

計畫編號：NHRI-EX96-9605NI

國家衛生研究院整合性醫藥衛生科技研究計畫

中樞神經系統在肥胖型高血壓所扮演的角色：
prolactin-releasing peptide 與 leptin

計畫名稱

96年度成果報告

執行機構：台北醫學大學

計畫主持人：黃玲玲 副教授

本年度執行期間 96 年 1 月 1 日 至 96 年 12 月 31 日

＊＊本研究報告僅供參考用，不代表本院意見＊＊

壹、96年度計畫研究成果摘要

計畫名稱：中樞神經系統在肥胖型高血壓所扮演的角色：prolactin-releasing peptide 與 leptin

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研究人員：黃尚誠、戴妤玟

關鍵字：交感神經系統、orexin、patch clamp、腦幹、下視丘

成果分類：☐ 癌症基礎與臨床研究(可複選，最多三項)

☐ 分子與基因醫學研究

☐ 臨床研究

☐ 生物技術與藥物研究

☐ 生物統計與生物資訊研究

☐ 醫療保健政策研究

☐ 環境衛生與職業醫學研究

☐ 醫學工程研究

☐ 老年醫學研究

☐ 精神醫學與藥物濫用研究

☐ 疫苗研究

☐ 幹細胞研究

☐ 奈米醫學研究

☒ 其他重要疾病或醫藥衛生問題研究

(1) 中文摘要

高血壓及其相關疾病是國人主要死因之一，根據流行病學資料顯示肥胖與高血壓之發生有密切關連性，再加上其他研究證據，我們提出之假說為，體內存在某些調節能量衡定之內生性分子同時具備調節心血管系統之能力，並且可能成為肥胖與高血壓發生之共同因子。我們在過去幾年的研究中，發現 cocain- and amphetamine-regulated transcript (CART), leptin, prolactin-releasing peptide (PrRP) 與 orexins 符合我們的假說，同時具備調控這兩種生理功能系統之能力，並證實他們可能透過對自主神經活性之作用，而調節血壓。在本計畫中，我們希望達成三項目標，分別為(1)以免疫組織染色法確認這些分子(特別是 leptin 和 PrRP)在腦幹各自主神經核區之可能作用位置，(2)以電生理學方法探討這些分子在腦幹各自主神經核區之作用及作用機轉。(3)分析這些分子在高血壓動物模式之生物活性及其與血壓間之關係。

在本年度中我們繼續前一計畫之研究，檢測 leptin 受器在腦幹各自主神經核區之分佈情形，以評估 leptin 之可能作用位置，並測試 leptin、PrRP 與 orexins 在腦幹自主神經核區之作用，同時測量 PrRP 及 CART 在自發性高血壓大鼠之生物活性。

***leptin* 受器在腦幹自主神經核區之分佈**

Leptin 是脂肪組織分泌的蛋白質荷爾蒙，經血液循環作用於中樞神經系統(主要作用位置為下視丘)，以負向回餽調節方式降低體脂肪儲存量。許多研究證據顯示，在肥胖高血壓個體，血中 leptin 濃度上升可能參與活化交感神經系統。在神經系統 ObR 主要分佈在下視丘以調節能量恆定，除此之外，在腦幹數個與心血管功能有關之自主神經核區亦發現其蹤跡，這些核區包括 dorsal raphe nucleus, NTS 與 dorsal motor nucleus of the vagus (DMNV)。因 leptin 可能對高血壓個體之上升的交感神經活性有所貢獻，因此我們利用組織免疫染色法再仔細檢視 ObR 在大鼠腦幹中數個參與調節自主神經活性及感壓反射之核區的分佈情形，這些核區包括 caudal ventrolateral medulla (CVLM)、rostral ventrolateral medulla (RVLM)與 NTS。在上一計畫之研究中，我們測得 ObR 存在於 CVLM 與 RVLM 核區。並於今年利用雙重染色方式進一步確認 ObR 存在於 CVLM 與 RVLM 核區內之 A1 與 C1 細胞上，這類神經細胞以 catecholamines 作為神經傳導物質，並早已被證實對心血管功能之衡定具有重要調節功能。這些結果提供解剖證據支持 leptin 可能因具備調節 A1 與 C1 細胞活性的能力而調控心血管功能，因此，我們也進一步以電生理學方法測試 leptin 對這類神經細胞之作用，將於下一段中討論。上述結果曾以海報方式發表於 IBRO World Congress of Neuroscience, 2007。

Leptin、*PrRP* 與 *orexins* 對 *RVLM* 神經細胞之影響

Prolactin-releasing peptides (PrRPs)被發現為位於下視丘的一類 neuropeptides，並被證實為 prolactin-releasing factors，且具有降低食慾功能。進一步研究發現 PrRP20 與 PrRP31 兩種具生物活性之 PrRPs 也具升高血壓作用。研究人類基因 PrRPs 的接受器 GPR10 polymorphisms，發現與血壓有關聯性。這些發現暗示 PrRPs 與血壓調控之可能關係。另外，*orexin A* and *orexin B* 是一類由下視丘神經細胞所合成之 neuropeptides，初期發現這類 neuropeptides 與調節進食有關。本計畫主持人與前工作夥伴曾因發現其影響中樞對血壓之調控能力。組織免疫染色亦證實含有 *orexins* 之神經纖維出現於 *RVLM*，根據這些功能性及結構性證據，因此 *RVLM* 可能是 *orexins* 調節心血管功能之中樞作用位置之一。*RVLM* 核區為維持基礎血壓最重要之腦區，它所發出的興奮性訊息維持了心血管交感神經基礎活性。

我們使用腦幹組織切片進行 *RVLM* 神經細胞活性記錄，測試 *Leptin*、*PrRP* 與 *orexins* 對 *RVLM* 神經細胞之膜電位的影響，並未測得 *Leptin* 與 *PrRP* 對膜電位之任何影響，但測得 *orexins* 對 *RVLM* 神經細胞之興奮作用。*orexins* 使 *RVLM* 神經細胞去極化，有時並引發動作電位，*orexins* 的興奮作用具有濃度依賴性，並且不受河豚毒素阻斷。河豚毒素可阻擾動作電位產生，因而能阻斷因動作電位而發生之突觸訊息傳遞，因此常用來區分作用劑對神

經細胞活性之影響來自直接作用於該細胞，亦或來自其他細胞透過突觸訊息傳遞方式而影響神經細胞之活性。因此 orexins 能直接作用於 RVLM 神經細胞而使之興奮性增加。除此之外，我們也探討究竟是何種 orexin 受器參與 orexin 對 RVLM 神經細胞之作用，初步結果顯示 orexin 1 受器與 orexin 2 受器可能都參與此反應。我們仍持續研究 orexins 在 RVLM 之興奮作用的詳細機轉。

高血壓鼠之血漿及腦脊髓液中 CART 與 PrRP 之濃度變化

PrRP 已於上一段落中介紹過，CART 是於急性投與 Cocaine 與 amphetamine 後，表現於 striatum 的一類 neuropeptides，CART 具有降低食慾功能，我們在上一計畫執行過程中已發表了 CART 尚可調控血壓及交感神經活性。CART 與 PrRP 都能透過中樞自主神經調節心血管功能。為了探討它們是否參與高血壓之病理變化，我們已在上一計畫中確認這兩類分子的濃度在肥胖鼠之血漿及腦脊髓液中是上升的，卻在高血壓患者血液中的濃度是下降的。由於肥胖鼠之血壓僅偏高，並未達高血壓程度，因此，我們進一步檢測這兩類分子在自發性高血壓鼠體內之變化情形，結果發現相較於正常血壓鼠這兩類分子在自發性高血壓鼠之血漿及腦脊髓液中的濃度並無有意義差異。部份結果曾發表於 IBRO World Congress of Neuroscience 之 2007 年會。

其他肥胖相關之生理功能變化-DIO 小鼠之學習行為變化

除了上述有關中樞自主神經活性對心血管調節機制之探討外，我們亦利用本計畫所建立高脂飲食誘發之肥胖鼠進行其他與肥胖相關之生理功能變化之研究，在上一計畫中，我們報告了雄性 DIO 小鼠對 step-down passive avoidance test 與 conditioned contextual fear 的學習行為能力較正常體重之對照組為低，同時雄性 DIO 小鼠海馬迴標本之 LTP 及 LTD 現象較對照組小鼠海馬迴標本差，但是這差異並未發生於母鼠。今年，持續的研究中，我們完成四組實驗小鼠之腦部組織收集，並進行組織蛋白質萃取及 2-DE proteomic 分析，確認了 12 個表現量有顯著差異的蛋白質，這些蛋白質與能量衡定及突觸功能有密切關係，同時也與多種神經退化性疾病有關，部份結果曾發表於 37th Annual Meeting, Society for Neuroscience。這一部分研究與多個實驗室合作，包括臺灣大學邱麗珠教授與梁庚辰教授，以及國家衛生研究院何英剛副院長與陳炯東研究員。

(2) 英文摘要

Hypertension and its related diseases have been one of the leading causes of death in Taiwan. As obesity and hypertension are closely correlated, our main hypothesis is that the neuronal signaling molecules of energy homeostasis link the pathogenic mechanisms of obesity and hypertension through their actions on the autonomic nervous system. Therefore, the aim of our study is to evaluate the effects of these molecules in central autonomic regulation of cardiovascular functions. We had demonstrated, in the previous project, that several of these molecules are involved in the central regulation of cardiovascular functions, including cocaine- and amphetamine-regulated transcript (CART), leptin, prolactin-releasing peptide (PrRP) and orexins. In the present project, we proposed to accomplish three aims, which are (1) to locate target sites of the molecules, especially PrRP and leptin, in the brainstem autonomic nuclei; (2) to examine the cellular mechanisms of the molecules in neurons of the rat brainstem autonomic nuclei; and (3) to evaluate the bioactivities of the molecules in hypertension models of rats.

