

doi: 10.13241/j.cnki.pmb.2019.08.005

光纤成像技术记录小鼠眶额皮层奖赏相关神经元活性的应用*

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摘要 目的:探讨光纤成像技术用于记录小鼠眶额皮层奖赏相关神经元活性变化的可行性。**方法:**应用光纤成像的方法记录自由活动小鼠在饮用糖水时,携带有钙离子荧光探针(GCaMP6m)的眶额皮层奖赏相关神经元的活性。首先,在小鼠的眶额皮层注射携带 GCaMP6m 的腺相关病毒,同时在相应位点植入提前做好的光纤陶瓷插芯;等待小鼠术后恢复,病毒表达 2 周。然后在记录前,给予小鼠 36 小时禁水处理并运用光纤成像记录接受糖水刺激的小鼠眶额皮层锥体神经元的反应活性。最后,记录数据读入 matlab 软件进行数据分析并对小鼠进行心脏灌注、取脑、脑组织冰冻切片并显微荧光成像观察记录位点是否正确,病毒是否正常表达。**结果:**成功记录到对小鼠施加糖水刺激时,其眶额皮层内与奖赏相关的神经元活性变化。数据分析结果用热度图和事件相关的平均线图来表示。组织学切片及成像结果证实记录位点正确,病毒正常表达。**结论:**光纤成像的记录方法可以监测自由活动的小鼠在饮用糖水时眶额皮层内奖赏相关神经元活性的变化。

关键词:光纤成像;GCaMP6m;眶额皮层;奖赏

中图分类号:R-33;R338;TB86 文献标识码:A 文章编号:1673-6273(2019)08-1420-05

Application of Fiber Photometry for Reward-related Neuronal Activity in the Orbital Frontal Cortex (OFC) of Mice*

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ABSTRACT Objective: To investigate the feasibility of fiber photometry technology in recording the activity changes of reward-related neurons in the orbital frontal cortex of mice. **Methods:** The fiber photometry method was used creatively to record the activity of reward-related neuronal that expressed calcium fluorescent probe (GCaMP6m) in orbital frontal cortex of freely moving mice which were stimulated by drinking syrup. Firstly, adeno-associated virus carrying GCaMP6m were injected into the OFC of postanesthetic mice precisely. Then, one pre-made fiber-optic ceramic ferrule was implanted into the corresponding site carefully, accurately and suitably. The mice that undergone mentioned surgery needed to regain them health and the injected viruses needed to express GCaMP6m efficiently in two weeks. In the second place, the mice that recovered health after surgery were given water-deprivation treatment which sustained 36 hours before recorded reward-related neuronal signal. The reaction activity of frontal cortex pyramidal neurons in mice that stimulated by syrup was recorded by means of fiber photometry system dynamically. In the end, the date that reflected the activity of recorded reward-related neuro in mice's OFC were analyzed scrupulously using matlab software. At the same time, the anaesthetic mice were subjected to heart perfusion, brain taken, frozen section of removed brain and fluorescence imaging using fluorescence microscope orderly and feelinglessly. The purpose of above complex operations were to affirm the validity of neuronal signal recording site and the level of injected virusee expression. **Results:** The variation of neuronal activity which associated with reward in OFC of syrup stimulated mice was recorded successfully and clearly using fiber photometry method. The results of data analysis that reflected changed neuronal activity were shown through a heat map and an event-related average plot scientifically. Meantime, the frozen section of mice brain and fluorescence imaging results all demonstrably indicated that the neuronal signal recorded sites were undoubted correct and the expression level of viruses were very high in correspond region. **Conclusions:** Fiber photometry was able to be used to measure the activity of reward-related neurons in the orbito frontal cortex of free-moving mice when they were given sugar water.

Key words: Fiber photometry; GCaMP6m; OFC; Reward

Chinese Library Classification(CLC): R-33; R338; TB86 **Document code:** A

Article ID: 1673-6273(2019)08-1420-05

* 基金项目:国家自然科学基金项目(31171051,31371108);北京市自然科学基金项目(5132007,5112008);

北京市教委科技发展项目(KM201110025001)

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(收稿日期:2018-11-16 接受日期:2018-12-13)

