animales anestesiados (ketamina 90 mg/kg, i.p. y xilazina hidroclorada 10 mg/kg, i.p.). Una vez aislado, el SCG fue separado del tejido conectivo y colocado en una caja Petri con solución fisiológica Ringer Krebs cuya composición en mM es: 136 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 1 KH2PO4, 12 NaHCO3, 11 glucosa, y 2 µM de atropina, 95% O_2, 5% CO_2, pH = 7.4).

Agentes farmacológicos

Las soluciones *stock* de β-NGF (GF028, Chemicon-Millipore, Billerica, MA) y de BDNF humano recombinante (B3795, Sigma-Adrich, St. Louis, MO) fueron almacenadas en alícuotas de 1 µg/µl y mantenidas a -80 °C por hasta 3 meses. Los compuestos tirofostina AG879 (Cat. No. 2617, Tocris Bioscience, Ellisville, MO), c_2-ceramida (sc-201375, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), XE991 (Cat. No. 2000, Tocris Bioscience, Ellisville, MO) y flupirtina (F8927, Sigma-Aldrich) fueron almacenados en congelación disueltos en DMSO de acuerdo con las instrucciones del fabricante. Las soluciones de trabajo se prepararon al día en solución Ringer Krebs y se aplicaron directamente en la cámara de registro a las concentraciones indicadas. En los experimentos en donde se utilizó DMSO como vehículo, la concentración final fue menor al 5% y corroboramos que no tuviera ningún efecto sobre la transmisión sináptica o sobre la LTP.

Inmunohistoquímica

El SCG fue disecado cuidadosamente de acuerdo al procedimiento mencionado anteriormente y post-fijado durante 12 horas en paraformaldehido (PFA 4%). Se obtuvieron cortes transversales de (14 µm de espesor) con un criostato, los cuales fueron inmersos en buffer de citratos (Bio SB 20x Inmuno/DNA Retriever) a 60 °C durante 1 hora. El tejido se bloqueó sumergiéndolo en una solución de albúmina sérica de bovino al 5% y Triton-X100 al 0.3% durante 1 h. Para revelar la localización de los receptores a neurotrofinas en el SCG, los cortes se incubaron durante 16 h a temperatura ambiente en presencia de los siguientes anticuerpos primarios diluidos en solución lavadora: anti-TrkB, conejo, policlonal (Santa Cruz, sc-12, 1:500), anti-TrkA, conejo, policlonal (Abcam, ab8871, 1:200) y anti-p75NTR, policlonal, cabra (Santa Cruz, sc-6188, 1:200). Posteriormente se realizaron lavados con PBS-Tx y los cortes se incubaron durante 2 h a temperatura ambiente con los siguientes anticuerpos secundarios diluidos en PBS-Tx: anti-conejo Alexa Fluor 594, burro (Jackson 1:400) y anti-cabra DyLight 488, burro (Jackson, 1:200). Después de incubar con el anticuerpo secundario y lavar, se utilizó el medio de montaje DAKO para preservar la fluorescencia. Las fotografías se tomaron con un microscopio de epifluorescencia (Nikon Eclipse E600).

Registros electrofisiológicos

Para estudiar la transmisión sináptica ganglionar realizamos registros electrofisiológicos del CAP. Para ello, estimulamos eléctricamente el tronco cervical simpático y registramos la respuesta postsináptica en el nervio carotídeo interno utilizando electrodos de succión. La estimulación basal se realizó con un estimulador Pulsar 6i (FHC Inc., Bowdoin, ME, USA) y consistió en pulsos cuadrados de voltaje de intensidad supra-máxima (9 – 12 V), 0.1 ms de duración y frecuencia de 0.2 Hz.

Las señales electrofisiológicas se amplificaron (x100) y se acondicionaron con un filtro pasabanda integrado al amplificador diferencial (DP-301, Warner Instruments, Hamden, CT, USA). Para determinar el efecto de las neurotrofinas sobre la transmisión basal construimos curvas entrada-salida (E/S) graficando la amplitud de los CAP (voltaje de salida) en función de una ronda de estímulos de amplitud creciente de 1 – 10 V.

Para inducir LTP estimulamos el tronco cervical simpático a 40 Hz durante 3 segundos con pulsos de amplitud supra-máxima en presencia de hexametonio (100 μM) (Cifuentes et al. 2004). El hexametonio se aplicó para bloquear parcialmente la transmisión nicotínica con el propósito de asegurar la activación del 100% de las fibras preganglionares con el estímulo supramáximo y para normalizar las respuestas sinápticas post-tren con la amplitud del potencial pre-tren (en presencia de hexametonio). Los agentes farmacológicos fueron aplicados disueltos en solución Ringer Krebs directamente en la cámara de registro a las concentraciones indicadas por lo menos 30 minutos antes de la aplicación del tren y se mantuvieron presentes durante todo el experimento.

Las señales bioeléctricas se digitalizaron con una tarjeta de adquisición (PCI-DAQ) convertidor A/D 16-bit y un programa de adquisición desarrollado por nosotros con LabView (v8.6, National Instruments, Austin, TX, USA).

Simulación computacional del potencial de acción compuesto ganglionar

Modelamos el CAP del SCG utilizando el software NEURON (Hines & Carnevale 2001). Para ello modelamos una neurona de 40 μm de diámetro con conductancias de sodio y potasio tipo Hodgkin y Huxley. En nuestra simulación del CAP promediamos las respuestas individuales de cinco mil neuronas de ese tipo estimuladas con una entrada sináptica supra-umbral (modelada), disparando

aleatoriamente con un retraso de 36 ms y con varianza variable (σ^2) de 13 o de 26 ms respectivamente. El promedio de los PA individuales en cada ensayo generó un CAP con características cinéticas equivalentes a aquellos obtenidos en presencia de BDNF (σ^2 = 13 ms) y dosis bajas de NGF (σ^2 = 26 ms).

Medición del potencial de acción compuesto

Realizamos un análisis digital de los CAP registrados a los 40 minutos en el caso de NGF y a los 60 minutos en el caso de BDNF. Los parámetros que medimos fueron: área bajo la curva (*area),* tiempo de inicio (*onset),* ancho al medio (*FWHM*), pendiente positiva (*+d/dt*) y pendiente negativa (*-d/dt*). Los CAP fueron normalizados con relación al CAP registrado en presencia de hexametonio. Para medir el *onset* se promedió la respuesta basal y se calculó la desviación estándar. Tomamos el tiempo de inicio como el valor de x cuando $y>y_0, + 2\sigma^2$.

Análisis de datos

Para evaluar las curvas E/S utilizamos la función: $V_{min}= V_{max}/[1+((V_{max}/V_{min})-1)e^{-aV}$. Donde V_{min} es la respuesta mínima (~0.0005), V_{max} es la respuesta máxima y α un coeficiente relacionado con la pendiente (Banks 1994). El valor de voltaje de entrada que produce la mitad de la respuesta máxima ($V_{1/2}$) fue estimado por interpolación en el eje *X*.

Para cuantificar la LTP, los registros de voltaje fueron normalizados utilizando la relación $(R_i-R_0)/R_0$, donde R_i es la amplitud del CAP al tiempo i y R_0 es la amplitud promedio de los CAP en presencia de hexametonio durante los 5 minutos previos a la aplicación del estímulo de alta frecuencia.

Dado que la cinética de decaimiento de la amplitud de los CAP registrados post-tren sigue una cinética bi-exponencial, utilizamos la función $f(t)=A_1e^{-t/\tau 1}+ A_2e^{-t/\tau 2}$ para estimar la duración y la magnitud de la LTP. El coeficiente A_1 y la constante de tiempo τ_1 de la primera exponencial corresponden a la potenciación post-tetánica (PTP), mientras que el coeficiente A_2 y la constante de tiempo τ_2 corresponden a la LTP (Briggs et al. 1985; Cifuentes et al. 2004; Vargas et al. 2007). Evaluamos la LTP calculando dos parámetros: el tiempo de decaimiento ($t_{0.2}$) en minutos (*LTP decay*) definido como el valor de tiempo en el que la amplitud promedio del CAP llega al 20% sobre la amplitud del valor en presencia de hexametonio, el cual se calcula resolviendo la función bi-exponencial para $f(t)=0.2$. La magnitud de la LTP (*LTP extent*) se calculó a partir del valor del área bajo la curva desde t=0 hasta $t=t_{0.2}$. Los datos se expresaron como el promedio ± error estándar y el análisis estadístico se realizó con el software *OpenOffice* (http://www.openoffice.org). Las diferencias fueron valoradas utilizando la prueba t-Student y se consideraron significativas para toda $p<0.05$.

RESULTADOS

Efecto de BDNF y NGF sobre la transmisión basal ganglionar

Para determinar si las neurotrofinas modulan la transmisión basal ganglionar evaluamos el efecto de la aplicación de BDNF o NGF sobre la transmisión sináptica evocada construyendo curvas E/S (ver métodos). Las curvas E/S dan información sobre la fuerza de la transmisión sináptica y permiten inferir si la eficacia sináptica aumenta o disminuye en respuesta a un tratamiento. Si la curva se desplaza sobre el eje de las abscisas hacia la derecha significa que la fuerza de la transmisión sináptica ha disminuido; por el contrario, si la curva se desplaza hacia el lado izquierdo significa que la eficacia de la transmisión sináptica ha aumentado (Johnston & Wu 1994). Al comparar las curvas de E/S obtenidas en presencia de BDNF 200 ng/ml ($BDNF_{200}$) y NGF 200 ng/ml (NGF_{200}) con las del grupo control, observamos que $BDNF_{200}$ o NGF_{200} no modifican la eficacia de la transmisión basal del SCG (Fig. 9).

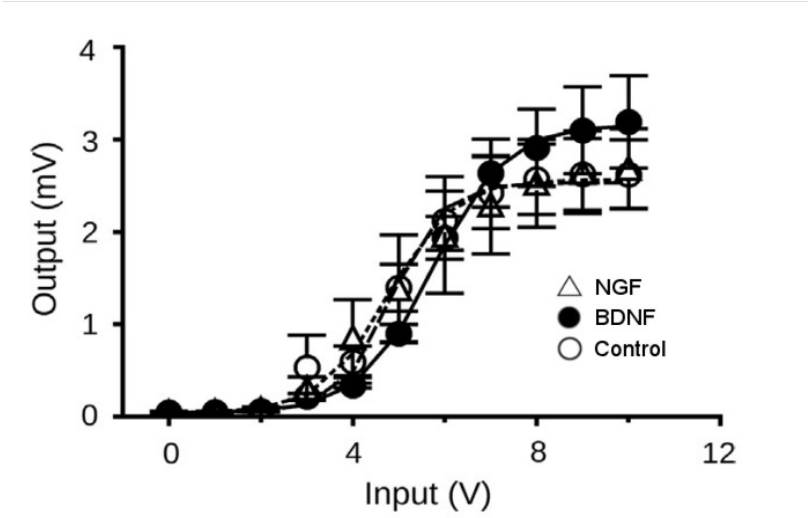

Figura 9: BDNF y NGF no modifican la transmisión basal ganglionar. Las curvas E/S muestran que no hay cambios significativos en la en respuesta ganglionar en presencia de $BDNF_{200}$ (circulo negro) o NGF_{200} (triángulo blanco) con relación al control (circulo blanco).

Los parámetros obtenidos fueron V_{max} = 2.5±0.3 mV para el grupo control, 3.0±0.3 mV para el grupo de $BDNF_{200}$ y 2.7±0.4 mV para el grupo de NGF_{200} ($p>0.2$, n=6); $V_{1/2}$ = 5.0±0.4 mV para el grupo control, 5.0±0.2 mV para el grupo de $BDNF_{200}$ y 5.3±0.6 mV para el grupo de NGF_{200} ($p>0.3$, n=6); y el exponente a = 1.8±0.2 u.a. para el grupo control, 1.5±0.2 u.a para el grupo de $BDNF_{200}$ y 1.3±0.2 u.a. para el grupo de NGF_{200} ($p>0.2$, n=6).

Efecto de BDNF y NGF sobre la LTP ganglionar

A continuación caracterizamos el efecto de BDNF y NGF sobre la LTP ganglionar y encontramos que $BDNF_{200}$ incrementa el tiempo de decaimiento y la magnitud de la LTP al doble del valor control (Fig. 10A; $p<0.01$, n=6). Al inducir LTP en presencia de distintas concentraciones de BDNF encontramos que 350 y 500 ng/ml también incrementaron significativamente el tiempo de decaimiento y la magnitud de la LTP (Fig. 10D, $p<0.05$, n=6).

Por el contrario, el tratamiento con NGF_{200} redujo significativamente la LTP ganglionar, el tiempo de decaimiento de la LTP disminuyó en un 52% y la magnitud en un 32% con respecto al valor control (Fig. 10B, $p<0.0001$, $p<0.05$, n=14). No obstante, con NGF_{500} el tiempo de decaimiento y la magnitud de la LTP aumentaron 1.7 y 1.5 veces respectivamente con respecto al control (Fig. 10C, $p<0.02$). En la curva dosis-respuesta de NGF se puede apreciar que el efecto sobre la LTP es bifásico y depende de la dosis (Fig. 10D, $p<0.001$, n=6).

Al analizar de manera individual los CAP para buscar cambios en la cinética que nos pudieran dar información sobre el mecanismo de acción de las neurotrofinas, encontramos que el aumento en la LTP producido por $BDNF_{200}$ estuvo acompañado de un incremento en $+d/dt$ (Fig. 11D). Por otra parte, en presencia de NGF_{200} se incrementó *FWHM* (Fig. 11C), mientras que $+d/dt$ y $-d/dt$ disminuyeron (Fig. 11D), mientras que el área no se modificó (Fig. 11B).

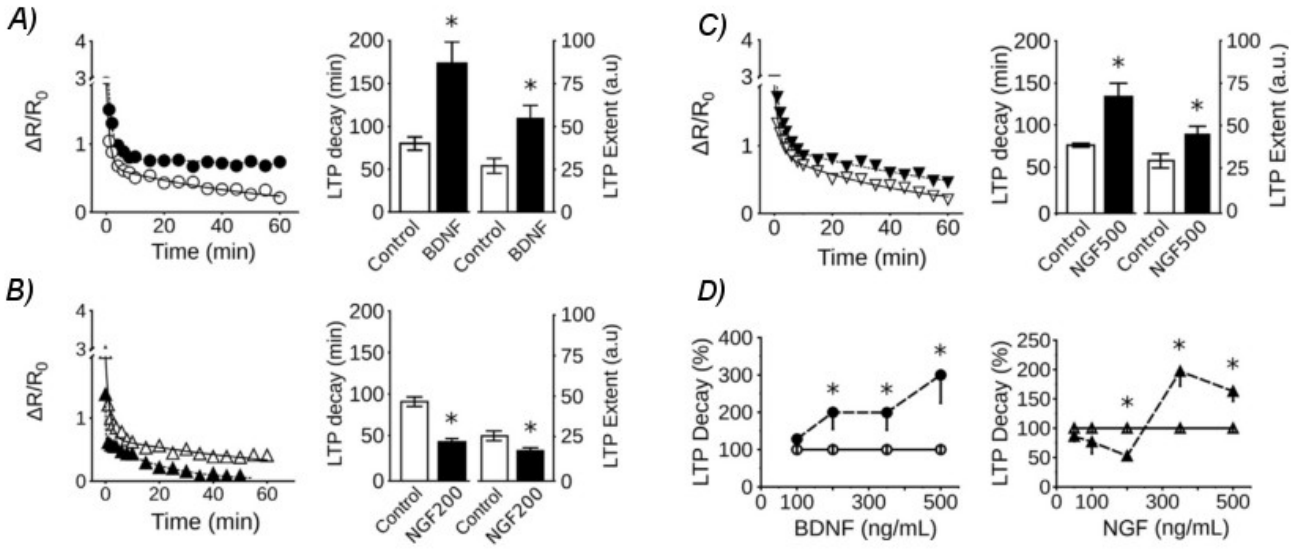

Figura 10. Efecto de BDNF y NGF sobre la LTP ganglionar. *A)* Curso temporal de un experimento típico de BDNF vs. control en el que se observa que el decaimiento de la relación ΔR/R0 es más lento en presencia de $BDNF_{200}$ (circulo negro) con respecto al control (circulo blanco). La gráfica de barras muestra que el tiempo de decaimiento (*LTP decay*) y la magnitud de la LTP (*LTP extent*) son significativamente mayores en presencia de BDNF que en el control. *B)* Curso temporal de *ΔR/R0* donde se aprecia que la amplitud de la respuesta ganglionar decae más rápidamente en presencia de NGF_{200} (triángulo negro) respecto al control (triángulo blanco). Las barras muestran la cuantificación de la reducción de la LTP por NGF_{200}. C) En presencia de NGF_{500} el decaimiento de *ΔR/R0* fue más lento (triángulo invertido negro) en comparación con el control (triángulo invertido blanco). Las barras muestran la cuantificación de la potenciación mediada por NGF_{500} con respecto al control. D) Curvas dosis respuesta para BDNF y NGF.