In the first year of the project, we have examined the location of leptin receptors in the brainstem autonomic nuclei, the effects of leptin, PrRP and orexins in the neuronal activity of brainstem autonomic nuclei, as well as the bioactivities of PrRP and CART in the plasma and cerebrospinal fluid (CSF) of spontaneous hypertensive rats (SHR). We have the following accomplishments.

Distribution of leptin receptors, ObR, in autonomic nuclei of the rat brainstem

Leptin is a negative feedback signal of body adiposity regulation through its actions in the hypothalamus. Evidences support that leptin is one of the important factors that contribute to over-activation of sympathetic nervous system in obese hypertensive subjects. Leptin receptors (ObR)-immunoreactivity has been detected in several brainstem nuclei relevant

to the autonomic homeostasis, which include the dorsal raphe nucleus, nuclei tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMNV). Since the contribution of leptin in elevating the sympathetic activity in obese hypertensive subjects was suggested, we re-examined the distribution of ObR-immunoreactivity in the brainstem nuclei involving in baroreceptor reflex circuitry, which include the caudal and rostral ventrolateral medulla (CVLM and RVLM) and NTS, of rats. In our previous project, we had detected ObR-immunoreactivity in the RVLM and CVLM. In this year, by using immunohistochemical double-staining approaches, we detected co-localization of ObR- and phenylethanolamine N-methyltransferase (PNMT)-immunoreactivity in the A1 and C1 areas of the CVLM and RVLM, respectively. These findings provide anatomical evidences for the catecholaminergic neurons in CVLM and RVLM as a potential target site of leptin in the regulation of cardiovascular functions. These results had been published as a conference abstract entitled "Distribution of leptin receptors in the brainstem nuclei involving in baroreceptor reflex circuitry" in IBRO World Congress of Neuroscience, 2007.

Effects of leptin, PrRP and orexins in the RVLM

Prolactin-releasing peptides (PrRPs) were originally isolated from hypothalamus as ligands of an orphan G protein-coupled receptor, GPR10 (human)/UHR (rat), and were functionally identified as prolactin-releasing factors. Soon after, Gatherine and colleagues reported catabolic actions of PrRP by reducing food intake and producing hyperthermia. Further studies revealed the involvement of PrRPs in cardiovascular homeostasis. PrRP administered intracerebroventricularly were found to increase mean arterial blood pressure in conscious rats. A family of hypothalamic neuropeptides, orexin A and orexin B,

was discovered to be associated with feeding behaviors. We have reported that intracisternal administration of orexin A or B increased arterial blood pressure and heart rate in the rat and a potential relationship between obese and hypertention statuses was suggested. In the brainstem, orexin A-like immunoreactive nerve fibers scattered in the RVLM. Therefore, orexins may act on neurons in the RVLM and in turn regulate the cardiovascular functions.

Cellular effects of leptin, PrRP and orexins in the neuronal activity of RVLM had been tested with whole-cell patch clamp recording in the brainstem slices. Very little or non-detectable effects of leptin and PrRP in the resting membrane potential of RVLM neurons was observed. Both orexin A and orexin B caused membrane depolarization and increased firing frequency on RVLM neurons in a concentration-dependent manner. The orexins-induced depolarization persisted in the presence of tetrodotoxin, which, by blocking the action potential-dependent synaptic transmission, diminishes the indirect effects from neurons synaptically affecting the recording neurons. This result indicates that orexins-induced depolarization is a direct effect of orexins acting on the recorded RVLM neurons. We also try to elucidate the receptor types involved in orexin-induced depolarization and found that both orexin 1 and orexin 2 receptors might be involved. We are still working on the detail mechanisms of orexins-induced excitatory effect on RVLM neurons.

Levels of PrRP and CART in the plasma and cerebrospinal fluid (CSF) of hypertensive rats

Cocaine- and amphetamine-regulated transcript (CART) was first discovered in the striatum of rats as an mRNA up-regulated by acute administration of cocaine or amphetamine (Douglass et al., 1995). CART

peptides have received much attention as regulators of feeding behavior (Kristensen et al., 1998). We have demonstrated cardiovascular pressor effects of CART peptides applied centrally in rats (Hwang et al., 2004).

To find out whether CART or PrRP are involved in the pathogenesis of hypertension, in the previous project, we examined the levels of CART and PrRP in high-fat diet-induced obese (DIO) rats and in hypertensive human subjects. We found a higher level of these peptides in the CSF and plasma of DIO rats and a lower plasma level of PrRP in hypertensive human subjects as compared to their counterparts. In the first year of this continuous project, we compared the plasma and CSF levels of CART and PrRP in spontaneous hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. However, there is no difference in the CSF and plasma levels of CART and PrRP between the hypertensive and normotensive rats. Part of the results had been published as a conference abstract as "Li, T.-L., Chiu, C.-Z., Wu, F. H. and Hwang L.-L. (2007) Altered levels of PrRP and CART in CSF and plasma of diet-induced obese rats" in IBRO World Congress of Neuroscience, 2007.

Other obesity-related physiological modifications in DIO animals

In addition to the studies regarding the central regulation of cardiovascular functions, we have investigated other obesity-related physiological alterations in DIO animals. In the previous project, we have reported an impaired learning performance of step-down passive avoidance and contextual fear conditioning and a poorer long-term potentiation and long-term depression in hippocampal Schaffer collateral-CA1 synapses in DIO male, but not female, mice. In this year, we performed proteomic assays and compared the expression levels of proteins in the brain tissues from the four groups of animals.

We identified 12 proteins in the mice hippocampus using 2-DE proteomics, but Western blot analysis has yet to confirm these alterations, possibly due to post-translational modification. The significantly altered proteins were found to be associated with energy metabolism and synaptic plasticity and involved in multiple neurodegenerative diseases. Our results suggest that high-fat diet feeding may alter processes of energy metabolism and synaptic plasticity in hippocampus to decline cognitive function via multiple enzymes. These results have been presented as a conference abstract in 37th Annual Meeting, Society for Neuroscience as "Lin, L-C, Hwang, L-L, Lee, H-J, Chen, C-T, Li, T-L, Ho, I-K, Chiou, L-C (2007) Proteomic study of the hippocampus in high-fat diet-induced obese mice". This study is in collaboration with Dr. Chiou, Lih-Chu and Dr. Liang, Keng-Chen at the National Taiwan University, and Dr. Ho, Ing-Kang and Dr. Chen, Chiung-Tong at the NHRI.