前言

神经元反应与动物行为相关联在神经环路的功能解剖以及行为神经科学的研究中起着重要作用^[1]。然而,以往的研究方法存在诸多的局限性,如:传统的电生理技术无法区分神经元类型^[2];双光子技术只能记录表浅的大脑皮层^[3,4];显微成像技术设备昂贵,实验难度大^[5,6]等。光纤成像技术提供了更为简单、有效的方法来记录行为动物深脑神经元活性^[7-10]。在特定脑区中,同种类型的神经元在受到外部或内部刺激时是同步活动的^[11-13],此时同步活动的神经元会引起相应脑区钙信号的显著性升高。因此光纤成像技术结合遗传编码的钙离子指示剂 (genetically encoded calcium indicators, GECIs), 如: GCaMP6m^[14-16],便可以记录特定类型神经元的活性。眶额皮层是奖赏处理的重要脑区^[17-19],那么光纤成像技术是否可以探测到给予糖水时,小鼠眶额皮层内奖赏相关神经元的活性变化?这不仅是对神经元活性的监测,也是对光纤成像系统性能的重要探索。本研究应用光纤成像技术记录小鼠在舔舐糖水时眶额皮层内神经元活性的变化,旨在探讨光纤成像技术用于监测小鼠眶额皮层奖赏相关神经元活性的可行性。

1 材料与方法

1.1 材料

实验动物为 SPF 级 C57BL/6J 小鼠,饲养于首都医科大学实验动物部。

立体定位仪购自美国 Siskiyou 公司;手术显微镜及微量注射器购自美国 World Precision Instruments 公司;电钻购自韩国世新公司;蠕动泵购自中国兰格恒流泵有限公司;冰冻切片机购自德国 Leica 公司;正置荧光显微镜购自日本尼康公司;腺相关病毒购自武汉恒睿科技有限公司;光纤陶瓷插芯及裸纤购自上海斐波光电;C&B-Metabond 购自美国 Parkell 公司;光纤成像记录与采集系统购自南京千奥星生物科技有限公司;视频采集系统购自美国 Black Rock 公司;Matlab 软件购自美 Mathworks 公司;水合氯醛购自天津市光复精细化工研究所;多聚甲醛购自天津市福晨化学试剂厂;矿物油购自 sigma-aldrich(上海)有限公司;光纤切割刀购自日本住友电气工业株式会社;全透明环氧树脂 AB 胶购自深圳市特固新材料有限公司。

1.2 方法

1.2.1 病毒注射 本实验选用的病毒为 rAAV-CaMK II a-Gcamp6m-WPRE-pA, 其同时携带表达在海马及大脑皮层兴奋性神经元的启动子 CaMK II a 和钙离子荧光探针 Gcamp6m。Gcamp6m 是单个绿色荧光蛋白(GFP)/钙调蛋白(CaM)和平滑肌肌球蛋白轻链激酶片段 M13 融合的产物^[14,20,21],神经元细胞内钙离子浓度升高会引起 GFP 生色基团环境改变,从而使 GCaMP6m 的荧光强度(F)增加,增加的荧光强度反应了升高的神经元活性^[14]。

质量分数为 6%水合氯醛,6 mL/kg 腹腔注射将小鼠麻醉,用剃毛器剔除头部毛发;将麻醉后小鼠的头部固定于小鼠头部立体定位仪,在手术显微镜下消毒并减掉颅顶部皮肤,暴露颅骨,清除颅骨表面的结缔组织。

根据小鼠脑图谱^[22],查找待记录脑区坐标值。本实验选取

的记录位置为外侧眶额皮层区域(Bregma 前方 2500 μm ,旁开 1500 μm),通过微操纵器进行定位,并在颅骨上做标记点,以标记点处为中心,用电钻轻轻将标记点处颅骨磨薄至一薄层骨片,打磨范围直径约 1000 μm ,显微镊轻轻拨除骨片。

在拉制好的玻璃电极中注满松柏油,并安装至微量注射器(Nanolitter2000),缓慢吸取适量腺相关病毒,操纵微量注射器再次定位至记录位点,微操纵器以中速使玻璃电极缓慢进入脑组织,深度 1500 μm ,等待脑组织回弹 10 min 后按动微量注射器注射病毒,病毒滴度为 $4.11\text{E}+12$ vg/mL,每次注射量为 32.2 nL,共注射 10 次。为防止病毒的溢出,注射之后留针十分钟,然后再以微操纵器最小速度将玻璃电极缓慢移出。