De forma notable, en presencia de $BDNF_{200}$ y NGF_{500} la sincronía de los CAP aumentó. Por otra parte, en presencia de NGF_{200} hubo una mayor dispersión en el *onset* de los CAP intragrupo (Fig. 11E).

Expresión de los receptores a neurotrofinas en el ganglio cervical superior

Caracterizamos la presencia de los receptores p75NTR, TrkB y TrkA en las neuronas del SCG de rata adulta por medio de inmunohistoquímica. Encontramos que 83±2% de las neuronas simpáticas ganglionares son positivas para TrkA (Fig. 12A), mientras que 52±3% son positivas para p75NTR (Fig. 12B) y un 18±1% son positivas para TrkB (Fig. 12C).

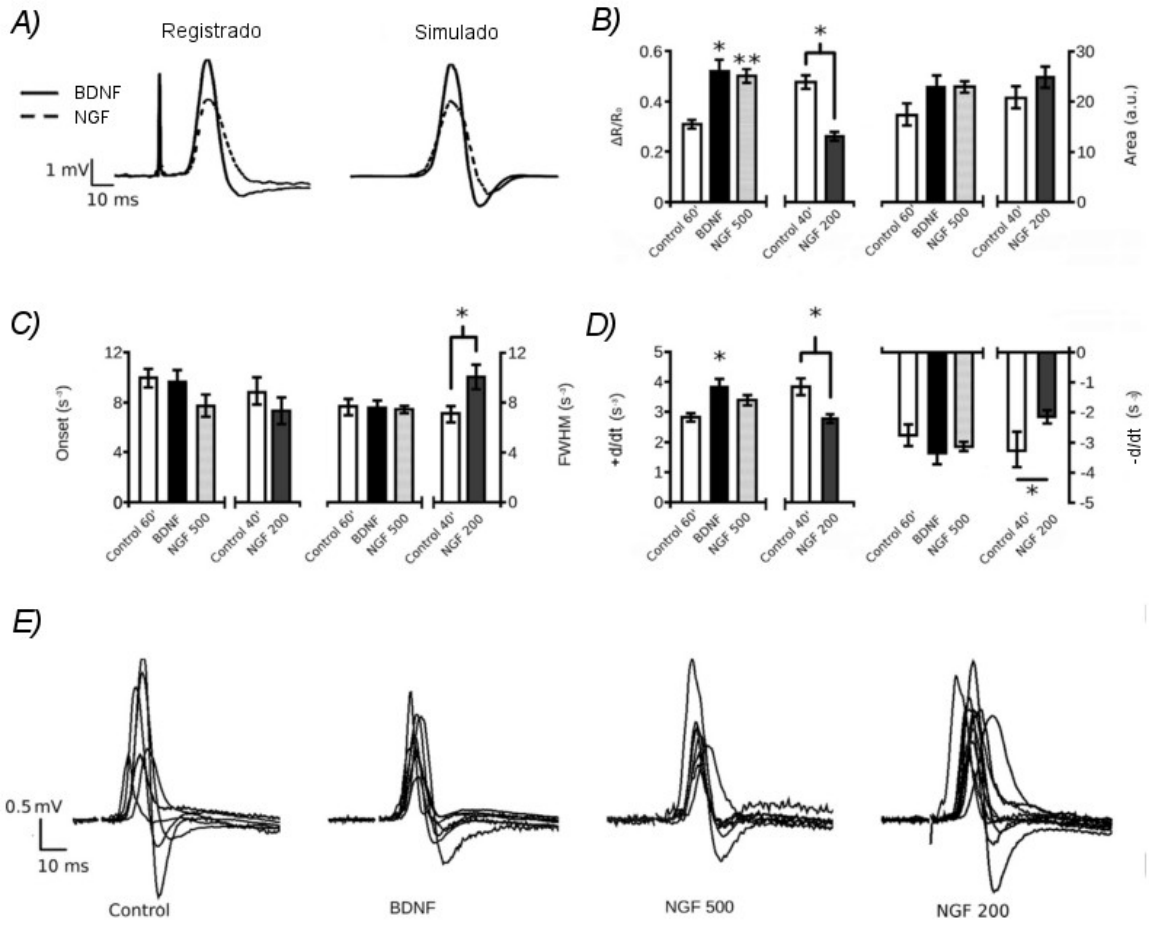

Figura 11. Análisis del potencial de acción compuesto del SCG en presencia de BDNF y NGF. A) Izquierda: CAP registrados a los 60 minutos (BDNF, línea continua) y a los 40 minutos (NGF_{200}, línea punteada) post-tren. Derecha: Simulación del potencial de acción compuesto (σ^2=13 ms, línea continua; σ^2=26 ms, línea punteada). B-D) Medidas del potencial de acción compuesto bajo diferentes condiciones experimentales. BDNF incrementa el valor de la relación $\Delta R/R_0$ (B, izquierda) y la pendiente positiva (D, izquierda), sin afectar el área (B, derecha), ni el FWHM (C, derecha). Por su parte, NGF_{200} diminuye el valor de la relación $\Delta R/R_0$ (B, izquierda), *FWHM* (C, derecha), *d/dx* (D, izquierda) y *-d/dx* (D, derecha). NGF_{500} aumentó el tiempo de decaimiento de $\Delta R/R_0$ (B, izquierda) y produjo una tendencia de aumento en *d/dx* (D, izquierda) sin afectar el área (B, derecha), ni el FWHM (C, derecha). E) Trazos superpuestos de varios CAP obtenidos en diferentes condiciones experimentales (sin normalizar), nótese que hay mayor sincronización en los grupos de BDNF y NGF_{500} con relación al control y al grupo de NGF_{200}.

Analizamos la funcionalidad de los receptores Trk utilizando anticuerpos contra fosfoTrk y fosfoAkt. Encontramos que la estimulación eléctrica aumentó 2.0 veces el número de neuronas inmunoreactivas a fosfoTrk y fosfoAkt. Al estimular en presencia de BDNF el número de neuronas marcadas aumentó 3.0 veces y en presencia a NGF aumentó 4.0 veces (Fig. 13).

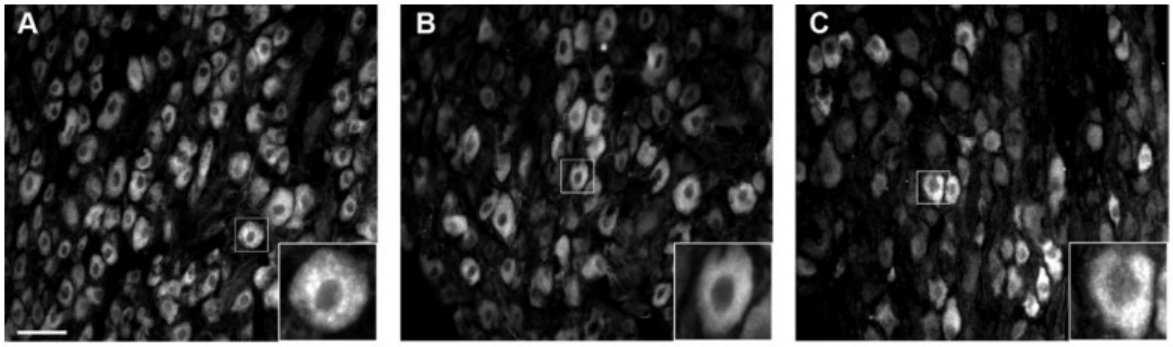

Figura 12. Expresión de los receptores a neurotrofinas en el ganglio cervical superior de la rata adulta. Micrografías de cortes de GCS donde se observa la presencia de neuronas simpáticas inmunomarcadas contra TrkA (A), p75NTR (B) y TrkB (C) donde se muestra una alta expresión de dichos receptores. 83% de la población neuronal expresa al receptor TrkA, 52% expresa a p75NTR y 18% expresa a TrkB. Los insertos en las fotografías (3x) muestran la expresión celular de la proteína. Escala 50 μm.

Papel de p75NTR y TrkA en la modulación de la LTP ganglionar por BDNF y NGF

Dado que las neuronas ganglionares expresan a los receptores p75NTR y TrkA de manera abundante, quisimos determinar la contribución relativa de ambos receptores por separado para la LTP ganglionar.

Para estudiar el papel de p75NTR en la LTP, primero aplicamos el compuesto c_2-ceramida, el cual se produce de manera endógena tras la activación del receptor p75NTR (Dobrowsky et al. 1994). Encontramos que el valor de la relación $\Delta R/R_0$ y la magnitud de la LTP aumentaron 4.1 y 2.1 veces respecto a los valores control en presencia de 100 μM de c_2-ceramida (Fig. 14A, $p<0.03$ y $p<0.02$, $n=6$ respectivamente).

Por otra parte, determinamos si la atenuación de la LTP ganglionar producida por NGF_{200} estaba mediada por el receptor TrkA aplicando NGF en forma conjunta con el compuesto tirofostina (1 μM), el cual es un inhibidor de tirosin cinasa (Ohmichi et al. 1993). Encontramos que el efecto de NGF_{200} sobre la LTP ganglionar se revierte en presencia de tirofostina, ya que el tiempo de decaimiento y la magnitud aumentan aproximadamente 2.0 veces con relación al grupo de NGF, resultando en valores

similares al control (Fig. 14B, $p<0.03$ y $p<0.02$, n=6). Al inducir LTP únicamente en presencia de tirofostina, la duración y la magnitud de la LTP aumentaron 2.4 veces y 1.7 veces respectivamente con respecto al grupo control (Fig. 14C, $p<0.005$ y $p<0.03$, n=6).

Confirmamos la participación del receptor TrkA en la disminución de la LTP mediada por NGF aplicando un anticuerpo dirigido contra el dominio extracelular del receptor TrkA junto con NGF. Encontramos que el anticuerpo revirtió parcialmente el efecto inhibidor de NGF sobre la LTP (Fig 14D, $p<0.005$ y $p<0.03$, n=4).

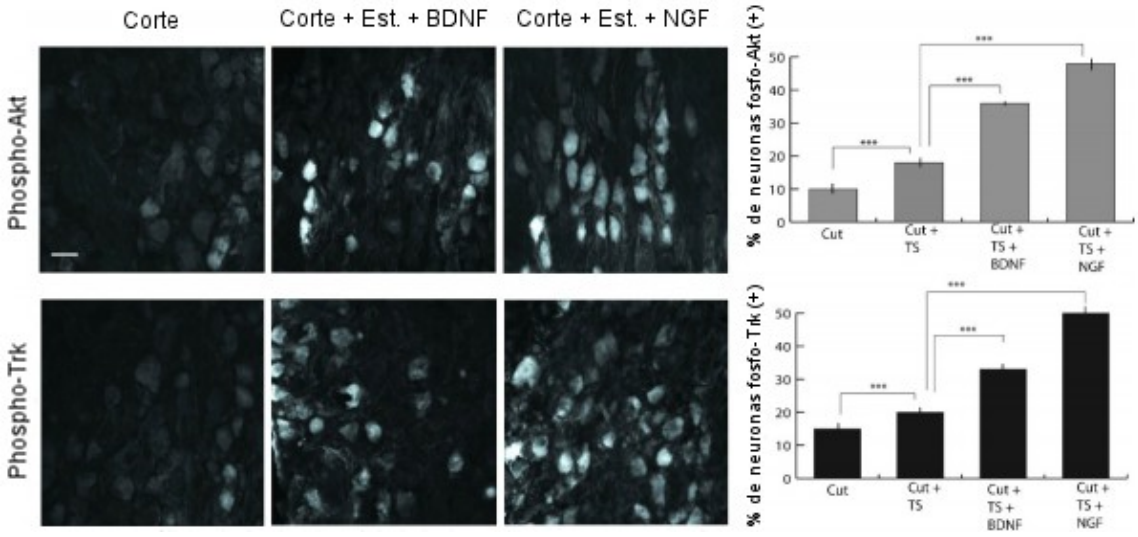

Figura 13. Análisis de la funcionalidad de los receptores a neurotrofinas en el ganglio cervical superior. En las micrografías de la izquierda se muestran cortes de SCG donde se observa la presencia de neuronas simpáticas inmunoreactivas a fosfoAkt y fosfoTrk en diferentes condiciones experimentales. El aumento en la inmunoreactividad producido por el estímulo inductor de la LTP es mayor en presencia de BDNF y NGF respectivamente. A la derecha se muestra la cuantificación de la inmunoreactividad para fosfoAkt y fosfoTrk bajo diferentes condiciones experimentales.

Rol de los canales KCNQ en la modulación de la LTP ganglionar por BDNF y NGF

Considerando que las neurotrofinas modulan la actividad de los canales KCNQ en neuronas simpáticas y en neuronas centrales y por ende la corriente de potasio tipo-M, la cual es fundamental para la regulación de la excitabilidad neuronal.

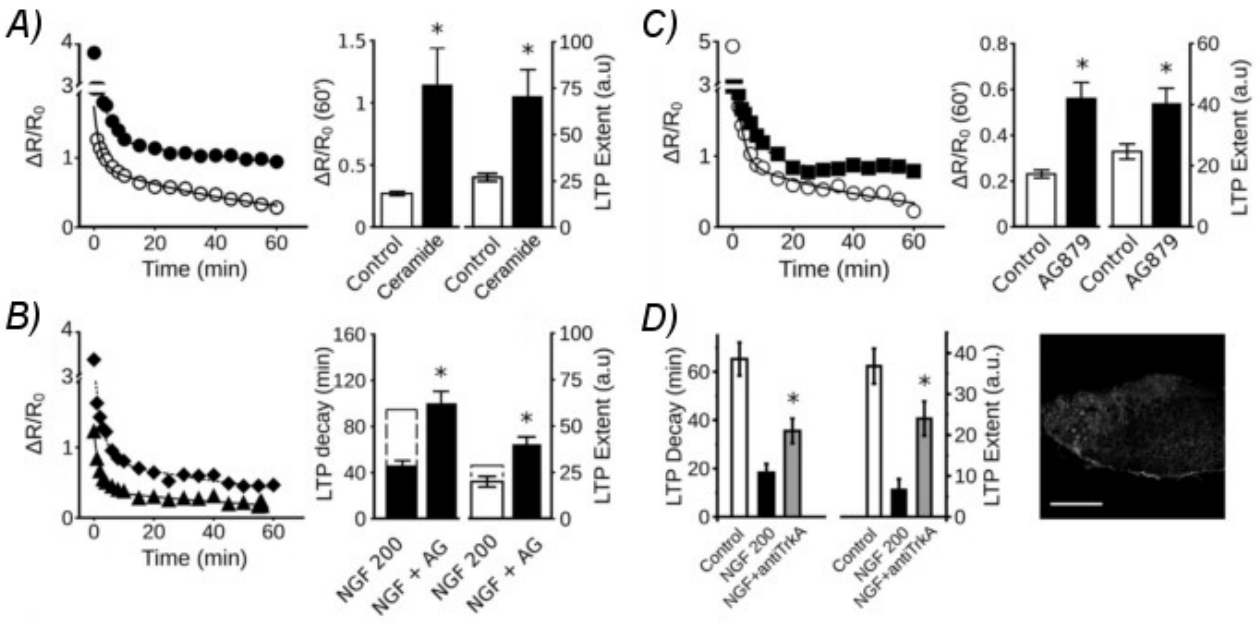

Figura 14. Participación de los receptores p75NTR y TrkA en la modulación diferencial de la LTP ganglionar mediada por neurotrofinas. *A)* Curso temporal de un experimento que muestra el efecto de c_2-ceramida (circulo negro) sobre la LTP ganglionar (control, circulo blanco). El valor de $\Delta R/R_0$ y la magnitud aumentaron con respecto al valor control. *B)* Experimento representativo en presencia de tirofostina + NGF_{200} (rombo negro). En presencia de ambos compuestos la LTP es de mayor magnitud en relación al grupo de NGF_{200} (triángulo negro), los valores fueron similares a los obtenidos en el grupo control (líneas punteadas). *C)* Experimento realizado en presencia de tirofostina (cuadro negro). Se observa un aumento significativo de la LTP con relación al experimento control (circulo blanco). *D)* La aplicación de un anticuerpo anti-TrkA revirtió parcialmente el efecto de bloqueo de NGF sobre la LTP ganglionar, en esta condición el tiempo de decaimiento y la magnitud de la LTP fueron mayores que al aplicar NGF solo. Micrografía de un ganglio seccionado donde se observa la difusión del anticuerpo en el sitio cercano al corte.

Para investigar si los canales KCNQ están implicados en el mecanismo de acción de las neurotrofinas estimulamos en presencia del compuesto XE991 (1 μM), el cual disminuye la actividad de los canales KCNQ. Encontramos que la LTP se atenúa significativamente en presencia del bloqueador (Fig. 15; $p<0.04$, n=5). En otra serie de experimentos aplicamos el compuesto flupirtina (5 μM), el cual aumenta la actividad de los canales KCNQ. Encontramos que la aplicación de flupirtina aumentó la duración y la magnitud de la LTP de manera semejante a $BDNF_{200}$, NGF_{500} y c_2-ceramida ($p<0.05$, n=5). Los experimentos de flupirtina se realizaron en presencia de bicuculina (1 μM) debido a que se ha reportado que flupirtina es un agonista de los receptores $GABA_A$, los cuales se expresan ampliamente en el ganglio.