貳、96年度計畫著作一覽表

Journal

序號	計畫產出名稱	產出型式	Impact factor	致謝對象
1	Li, T.-L., Chiou, L.-C., Lin, Y. S., Hsieh, J.-R. and Hwang, L.-L. Electrophysiological study on the effects of leptin in rat dorsal motor nucleus of the vagus.. Am J Physiol Regul Integr Comp Physiol 2007;292: R2136-R2143. Supported by NHRI95-9325NC (SCI)	Foreign	3.685	NHRI
2	*Hwang, L.-L., Wang, C.-H., Chen, C.-T., Li, T.-L., Chen, C.-P., Chang, S.-D., Liang, K.-C. and Chiou, L.-C. Impairment of hippocampal synaptic plasticity and learning memory performance in high-fat diet-induced obese male, but not female, mice.. 2007; in Preparation	Foreign		
3	Chiung-Zuan Chiu, Li, TL, Wu, FH, and **Hwang, L.L. Plasma Levels of PrRP-like and CART peptides-like Immunoreactivity in Hypertensive and Normotensive Human Subjects and Rats. 2007; in Preparation	Foreign		

Patent

序號	計畫產出名稱
	無

Book

序號	計畫產出名稱
	無

Conference Paper

序號	計畫產出名稱
1	Hsieh, M.-C., Hwang, L.-L., Shieh, K.-R. (2007) The

	circadian related genes expression on peripheral tissues in high fat diet-induced obesity mice. 22th Annual Meeting of Joint Biomedical Sciences, Taipei, Taiwan, Republic of China, March 17-18. (page164, P248) 2007
2	Li, T.-L., Li, Y.-H., Huang, S.-C. and Hwang L.-L. (2007) Distribution of leptin receptors in the brainstem nuclei involving in baroreceptor reflex circuitry. IBRO World Congress of Neuroscience, July 12-17, Melbourne Australia. 2007
3	Li, T.-L., Chiu, C.-Z., Wu, F. H. and Hwang L.-L. (2007) Altered levels of PrRP and CART in CSF and plasma of diet-induced obese rats. IBRO World Congress of Neuroscience, July 12-17, Melbourne Australia. 2007
4	Lin, L-C, Hwang, L-L, Lee, H-J, Chen, C-T, Li, T-L, Ho, I-K, Chiou, L-C (2007) Proteomic study of the hippocampus in high-fat diet-induced obese mice. 37th Annual Meeting, Society for Neuroscience, San Diego, November 3 to 7. 2007

Technical Report

序號	計畫產出名稱
	無

參、96年度計畫重要研究成果產出統計表

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
(係指執行96年度計畫之所有研究產出結果)

科技論文篇數			技術移轉			技術報告 1 項		
發表地點 類 型	國 內	國 外	類 型	經 費	項 數	技術創新 1 項		
期 刊 論 文	0 篇	1 篇	技 術 輸 入	100 千元	0 項	技術服務 1 項		
研討會 論 文	1 篇	3 篇	技 術 輸 出	100 千元	0 項	專利權	國內	1 項
							國外	1 項
專 著	0 篇	0 篇	技 術 擴 散	100 千元	0 項	著作權	國內	1 項
							國外	1 項

[註]：

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部份，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內。

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者。

專 著：為對某項學術進行專門性探討之純學術性作品。

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者。

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散。

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者。

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種。

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）。

技術創新：指研究執行中產生的技術，且有詳實技術資料文件者。

技術服務：凡有關各項研究計畫之規劃與評審、技術督察與指導及專業技術服務事項等。

肆、96年度計畫重要研究成果

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

計畫之新發現、新發明或對學術界、產業界具衝擊性(impact)之研究成果，請依性質勾選下列項目。

- ☐ 1. 研發或改良國人重要疾病及癌症的早期診斷方式及治療技術
- ☐ 2. 發展新的臨床治療方式
- ☐ 3. 發展新生物製劑、篩檢試劑及新藥品
- ☐ 4. 瞭解常見疾病及癌症之分子遺傳機轉
- ☐ 5. 瞭解抗癌藥劑對癌細胞之作用機制
- ☐ 6. 提供有效的疾病預防策略
- ☐ 7. 利用生物統計與生物資訊研究，推動台灣生技醫藥研究，促進生物技術與基因體醫學之發展
- ☐ 8. 醫療保健政策相關研究
- ☐ 9. 瞭解環境毒理機制及重金屬對人體健康的影響
- ☐ 10. 研發適合臨床使用的人造器官及生醫材料
- ☐ 11. 縮短復健流程並增加復健效果的醫療輔助方式或器材之研究應用
- ☐ 12. 改進現有醫療器材的功能或增加檢驗影像的解析能力
- ☒ 13. 其他重要疾病或醫藥衛生問題研究

中樞神經對血壓之調節

- 一、計畫之新發現、新發明或對學術界、產業界具衝擊性 (impact) 之研究成果，請敘述其執行情形。

此計畫之年度研究成果，提供學術界(1)Leptin receptor 分佈於腦幹自主神經核區，並確認分佈於 A1 與 C1 區域之 catecholaminergic neurons 上。(2)測試 Leptin 與 PrRP 對 rostral ventrolateral medulla (RVLM)核區內神經細胞之活性，並未測得明顯作用。(3)自發性高血壓鼠與正常血壓鼠血漿中 CART 及 PrRP 濃度並無差異。(4)Orexins 影響 RVLM 核區活性，機轉仍在探討中。

- 二、計畫對民眾具教育宣導之研究成果 (此部份將為規劃對一般民眾教育或宣導研究成果之依據，請以淺顯易懂之文字簡述研究成果，內容以不超過 300 字為原則)

體重過重或肥胖嚴重威脅人體健康，它增加許多慢性疾病的罹患率，包括本研究探討的高血壓，根據此研究計畫的成果，我們發現許多與體重調節(或能量恆定)有關的機制也同時參與調節其他重要生理功能，因此當能量恆定失去平衡至使過重或肥胖發生時，許多重要生理功能也同時逐漸失衡，例如我們在此研究中所發現的血壓上升、交感神經活性上升以及學習能力的減退等，因此除了藉生物醫學研究努力了解其詳細機轉外，希望也能讓民眾了解肥胖的確會使身體的許多功能失常，最後導致嚴重疾病，並希望提醒大家改變飲食及生活習慣來防範未然。

- 三、簡述年度計畫成果之討論與結論，如有技術移轉、技術推廣或業界合作，請概述情形及成效

本年度計畫之主要成果及討論與結論包括

- 1、Leptin 受器分佈於腦幹 A1 與 C1 腦區之 catecholamine 神經細胞
A1 與 C1 腦區之神經細胞以 catecholamines 作為神經傳導物質，

並早已被證實對心血管功能之衡定具有重要調節功能。這項結果提供解剖證據支持 leptin 可能因具備調節 A1 與 C1 細胞活性的能力而調控心血管功能

2、Orexins 對 RVLM 神經細胞之影響

RVLM 核區為維持基礎血壓最重要之腦區，它所發出的興奮性訊息維持了心血管交感神經基礎活性，orexin 能直接興奮 RVLM 神經細胞，可因此推論 orexin 可藉由對 RVLM 神經細胞的作用調節心血管系統之交感神經活性，進而影響血壓。

3、高血壓鼠血漿中 CART 與 PrRPs 濃度變化

許多證據證實 CART 與 PrRPs 與血壓調控之關係，但其是否參與高血壓發生之生理或病理變化仍然未知。我們已證實高血壓患者之血漿 PrRPs 濃度異於對照組，暗示其可能之生理或病理角色，然而高血壓鼠血漿中 CART 與 PrRPs 濃度並未如高血壓患者般變化，因此我們仍需進一步研究才能作出正確結論。

4、DIO 小鼠之學習能力

高血壓與肥胖除了是心血管疾病的重要危險因素外，也有研究指出它們也會對認知及學習行為產生負面影響，根據我們這數年來的實驗結果顯示雄性 DIO 小鼠的學習能力較對照組小鼠差，在海馬迴的神經電生理研究也有相同的結果，此現象在母鼠較不明顯。今年起 proteomic 分析結果，提供我們許多訊息，可作為深入探討這些現象之分子機轉之基礎。

四、成效評估（技術面、經濟面、社會面、整合綜效）

本年度研究成果讓我們更清楚瞭解這些內生性分子參與調控心血管功能之作用機轉。近年來肥胖的盛行率快速攀升，肥胖又易導致許多

慢性病，使得與肥胖相關的疾病將是未來公共健康重要課題。肥胖所造成的社會負擔是相當重的，本計畫旨在研究肥胖相關的生理及病理變化之機轉，期能提供生物醫學界更加瞭解肥胖衍生的疾病背後的詳細機制，以發展有效的防治與治療方法，則將有助於國人甚至全人類的健康。

五、 下年度工作構想及重點之妥適性

下年度工作將承續今年度的進展，主要包括

- 5、 持續測試 leptin 及 PrRP 在腦幹自主神經核區之作用。
- 6、 繼續研究 orexins 在腦幹 RVLM 核區之作用機轉。
- 7、 利用 track-tracing 神經連繫追蹤技術，探討位於 lateral hypothalamus 之 orexin 神經細胞之上游調控機轉。
- 8、 繼續 proteomic assays，研究 DIO 小鼠之學習行為變化之分子機轉。