1.2.2 光纤陶瓷插芯植入 光纤陶瓷插芯的制作,光纤切割刀将直径为 230 μm 的裸纤切割成末端平整的长度为 13 mm 的小段,用无水乙醇清洗后将切好的光纤小段插入内径为 230 μm 的 SC 型陶瓷插芯(数值孔径 0.37)中,从陶瓷插芯有凹槽端插入,使其末端刚好伸出陶瓷插芯平面端(<1 mm)。一手用镊子夹持陶瓷插芯,另一只手用牙签在凹槽端涂抹混合好的环氧树脂胶。涂抹后的胶呈锥体,高度在 0.5 mm-1 mm 间。取干净载玻片,将陶瓷插芯平面端置于载玻片上,在体视镜下观察并做调整使光纤末端与陶瓷插芯的平面端平齐,并小心地将其横向放置在干净的 A4 纸上。待环氧树脂胶变硬后即可将光纤固定在陶瓷插芯内。为了保证传光效率,必须保证光纤末端和陶瓷插芯末端平齐。

最后在记录位点植入制做好的光纤陶瓷插芯(图 1B),然后在其周围颅骨上均匀涂抹上 C&B-Metabond 使光纤插芯牢固固定在颅内。注意涂抹 C&B-Metabond 之前要保证脑组织表面不得有血液渗出,颅骨表面不得有结缔组织的存在并保持干燥。术后将小鼠单只笼内饲养。病毒表达以及小鼠身体恢复约需 2 周。

1.2.3 光纤成像记录钙信号 根据光纤记录系统原理,可将其分为荧光激发系统和荧光收集系统(图 1A)。激光器发出波长为 488 nm 的激发光,经过分光镜反射后,反射光从垂直角度射出。射出的光束经过一个放大 10 倍的物镜(数值孔径 0.3)后被聚焦到光纤跳线(图 1C)的纤芯端面上。光纤跳线通过陶瓷插芯连接器与动物头部固定的光纤陶瓷插芯连接,激发光经过光纤跳线和小鼠头部陶瓷插芯后到达特定脑区,从而激发绿色荧光信号。被激发的荧光信号的强度可以被植入动物脑内的光纤末端收集,反向沿着光纤跳线和物镜传播,到达并直接穿过分光镜,经过 GFP 带通滤波片后,荧光强度信号即可由光电倍增管检测到^[23-25]。

在光纤记录之前给予小鼠 36 小时禁水处理,记录当天将盛有 10%浓度糖水的装置置于鼠笼内让其识别糖水装置,之后将光纤跳线的陶瓷插芯连接器插到小鼠头部固定的光纤陶瓷插芯上。将小鼠置于实验箱(图 1D)内适应 10-15 分钟,放入糖水装置,在小鼠识别糖水并饮用的过程中同时进行神经元钙信号的记录。每次放入糖水装置时,仅允许小鼠饮用 3-5 秒便立即取出,以保持小鼠对糖水较长时间的敏感性。每次实验间隔 15 秒。

1.2.4 数据采集与分析 包括行为学视频以及钙信号的采集与分析。

行为学视频采集与分析:应用 neural motive 采集摄像头及记录软件进行采集,采样频率为 50HZ。对离线视频逐帧进行分析,将小鼠舔水的时间记录下来用于在钙信号数据上做时间标记。

钙信号的采集与分析:光电倍增管探测到小鼠脑区内发射的荧光信号后将其转换成电流信号,电流信号经过与光电倍增管配套的放大器后变成模拟电压信号,模拟电压信号进一步经过一个低通滤波器(40 Hz)并输入到数据采集系统。以 200-500 Hz 的采样频率记录保存。

光纤记录的结果读入 Matlab。然后截取小鼠舔水行为发起之前 5 秒和舔水后 10 秒这一时间窗内的荧光强度变化的数据并使其按照舔水行为发起时间为时间零点对应整齐。我们用当

前荧光强度 F 和基线荧光强度 F_0 的差值再除以基线荧光强度 F_0 的值即 $\Delta F/F = (F-F_0)/F_0$ 的值来表征上述时间窗内小鼠舔水这一行为前后的荧光强度变化。基线荧光强度 F_0 的选取为小鼠舔水行为发起之前第 5 秒至第 2 秒这一时间段的荧光信号强度。分析的结果用两种图表征:一种是热度图,另外一种是事件相关的线图。在热度图中,按照小鼠开始舔水时间作为零点对齐,将所有次数的实验结果按顺序排列呈现。不同的颜色表征实验结果的幅度值大小。我们通常用暖色调表征较高的荧光强度变化,冷色调表征较低的或者反向的