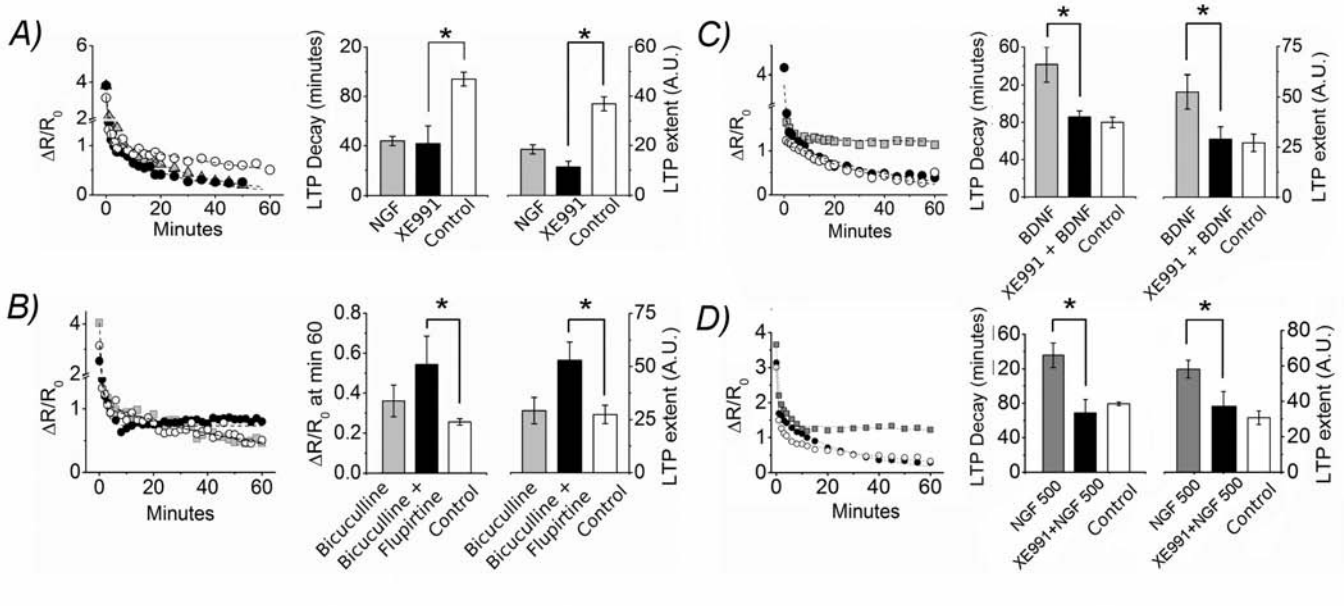

Figura 15. Contribución de los canales KCNQ para el efecto de las neurotrofinas sobre la LTP ganglionar. *A)* Experimento de LTP en condición control (blanco), en presencia de XE991 (negro) y en presencia de NGF_{200} (gris). La atenuación de la LTP observada en presencia de XE991 es similar a la que se produce en presencia de NGF_{200}. *B)* Experimento típico de LTP en presencia de bicuculina (gris), bicuculina+flupirtina (1 μM y 5 μM) y control (blanco). *C)* Experimento de LTP en presencia de $BDNF_{200}$ (gris), XE991+$BDNF_{200}$ (negro) y control (blanco). La incubación de los ganglios con XE991 previene el efecto facilitador de $BDNF_{200}$ sobre la LTP ganglionar, resultando en valores similares al control. *D)* Experimento representativo de LTP en presencia de NGF_{500} (gris), XE991+NGF_{500} (negro) y control (blanco). Se observa como el tratamiento con XE991 previene el efecto facilitador de NGF_{500} sobre la LTP ganglionar, resultando en valores similares al control.

Nuestros resultados muestran que aunque la bicuculina tiende a incrementar la LTP *per se* (Fig. 15B), sin embargo, el aumento de la LTP es mayor cuando se aplica junto con flupirtina (Fig 15B, $p<0.05$, n=5). Por último, determinamos si los canales KCNQ contribuyen para el efecto de las neurotrofinas sobre la LTP ganglionar. Para ello aplicamos $BDNF_{200}$ o NGF_{500} en presencia del inhibidor XE991. Encontramos que en presencia de XE991 el tratamiento con $BDNF_{200}$ no tuvo ningún efecto sobre la LTP ganglionar, de la misma forma, el tratamiento con NGF_{500} tampoco produjo ningún cambio en la LTP, en ambos casos la LTP resultante fue semejante a los experimentos control (Fig. 15C y 15D, $p<0.01$ y $p<0.05$, n=5).

DISCUSIÓN

Nuestros resultados muestran que BDNF y NGF no modifican la transmisión basal ganglionar, sin embargo, tienen un efecto diferencial sobre la LTP del SCG. Esta diferencia podría deberse a que es necesario que se acople la señalización por neurotrofinas con el aumento en la actividad presináptica. Estudios previos realizados por otros grupos de investigación han reportado que la síntesis y secreción de BDNF depende de la actividad sináptica (Kuczewski et al. 2009; Edelmann et al. 2014), lo que podría explicar porque no tiene efecto sobre la transmisión basal.

Nuestros experimentos de inmunodetección muestran que las neuronas simpáticas expresan a los receptores p75NTR, TrkA y TrkB, y que son funcionales y responden al tratamiento con BDNF y NGF más la estimulación eléctrica de alta frecuencia que induce la LTP, indicando que los receptores Trk del ganglio son funcionales y responden al aumento en la actividad presináptica y al tratamiento con neurotrofinas.

La activación de p75NTR (o TrkB) por BDNF o por NGF_{500} cuando la concentración de neurotrofinas aumenta en el medio extracelular debido a un aumento en la actividad sináptica podría ser el estímulo que sensibiliza la postsinapsis para acoplarla con la descarga presináptica. Tal fenómeno podría deberse a una variedad de mecanismos como la sensibilización de los receptores nicotínicos o la actividad de canales iónicos a través de mecanismos alostéricos mediados por los receptores p75NTR y TrkA.

Nuestros datos sobre la modulación diferencial de la LTP concuerdan con los presentados en otros reportes donde se muestra que BDNF es un regulador positivo de la LTP (Jia et al. 2010; Akaneya et al. 1997) y que NGF tiene un efecto diferencial dependiente de la dosis (Pesavento et al. 2000; Brancucci et al. 2004; Tancredi et al. 1993). La diferencia en el efecto de NGF podría

deberse a que es una molécula capaz de activar tanto a p75NTR como a TrkA, por lo tanto, las afinidades relativas de los receptores son las que determinan cuál de ellos predominará en el efecto final.

En nuestro modelo el efecto de NGF_{200} sobre la LTP es muy robusto, probablemente debido a que una gran cantidad de neuronas simpáticas expresan al receptor TrkA y son potencialmente sensibles a sus efectos. En dosis mayores, NGF podría activar también la vía de p75NTR e incluso activar receptores TrkB de manera no especifica. Para distinguir entre dichas posibilidades es necesario realizar más estudios farmacológicos o incluso experimentos con animales *knock-out*, sin embargo, tales interrogantes quedarán abiertas para su exploración futura.

Al caracterizar las contribuciones de TrkA y p75NTR para la modulación de la LTP ganglionar por neurotrofinas encontramos que la mimetización de la activación del receptor p75NTR por c_2-ceramida genera un aumento de la LTP ganglionar que podría contribuir de manera importante para la facilitación de la plasticidad producido por BDNF y NGF_{500}, aunque no podemos descartar la posible contribución del receptor TrkB, ya que también podría ser activado en una pequeña sub-población de neuronas ganglionares o en las terminales presinápticas, donde facilitaría la liberación de acetilcolina (Fig. 16).

En el caso del receptor TrkA encontramos que al aplicar NGF_{200} en presencia de tirofostina, el efecto de NGF_{200} sobre la LTP se revirtió igual que al aplicarlo en presencia del anticuerpo anti-TrkA. De manera interesante encontramos que la aplicación de tirofostina *per se* aumenta la LTP ganglionar, lo cual indica que en efecto el NGF endógeno limita la capacidad plástica del sistema y que al antagonizar la actividad del receptor TrkA el sistema aumenta su actividad tónica. Estos resultados sugieren que NGF funciona como un regulador negativo de la LTP en el SCG de la rata cuando actúa a través del receptor TrkA *in vivo*.

Las concentraciones de neurotrofinas utilizadas en este estudio son similares a las

reportadas en trabajos previos donde se utilizaron concentraciones del orden de 50 - 100 ng/ml de NGF para tratar neuronas simpáticas en cultivo (Maguire et al. 1999; Conner et al. 2009). No obstante, en otros trabajos realizados en el hipocampo se utilizaron concentraciones del orden de 0.01 ng/ml (Lockhart et al. 1997; Luther et al. 2013; Pesavento et al. 2000; Brancucci et al. 2004). Tales diferencias podrían deberse a la expresión y afinidad de los receptores en diferentes estructuras o incluso a la presencia de barreras físicas que dificultan la difusión a los sitios en donde se encuentran las sinapsis.

Finalmente, estudiamos el papel de los canales KCNQ en el efecto diferencial de las neurotrofinas sobre la LTP. Nuestros experimentos farmacológicos revelaron que el bloqueo de los canales KCNQ con XE991 atenúa la LTP y que la activación de los canales KCNQ con flupirtina la aumenta, y adicionalmente, al aplicar BDNF o NGF_{500} en presencia de XE991 el efecto de facilitación no se produce. Por lo tanto, estos resultados sugieren que los canales KCNQ son importantes para regular la duración de la LTP ganglionar y que participan en el efecto diferencial de las neurotrofinas como efectores.

Por un lado, BDNF y NGF_{500} podrían regular la amplitud de la corriente M en las neuronas ganglionares a través del receptor p75NTR favoreciendo el disparo fásico (Jia et al. 2008; Nieto-Gonzalez & Jensen 2013), generando un aumento en el umbral de disparo (Luther & Birren 2009) y una disminución en la primera latencia (Springer et al. 2015). En nuestro modelo, el incremento en el umbral de disparo no afectaría porque utilizamos estimulación supramáxima, por ende, aseguramos que todas las neuronas disparen aunque lo hacen a baja frecuencia. La probabilidad de que los potenciales de acción coincidan temporalmente y se sumen al CAP es mayor cuando las neuronas disparan de forma fásica debido a que la latencia de disparo disminuye, lo que resulta en un CAP de mayor amplitud como se observa en nuestras simulaciones (Fig. 16).

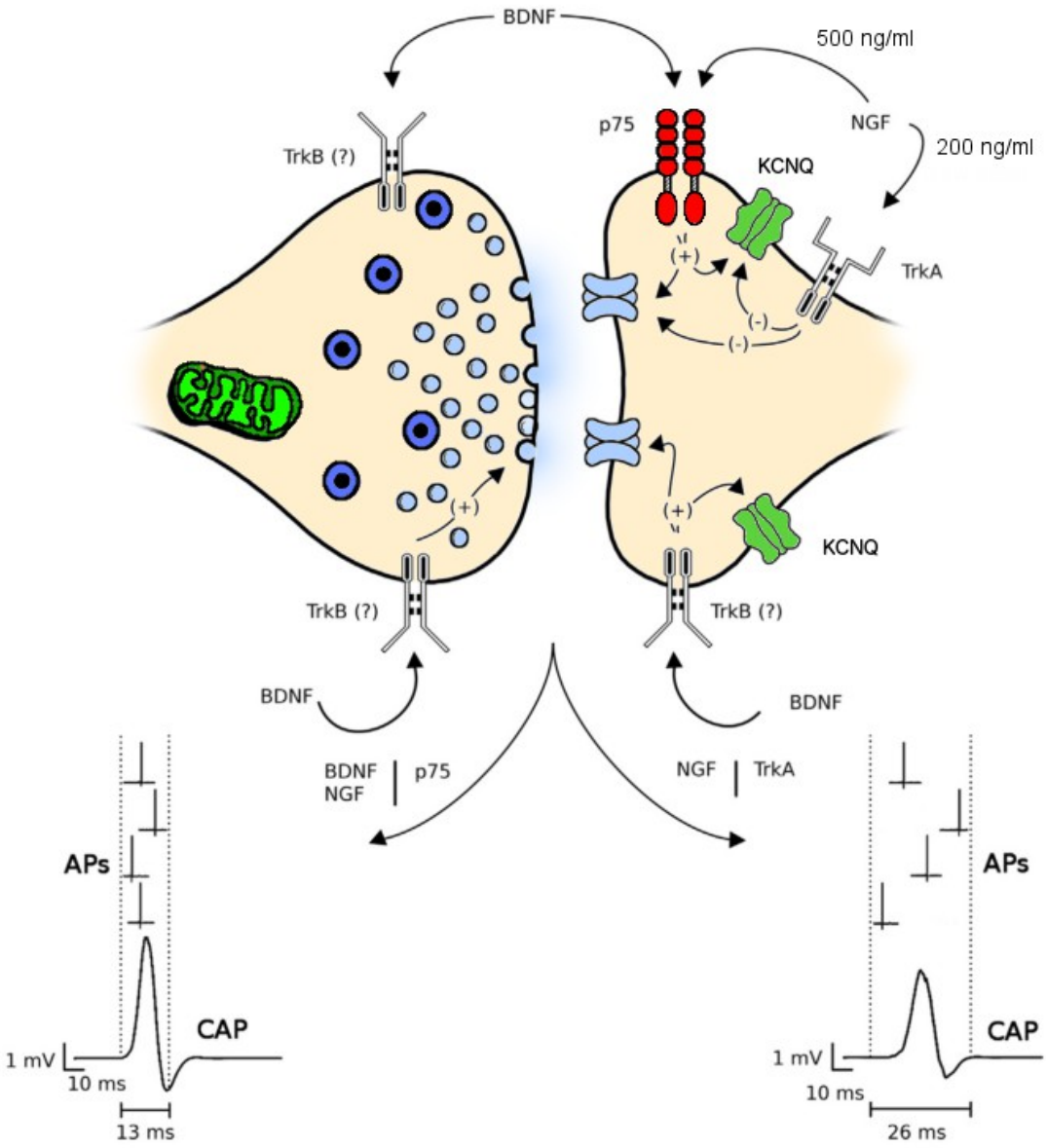

Figura 16: Mecanismo propuesto para explicar el efecto de las neurotrofinas sobre la LTP ganglionar. El esquema ejemplifica las posibles acciones de BDNF y NGF sobre la función sináptica. BDNF podría incrementar la liberación de acetilcolina a través de la activación de receptores TrkB presinápticos. Adicionalmente, BDNF o dosis altas de NGF podrían incrementar la excitabilidad de la membrana de la neurona post-sináptica actuando a través de receptores TrkB o p75NTR post-sinápticos que modulan la actividad de los canales KCNQ. Nuestra hipótesis es que la activación de p75NTR (y TrkB) aumenta la sincronía de los potenciales de acción unitarios, probablemente al reducir su latencia, generando mayor sincronía y por ende un aumento en la amplitud del CAP. Por otra parte, NGF en dosis baja podría actuar a través de receptores TrkA en la post-sinapsis e inducir un decremento en la amplitud del CAP provocado por la desincronización temporal de los potenciales de acción unitarios. En la parte inferior de la figura se muestra la simulación de cinco mil potenciales de acción disparados con diferente varianza, el rango de la varianza se especifica debajo de cada experimento (13 y 26 ms respectivamente). Con una varianza de 13 ms (izquierda) la cinética del potencial de acción compuesto es similar a la de BDNF, mientras que con varianza de 26 ms produce un potencial de acción compuesto con una cinética que asemeja a la de los registros realizados en presencia de NGF.

Por otra parte, proponemos que la atenuación de la LTP producida por NGF vía TrkA es resultado de la desensibilización de las neuronas postsinápticas a la acción de la acetilcolina y a la disminución en la probabilidad de coincidencia temporal de los potenciales de acción post-sinápticos.

El mecanismo de NGF-TrkA sobre la LTP ganglionar es exactamente el caso opuesto al de BDNF/NGF_{500}-p75NTR, ya que el bloqueo de los canales KCNQ incrementaría la excitabilidad de las neuronas al reducir su umbral de disparo a costa de una disminución en la sincronía temporal entre los PA unitarios, dando como resultado un CAP de menor amplitud y mayor duración con relación al control, debido a la dispersión de los PA unitarios en el tiempo. Los resultados de nuestras simulaciones son consistentes con nuestra propuesta ya que cuando las neuronas disparan con una varianza de 13 ms se produce un CAP de mayor amplitud y de menor duración que cuando disparan con una varianza de 26 ms (Fig. 16).