六、 檢討與展望

神經電生理學方法是本計畫中主要的研究方法，優點是其為測量神經細胞之功能性活性之直接方法，缺點是這類實驗不但執行上耗時，同時人員之技術與知識訓練過程亦十分耗費時日，另外，受過這類型研究訓練的人員相當稀少，因此人力不足及計畫進展速度緩慢是我們最大的困難。然而，電生理學方法是神經科學領域中不可或缺的一種研究方法，所幸目前有數位研究生有意願投入學習這種耗時費工的研究方法，期待在未來幾年能看到他們的成果。

伍、96年度計畫所培訓之研究人員

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

種 類				人數	備 註
專任人員	1.	博士後 研究人員	訓練中	0	
			已結訓	0	
	2.	碩士級 研究人員	訓練中	1	
			已結訓	0	
	3.	學士級 研究人員	訓練中	0	
			已結訓	0	
	4.	其他	訓練中	0	
			已結訓	0	
兼任人員	1.	博士班 研究生	訓練中	3	含醫師一名
			已結訓	0	
	2.	碩士班 研究生	訓練中	0	
			已結訓	0	
醫 師		訓練中	1		
		已結訓	0		

特殊訓練課程（請於備註欄說明所訓練課程名稱）

陸、參與96年度計畫所有人力之職級分析

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	0 人
第二級	副研究員、副教授、總醫師、助教授	1 人
第三級	助理研究員、講師、住院醫師	2 人
第四級	研究助理、助教、實習醫師	1 人
第五級	技術人員	0 人
第六級	支援人員	0 人
合計		4 人

〔註〕：

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者。

第二級：副研究員、副教授、助研究員、助教授、總醫師、薦任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者。

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士、或學士滿三年以上之研究經驗者。

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士、或專科滿三年以上之研究經驗者。

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者，或專科畢業目前從事研究發展，經驗未滿三年者。

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事、會計、秘書、事務人員及維修、機電人員等。

柒、參與96年度計畫所有人力之學歷分析

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

類別	學 歷 別	參與人次
1	博士	1 人
2	碩士	0 人
3	學士	0 人
4	專科	0 人
5	博士班研究生	3 人
6	碩士班研究生	0 人
7	其他	0 人
合計		4 人

捌、參與96年度計畫所有協同合作之研究室

註：群體/中心計畫者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

機構	研究室名稱	研究室負責人
無		

玖、九十六年度計畫執行情形

一、請簡述原計畫書中，九十六年預計達成之研究內容

根據原計畫書九十六年預計達成之研究內容包括

1. 研究 PrRP、leptin 或其他能量衡定相關分子在腦幹自主神經核區之可能作用位置，例如受器分佈情形。
2. 研究這些具有 PrRP、leptin 或其他能量衡定相關分子受器之神經細胞種類。
3. 研究 PrRP、leptin 或其他能量衡定相關分子對這些具有其受器之核區內神經細胞之影響。
4. 建立數個高血壓鼠模式，以供探討這些分子在高血壓動物模式中之變化情形或作用情形。

根據計畫評審委員的建議及我們的研究成果，我們對研究方針作了一些調整。其一是，因上述第三項之測試結果，發現 PrRP 及 leptin 對我們所研究的多數核區內神經細胞膜電位影響不明顯，而其他分子例如 orexins 則有顯著作用，因此將研究重心移轉至 orexin 系統。其二是，我們在計畫書中提出的高血壓鼠模式其可能病理原因太過複雜，計畫評審委員建議仔細評估衡量，因此我們選用自發性高血壓鼠，因其受研究界關注探討，因此有許多文獻資料可作參考。

二、請詳述九十六年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

九十四年度計畫執行情形如下

1. 完成 leptin 可能作用位置之分析。證實其受器分佈於 CVLM 與 RVLM

之 A1 與 C1 神經細胞上。(及上述第一及第二項)

2. 使用電生理學方法持續探討 orexins 在大鼠 RVLM 核區之作用與機轉。(及上述第三項)

3. 完成自發性高血壓鼠血漿及腦脊髓液中 CART 與 PrRP 之濃度分析

4. 繼續飲食誘發肥胖小鼠之學習能力之機轉探討，成果已發表為研討會論文，並正在準備文稿發表於學術性雜誌。

5. 評估

計畫執行情形達成多數預期目標，唯電生理實驗耗時，進度無法加速，整體而言九十六年度計畫執行情形，其達成率達到原預期目標之 90%。

拾、附錄

無

拾壹、本年度之著作抽印本或手稿

Electrophysiological study on the effects of leptin in rat dorsal motor nucleus of the vagus

Tzu-Ling Li,^{1,2,3} Lih-Chu Chiou,⁴ You Shuei Lin,¹ Jing-Ru Hsieh,² and Ling-Ling Hwang^{1,2,3}

¹Department of Physiology, ²Graduate Institute of Medical Sciences, and ³Graduate Institute of Neuroscience, College of Medicine, Taipei Medical University, Taipei, Taiwan; and ⁴Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan

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Li T-L, Chiou L-C, Lin YS, Hsieh J-R, Hwang L-L. Electrophysiological study on the effects of leptin in rat dorsal motor nucleus of the vagus. *Am J Physiol Regul Integr Comp Physiol* 292: 2136–2143, 2007. First published February 15, 2007; doi:10.1152/ajpregu.00563.2006.—Immunoreactivity of leptin receptor (Ob-R) has been detected in rat dorsal motor nucleus of the vagus (DMNV). Here, we confirmed the presence of Ob-R immunoreactivity on retrograde-labeled parasympathetic preganglionic neurons in the DMNV of neonatal rats. The present study investigated the effects of leptin on DMNV neurons, including parasympathetic preganglionic neurons, by using whole cell patch-clamp recording technique in brain stem slices of neonatal rats. Leptin (30–300 nM) induced membrane depolarization and hyperpolarization, respectively, in 14 and 15 out of 80 DMNV neurons tested. Both leptin-induced inward and outward currents persisted in the presence of TTX, indicating that leptin affected DMNV neurons postsynaptically. The current-voltage (I–V) curve of leptin-induced inward currents is characterized by negative slope conductance and has an average reversal potential of -90 ± 3 mV. The reversal potential of the leptin-induced inward current was shifted to a more positive potential level in a high-potassium medium. These results indicate that a decrease in potassium conductance is likely the main ionic mechanism underlying the leptin-induced depolarization. On the other hand, the I–V curve of leptin-induced outward currents is characterized by positive slope conductance and has an average reversal potential of -88 ± 3 mV, suggesting that an increase in potassium conductance may underlie leptin-induced hyperpolarization. Most of the leptin-responsive DMNV neurons were identified as being parasympathetic preganglionic neurons. These results suggest that the DMNV is one of the central target sites of leptin, and leptin can regulate parasympathetic outflow from the DMNV by directly acting on the parasympathetic preganglionic neurons of the DMNV.

Ob-R; brain slice; potassium channel; whole cell patch-clamp; immunohistochemistry

LEPTIN, A CIRCULATING PROTEIN of 16 kDa secreted primarily from white adipose tissue, is one of the key regulators of energy homeostasis (11, 37). It serves as a negative feedback signal of body adiposity through its actions in the central nervous system (CNS), particularly the hypothalamus, to regulate food intake and energy expenditure. At least 6 leptin receptors isoforms, Ob-R_{a-f} (23, 42, 43), have been identified, and the Ob-R_b isoform is believed to be pivotal in the regulation of energy homeostasis. Intense Ob-R immunoreactivity was detected not only in the hypothalamus but also in other brain regions, including many nuclei in the brain stem (39). Hosoi and coworkers (17) demonstrated that leptin intravenously applied activated Ob-R_b in the nucleus tractus solitarius,

lateral parabrachial nucleus, central gray and dorsal motor nucleus of the vagus (DMNV) of the brain stem, by detecting two biochemical markers of the activation of Ob-R_b, that is, the activation of signal transducers and activators of transcription (STAT3) and the expression of suppressor of cytokine signaling 3 (SOCS3) (17). They, therefore, proposed that the brain stem is a direct target site of leptin in the brain. Several lines of evidence are in agreement with this hypothesis. First, when injected into the fourth ventricle, leptin was found to inhibit gastric emptying in rats (40) and to reverse fasting-induced anestrus in hamsters (36). Further, Grill and colleagues (13) showed that leptin microinjected into the dorsal vagal complex suppressed food intake in rats. A recent report of Williams and Smith (44) demonstrates that leptin directly hyperpolarized NTS neurons, providing direct functional evidence that supports the hypothesis proposed by Hosoi et al. (17).