Al analizar los registros de los CAP de manera individual encontramos que tanto en la condición control como en presencia de NGF_{200} hubo una mayor dispersión en el parámetro *onset* respecto a los grupos de BDNF y NGF_{500}, lo que también apoya nuestra propuesta sobre la sincronía.

Además de la sincronía, otros mecanismos que podrían contribuir para modular la LTP ganglionar mediada por neurotrofinas y que sería interesante explorar son el aumento en la liberación de neurotransmisores y el incrementó de los sitios de anclaje para las vesículas sinápticas en la zona activa mediados por TrkB.

CONCLUSIONES

BDNF y NGF tienen la capacidad de modular la eficacia de la transmisión colinérgica en el SCG de la rata. Sin embargo, para que BDNF y NGF puedan ejercer su acción neuromoduladora sobre las neuronas ganglionares es necesario que la señalización se acople temporalmente con un aumento concomitante de la actividad presináptica.

La activación preferencial de los receptores p75NTR y TrkA genera respuestas opuestas sobre la LTP ganglionar. Por un lado, p75NTR favorece el mantenimiento de la LTP ganglionar, mientras que TrkA genera el efecto opuesto e inhibe la LTP.

Los canales KCNQ participan en la regulación diferencial de la LTP ganglionar por neurotrofinas, ya que el efecto de facilitación sináptica producido por $BDNF_{200}$ y NGF_{500} se cancela en presencia del bloqueador XE991.

En conclusión, las neurotrofinas BDNF y NGF regulan la plasticidad de la transmisión sináptica colinérgica en el SCG aislado de la rata a través de la modulación diferencial de los canales KCNQ vía p75NTR y TrkA.

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APÉNDICE: ARTÍCULOS PUBLICADOS

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Differential contribution of BDNF and NGF to long-term potentiation in the superior cervical ganglion of the rat

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ABSTRACT

Synaptic transmission in the sympathetic nervous system is a plastic process modulated by different factors. We characterized the effects of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) on basal transmission and ganglionic long-term potentiation (LTP) in the rat superior cervical ganglion. LTP was elicited by supramaximal tetanic stimulation (40 Hz, 3 s) of the sympathetic trunk and was quantified by measuring LTP decay time and LTP extent. Neurotrophins did not affect basal transmission, however, they differentially affected LTP. BDNF (200 ng/ml) increased LTP decay time and LTP extent 2.0-fold ($p < 0.01$). In contrast, NGF showed a dual effect: 200 ng/ml NGF reduced LTP decay time and LTP extent to 53% and to 32% of control value ($p < 0.0001$ and $p < 0.02$; respectively), whereas >350 ng/ml NGF significantly increased LTP decay time and LTP extent ($p < 0.02$). Digital analysis of compound action potentials suggests that neurotrophins could change the synchronization of unitary action potentials. Pharmacological data obtained in intact ganglia show that C_2-ceramide produced a 2-fold enhancement in LTP, whereas tyrphostin AG879, an inhibitor of tyrosine kinase activity, reversed the NGF blockade and produced by itself an enhancement in LTP. In sliced ganglia we observed that an anti-TrkA antibody reversed the NGF-induced LTP blockade. Immunohistochemistry studies revealed that 83% of ganglionic neurons express TrkA, whereas 52% express p75 receptor, and 18% express TrkB receptor. We propose that p75 neurotrophin receptors and probably TrkB signaling enhance LTP, whereas TrkA signaling reduces it.

1. Introduction

The sympathetic nervous system (SNS) controls diverse functions in mammals and vertebrates including blood pressure, cardiac contractility, and some glandular secretions (Livingston, 1990). SNS activity is modulated by neurotrophic factors secreted by the target organs, either in physiological (Luther and Birren, 2009a, 2009b; Luther et al., 2013) or in patho-physiological conditions (Kanazawa et al., 2010). A remarkable characteristic of SNS activity is its plasticity (Kuba and Kumamoto, 1990). One form of synaptic plasticity is the long-term potentiation in the sympathetic ganglia (gLTP), which is characterized by a long-lasting increase in synaptic efficacy (Alkadhi et al., 2005; Alonso-deFlorida et al., 1991; Brown and McAfee, 1982; Dunant and Dolivo, 1968). The mechanisms underlying this phenomenon include calcium entry and calcium release (Briggs et al., 1985; Cifuentes et al., 2004, 2013; Cong et al., 2004; Vargas et al., 2007).

In the central nervous system, some neurotrophins (Nts) have been involved in a rapid regulation of neuronal excitability (Kovalchuk et al., 2004) and also in LTP (Kovalchuk et al., 2002). In the rat hippocampus and dorsal striatum, it has been shown that brain-derived neurotrophic factor (BDNF) promotes LTP (Akaneya et al., 1997; Figurov et al., 1996; Jia et al., 2010; Kang and Schuman, 1995). In contrast, the effects of nerve growth factor (NGF) on LTP are controversial. Some reports showed that NGF blocks the maintenance phase of *in vitro* LTP in rat visual cortex (Brancucci et al., 2004; Pesavento et al., 2000) and in the CA1 region of hippocampus (Tancredi et al., 1993). On the contrary, Conner et al. (2009) found that NGF augmentation facilitates induction of hippocampal LTP, while blockade of endogenous NGF significantly reduces it. A similar conclusion was previously raised by Maguire

Abbreviations: BDNF, Brain-derived neurotrophic factor; CAPs, Compound action potentials; CNS, Central nervous system; FWHM, Full width at half maximum; LTP, Long-term potentiation; NGF, Nerve growth factor; Nts, Neurotrophins; p75NTR, pan-neurotrophin receptor; SNS, Sympathetic nervous system; Trk, Tropomyosin-related kinase.

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et al. (1999) who suggested that endogenous NGF *in vivo* contributes to LTP expression in the dentate gyrus.

In the SNS Nts modulate synaptic transmission, in sympathetic neurons co-cultured with cardiomyocytes NGF enhances noradrenergic transmission (Lockhart et al., 1997), whereas BDNF rapidly changes the neurotransmitter released from noradrenaline to acetylcholine (Yang et al., 2002). Furthermore, these Nts also regulate neuronal firing pattern and some ionic currents in sympathetic neurons, which may underlie the neurotrophic regulation of neuronal function (Luther and Birren, 2006, 2009a, 2009b). Recently, Alzoubi et al. (2013) suggest a possible correlation between reduced BDNF levels and the lack of gLTP in ganglia from streptozocin-induced diabetic rats. In this work, we have examined the effects of Nts on sympathetic synaptic transmission, and on LTP, in the rat superior cervical ganglion *in vitro*. We found that Nts do not affect basal transmission, however, low concentrations of NGF inhibited LTP, whereas BDNF and high concentration of NGF enhanced LTP. We propose that BDNF, throughout p75 neurotrophin receptors (p75NTR) and probably TrkB receptors activation, and high dose of NGF, via activation of p75NTR, increase the synchronization of action potentials firing. These results suggest that the relative levels of signaling through these types of receptors could dynamically set the synaptic plasticity, and possibly the functional output, of the sympathetic system.

2. Materials and methods

2.1. Animals

Experiments were carried out in male Wistar rats (230–280 g) used in accordance with the Ethical Guidelines for the Care and Use of Laboratory Animals from the National Academy of Sciences of the United States and approved by our local Bioethical Committee. All efforts were made to minimize the number of animals used and their stress. The rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.). The ganglia were rapidly excised and carefully desheathed, then the preganglionic and post-ganglionic nerve roots were trimmed to a length of 3–5 mm, and the ganglia were transferred to a recording chamber (Warner Instruments, Hamden, CT, USA) and bathed with oxygenated (95% O_2, 5% CO_2) Ringer Krebs solution, pH 7.4, containing (in mM): 136 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 KH_2PO_4, 12 $NaHCO_3$, 11 glucose, and 2 μM atropine. All experiments were carried out at controlled temperature of 24.0 ± 0.5 °C.

2.2. Immunohistochemistry

After deep anesthesia with sodium pentobarbital (125 mg/kg i.p.), rats were perfused through the ascending aorta with ice-cold phosphate-buffered saline (0.01 M PBS, pH 7.4) followed by an ice-cold fixative solution. Superior cervical ganglion (SCG) was carefully dissected out and postfixed for 12 h in the same solution. Transversal SCG sections (14 μm thickness) were obtained on a cryostat. Sections were immersed in citrate buffer (Bio SB 20x Inmuno/DNA Retriever) at 60 °C for 1 h. Tissue sections were blocked with a solution containing 5% donkey serum, 5% bovine serum albumin and 0.3% Triton-X100 for 1 h. To study the localization of neurotrophin receptors in the SCG, sections were incubated with one of the following primary antibodies (diluted in blocking solution) for 16 h at room temperature: Rabbit polyclonal anti-fl-TrkB (Santa Cruz, sc-12, 1:500), rabbit polyclonal anti-TrkA (Abcam, ab8871, 1:200) and goat polyclonal anti-p75NTR (Santa Cruz, sc-6188, 1:200). Treatment with the primary antibody was followed by PBS-Tx washes and incubation with the following fluorescent secondary antibodies (in PBS-Tx) for 2 h: Donkey anti-rabbit Alexa Fluor 594 (Jackson, 1:400) and Donkey anti-goat DyLight 488 (Jackson, 1:200). After washing the secondary antibody, DAKO mounting medium was used to preserve fluorescence and pictures were taken with an epifluorescence microscope (Nikon Eclipse E600).

2.3. Electrophysiological recording

For recording and stimulation, the cervical sympathetic trunk (preganglionic) and the internal carotid nerve (postganglionic) were pulled enough into glass suction electrodes to keep stable the seal and recordings. Stimuli were applied by a Pulsar 6i Stimulator (FHC Inc., Bowdoin, ME, USA) and consisted of supramaximal square voltage pulses (9–12 V) of 0.1 ms duration at 0.2 Hz. Compound action potentials (CAPs) were recorded from the postganglionic nerve, voltage traces were amplified (x100) and bandpass filtered by a differential amplifier (DP-301, Warner Instruments, Hamden, CT, USA), and digitized with a multifunction data-acquisition (PCI-DAQ) board with 16-bit A–D converter using a custom-made acquisition program written in LabView v8.6 (National Instruments, Austin, TX, USA). The basal CAP amplitude was stable during 3–4 h. To avoid CAP signal saturation by the recruitment of all the postganglionic fibers during LTP, we partially blocked nicotinic transmission (70–80%) by adding 100 μM hexamethonium to the bath (Bachoo and Polosa, 1991). After reaching a stable level of blockade by hexamethonium, 25–30% of the basal CAP amplitude, reagents were added directly to the bath 30 min before the conditioning train and they were left for the duration of the experiment. LTP was induced by a train of supramaximal pulses applied at 40 Hz for 3 s. This conditioning train produced an immediate increase in the amplitude of the postsynaptic CAPs evoked by test pulses. Both ganglia of each rat were used, typically one for the experimental treatment and the other as a control. The experiments with neurotrophins and drugs were randomly performed. Since SCG are enclosed by several layers of connective tissue, which prevents drug accessibility, in some trials (control, NGF 200 ng/ml, NGF 200 ng/ml + anti-TrkA antibody) we increased the accessibility by lightly slicing the ganglion. To determine the diffusion of the anti-TrKA antibody, we fixed sliced ganglia in ice-cold fixative solution during 12 h, and detected the anti-TrKA antibody using an Alexa Fluor conjugated secondary antibody (1:300), according to the procedure described in the Immunohistochemistry section.

2.4. Pharmacological agents

Stock solutions of recombinant human β-NGF (GF028, Chemicon-Millipore, Billerica, MA) and recombinant human BDNF (B3795, Sigma–Aldrich, St. Louis, MO) were aliquoted, maintained at −80 °C, and used for up to 3 months after they were prepared. Working solutions were prepared fresh in Ringer–Krebs solution and added directly to the bath at the concentration indicated (100 ng/ml–500 ng/ml) at least 30 min before the onset of the experiment. The compounds tyrphostin AG879 (Cat. No. 2617, Tocris Bioscience, Ellisville, MO) and C_2-ceramide (sc-201375, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) were aliquoted and stored frozen in DMSO following the directions on the supplier's technical datasheet. Final concentration of DMSO always was <0.5% and we checked that it produces no effect on LTP. Tyrphostin AG879 and C_2-ceramide were added directly to the bath at final concentrations of 1 μM and 200 μM, respectively. Anti-TrkA antibody (Abcam, ab8871) was used at 1:500 dilution.

2.5. Quantification and statistics

After stabilization for approximately 30 min, input–output (I/O) curves were constructed by recording the amplitude of CAPs (output) obtained in response to progressively increasing stimulus intensities (input). We evaluated these curves by fitting a logistic function to the data, $V_o = V_*/[1 + ((V_*/V_0) - 1)^* e^{-aVi}]$ (Banks, 1994), using the following parameters: V_0 = minimal response (in our case $V_0 \approx 0.005$), V_* = maximal response, and coefficient *a*, related to the slope. We obtained the input value at which the half response occurs by interpolating from the graphs.

To quantify LTP, the voltage data were expressed as the ratio $(R_i - R_0)/R_0$ as a function of time (R_0: control CAP before the train, R_i: CAP at time = i after the train). The time course of CAP amplitudes recorded in the sympathetic ganglion in response to train pulses has been described as bi-exponential (Briggs et al., 1985; Cifuentes et al., 2004). Therefore, we fitted the function $f(t) = \alpha\, e^{-t/\tau 1} + c\, e^{-t/\tau 2}$ to the data of each experiment. The rapid component (α and time constant τ1) corresponds to post-tetanic potentiation (PTP), while the slow component (c and time constant τ2) corresponds to LTP (Briggs et al., 1985; Cifuentes et al., 2004; Vargas et al., 2007). To avoid the intrinsic variability observed in the decaying kinetics of the slow component, that limits the proper assessment of LTP differences, we evaluated LTP by calculating two parameters: LTP decay time, defined as the elapsed time it takes for potentiated response to fall to 20% above control value, i.e., solving the equation for $f(t) = 0.2$, and LTP extent, which is defined as the area under the curve of the potentiated response from $t = 0$ to LTP decay time. The data are expressed as the mean ± SEM. Data analysis was performed using the OpenOffice software suite (http://www.openoffice.org/). The significance of differences between means was tested with an unpaired Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

2.6. Compound action potential analysis

To determine whether neurotrophins affect CAP kinetics that could explain, at least partially, their effects on LTP, we carried out digital signal analysis of CAPs recorded 40 min (in the case of LTP reduction) or recorded 60 min (in the case of LTP enhancement) after the train. To increase signal to noise ratio we averaged five consecutive CAP traces to obtain the values at 40 and 60 min. The area of CAPs, onset time, full width at half maximum (FWHM), raising slope (+d/dt), and decaying slope (−d/dt) of normalized CAPs in relation to the CAPs obtained in the presence of hexamethonium before the train were measured. Note that the result of differentiation of normalized CAPs has a scale of s^{-1}. To measure onset time we calculated the average and standard deviation of basal line (y0, previous to stimulus) and defined the onset time when y > y0 + 2 SD.

2.7. Compound action potential modeling

To simulate action potentials we used NEURON software (Release 7.1; Carnevale and Hines, 2006). We modeled a neuron with a 40 μm soma stimulated by a synaptic input that activates sodium and potassium channels producing classic Hodgkin and Huxley action potentials. To compare the model with actual action potentials records

we averaged and scaled five thousand action potentials (APs) fired with a delay of 36 ms with variance (σ) of 13 ms or 26 ms, which produces an averaged population potential with FWHM equivalent to those obtained in the presence of BDNF and low dose of NGF, respectively.

3. Results

3.1. Neurotrophins differentially affect LTP

We found that BDNF significantly increased LTP, whereas NGF showed a differential effect, at low dose diminished LTP, whereas at high dose (>350 ng/ml) enhanced potentiation.

The addition of BDNF clearly enhanced LTP (Fig. 1D left), as shown in Fig. 1A (left), $\Delta R/R_0$ was larger in the presence of BDNF (200 ng/ml) than the corresponding control value. BDNF significantly increased both LTP decay time and LTP extent 2.0-fold above control ($p < 0.01$; Fig. 1A right). We explored different BDNF concentrations and found, as the dose–response curve shows, that BDNF at 350 and 500 ng/ml also enhanced LTP (Fig. 1D left). In contrast, NGF had a dual effect on LTP (Fig. 1D right), at 200 ng/ml NGF reduced LTP, while at >350 ng/ml significantly enhanced LTP. Fig. 1B (left) shows that in the presence of NGF (200 ng/ml) the ratio $\Delta R/R_0$ clearly decreased more rapidly, reaching basal levels approximately 40–45 min after the train. Both LTP decay time and LTP extent diminished to 52% and to 32% of the control values ($p < 0.0001$ and $p < 0.02$, respectively; Fig. 1B right). Since it has been shown that in cultured sympathetic neurons neurotrophins can activate high affinity TrkA or low affinity p75NTR, we tried different NGF concentrations and interestingly found that at concentration >350 ng/ml, instead of blocking LTP, NGF significantly enhanced the ganglia response after the train ($p < 0.001$; Fig. 1D right). At 500 ng/ml LTP decay time and LTP extent increased 1.7- and 1.5-fold, respectively, compared to control values ($p < 0.02$; Fig. 1C right). NGF at 50 and 100 ng/ml did not affect gLTP (Fig. 1D right).

3.2. Neurotrophins could affect synchronization of neurons rather the number of neurons firing

Considering that CAP size reflects the number and timing of firing ganglionic neurons, the differential effects of neurotrophins on LTP may be explained by a change in the number of firing neurons, by a change in their firing synchronization, or by a combination of both effects. Our rationale is that if there were a change in the number of neurons firing, both CAP amplitude and area would change accordingly. On the other hand, if the number of firing neurons remained unchanged but firing with more synchrony, CAP amplitude would be increased without a change in the area, while CAP width would be reduced and the rising and decaying slopes would change concurrently. To assess if there were a change in the number and synchronization of the underlying single action potentials, we carried out digital signal analysis of CAPs (Fig. 2A left). We found that the increase in CAP size produced by BDNF (Fig. 2B, left) was accompanied by a significant increase in the rising slope (+d/dt Fig. 2D, left) of CAPs. The other kinetics parameters, onset time, FWHM, the decay slope (Fig. 2C, 2D right), and the area of CAPs (Fig. 2B right) remained unchanged in presence of BDNF. The changes on CAPs kinetics produced by NGF (500 ng/ml) were similar, but smaller, to those produced by BDNF, there is a tendency to increase rising and decaying slopes but these changes were not significant. On the other hand, besides the reduction in CAP size NGF (200 ng/ml) did produce significant and opposing changes on CAPs kinetics than BDNF and NGF 500 did, at 200 ng/ml NGF increased significantly FWHM (Fig. 2C, right) and reduced the rising and decaying slope of CAPs (Fig. 2D), but area

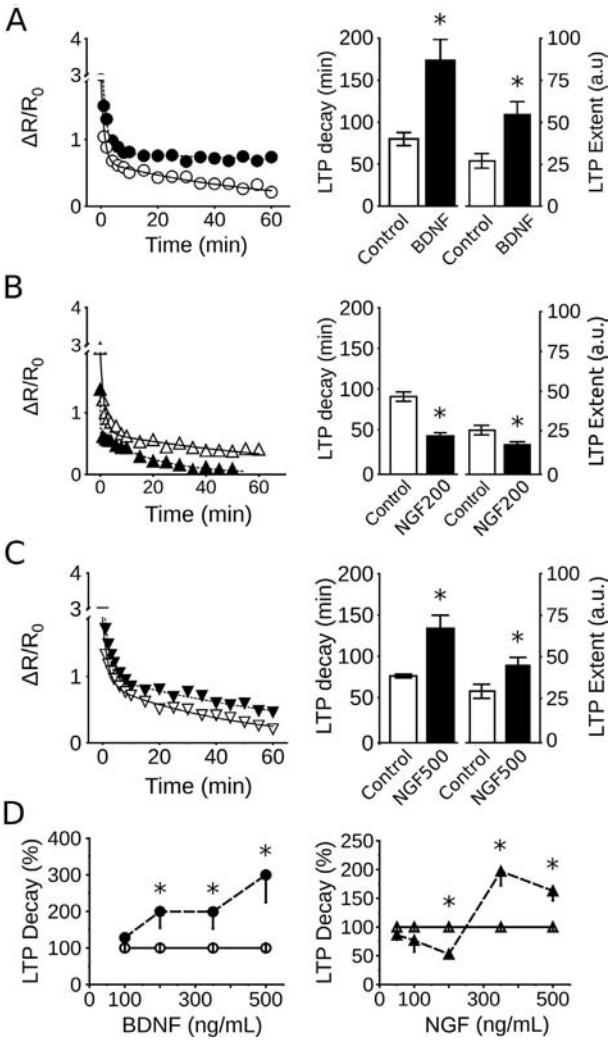

Fig. 1. Neurotrophins differentially affect LTP. **A**, BDNF enhanced LTP in sympathetic ganglia. **A** left: Time course of $\Delta R/R_0$ of a representative experiment showing that the ganglia response was larger in the presence of 200 ng/ml BDNF (●) than in the control conditions (○). **A** right: Bar plots of LTP decay time and LTP extent showing that BDNF significantly enhanced LTP in the sympathetic ganglia ($n = 6$; $p < 0.01$, Student's t-test, unpaired). **B**, NGF, at low concentration, blocked LTP in sympathetic ganglia. **B** left: Time course of $\Delta R/R_0$ showing that the ganglia response was smaller and end earlier in the presence of 200 ng/ml NGF (▲) than in the control conditions (△). **B** right: Bar plots showing that at this concentration NGF significantly decreased LTP decay time and LTP extent in sympathetic ganglia ($n = 11$; $p < 0.0001$ and $p < 0.02$, respectively, Student's t-test, unpaired). **C**, NGF, at high concentration, enhanced LTP in sympathetic ganglia. **C** left: Time course of $\Delta R/R_0$ showing that the ganglia response was larger in the presence of 500 ng/ml NGF (▼) than in the control conditions (▽). **C** right: Bar plots showing that at this concentration NGF significantly enhanced LTP decay time and LTP extent in sympathetic ganglia ($n = 6$; $p < 0.02$, Student's t-test, unpaired). **D**, Dose–response curves for neurotrophins. **D** left: Plot showing that BDNF (●) significantly enhanced LTP decay time at concentration >200 ng/ml. **D** right: Plot showing that NGF (▲) significantly decrease LTP at 200 ng/ml, while at >350 ng/ml significantly enhanced LTP decay time. * = $p < 0.05$ for all graphs.

remained unchanged (Fig. 2B right). The fact that area did not change in presence of Nts indicate that they most likely affect the synchronization of neuronal firing but not the number of firing neurons.

To test if a change in firing synchronization would account for the differences observed in CAP amplitude we simulated five thousand action potentials randomly fired with different synchrony (see Methods). We found that action potentials fired synchronously produced a population potential (i.e., CAP) with large amplitude, short duration, and larger slopes, while action potentials fired

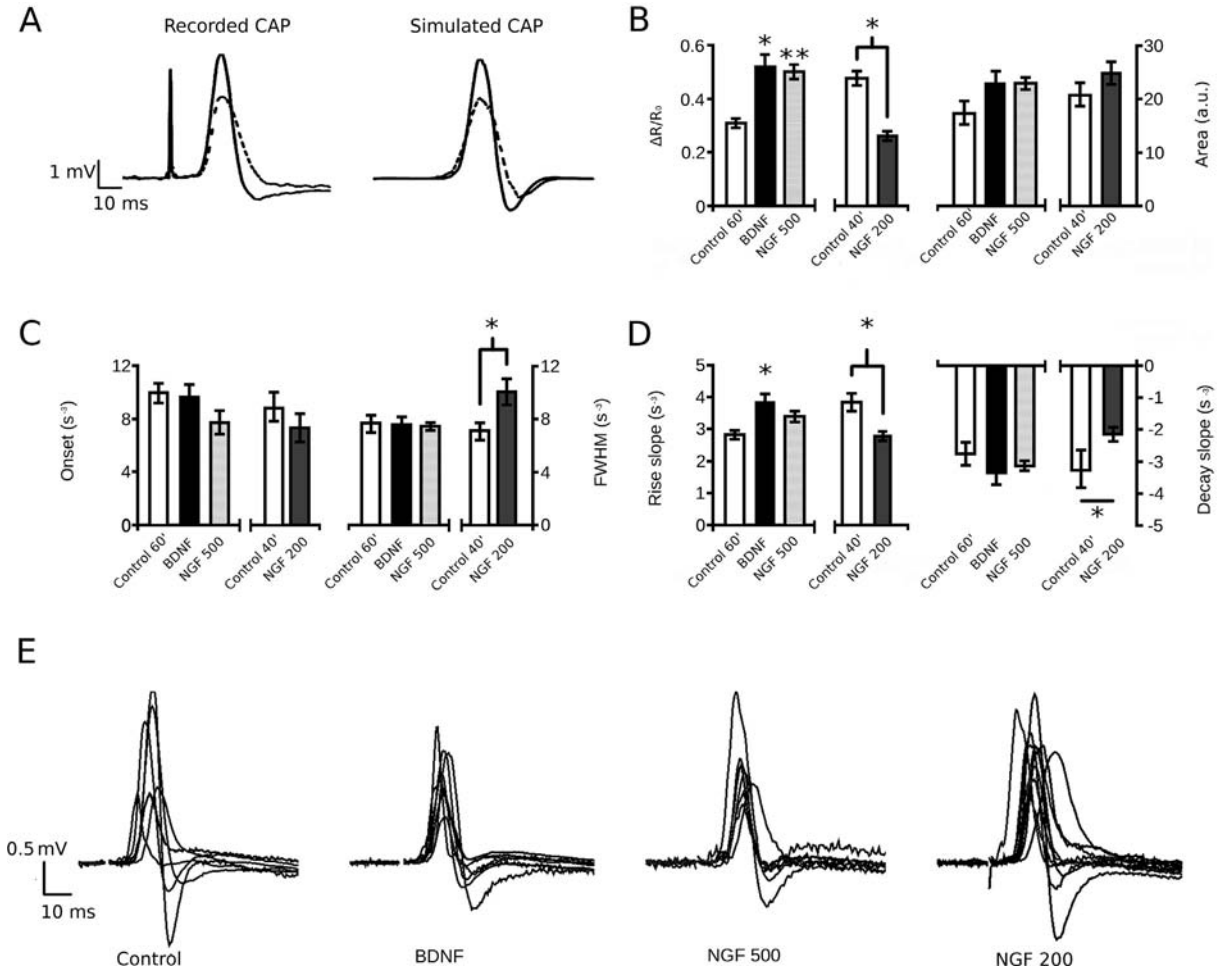

Fig. 2. Neurotrophins most likely affect synchronization rather than the number of neurons firing. **A** left: Superimposed traces of compound action potentials recorded 60 and 40 min after the train in the presence of BDNF (continuous line) and NGF 200 (dashed line). **A** right: It shows the results of our compound action potential modeling, where five thousand action potentials fired with a variance of 13 ms produced a large population potential (continuous line), while if they were fired with a variance of 26 ms they produced a small population potential (dashed line). **B**–**D**: Digital analysis of CAPs under the different experimental conditions: control at 60 min, BDNF, NGF at 500 mg (NGF 500), control at 40 min (control 40) and NGF at 200 mg (NGF 200). While BDNF in parallel to increase $\Delta R/R_0$ (**B** left) produced an enhancement in the rise slope of CAPs (**D** left), without affecting area (**B** right) or FWHM (**C** right), NGF 200 produced the opposite a decrease in $\Delta R/R_0$ (**B** left) and enhancement in FWHM (**C** right) and a decrease in rise slope (**D** left). NGF 200 also reduced decay slope (**D**, right). NGF 500, like BDNF, increased $\Delta R/R_0$ (**B** left) and showed a tendency to increase rise slope (**D** left) without affecting area (**B** right) and FWHM (**C** right). **E**: Superimposed traces of several compound action potentials (not normalized) evoked in each ganglion of the different experimental groups. It shows that BDNF and NGF (500 ng/ml) produced a synchronization of CAP between ganglia, while in control conditions and NGF (200 ng/ml) there was a dispersion of records.

asynchronously resulted in a population potential with small amplitude, longer duration, and smaller slopes (Fig. 2A, right).

We analyzed in more detail the change produced by Nts by looking at each CAP record and found that in control conditions and 200 ng/ml NGF there was a large dispersion within the group in the onset of CAPs (Fig. 2E), but remarkably, this dispersion disappeared in the presence of BDNF or 500 ng/ml NGF. In these conditions, CAPs showed an unexpected degree of synchronization (Fig. 2E).

The fact that BDNF and NGF (500 ng/ml) produce the same effects on LTP and opposite effects than NGF (200 ng/ml) on CAP kinetics suggests they would probably have the same mechanism of action to enhance LTP (see below).

3.3. Neurotrophins do not affect basal transmission

An enhancement or a reduction of synaptic transmission could explain the effects of Nts on LTP; therefore, we studied whether Nts produce any changes in the input–output (I/O) curve of base line postganglionic responses. This curve reflects the number of fibers activated at various stimulus intensities and shifts position when the strength of synaptic transmission is changed (Johnston and Wu, 1995). If synaptic transmission is depressed the I/O curve is expected to shift to the right along the stimulus intensity axis, implying that at a given intensity the resulting CAP is smaller in amplitude. A shift to the left indicates facilitation in synaptic transmission. Fig. 3 shows the I/O curves obtained in the presence of BDNF (200 ng/ml) or NGF (200 ng/ml) and in control conditions. It is clear that Nts have no effect on basal synaptic transmission. The results of fitting a logistic function to the data (see Methods) confirm that there was no difference between the groups (Fig. 3). Thus, the values for the maximal response, V_*, were 3.5 ± 0.3, 3.0 ± 0.3, and 2.7 ± 0.4 mV for the control, BDNF, and NGF, respectively ($p > 0.25$). The input values at which the half response occurs ($V_{0.5}$) were 5.0 ± 0.4, 5.0 ± 0.2, and 5.3 ± 0.6 V for the control, BDNF, and NGF, respectively ($p > 0.3$). The values for parameter a were 1.8 ± 0.2, 1.5 ± 0.2, and 1.3 ± 0.2 in the control, BDNF, and NGF, respectively ($p > 0.2$). These results indicate that basal transmission was not changed in the presence of neurotrophins.

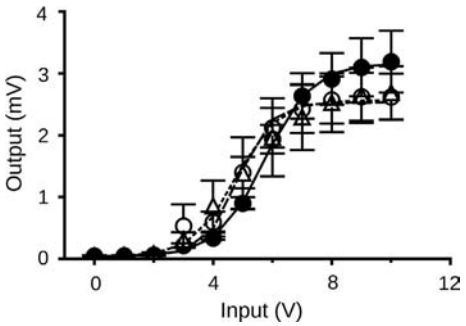

Fig. 3. Neurotrophins have no effect on control synaptic transmission. The I/O curves show that at any input voltage the output voltage was similar in control conditions (○), in the presence of 200 ng/ml BDNF (●), and in the presence of 200 ng/ml NGF (△). The logistic function fitted to the data confirms there was no difference in the parameters maximal response (V_*) ($p > 0.25$), $V_{0.5}$ ($p > 0.3$), and slope a ($p > 0.2$) between the conditions. Each point represents the mean ± S.E.M. from 5 to 7 ganglia.

3.4. Immunohistochemical detection of neurotrophin receptors

Considering that it has been postulated and assumed that sympathetic neurons only express TrkA and low affinity p75NTR (Dixon and McKinnon, 1994; Wetmore and Olson, 1995), to better interpret our results, we characterized by immunohistochemistry the presence of neurotrophin receptors in adult sympathetic neurons. We found that 83 ± 2% of neurons express TrkA, while 52 ± 3% express p75NTR, and merely 18 ± 1% of neurons express TrkB receptor (Fig. 4). Therefore we postulate that it is likely that NGF at ≤200 ng/ml activates mainly TrkA, and NGF at >350 ng/ml triggers p75NTR signaling pathway, whereas BDNF acts mainly via p75NTR and marginally on TrkB receptors.

3.5. Differential regulation of LTP by p75NTR and TrkA signaling

Given the opposing effects produced on LTP and CAPs kinetics by BDNF and NGF (500 ng/ml) in comparison to NGF (200 ng/ml), we hypothesized that LTP enhancement would involve a common signaling pathway (likely p75NTR), whereas LTP blockade would involve a different pathway, probably TrkA. Trying to elucidate the signaling pathways involved we explored the effects of C_2-ceramide, a putative second messenger produced by the activity of p75NTR-activated sphingomyelinase, tyrphostin AG879, a tyrosine kinase inhibitor, and of an anti-TrkA antibody. We found that C_2-ceramide significantly enhanced LTP (Fig. 5A). Fig. 5A left shows that $\Delta R/R_0$ was larger in the presence of C_2-ceramide than the corresponding control values. Since the response to this drug was always activating but variable, LTP decay time ranged from 90 to 500 min, we decided to evaluate the effect of the drug at a fixed post-train time. Therefore, we measured the ratio of $\Delta R/R_0$ and calculated LTP extent at 60 min post-train (Fig. 5A right). We determined that both measurements increased significantly (4.1- and 2.1-fold) in the presence of C_2-ceramide ($p < 0.03$).