Several electrophysiological studies have been conducted in the hypothalamus, a primary site of leptin's action to influence energy homeostasis, and demonstrate that leptin acts in several hypothalamic areas by changing neuronal excitabilities (9, 16, 34, 41). However, little electrophysiological study was carried out in the brain stem nuclei, which are considered to be potential action targets of leptin. The DMNV is one of the brain stem nuclei that exhibit Ob-R immunoreactivity (39) and a crucial area involved in the regulation of feeding. It is where the parasympathetic preganglionic neurons are located and provides the major source of parasympathetic efferents to subdiaphragmatic targets (25). The present study, therefore, investigated how leptin affects the neuronal activities of DMNV neurons, including the retrograde-labeled parasympathetic preganglionic neurons, using whole cell patch-clamp recording technique in brain stem slices.

METHODS

Animals. Pregnant Sprague-Dawley (SD) rats were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and delivered to the Animal Center of Taipei Medical University 5 days before the due date. The use of animals was approved by the Institutional Animal Care and Use Committee of the Taipei Medical University. Neonatal rats of either sex were used.

Retrograde labeling of parasympathetic preganglionic neurons. Parasympathetic preganglionic neurons in the DMNV were retrograde-labeled with a systemically applied retrograde tracer, Fluorogold (Fluochrome, Denver, CO), as described in our previous study (20). Briefly, neonatal SD rats received an intraperitoneal injection of Fluorogold of 10 μ g in 0.1 ml saline 2 or 3 days before the rats were

Address for reprint requests and other correspondence: L.-L. Hwang, Dept. of Physiology, College of Medicine, Taipei Medical Univ., 250 Wu-Hsing St., Taipei 110, Taiwan (e-mail: llhwang@tmu.edu.tw).

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killed for the immunohistochemical and electrophysiological experiments.

Double-immunostaining of Ob-R and retrograde-Fluorogold in the DMNV. Three days after Fluorogold was injected, 10-day-old neonatal SD rats were transcardially perfused with cold PBS of 0.1 M followed by 4% paraformaldehyde/PBS. The brain was removed, postfixed in the same fixative for 2 h, and then immersed in 30% sucrose/PBS for 3 days. The brain stem containing the DMNV was coronally sectioned into 30- μ m sections with a cryostat (Cryotome SME, Shandon, Astmoor, UK). Free-floating brain stem sections were processed for Ob-R and Fluorogold immunostaining using the avidin-biotin enzyme complex method (21). Briefly, sections were blocked with 10% normal horse serum and then incubated with goat polyclonal anti-Ob-R IgG (1:100, cat. no. sc-1835; Santa Cruz Biotechnology) for 24 h at room temperature followed by 24 h at 4°C with gentle agitation. After several washes with PBS, the sections were incubated with biotinylated horse anti-goat IgG (1:100, Vector Laboratories, Burlingame, CA) for 2 h followed by incubation with the avidin-biotinylated-alkaline phosphatase complex (1:100, Vector Laboratories) for 1 h at room temperature. After washing, sections were developed with a Vector Blue Kit (Vector Laboratories). The same sections were then processed for Fluorogold immunoreactivity with similar procedures of Ob-R immunostaining except that the following reagents differed: 1) 10% normal goat serum for blocking, 2) rabbit polyclonal Fluorogold antisera (1:3,000, Chemicon International, Temecula, CA) as the primary antibody, 3) biotinylated goat anti-rabbit IgG as the secondary antibody, 4) avidin-biotinylated peroxidase complex for signal amplification, and 5) Vector NovaRED kit for detection. The sections were examined with an upright microscope (Eclipse 80i, Nikon, Japan). The regions of interest were recognized by comparing section images with the atlases in *The Rat Brain in Stereotaxic Coordinates* (31). Cell counts were performed bilaterally using a $\times 20$ objective at a 90- μ m intervals (one-in-three series) from the level 400 μ m caudal to the obex to the level 400 μ m rostral to the obex. The ratio of Ob-R-labeled parasympathetic preganglionic DMNV neurons was calculated for each animal, and an averaged ratio was obtained from three neonatal rats. Immunohistochemical control experiments consisted of preadsorption of the antiserum with an excess of antigen or omission of the primary antiserum. Staining was absent in all of the immunohistochemical control experiments.

Preparation of brain stem slices for electrophysiological experiments. Brain stem slices containing the DMNV were prepared from neonatal SD rats of 8 to 14 days old. After anesthetization with ether, the brain stem was rapidly removed from the animal and placed in a chilled, oxygenated artificial cerebral spinal fluid (ACSF) containing the following compositions (in mM): 127 NaCl, 1.9 KCl, 1.2 KH_2PO_4 , 2.4 CaCl_2 , 1.3 MgCl_2 , 26 NaHCO_3 , and 10 glucose. Three coronal sections of 400 μ m, starting from about 600 μ m caudal to the obex and moving rostrally, were sliced with a vibratome (Vibratome 1000 classic; St. Louis, MO). The brain stem slices were then incubated in an oxygenated ACSF at room temperature for at least 1 h before the start of the recording.

Electrophysiological recording. After a 1-h recovery in oxygenated ACSF, one slice was transferred to a recording chamber and continuously perfused with an oxygenated ACSF at a rate of 1 to 3 ml/min throughout the entire period of the experiment. All reagents were applied in the perfused ACSF. A high-potassium ACSF was prepared by substituting NaCl with KCl for some experiments. All experiments were conducted at room temperature ($21 \pm 1^\circ\text{C}$).

The whole cell patch-clamp recording technique employed was similar to that described previously (18–20). Glass microelectrodes filled with an electrode solution had a resistance of 2 to 5 M Ω . The electrode solution contained (in mM) 125 K⁺ gluconate, 5 KCl, 1 MgCl_2 , 0.4 CaCl_2 , 5 ATP, 0.3 GTP, 2 EGTA, 10 HEPES, 10 sucrose, and in some cases, 0.2% Lucifer yellow, with a pH of 7.2. Signals were recorded with an Axopatch-1D (Axon Instruments, Foster City, CA), low pass filter at 2 kHz, acquired using a personal computer and

pClamp software (ver. 9.0, Axon Instruments) for later analysis. Membrane potentials reported have been corrected for a liquid-junction potential of -8 mV. The access resistance was less than 25 M Ω .

Whole cell patch-clamp recording was performed in both current-clamp and voltage-clamp modes. In the voltage-clamp experiments, the steady-state current-voltage (I–V) relationship was obtained before and during the application of leptin (PeproTech EC, London, UK) by applying a series of 1-s voltage command steps every 5 s from the holding potential of -60 mV to different potentials (-120 to 0 mV) at 10-mV increments in a TTX (0.5–1 μM)-containing ACSF (TTX was purchased from Sigma, St. Louis, MO). The current value was measured at the end of each command step. Currents elicited by such voltage commands in a control medium were subtracted from their counterparts in the presence of leptin to yield steady-state I–V curves of leptin-induced currents.

Identification of parasympathetic preganglionic neurons in recorded neurons. To identify whether the recorded neurons were parasympathetic preganglionic neurons, rats received an intraperitoneal injection of Fluorogold 2 to 3 days before the electrophysiological experiments were carried out (20). During the electrophysiological recordings, the patch electrodes were filled with a solution containing the fluorescent dye, Lucifer yellow (0.2%), which was allowed to diffuse into the recorded neuron, to identify the recorded neuron. At the end of the recordings, the slice was immersed in a solution of 4% paraformaldehyde/PBS overnight, and then transferred to a solution of 30% sucrose/PBS until further processing. The fixed slice was sectioned into 50- μ m sections with a cryostat. Sections were viewed under an upright Nikon 80i epifluorescence microscope and the section containing Lucifer yellow-labeled neuron was selected for immunostaining of Fluorogold, as described previously (20). Briefly, the signal of the retrograded Fluorogold was amplified by immunostaining with rabbit polyclonal Fluorogold antisera (Chemicon International, Temecula, CA) followed by detection with biotinylated goat anti-rabbit IgG and then avidin-Texas red (Vector Laboratories). Fluorescent staining of the Lucifer yellow and Texas red was visualized using the fluorescent microscope.

Data analysis. Data are expressed as the means $\pm$ SE. Statistical analysis of the data was performed using Student's *t*-test or one-factor ANOVA followed by multiple comparisons using the Student-Newman-Keuls test at $P = 0.05$.