To study the possible role of TrkA signaling on LTP, we applied 1 μM tyrphostin in presence of 200 ng/ml NGF. In this condition, we observed that the inhibitory effect of NGF is reversed; $\Delta R/R_0$ decayed slower in the presence of tyrphostin plus NGF (Fig. 5B, left), LTP decay time and LTP extent increased 2.2- and 2.0-fold, respectively, in relation to NGF 200 ng/ml alone ($p < 0.003$; Fig. 5B, right), resulting in values similar to control LTP (dashed lines, Fig. 5B, right). Next, in another series of experiments we applied an anti-TrkA antibody trying to assert the involvement of TrkA pathway. Previously, we tested whether an Alexa Fluor conjugated IgG can diffuse into the ganglion, as expected, we found that in intact ganglion the IgG did not permeate, whereas in a lightly sliced ganglion the antibody diffused to a portion of the ganglion (not shown). We did control and NGF (200 ng/ml) experiments to validate this preparation of lightly sliced ganglia, we found that there was no significant difference in control condition between intact and sliced ganglion, however, NGF was significantly more effective in inhibiting LTP in sliced ganglion than in intact one (Fig. 5D left). Then, we tried NGF (200 ng/ml) in presence of an anti-TrkA antibody and found that the antibody was able to revert significantly (but partially) NGF effect (Fig. 5D left). The partial effect of the anti-TrkA antibody could be explained by its limited diffusion (Fig. 5D right). These results suggest the participation of TrkA signaling in the blocking effect of LTP.

3.6. Tyrphostin AG879 discloses an effect of endogenous NGF on LTP

We were aware of the partial inhibitory effect of NGF on LTP (Fig. 1B) and the reversal effect produced by tyrphostin (Fig. 5B), therefore, we wondered if tyrphostin had an effect on LTP that could indicate that endogenous NGF is likely down-regulating LTP in the sympathetic ganglia. We found that tyrphostin AG879 at 1 μM significantly enhances LTP (Fig. 5C, left). LTP decay time and LTP extent increased 2.4- and 1.7-fold, respectively, in the presence of tyrphostin ($p < 0.005$ and $p < 0.03$, respectively, Fig. 5C, right). This result suggests the participation of endogenous NGF in the regulation of LTP in sympathetic ganglia.

4. Discussion

We have found that NGF and BDNF have differential effects on LTP in sympathetic ganglia and that endogenous NGF is most likely participating in the regulation of ganglionic LTP. These results,

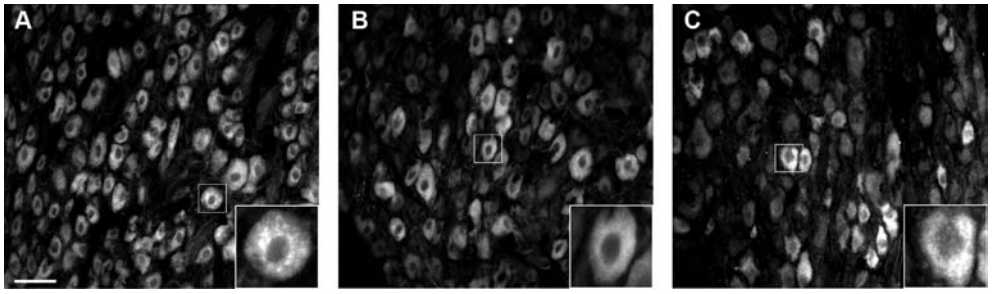

Fig. 4. TrkA, p75NTR and TrkB receptor expression in rat SCG. Photomicrographs of sympathetic neurons immunolabeled against TrkA (A), p75NTR (B) and TrkB (C) show a strong expression of these neurotrophin receptors; 83% of neuronal population of SCG expressed TrkA, 52% expressed p75NTR, and 18% expressed TrkB. The inset (3x magnification) shows the cellular expression of each protein. There is a homogeneous cytoplasmic expression of p75NTR, TrkA has a thicker perinuclear distribution, whereas TrkB has a more diffuse distribution pattern. Scale bar = 50 μm.

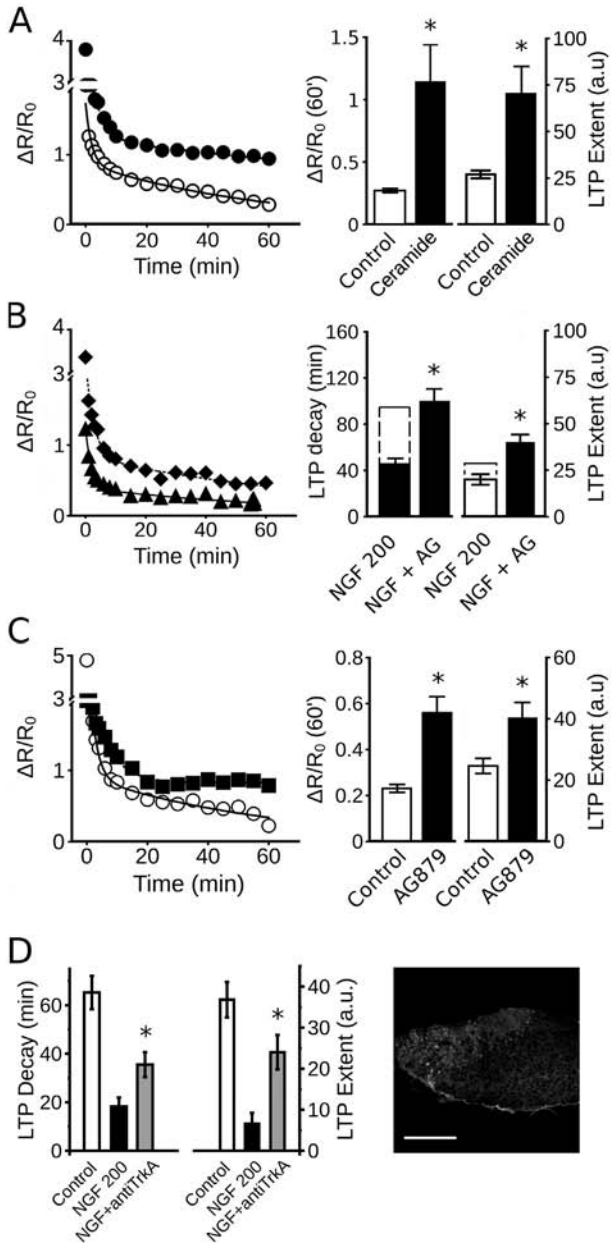

Fig. 5. Probable signaling pathways involved in neurotrophin-induced LTP regulation. **A** left: Time course of $\Delta R/R_0$ of a representative experiment showing that C_2-ceramide (●) provoked a LTP that was significantly larger than control (○). **A** right: Bar plots showing that $\Delta R/R_0$ and LTP extent, measured 60 min after the train, were significantly larger in presence of C_2-ceramide ($p < 0.03$ and $p < 0.02$, respectively). **B** left: Time course of $\Delta R/R_0$ of a representative experiment showing that CAPs recorded in presence of tyrphostin AG879 plus NGF (200 ng/ml) (◆) were larger than those recorded with NGF (200 ng/ml) alone (▲). **B** right: Bar plots showing that LTP decay time and LTP extent in the presence of tyrphostin AG879 plus NGF (200 ng/ml) were significantly larger than in the presence of NGF (200 ng/ml) alone ($p < 0.003$ and $p < 0.006$, respectively, $n = 6$; Student's *t*-test, unpaired) and were similar to control values (dashed lines; $p > 0.5$). **C** left: Time course of $\Delta R/R_0$ of a representative experiment showing that tyrphostin AG879 (■) provoked a significant LTP enhancement above control (○). **C** right: Bar plots showing that $\Delta R/R_0$ and LTP extent, measured 60 min after the train, were significantly larger in the presence of tyrphostin AG879 ($p < 0.005$ and $p < 0.03$, respectively, $n = 6$; Student's *t*-test, unpaired). **D** left: Bar plots showing that an anti-TrkA antibody partially reverted NGF blocking effect. LTP decay time and LTP extent in the presence of the antibody plus NGF (200 ng/ml) were significantly larger than in the presence of NGF (200 ng/ml) alone. **D** right: Photomicrograph of a sliced ganglion showing that the anti-TrkA antibody diffused only in the periphery of the ganglion. Scale bar = 500 μm.

along with our pharmacological data obtained with C_2-ceramide, tyrphostin AG879, and anti-TrkA antibody suggest that signaling likely through p75NTR or TrkB receptors enhances LTP, while signaling probably through TrkA receptors reduces LTP. We propose that the signaling responsible of LTP enhancement synchronizes unitary action potentials enlarging CAPs, while the signaling responsible of LTP reduction disrupts the timing of action potentials producing CAPs reduction. Neurotrophins have no effect on basal synaptic transmission.

The range of concentration of neurotrophins used here is similar to that used by Pesavento et al. (2000) and Brancucci et al. (2004) who used 100 ng/ml of NGF in their studies of the action of NGF on LTP in the primary visual cortex slices, and more relevant, the range is similar to those used by Lockhart et al. (1997) and Luther et al. (2013) to study the regulation of synaptic transmission in sympathetic neurons in culture. However, it is completely different to 0.01 ng/ml of NGF used by Tancredi et al. (1993) in hippocampal slices. Differences in receptor's affinity among regions/tissues could explain these results, thus, the affinity in neurons of sympathetic ganglia and primary visual cortex would be lower than in hippocampal neurons. We also found that NGF was more efficient in blocking LTP in sliced ganglion than in intact one, which could be explained by the presence of a diffusion barrier (several layers of connective tissue that surround ganglionic neurons) in the intact ganglion, and by the molecular weight of neurotrophins (ca. 13 kDa).

Our results showing opposite effects of BDNF and NGF on ganglionic LTP agree with the reports showing that LTP depends on BDNF in the dorsal striatum (Jia et al., 2010) and in the hippocampus (Akaneya et al., 1997; Figurov et al., 1996; Kang and Schuman, 1995; Korte et al., 1998), and that NGF blocks LTP in rat visual cortex *in vitro* (Brancucci et al., 2004; Pesavento et al., 2000) and in the CA1 region of the hippocampus (Tancredi et al., 1993). However, there are studies showing that *in vivo* NGF increases LTP in dentate gyrus (Maguire et al., 1999) and facilitates induction of hippocampal LTP (Conner et al., 2009). A preferential activation of TrkA or p75NTR, which are unevenly distributed in the central nervous system (Aboulkassim et al., 2011), could explain these opposite results.

It has been shown that the activation of p75NTR by BDNF and of TrkA by NGF, results in opposite effects on neurotransmission between sympathetic neurons and cardiac myocytes (Yang et al., 2002). Further, Birren's laboratory demonstrated that NGF poses a dual role, thus, via p75NTR increases the number of sympathetic neurons firing in phasic pattern (Luther and Birren, 2009a) and potentiates cholinergic transmission between sympathetic neurons (Luther et al., 2013), conversely, NGF via TrkA promotes tonic firing (Luther and Birren, 2009a). Therefore, we tried to figure out the receptors differentially activated by NGF and BDNF that could explain their opposite effects on LTP. We found that LTP inhibition produced by low dose of NGF was abolished by tyrphostin AG879 and partially reversed by an anti-TrkA antibody. We also found that C_2-ceramide, which produces p75NTR activation-like effects in cultured sympathetic neurons (Luther and Birren, 2009a; Luther et al., 2013; Yang et al., 2002) and it is produced by the activation of p75NTR (Dobrowsky et al., 1994, 1995), enhanced LTP more efficiently than BDNF. All these results along with our immunohistochemical data, which clearly indicate that, besides TrkA and p75NTR, sympathetic neurons express TrkB receptors (Fig. 4), support the hypothesis that NGF via TrkA signaling may be underlying the LTP blockade, whereas BDNF would activate preferentially p75NTR, and TrkB receptors in a small extent. However, since tyrphostin AG879 blocks TrkA-mediated NGF effect in sympathetic neurons (Jia et al., 2008) and in other cell systems (Ohmichi et al., 1993; Plouffe et al., 2006; Rende et al., 2000), but

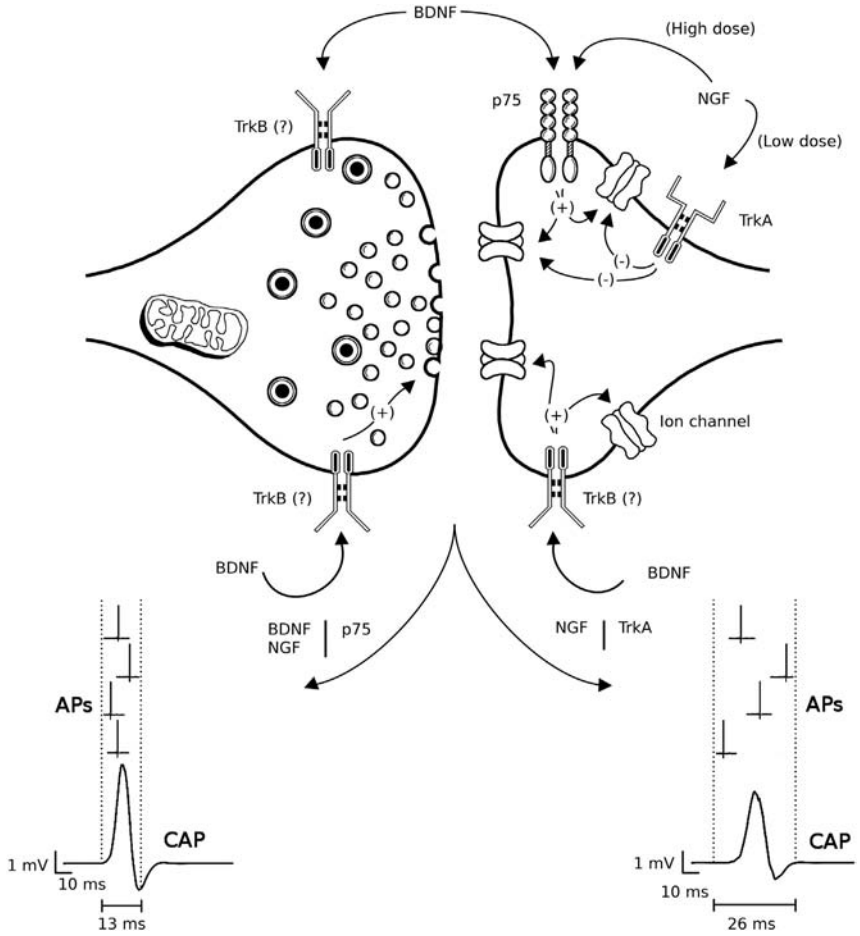

Fig. 6. Proposed mechanisms for the neurotrophin effects on ganglionic LTP. Scheme of a ganglionic synapse depicting the possible course of BDNF and NGF actions on synaptic function. BDNF through presynaptic TrkB receptors (Causing et al., 1997; Schober et al., 1999) can increase ACh release, thereby potentiating synaptic transmission. In addition, BDNF or high dose of NGF, via postsynaptic TrkB or p75NTR receptors, acting on ion channels or nicotinic receptors would increase postsynaptic membrane excitability. We hypothesize that the activation of p75NTR (or TrkB) produces synchronization in the latencies of action potentials (APs), which can lead to an enlargement in the amplitude and a reduction in the duration of evoked CAPs. NGF at low doses can activate postsynaptic TrkA receptors to induce a decrease in the amplitude and an increase in the duration of CAPs due to the asynchronous summation of action potentials. The simulation of five thousand action potential fired with different synchronization (variance) is shown in the lower part of the figure. The variance used is indicated below each CAP and by the broken line. With a variance of 13 ms (left) CAP kinetics is similar to BDNF, while a variance of 26 ms produces a CAP with kinetics that resemble those recorded in the presence of NGF.

also blocks other tyrosine kinases (Lee et al., 2008), we cannot completely rule out the participation of another tyrosine kinase-dependent signaling pathway in the NGF blocking effect. Alternatively, the opposite effects of NGF on LTP can be explained by an interaction between NGF and the ganglionic cholinergic system. Thus, depending on the concentration of NGF used, it can increase or can reduce LTP through a regulation of the intracellular signals underlying potentiation, possibly by interfering with the cholinergic modulated intracellular pathway (Pesavento et al., 2000).

Our immunohistochemical results disagree the assumption that sympathetic neurons only express TrkA and p75NTR with almost no expression of TrkB receptors (Dixon and McKinnon, 1994; Wetmore and Olson, 1995), although TrkA and p75NTR prevail, TrkB is also expressed but in small extent. Interestingly, we found that tyrphostin AG879 itself enhanced LTP suggesting that endogenous NGF down regulates synaptic plasticity and that TrkA likely prevails over p75NTR/TrkB signaling in sympathetic ganglia.