RESULTS

Ob-R immunoreactivity in the DMNV of neonatal rats. Previous studies have demonstrated the existence of the Ob-R in the DMNV of adult rats (39). The use of neonatal rats in the present study was partly due to technical limitations of the blind patch-clamp recording in brain stem tissue. High plasma concentration of leptin is present in neonates of rodents and humans (1, 10, 15, 35), implying leptin as being a physiological regulator in newborn lives. To examine whether the Ob-R is present in parasympathetic preganglionic neurons of the DMNV of neonatal rats used in the present study, Fluorogold was administered systemically (ip) to labeled parasympathetic preganglionic neurons of the DMNV. Fluorogold is a retrograde tracer that can be taken up by nerve terminals but does not penetrate the blood-brain barrier. Therefore, systemic application of Fluorogold is a convenient method to label CNS neurons projecting to areas supplied by fenestrated capillaries or to the periphery (2, 24, 26). The distribution of the Ob-R in the DMNV neurons that project to the periphery was examined by the double immunostaining of Ob-R and Fluorogold. The result shows that most of Ob-R immunoreactive cells in the DMNV were Fluorogold-labeled, that is, parasympathetic

preganglionic neurons. However, not all parasympathetic preganglionic neurons exhibited immunoreactivities to the Ob-R (Fig. 1). Ob-R immunoreactivity was detected in $45.6 \pm 5.1\%$ ($n = 3$) of the parasympathetic preganglionic neurons in the DMNV.

Passive membrane properties of DMNV neurons. Stable recordings were made from 80 DMNV neurons in brain stem slices harvested from 58 rats. The mean resting membrane potential, input resistance, and cell capacitance of the recorded DMNV neurons were -52 ± 1 mV, 366 ± 25 M Ω , and 31 ± 2 pF.

Effects of leptin on DMNV neurons. Under the current-clamp recording mode, leptin (30–300 nM) applied in the perfusion solution for 5 min caused membrane depolarization and hyperpolarization in 14 (17.5%) and 15 (17.6%) out of 80 recorded DMNV neurons, respectively (Fig. 2) and had no effect in the rest neurons. Both depolarization and hyperpolarization responses developed quickly and were often accompanied by changes in the activities of neuronal discharges. The passive membrane properties of the DMNV neurons with different responses to leptin are listed in Table 1, and no significant difference was found among the three groups. Three slices were obtained from each animal, and not more than one neuron was recorded in a slice. All responsive neurons of each experiment in the present study were from different animals.

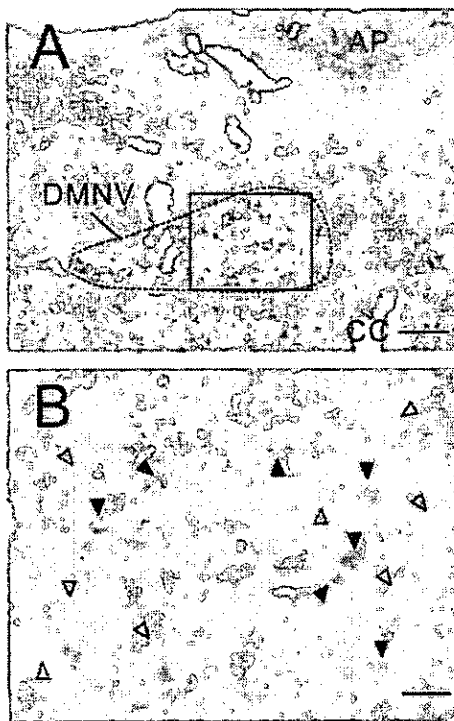


Fig. 1. Presence of Ob-R-immunoreactivity on Fluorogold-labeled parasympathetic preganglionic neurons in the dorsal motor nucleus of the vagus (DMNV). A: A photomicrograph of a brain stem section containing the DMNV neurons double-stained with Fluorogold- and Ob-R-immunoreactivities in red and blue, respectively. B: A higher magnification of the rectangle area outlined in A. Solid arrowheads point to representative double-stained neurons, and open arrowheads indicate neurons stained only with Fluorogold immunoreactivities. AP, area postrema; CC, central canal; DMNV, dorsal motor nucleus of the vagus nerve. Scale bar: 100 μ m in A; 25 μ m in B.

Because the experiments were carried out in brain stem slices obtained from rats with ages ranging from 8 to 14 days old, the effect of animal age on differential responses of DMNV neurons to leptin was analyzed, and no significant effect was found among the three groups.

The concentration of leptin tested was from 10 to 300 nM. The lowest effective concentration of leptin was 30 nM. For a given concentration, the size of the response varied considerably among the different cells tested. However, a concentration-dependent manner of leptin-induced depolarization was observed for individual neurons. An example is shown in Fig. 2, A and B. The depolarization induced by leptin at 30–300 nM ranged from 4 to 16 mV, which could evoke firing activity in a silent neuron (Fig. 2, A and B). The hyperpolarization induced by leptin at 30–300 nM ranged from 4 to 28 mV, which could abolish the spontaneous neuronal activity (Fig. 2D). The duration of leptin-induced hyperpolarization was significantly longer than that of leptin-induced depolarization (Fig. 2). The duration of 90% recovery from leptin (300 nM)-induced responses was 18.3 ± 1.1 ($n = 3$) and 113.0 ± 22.1 ($n = 3$) min for depolarization and hyperpolarization, respectively. The long-lasting hyperpolarization response of leptin renders a repetitive application of leptin in a single neuron to evaluate the concentration-response relationship of leptin-induced hyperpolarization impracticable.

Under voltage-clamp recording mode, the leptin-induced depolarization and hyperpolarization were exhibited as an inward and outward currents, respectively, at a holding potential of -60 mV. TTX (0.5–1 μ M), which eliminates action potentials by blocking voltage-gated sodium channels, was added to the ACSF to block any indirect effects due to the action of leptin on neighboring neurons in the brain stem slice. In the presence of TTX, leptin of 100 nM induced an inward current of 10 ± 3 pA in six tested DMNV neurons and an outward current of 19 ± 8 pA in four tested DMNV neurons. These results indicate that leptin affected neuronal activity of the DMNV via a direct action on DMNV neurons.

Ionic basis of leptin-induced depolarization and hyperpolarization. In this series of experiments, DMNV neurons were voltage-clamped to a holding potential of -60 mV. The steady-state I–V relationship was measured before (control curve) and during (leptin curve) the application of leptin (100 nM) by applying a series of command steps from the holding potential of -60 mV to different potentials (-120 to 0 mV) in a TTX-containing ACSF. The steady-state I–V curve of the leptin-induced inward current was obtained by subtracting the control curve from the leptin curve in the neurons, where leptin induced inward current (depolarized neurons) and outward current (hyperpolarized neurons), respectively.

Depolarized neurons. A representative steady-state I–V curve of leptin-induced inward currents at 100 nM is shown in Fig. 3. The I–V curve is characterized with a negative slope conductance, indicating that leptin caused a decrease in membrane conductance. The average reversal potential of leptin-induced inward current was -90 ± 3 mV ($n = 6$), which is close to the calculated equilibrium potential of potassium ions, -94 mV, under our experimental conditions, suggesting that potassium is the main ion involved in the leptin-induced depolarization/inward current. In a high-potassium (10 mM) medium, the amplitude of the leptin-induced inward currents was reduced, and the reversal potential of the steady-state I–V

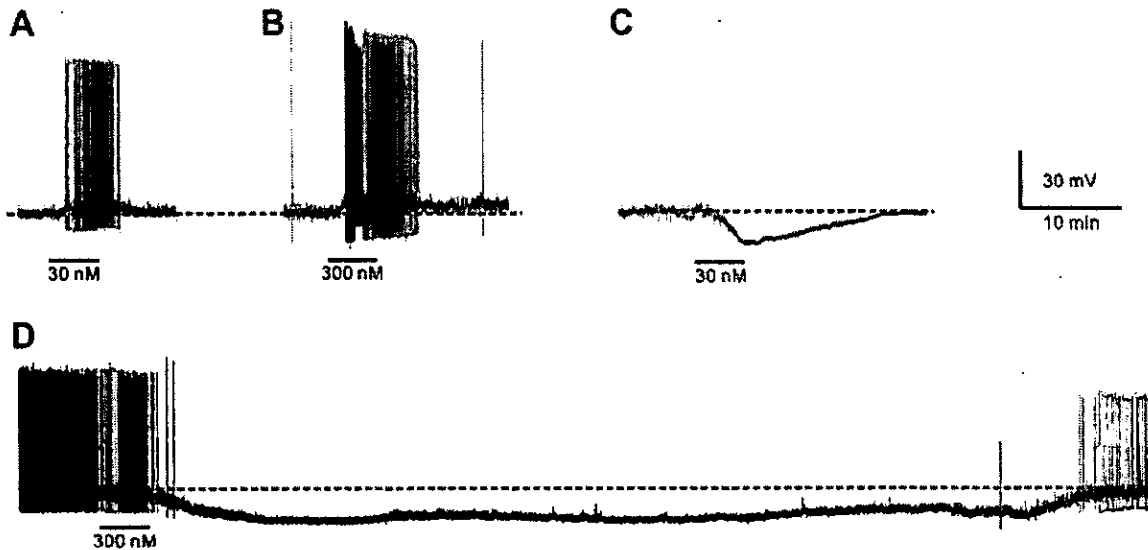


Fig. 2. Leptin-induced depolarization and hyperpolarization in DMNV neurons. *A* and *B*: leptin of 30 and 300 nM applied in a bath solution caused membrane depolarization accompanied by intensified spontaneous discharges in a DMNV neuron. The resting potential of this neuron was -55 mV. *C* and *D*: leptin of 30 and 300 nM applied in bath solution caused membrane hyperpolarization associated with cessation of the spontaneous discharges in 2 DMNV neurons. The resting levels of neuron in *C* and *D* were -53 and -45 mV, respectively.

curve was shifted to -57 ± 7 mV ($n = 3$), a more-positive potential level, as predicted by the Nernst equation for a potassium-selective conductance. Therefore, a decrease in potassium conductance is likely the main ionic mechanism underlying leptin-induced depolarization/inward current. Significant outward rectification, which is manifested by increased slope conductance in the direction of membrane depolarization, was noticed in the I-V relationship of leptin-induced inward current (Fig. 3*B*).