The mechanism of Nts actions on gLTP remains to be elucidated. The sole antecedent known has been recently provided by Alzoubi et al. (2013), who found reduced BDNF levels in ganglia from streptozocin-induced diabetic rats, which lack of gLTP. In cultured hippocampal neurons BDNF potentiates synaptic transmission by increasing transmitter release (Schinder et al., 2000; Tyler and Pozzo-Miller, 2001) and by increasing the number of docked vesicles at the active zones (Tyler and Pozzo-Miller, 2001). Therefore, BDNF may increase the amount of acetylcholine released, or BDNF and NGF, via post-synaptic p75NTR/TrkB or TrkA, may act on nicotinic receptors or on ion channels to modify the excitability of sympathetic neurons (Luther and Birren, 2009a), enhancing or decreasing LTP (Fig. 6). Taking into account that rat ganglion cells show different spike delays at the same stimulus strength (Perri et al., 1970), and that spikes occur with shorter latency after tetanization in hippocampal slices (Andersen et al., 1977), and that NGF-activated neurons firing in a tonic pattern show a delay in their initial spike (Fig. 2, Luther and Birren, 2009a), we propose that Nts modify LTP by regulating the synchronization of underlying action potentials. Modeling five thousand CAPs fired with different synchronization (Fig. 2A), we found that neurons firing synchronously will produce a large, short population potential due to summation of the action potentials (Fig. 6, lower left). In contrast, neurons firing asynchronously, with different latencies, will decrease the amplitude but will increase the duration of population potential (Fig. 6, lower right). In both cases the area of CAPs will remain unchanged. This model agrees with our results that BDNF

increased the CAP rising slope; while NGF 200 increased the duration and reduced the rising and decaying slope of CAPs (Fig. 2). Furthermore, looking at each CAP recorded we found that in control conditions and with 200 ng/ml NGF there was a large dispersion within the group in the onset of CAPs, but remarkably, this dispersion disappeared in the presence of BDNF or 500 ng/ml NGF, in these conditions, CAPs showed an unexpected degree of synchronization (Fig 2E). These differences cannot be attributed to different pre-ganglionic nerve length that would unmask different conduction velocities because we stimulated the nerve as a whole regardless of its length, precluding the effects of differences in conduction velocity (Perri et al., 1970).

One possible mechanism of synchronization was shown by Fujisawa et al. (2004) who demonstrated that BDNF led to rhythmical firing of hippocampal neurons. They showed that the incorporation of a $Na_V1.9$-like conductance in a simple model neuron reproduces the effect of BDNF, interestingly this conductance has been shown to underly the neurotrophin-evoked excitation effect (Blum et al., 2002). It remains to be elucidated if this type of Na-conductance is present in the ganglion. A simpler manner to synchronize neurons is through electrical synapsis, however, this is not feasible in ganglia since the majority of neuron somas are surrounded by satellite cells (Gibbins and Morris, 2006).

It is known that SNS activity is rhythmic and that its frequency may vary depending on physiological requirements (Malpas, 1998; Montano et al., 2009). Therefore, the balance of p75NTR/TrkB and TrkA signaling would define the sympathetic tone in physiological or patho-physiological conditions. The neurotrophic mechanisms could play a role in maladaptive changes that occur following heart attacks (Chaldakov et al., 2004) or in congestive heart failure (Kanazawa et al., 2010), where sympathetic neural tone is upregulated.

Disclosure statement

The authors Erwin R. Arias, Pablo Valle-Leija, Miguel A. Morales, and Fredy Cifuentes all declare no conflict of interest.

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Review

Long-term potentiation in mammalian autonomic ganglia: An inclusive proposal of a calcium-dependent, trans-synaptic process☆

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ABSTRACT

Ganglionic synapses have the capability to express long-term potentiation (gLTP) after application of a brief high-frequency stimulus. It has been suggested a possible role of gLTP in some cardiovascular diseases. Although a number of characteristics of gLTP have been described, the precise locations and mechanisms underlying gLTP are not completely known. Current findings support two major conflicting presynaptic and postsynaptic hypotheses. The presynaptic hypothesis posits a presynaptic increase in acetylcholine (ACh) release, whereas the postsynaptic hypothesis proposes a long-lasting enhancement of the nicotinic response on the postsynaptic membrane. An alternative trans-synaptic hypothesis proposes the presynaptic release of a cotransmitter from large dense core vesicles, which postsynaptically enhances synaptic efficacy and accounts for gLTP. Here, we review the studies of LTP, with emphasis on gLTP in mammals, and we examine the findings that support the presynaptic, the postsynaptic and the trans-synaptic hypotheses. We then review our data on the contribution of calcium to gLTP as an approach to elucidate the mechanisms of gLTP. Data on the contribution of calcium to gLTP and on prolonged high-frequency stimulus-dependent fading of LTP have led us to support the trans-synaptic process as responsible for gLTP. Finally, we present a formal working model for the mechanisms of gLTP.

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1. Introduction

A brief train of high-frequency presynaptic stimulation induces a prolonged enhancement in the efficacy of synapses. This phenomenon, known as long-term potentiation (LTP), was first described in the glutamatergic synapses of the rabbit hippocampus (Bliss and Gardner-Medwin, 1973; Bliss and Lömo, 1973). Hippocampal LTP has acquired much attention for its potential role in the cellular mechanisms of learning and memory (Bliss and Collingridge, 1993). Subsequently, a similar phenomenon of

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strengthened synaptic transmission was found in other vertebrate synapses, including peripheral synapses (Brown and McAfee, 1982; Dolphin, 1985; Kuba and Kumamoto, 1990). The first report of synaptic potentiation of ganglionic transmission appeared before the first formal report of hippocampal LTP. Dunant and Dolivo (1968) described a long-lasting increase in ganglionic transmission in the rat superior cervical ganglia (scg) resulting from a brief preganglionic tetanus. This potentiation of transmission in the rat scg was again observed and analyzed in more detail by Brown and McAfee (1982), who referred to this potentiation as LTP because of its similarity with hippocampal LTP. Equivalent phenomena were later demonstrated in other autonomic ganglia in different species using *in situ* (Alonso-deFlorida et al., 1991; Bachoo and Polosa, 1991; Morales et al., 1994) or *in vitro* preparations (Koyano et al., 1985; Scott and Bennett, 1993). Furthermore, muscarinic (Libet and Mochida, 1988) and non-cholinergic (Ashe and Libet, 1981) ganglionic LTP have also been demonstrated. Other forms of potentiation of ganglionic transmission have been described, such as the potentiation induced by the administration of adrenergic agonists (Brown and Dunn, 1983; Kuba et al., 1981) or by the exposition to specific antigens (Weinreich et al., 1995). High-frequency preganglionic stimulation also induces a type of potentiation known as rebound acetylcholine (ACh) (Collier et al., 1983).

It is known that ganglionic synaptic transmission involves sequential changes in membrane potential. It initiates with a nicotinic depolarization responsible of a fast excitatory postsynaptic potential followed by a muscarinic hyperpolarization that gives rise to an inhibitory postsynaptic potential. Then, a late muscarinic depolarization produces a slow excitatory potential, and finally a non-cholinergic depolarization, likely peptidergic, results in a late slow excitatory postsynaptic potential (Karczmar et al., 1986; Nishi and Koketsu, 1968). This review focuses on LTP of nicotinic transmission, mainly in mammalian ganglia.

2. Physiological relevance of gLTP

Like implications of hippocampal LTP in functions such as learning and memory (Bliss and Collingridge, 1993) it is possible that gLTP may play a role in the modulation of peripheral autonomic nervous system activity. It is expected that gLTP enhances tonic efferent impulses to targets, which would modify normal function of diverse organs, including heart, blood vessels and glands. Although the most efficient stimulus frequency to induce gLTP is over 20 Hz, it is possible to induce gLTP with lower frequencies ranging from 5 to 8 Hz (Briggs and McAfee, 1988), which certainly are within the range of spontaneous activity of sympathetic neurons (Jänig et al., 1983; Polosa, 1968). Sympathetic preganglionic neurons (SPN) that usually fire at 5 Hz, can increase their firing rate to bursts of 20 Hz for 1 s in response to an increase in end-tidal CO_2 (Preiss and Polosa, 1977). Therefore, it is reasonable to expect that gLTP may be present *in vivo* in sympathetic ganglia under stress conditions, such as hypercapnia.

Evidence associating expression of gLTP to the development or aggravation of hypertension in animal models have also been presented (Alkadhi et al., 2001b; Gerges et al., 2002). These evidence suggest that most of the neurogenic forms of hypertension are originated and sustained by an increase in the sympathetic-adrenal tone (Guyenet, 2006), which gives rise to elevated plasma levels of norepinephrine and larger electrical activity in the sympathetic efferent nerves (Mancia et al., 1999). It is likely that pathologically enhanced activity of the sympathetic outflow reaching sympathetic ganglia would produce a gLTP *in vivo* (Alkadhi and Alzoubi, 2007). Then, the presence of sustained gLTP in sympathetic ganglia *in vivo* could reinforce the postganglionic outflow to all target organs including blood vessels. This persistent larger efferent activity in blood vessels would increase peripheral vessels resistance leading to elevated blood pressure. In another form of hypertension, elicited by ouabain, it has been shown that the increase in sympathetic nerve activity is associated to the prolongation of gLTP (Aileru et al., 2001). Considering the link between gLTP and hypertension, this plastic phenomenon could be used as a bioassay to monitor installation of hypertension in animal models, such as spontaneous hypertensive rats. It has also been shown that there is a selective age-dependent decline in the capacity for sympathetic ganglia to generate long-term changes in synaptic efficacy (Alzoubi et al., 2010; Wu et al., 1991).

3. Mechanisms underlying ganglionic LTP

The diverse reports addressing the mechanisms underlying gLTP clearly indicate that this phenomenon results from a complex set of events involving enzymes, co-transmitters and second messengers arising from both presynaptic and postsynaptic locations (Alkadhi et al., 1996; Bachoo et al., 1992a; Briggs et al., 1985a; Gonzalez-Burgos et al., 1995; Heppner and Fiekers, 2003; Hogan et al., 1998; Southam et al., 1996). Recently, we have reported that neurotrophins likely participate in gLTP (Arias et al., 2011). Despite all these efforts, the differential contribution of presynapsis and postsynapsis has not been completely defined. In fact, from experiments in mammalian scg, two conflicting hypotheses have been proposed: (i) a presynaptic mechanism that postulates an increase in evoked ACh release as a result of presynaptic Ca^{2+} accumulation, triggered by high-frequency stimulation (Briggs et al., 1985a,b; Briggs and McAfee, 1988; Brown and McAfee, 1982), and (ii) a postsynaptic hypothesis, which states that postsynaptic nicotinic receptors increase in number, affinity for ACh, or ionic conductance by a phosphorylation-mediated mechanism (Collier, 1996; Morales et al., 1994).

4. Presynaptic mechanisms of gLTP

In their pioneer study, McAfee's group first proposed that the presynapsis is the locus of gLTP. In a simple schema, they stated that tetanic stimulation increases $[Ca^{2+}]$ in the preganglionic terminals, leading to a larger release of ACh that is responsible for gLTP (Brown and McAfee, 1982). The presynaptic hypothesis was also based on a collection of findings described in a series of further articles (Briggs et al., 1985a,b; Briggs and McAfee, 1988; reviewed in McAfee et al., 1987; Briggs, 1995): (1) potentiation was elicited by preganglionic tetanic stimulation, but not by antidromic postganglionic stimulation (Brown and McAfee, 1982), (2) potentiation was not induced by non-synaptic stimulation (intracellular depolarization) of individual postganglionic neurons (Briggs and McAfee, 1988), and (3) release of endogenous ACh into the bath medium was enhanced in parallel with synaptic transmission after tetanic stimulation (Briggs et al., 1985b).

Briggs et al. (1985a) were interested in knowing whether activating nicotinic receptors during the conditioning tetanus is important for the expression of gLTP. They found that gLTP induction did not depend on the release of ACh during the train because carbachol, a cholinergic agonist, was unable to mimic tetanic stimulation in producing gLTP. Furthermore, the use of selective nicotinic or muscarinic antagonists during the train did not affect gLTP. In the same work these authors explored whether neurotransmitters besides ACh could induce gLTP. They found that α- and β-adrenergic antagonists applied during the train did not affect gLTP (Briggs et al., 1985a). To further rule out the involvement of a chemical mediator released during the train, Briggs et al. (1985a) explored whether potentiation can be heterosynaptically elicited, *i.e.* induced by the application of a tetanizing train to

presynaptic terminals different to those used to apply the test pulses. They found that gLTP was produced only when both test pulses and tetanizing train were applied to the same presynaptic terminals. Accordingly, the absence of heterosynaptic gLTP persuaded Briggs et al. (1985a) to reject the possibility that a mediator released during the train was responsible for gLTP. In a subsequent work, to rule out a postsynaptic change in the responsiveness of nicotinic receptors, Briggs and McAfee (1988) tested whether ganglionic responses to exogenous administration of 1.1-dimethyl-4-phenylpiperazinium (DMPP), a nicotinic agonist, were increased after tetanic stimulation. They found that the response to this agonist was not increased after tetanus, supporting that preganglionic high-frequency stimulation induces a post-tetanic enhancement of the amount of ACh released (Briggs and McAfee, 1988). Later, Alkadhi's group (most likely the most active in the field) pursuing the presynaptic hypothesis of gLTP proposed the contribution of serotonin (Alkadhi et al., 1996). They found that 5-hydroxytryptamine (5-HT) and the serotonin-reuptake inhibitor fluoxetine enhanced the magnitude of gLTP in the rat scg. Ganglia depleted of serotonin by reserpine failed to express gLTP, whereas 5-HT3 receptor agonists and antagonists induced and blocked LTP, respectively. The authors concluded that tetanus-induced gLTP requires activation of 5-HT3 receptors, both for induction and maintenance (Alkadhi et al., 1996). They also suggested that during the tetanus, serotonin released from small intensely fluorescent (SIF) cells activates presynaptic 5-HT3 receptors and provides an influx of Ca^{2+} to the presynaptic terminals, inducing the enhancement of ACh release and gLTP (Alkadhi et al., 2005). Alkadhi's group also showed evidence of the participation of nitric oxide (NO; Altememi and Alkadhi, 1999) and carbon monoxide (CO; Alkadhi et al., 2001a) in the maintenance and induction of gLTP, respectively. With these findings, Alkadhi et al. (2005) proposed a comprehensive model of the mechanisms of gLTP. Although this model covers several sequential presynaptic and postsynaptic steps presumably involved in gLTP, one of its basic assumptions, the involvement of SIF cells in ganglionic transmission and therefore in gLTP, is questionable based on previous findings (Hadjiconstantinou et al., 1982; Kondo, 1980; Madariaga-Domich and Taxi, 1986/1987; Matthews, 1989).

5. Postsynaptic mechanisms of gLTP

Postsynaptic contribution to mammalian gLTP was initially proposed by Polosa's group. They worked either in the *in situ* perfused cat stellate ganglia or cat scg, which were stimulated through bipolar electrodes placed in the thoracic or in the cervical sympathetic trunk and recorded by measuring both compound action potentials (CAPs) in the postganglionic nerves or the activity of target organs, heart rate or nictitating membrane (NM) contractions, respectively. To assess the participation of ganglionic neurons in LTP by measuring target responses to testing pulses applied to the postganglionic nerve were also recorded. Polosa and his group drew the opposite conclusion of McAfee's group, since they found that high-frequency stimulation produced an increase in ACh release that lasted only 9 min, in contrast with the enhancement of the amplitude of contractions of the NM, which continued for over 60 min (Morales et al., 1994). These authors also showed that after the stimulus train, there was a marked and prolonged increase in the response to intra-arterial injection of ACh or DMPP that lasted over 60 min. Based on these evidence, Polosa and collaborators suggested that gLTP in the cat scg is due to a mechanism that increases nicotinic receptor sensitivity (Morales et al., 1994). This enhancement of receptor sensitivity could be the result of an increase in the number of receptors, the affinity for ACh, or the ion conductance of individual nicotinic receptors. Further evidence of the role of the postsynapsis in gLTP came from the work of Bachoo et al. (1992a), who demonstrated that the PKC inhibitor 1(5-isoquinolinylsulfonyl)-2 methyl-piperazine (H-7) antagonized gLTP. Based on the data that H-7 depresses the amplitude of miniature endplate potentials (Molgó and Van der Kloot, 1991) whereas has no effect on K^+-evoked ACh release from synaptosomes (Guitart et al., 1990) they proposed that H-7 acts postsynaptically (Bachoo et al., 1992a). Since Bachoo et al. (1992b) and Bachoo and Polosa (1991) found that cat sympathetic ganglia express heterosynaptic gLTP, they discard the exclusive contribution of presynaptic mechanisms in gLTP. Based on these findings, Polosa and his group suggested that the main mechanism of gLTP is an increase in postsynaptic responsiveness.

Additional support for a postsynaptic contribution to gLTP was provided by Kadota and Kadota (2002), who described a structural remodeling of shaft synapses associated with gLTP in the cat scg, that they consider compatible with the pharmacological proposal of postsynaptic mechanisms of Morales et al. (1994). Kumamoto and Kuba (1983) had reported that long-term synaptic potentiation occurs postsynaptically in a Ca^{2+}-dependent manner in bullfrog sympathetic ganglia. Either an orthodromic preganglionic or an antidromic postganglionic tetanus (20 Hz for 5 s) potentiated the amplitude of the fast excitatory postsynaptic potential (EPSP), lasting over 2 h. They detected an increase in quantal size but not in quantal content and suggested three possible mechanisms: (1) a release of an unidentified transmitter that raises the efficacy of the nicotinic action of ACh, (2) Ca^{2+} entering the cell may directly activate biochemical steps, like calmodulin, that are responsible for gLTP, and (3) the subsynaptic membrane may increase its sensitivity to ACh by increasing the number of nicotinic cholinergic receptors or unmasking "silent" receptors. Kumamoto and Kuba (1983) acknowledge that potentiation of fast EPSPs by antidromic tetanus was not observed by Brown and McAfee (1982) in the rat scg, and attributed this discrepancy to a species difference.