Hyperpolarized neurons. Under voltage-clamp recording, leptin-induced hyperpolarization was demonstrated to be an outward current. The steady-state I-V curve of the leptin (100 nM)-induced outward currents is characterized with a positive slope conductance and has a reversal potential of around -90 mV (Fig. 4). The average reversal potential of the leptin-induced outward current was -88 ± 3 mV ($n = 4$). The results indicated that an increase of potassium conductance might underlie leptin-induced hyperpolarization. No evident inward or outward rectification was observed in the I-V relationship of leptin-induced outward current (Fig. 4*B*).

Most of leptin-responsive neurons are parasympathetic preganglionic neurons. The DMNV contains parasympathetic preganglionic neurons that send projection to the vagal ganglia and are the major source of vagal efferents to the visceral organs in the thorax and upper abdomen (25). Parasympathetic preganglionic neurons in the DMNV were identified in leptin-responsive neurons with a positive immunostaining of Fluoro-gold in the cytosol. Among 7 leptin-responsive DMNV neurons, five neurons, including three leptin-depolarized and two leptin-hyperpolarized neurons, were identified as parasympathetic preganglionic neurons (Fig. 5). The other two nonparasympathetic preganglionic neurons were leptin-depolarized.

DISCUSSION

The discovery of leptin, a product of the ob gene (46), has been a critical breakthrough toward fully understanding energy

homeostasis. Effects of leptin in the hypothalamus to control energy balance and food intake have been greatly explored both in rodents and humans. However, the Ob-R is widely distributed in the CNS, and leptin may have functions on multiple target sites, including the DMNV. The DMNV contains parasympathetic preganglionic neurons that are the major source of vagal efferents to the visceral organs in the thorax and upper abdomen and are involved in the regulation of gastrointestinal, cardiovascular, and pancreatic secretions, and many other autonomic functions (7, 25). Ob-R immunoreactivity has been detected in choline acetyltransferase-containing neurons, that is, the parasympathetic preganglionic neurons, in the DMNV of adult rats (40). Hosoi et al. (17) demonstrated that systemically applied leptin is able to activate Ob-R_b in the DMNV of adult mice by detecting two biochemical functional markers of Ob-R_b receptors, STAT3 activation and of SOCS3 expression. The finding of Hosoi's suggests a functional role of leptin in the regulation of parasympathetic activity through acting on the Ob-R in the DMNV of adult rats. In the present study, we demonstrated that, in neonatal rats, the Ob-R is also located on parasympathetic preganglionic neurons in the DMNV, and leptin can directly act on these neurons to cause membrane depolarization and hyperpolarization and, in turn, to increase or decrease neuronal excitability in different subgroups of DMNV neurons, most of which are parasympathetic preganglionic neurons. To our best knowledge, this is the first

Table 1. Passive membrane properties of DMNV neuron subgroups with different responses to leptin

Neuronal Responses to Leptin	Resting Membrane Potential, mV	Input Resistance, M Ω	Capacitance, pF
Depolarization	-54 ± 3	335 ± 80	31 ± 6
Hyperpolarization	-52 ± 1	395 ± 61	33 ± 3
No response	-52 ± 1	367 ± 29	30 ± 3

Values are expressed as means $\pm$ SE.

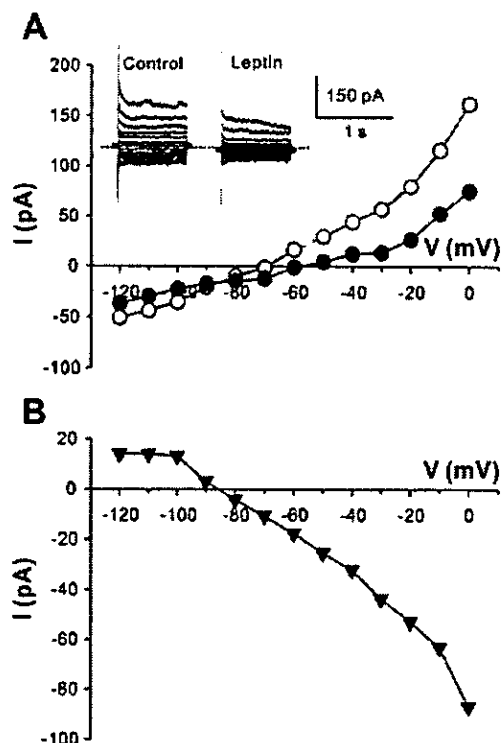


Fig. 3. Steady-state I-V relationship of leptin-induced inward currents in a DMNV neuron. The insets in A are original current traces in response to a series of command steps (from -60 mV to -120 to 0 mV) recorded before and during the application of leptin (100 nM). A: I-V curves measured before (open circles) and during (solid circles) the application of leptin (100 nM). Leptin decreased the neuronal input resistance, as evidenced by a decreased slope of the I-V curve. B: I-V relationship of leptin-induced currents (solid triangles) with a reversal potential close to -90 mV. The experiments were performed in the presence of TTX.

report providing functional evidence supporting that leptin directly affects the neuronal activity of parasympathetic preganglionic neurons of the DMNV.

Several *in vivo* studies have been performed to investigate the functional roles of leptin in the DMNV. Leptin when applied locally into the fourth ventricle inhibited gastric emptying in rats (40) and prevented fasting-induced anorexia in hamsters (36). Microinjection of leptin into the dorsal vagal complex, which contains the DMNV, caused an inhibition of food intake in rats (13). Moreover, increased leptin expression in the dorsal vagal complex reduced body weight and adiposity (4). Therefore, multiple functional roles are implicated for the leptin-responsive DMNV neurons, including the regulation of gastric functions, endocrine system, and energy homeostasis. However, the vagal efferents from the DMNV reach most of the visceral organs and tissues in the thorax and abdomen. Diverse vagal functions of the DMNV have only been partly explored for leptin's involvement. Further studies will be needed to fully verify all physiological functions in which the different subgroups of leptin-responsive DMNV neurons are involved. In addition, Peters and coworkers (32, 33) have found that leptin selectively excites nodose ganglion neurons innervating the stomach and duodenum. Therefore, both the vagal afferents and efferents are sites of leptin's actions.

Browning and colleagues (5) have found differential electrophysiological properties among the neuronal groups that project to different parts of the gastrointestinal tract. For example, the input resistance of fundus-projecting neurons, being 400 ± 25.3 M Ω , was significantly higher than that of other stomach-projecting neurons and cecum-projecting neurons, which are in the range of 291 ± 23 and 302 ± 22 M Ω . The input resistance (366 ± 25 M Ω) of the DMNV neurons recorded in the present study, are comparable to that reported by Browning et al. (5). However, no significant difference was found in the passive membrane properties among the leptin-depolarized, leptin-hyperpolarized and leptin-nonresponsive DMNV neurons. Therefore, it is infeasible to correlate the DMNV neuron by its responses to leptin (depolarization, hyperpolarization, or no response) to the functional subgroups identified by Browning and coworkers. Functional diversity of leptin-responsive neurons might account for the insignificant difference among the groups.