6. Approaching the controversy: presynaptic or postsynaptic source of gLTP?

It is remarkable that experimental data may favor both presynaptic and postsynaptic mechanisms responsible for gLTP. It is worth mentioning that the presynaptic results were obtained in the *in vitro* superfused rat sympathetic ganglia, whereas the postsynaptic results were obtained in cat sympathetic ganglia, either *in vivo*, which preserves the natural blood supply, or in perfused ganglia *in situ*, where physiological solutions bathe the ganglionic neurons throughout its own vascularization. The species difference and experimental conditions could explain these disparate results (Alkadhi et al., 2005; Morales et al., 1994). A second and more interesting possibility is that both presynaptic and postsynaptic mechanisms are indeed involved in gLTP. Accordingly, gLTP could result from presynaptic release of a non-cholinergic, non-adrenergic co-transmitter, which binds to specific receptors, changing the efficacy of postsynaptic nicotinic responses.

This trans-synaptic mechanism of gLTP was proposed by Polosa's group. Considering that exogenous peptides produce prolonged facilitation of ganglionic transmission (Kawatani et al., 1985; McKenna et al., 1987), and that prolonged stimulation exhausts presynaptic terminals of some peptides such as neurotensin and leu-enkephalin (Bachoo et al., 1987), Polosa and collaborators wondered whether neuropeptides could act as gLTP inducers. They tested if a prolonged stimulation (2–3 h) can abolish gLTP in the *in situ* cat scg, and found that homosynaptic gLTP disappeared, but heterosynaptic gLTP remained after the prolonged stimulation (Bachoo and Polosa, 1991). Next, these authors characterized the time course of recovery to provide some information

about likely mechanisms involved in this process, they demonstrated that gLTP was recovered to 90% of control values five days after prolonged stimulation, this recovery was prevented by disrupting axonal transport with colchicine, which would be consistent with the involvement of peptides (Bachoo et al., 1992b). Considering that peptides are usually stored in large dense core vesicles (LDCVs; Morales et al., 1993; Thureson-Klein and Klein, 1990), Weldon et al. (1993) explored if prolonged stimulation produces similar changes in LDCVs. They found a similar time course of fade and recovery between gLTP and LDCVs (Weldon et al., 1993). Later, Maher et al. (1994) reported that neurotensin has a time course of fade-recovery similar to gLTP. These finding prompted this group to hypothesize that an inductor (most likely a peptide or a combination of peptides) synthesized in the cell body, transported to the axon endings, and stored in LDCVs is released by the tetanizing train and acts on specific postsynaptic receptors, inducing gLTP.

7. Determining the contribution of calcium, a complementary approach to investigate the mechanisms of gLTP

In both proposals, presynaptic and postsynaptic, calcium plays a crucial role. Initially, Briggs et al. (1985a) demonstrated that gLTP was not observed when Ca^{2+} influx was prevented during the tetanus. Later, Bennett's group explored calcium kinetics in boutons of the rat scg using a calcium indicator dye to monitor changes in calcium levels. They found that the increase in calcium concentration after a long train (200 impulses at 30 Hz) declines with a time constant of 127 ± 34 s (Lin et al., 1998). Accordingly, it is unlikely that ACh release were maintained elevated during gLTP. The increase in calcium would produce a short (2–3 min) enhancement in ACh release, and might initiate other presynaptic Ca^{2+}-dependent mechanisms responsible for the remaining potentiation.

We started to determine the contribution of the different voltage-operated calcium channels (VOCCs) to gLTP in the *in vitro* rat sympathetic ganglia. We found that selective blockers of P/Q-, N-, and L-type calcium channels, synthetic funnel-web spider toxin (sFTX), ω-conotoxin GVIA, and nifedipine, respectively, reduce gLTP, indicating that these calcium channels participate in gLTP (Cifuentes et al., 2004). P/Q- and N-type channels blockers reduced basal transmission, whereas L-type channels blocker did not. L-type channels showed a reduced participation only when basal transmission was partially blocked with hexamethonium. The marginal participation of L-type channel in basal transmission agrees with Gonzalez-Burgos' data showing that these channels are responsible for just 14% of basal ganglionic transmission (Gonzalez-Burgos et al., 1995). Therefore, we postulate that like in chromaffin cells where the stimulus train recruits L-type calcium channels to improve the stimulus-secretion efficiency (Polo-Parada et al., 2006), in the scg the stimulus train would recruit L-type calcium channels to participate in gLTP.

Both our results and those of Lin et al. (Cifuentes et al., 2004; Lin et al., 1998) challenge the hypothesis that an increase in ACh release is solely responsible for gLTP. If the enhancement in ACh release were the unique responsible for gLTP, the increase in presynaptic calcium concentration responsible for that enhancement would last for most of the gLTP duration. Analogously, the blockade of L-type calcium channels, which has a marginal contribution to basal transmission (ACh mediated), would have a similar marginal effect on gLTP. It is clear that in addition to the initial increase in ACh release, calcium participates in other processes, such as the release of cotransmitters from LDCV. Calcium could also trigger postsynaptic calcium-activated second messenger-dependent mechanisms that lead to an increase in postsynaptic responses. Considering that L-type calcium channels are generally responsible for the release of co-transmitters, such as neuropeptides, from LDCVs during high-frequency stimulation (Agoston and Lisziewicz, 1989; Maher et al., 1994; Pécot-Dechavassine and Brouard, 1997; Polo-Parada et al., 2006; Zetina et al., 1999), it is probable that L-type calcium channels contribute to gLTP by evoking the release of a gLTP inducer during the conditioning train.

Calcium-induced calcium release (CICR; Berridge, 1993; Verkhratsky and Shmigol, 1996) is another cellular process likely involved in gLTP. Intracellular Ca^{2+} release channels, ryanodine receptors (RyR) and inositol trisphosphate receptors (IP3R), and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) likely participate in gLTP. In fact, it has been shown that a CICR mechanism contributes to gLTP in bullfrog ganglia (Cong et al., 2004; Peng, 1996). To determine whether a similar mechanism participates in rat gLTP, we explored if pharmacological inhibition of CICR process affects gLTP. We found that over-activation or blockade of RyRs with caffeine and dantrolene, blocking IP3R with Xestospongin C, or blocking SERCA with thapsigargin impaired gLTP (Vargas et al., 2007). Considering that CICR process can occur either in the presynapsis or in the postsynapsis, we decided to differentiate between presynaptic and postsynaptic CICR contribution to gLTP (Vargas et al., 2011). We applied heparin, a membrane-impermeant IP3R blocker, to preganglionic or postganglionic nerve trunks (or simultaneously in both), supposing that given its impermeant nature it will diffuse from the cut ending nerves to the presynapsis or to the postsynapsis. We found that both preganglionic and postganglionic blockade of IP3 receptors impairs gLTP in the same degree. Accordingly, we concluded that calcium release from presynaptic and postsynaptic IP3-sensitive reservoirs contribute almost equally to gLTP (Vargas et al., 2011).

8. End of the "presynaptic *vs.* postsynaptic" controversy? A trans-synaptic model of gLTP

Since the initial studies on gLTP, a controversy has persisted regarding the differential contributions of presynaptic and postsynaptic mechanisms. As we discussed in Sections 3–5, convincing evidence support both possibilities. Briggs and Alkadhi support the hypothesis of a presynaptic origin of gLTP, with almost no postsynaptic participation (Alkadhi et al., 2005; Briggs et al., 1985a,b; Briggs, 1995). On the contrary, Polosa's group (Bachoo et al., 1992b; Bachoo and Polosa, 1991; Morales et al., 1994; Weldon et al., 1993) supports a postsynaptic contribution to gLTP, and proposed a trans-synaptic model.

By extending our study of gLTP mechanisms in the *in vitro* preparation of rat scg (the same used by Briggs and co-workers) we found evidence that support the idea of a trans-synaptic process. We explored whether there is a reduction of gLTP after a prolonged stimulation. We found that, similar to cat scg *in situ*, the application of a preganglionic high-frequency train (40 Hz) for 20 min almost abolishes the capability of the *in vitro* rat scg to express gLTP (Fig. 1). These results agree with preliminary data of Wu et al. (1989), and with Weinreich et al. (1995) who reported that prolonged presynaptic stimulation blocked gLTP in guinea pigs. Previously, we had found that a 1-min train (40 Hz) reduces the content of LDCVs by 41% (Cifuentes et al., 2008), so we postulate that a 20-min stimulation in rat scg would deplete LDCVs, similar to the cat scg (Weldon et al., 1993). Our data showed that reduction in gLTP and in the population of LDCVs were both induced by prolonged high-frequency stimulation. In addition, results showing a similar contribution of presynaptic and postsynaptic intracellular calcium reservoirs to gLTP (Section 7) prompted us to propose the following trans-synaptic model of the mechanisms underlying gLTP.

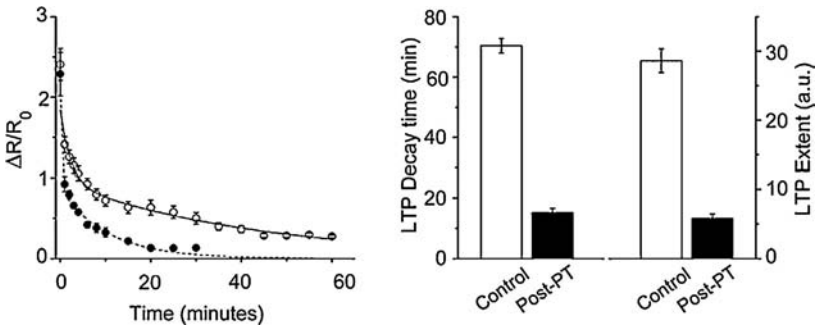

Fig. 1. Use- and frequency-dependent reduction of gLTP of the *in vitro* rat scg. The application of a preganglionic prolonged high-frequency train of stimuli (40 Hz, 20 min) significantly reduced the capability of the ganglion to express gLTP. (A) Time course of gLTP prior (○) and 90 min after (●) the application of a 20 min, 40 Hz train. (B) Bar graphs show the values (mean ± SEM) of LTP decay time (time when the potentiation falls 20% above basal value) and LTP extent (area under the curve from time 0 to decay time) from control ganglia and from ganglia stimulated with a prolonged high-frequency train.

8.1. Model of a trans-synaptic process involving cotransmitter release to explain gLTP

In sympathetic ganglionic synapses, the application of a presynaptic conditioning train (20–40 Hz, 3–30 s) provokes the activation of P/Q- and N-type calcium channels located in the presynaptic active zone near the site of exocytosis of small clear vesicles, and the recruitment of presynaptic L-type calcium channels (Polo-Parada et al., 2006) present far from the active zone. Calcium influx through P/Q and N-type calcium channels produces a transient

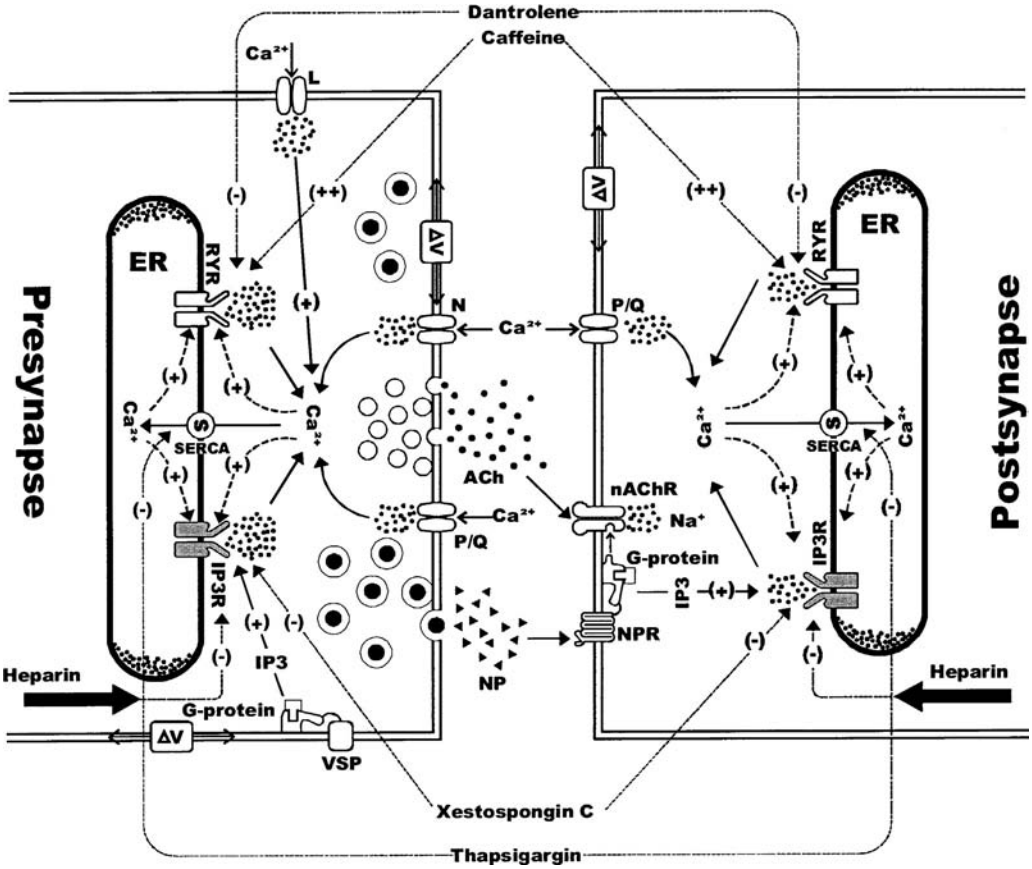

Fig. 2. A proposed model of trans-synaptic mechanisms to sustain gLTP. Presynaptically, calcium influx through P/Q- and N-type calcium channels evokes acetylcholine (ACh) release from small clear vesicles. The train also opens L-type calcium channels that contribute to the increase of presynaptic cytoplasmic calcium concentration, inducing the release of neuropeptides (NPs) from large dense core vesicles (LDCVs) that act as LTP inductors (Cifuentes et al., 2004). Concomitantly, the calcium-induced calcium release (CICR) process also potentiates the rise in cytoplasmic calcium levels (Vargas et al., 2007), thus significantly reinforcing LDCV exocytosis. Presynaptically, it is possible that IP3 is produced either by activation of a voltage-sensing protein (VSP) coupled to a G-protein (Ryglewski et al., 2007) or by the activation of presynaptic metabotropic G-protein-coupled receptors (Boehm and Kubista, 2002). Presynaptic IP3 activates even more the calcium release from intracellular stores. At the postsynapsis, ACh binds nicotinic receptors (nAChR) causing a membrane depolarization that allows calcium entry through voltage-operated calcium channels. In addition, the binding of the putative LTP-inducing neuropeptide to a G-protein coupled receptor (NPR) produces IP3 *via* phospholipase C. In this way, postsynaptic cytoplasmic calcium increases from both extracellular and intracellular sources, and through activation of second messenger pathways (protein kinase C, or calcium calmodulin kinase II) enhances the efficacy of nicotinic receptors responsible for sustained LTP. The effect of heparin, which decreases LTP by diminishing the CICR process through both presynaptic and postsynaptic blockade of IP3R, is also shown.

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(2–5 min) enhancement of ACh release, reflected in a brief potentiation of the CAPs. The activation of these calcium channels initiates the CICR process. Calcium entry through L-type calcium channels, in addition to induce extrasynaptic release of cotransmitters from LDCVs, reinforces the CICR process, which potentiates the rise in cytoplasmic calcium levels and significantly enhances LDCV exocytosis. Additionally, the production of presynaptic IP3 during train stimulation induces even more calcium release from intracellular stores. This increase in intracellular calcium concentration would release more ACh, which triggers a larger postsynaptic membrane depolarization. This postsynaptic depolarization allows more influx of calcium through postsynaptic N- and L-type calcium channels. In parallel, cotransmitters released from LDCVs bind to their postsynaptic G-protein coupled receptors, and *via* phospholipase C produce IP3 that releases Ca^{2+} *via* IP3R. Finally, superimposed onto the potentiation caused by the initial increase in ACh, the postsynaptic increase in [Ca^{2+}] resulting from both extracellular and intracellular sources activates second messenger pathways, such as PKC (Bachoo et al., 1992a) or calcium calmodulin kinase II (Alzoubi and Alkadhi, 2009). These second messengers produce a long-lasting increase in the efficacy of the nicotinic receptors, which sustains the enduring enhancement of the compound action potentials that characterize gLTP (Fig. 2).

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