The plasma leptin concentrations are high in the neonates of rodents (1, 10) and humans (15), compared with adult animals or with Tanner stages 1 and 2 children, implying significant roles of leptin in the neonatal period. Functional studies strengthen this hypothesis. For example, leptin administered systemically reduce body weight and fat deposition in newborn rats (22, 45). In addition, in C57BL/6J mice, leptin-induced enhancement of the metabolic rate was detected at 17 days

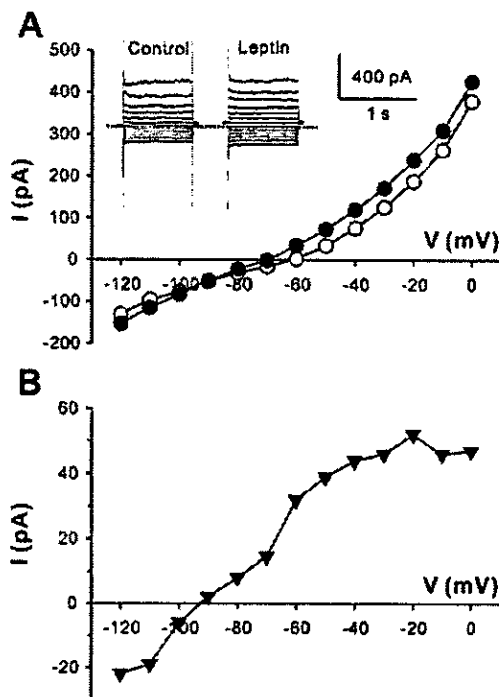


Fig. 4. Steady-state I-V relationship of leptin-induced outward currents in a DMNV neuron. The insets in A are original current traces in response to a series of command steps (from -60 mV to -120 to 0 mV) recorded before and during the application of leptin (100 nM). A: I-V curves measured before (open circles) and during (solid circles) the application of leptin (100 nM). Leptin increased the neuronal input resistance, as evidenced by an increased slope of the I-V curve. B: I-V relationship of leptin-induced currents (solid triangles) with a reversal potential close to -90 mV. The experiments were performed in the presence of TTX.

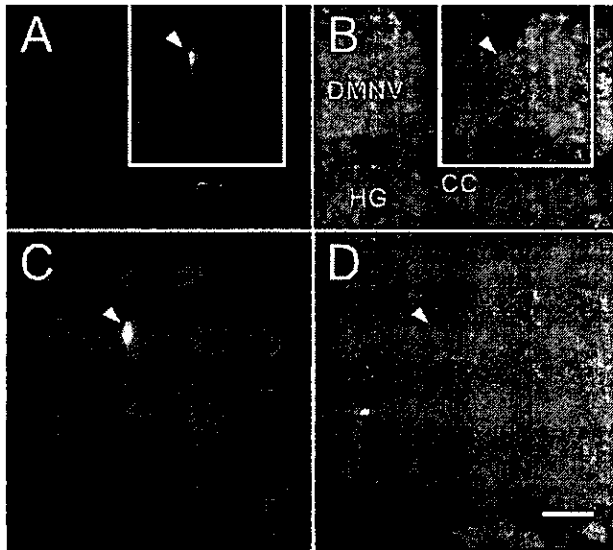


Fig. 5. A leptin-responsive neuron identified as a parasympathetic preganglionic neuron in the DMNV. The rat from which this brain stem section was taken had received an injection of a retrograde tracer, Fluorogold, 3 days before the recordings were made. *A*: micrograph of a leptin-responsive neuron intracellularly labeled with Lucifer yellow (green-yellow color). *B*: Fluorogold-filled parasympathetic preganglionic neurons in the DMNV (red color) of the same area in the brain stem slice shown in *A*, demonstrating that the leptin-responsive neuron is a parasympathetic preganglionic neuron in DMNV. *C* and *D*: higher magnification of the rectangle areas outlined in *A* and *B*, respectively. DMNV, dorsal motor nucleus of the vagus nerve; HG, hypoglossal nucleus; CC, central canal. Scale bar: 100 μ m in *A* and *B*; 50 μ m in *C* and *D*.

postnatal age, whereas the inhibition of food intake by leptin developed later, after 28 days old (28). Evidence also supports that leptin plays a critical role in sexual development of humans and rodents (3, 6, 8). Thus, leptin's direct effects on neonatal parasympathetic preganglionic DMNV neurons found in the present study might contribute to the regulation of neonatal development.

The circulating leptin is reported in a range from several to 50 ng/ml in neonatal rodents (1, 10). The lowest effective concentration of leptin in the present study is 30 nM, which is about 480 ng/ml, and is considerably higher compared with the reported physiological concentration of leptin. However, successful recordings are usually made on neurons located deep inside the brain stem slices since the neurons on surface are commonly damaged during the slicing process. Leptin is a big molecule with a molecular weight of 16 kDa and might not easily penetrate into the brainstem slice during a brief perfusion of leptin. The concentration of leptin reaching the recorded neuron is hard to be estimated yet it should be much lower than that in the bath solution.

Approximately 80% of the DMNV neurons are believed to be parasympathetic preganglionic neurons, and the remaining 20% neurons are likely to be local circuit interneurons or centrally projecting neurons, as suggested by Loewy and Spyer (25). Our results demonstrated that 29% (2/7) of the leptin-sensitive neurons are not Fluorogold retrograde-labeled neurons. This result suggests that leptin also affects nonparasympathetic neurons in the DMNV. Furthermore, the ratio of Ob-R-labeled parasympathetic preganglionic neurons, $45.6 \pm 5.1\%$, is a little higher than the ratio of leptin-responsive cells,

36% (29/80), which includes the responsive neurons of non-parasympathetic preganglionic neurons. Whether leptin is involved in other regulatory mechanisms without affecting resting membrane potential needs further verification.

Our results demonstrate that potassium conductance(s) is likely the main underlying ionic mechanism of leptin-induced depolarization and hyperpolarization in the DMNV. Leptin was reported to cause membrane depolarization by activating a nonspecific cation channel in neurons of hypothalamic paraventricular nucleus (34) and proopiomelanocortin neurons of the arcuate nucleus in rats (9). Herein, we report a different mechanism, a reduction of potassium conductance, by which leptin induces membrane depolarization in DMNV neurons. On the other hand, the mechanism of leptin-induced hyperpolarization is consistent with the findings of others that an increase of potassium conductance underlies the inhibitory effects of leptin in neurons of other central areas, which include the glucose-sensitive neurons in ventromedial and arcuate nuclei of the hypothalamus (41) and hippocampal neurons (38).

The depolarization induced by leptin faded out rapidly upon removal of leptin. In contrast, after the washout of leptin, the leptin-induced hyperpolarization lasted for more than an hour. The latter result suggests that the potassium channels involved in leptin-induced hyperpolarization are likely activated by intracellular signaling cascades. Ob-R is a member of the cytokine receptor superfamily (42). The major brain-intrinsic leptin receptor isoform, Ob-R_b, is reported to couple with JAK/STAT3 transductional pathway (12). Activating Ob-R_b results in JAK2 activation, which, in turn, leads to the activation of other downstream kinase cascades, including MAPK and phosphatidylinositol 3-kinase (PI3K). It has been reported that leptin induces a long-lasting or irreversible hyperpolarization by activating an ATP-sensitive potassium channel (K_{ATP}) in neurons of the ventromedial and arcuate nuclei in rat hypothalamus (41). A further study, using isolated rat arcuate neurons and mouse hypothalamic cell line GT1-7, demonstrates that leptin activates K_{ATP} via a signaling pathway of PI3K-dependent depolymerization of actin filaments (27). A similar mechanism, by which leptin activates K_{ATP} by a PI3K-dependent cortical actin rearrangement, has been reported for rat CRI-G1 insulinoma cells (14). A more recent study of Ning and coworkers' (29) verifies that leptin inhibits PTEN (phosphatase and tension homology deleted on chromosome ten) activity, which in conjunction with increased $\text{PtdIns}(3,4,5)\text{P}_3$ (phosphatidylinositol 3,4,5-trisphosphate, i.e., the main products of PI3K activation) levels results in actin depolymerization and K_{ATP} activation. The mechanism of leptin signaling has also been explored in hippocampal neurons, in which leptin activates BK channels via PI 3K-dependent reorganization of actin filaments (30, 38). It remains to be elucidated the signaling cascades and the associated potassium channels underlying the long-lasting hyperpolarization induced by leptin in DMNV neurons.

In summary, the present study demonstrates that leptin acts directly on parasympathetic preganglionic neurons in the DMNV of neonatal rats. This effect of leptin might contribute to its regulation of the parasympathetic outflow from the DMNV in neonatal rats and, perhaps, in adult animals too. Therefore, parasympathetic activity may play an important role in the physiological aspects of leptin. In

the future studies, it will be crucial to clarify the physiological functions in which leptin-responsive parasympathetic neurons in the DMNV are involved, as well as to identify the underlying ion channels and intracellular signaling of leptin-induced responses.

GRANTS

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