荧光强度变化。事件相关的线图描述的是所有次数实验结果的平均值。

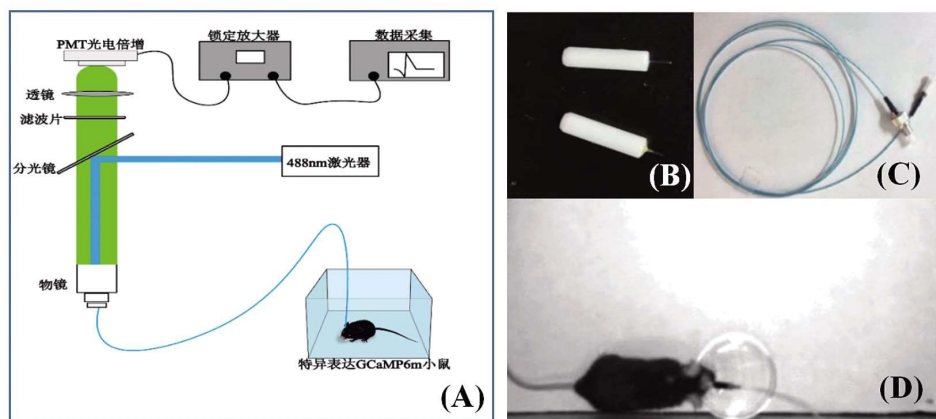


图 1 光纤成像记录系统及实验装置

Fig.1 Fiber photometry recording system and experimental setup

注:A.激光器发出波长为 488 nm 的激发光,经分光镜反射后垂直射出,经过一个放大十倍的物镜聚焦到光纤跳线进而激发出绿色荧光信号。被激发的荧光信号沿着光纤跳线和物镜反向传播,到达并直接穿过分光镜,经过 GFP 带通滤波片后,荧光强度信号即可由光电倍增管检测到;B.光纤陶瓷插芯;C.芯径 230 um,数值孔径 0.37 的光纤跳线;D.测试过程中实验箱内正在饮用糖水的小鼠。

Note: A. the laser emitted a excitation light of 488 nm wavelength and was reflected by a dichroic mirror. After passing through a 10-fold magnified objective lens, then stimulated the green fluorescent signal. The intensity of the excited fluorescence signal could propagated reversely along the optical fiber jumper and objective lens. After reaching the dichroscope, it passes through directly. After passing the GFP bandpass filter, the fluorescence intensity signal is detected by the photomultiplier tube; B. fiber optic ceramic ferrule; C. fiber jumper with 230 um in diameter and a numerical aperture of 0.37; D. the mouse that were being recorded were placed in a test box and were licking sugar water.

1.2.5 验证病毒表达及记录位点 实验的小鼠在完成光纤成像记录后,水合氯醛过量麻醉,用 0.9%(质量分数)氯化钠注射液及 4%(质量分数)多聚甲醛溶液灌注取脑,30%(质量分数)蔗糖溶液过夜,待脑组织沉底后直接进行冰冻切片,通过荧光显微镜观察病毒携带的钙离子荧光探针在脑组织中表达情况及在脑区的分布情况。

2 结果

2.1 光纤成像记录

光纤成像记录到与小鼠饮水行为相关的神经元反应,分别以热度图(图 2A)和事件相关的平均线图(图 2B)表示。

图 2A 为热度图,将小鼠饮水行为发起的时间作为时间零点,取零点前 5 秒至后 10 秒的时间窗,将各个试次按照时间零点对应整齐,不同的颜色代表神经元反应的强弱,其中暖色调代表较强的反应,冷色调代表抑制的或较弱的反应。从图中可在小鼠饮水行为发起的时间周围神经元的反应是明显升高的,

且各个试次对应整齐。可以反映每次实验的细节信息。

图 2B 为事件相关的平均线图。它是所有实验次数结果的平均。图中在时间零点即饮水行为发起之前神经元活性已经开始升高。

2.2 GCaMP6m 在眶额皮层的表达及记录位点

GCaMP6m 在眶额皮层高表达,且记录位点恰好为眶额皮层 GCaMP6m 稳定表达的脑区内,见图 3。

3 讨论

我们应用光纤成像的方法成功地记录到了小鼠眶额皮层中与奖赏相关神经元的活性。我们还发现:小鼠的神经元活性开始升高的时间位点并非是在开始舔水的瞬间,而是在靠近盛水的器皿时或者说有了要舔水的冲动时神经元活性即开始升高,在小鼠舔到糖水的瞬间(时间零点),神经元的活性达到峰值。这是对神经元活性监测的一个有趣的发现。

技术的实现依赖于钙离子。应用基因编码的钙离子敏感的

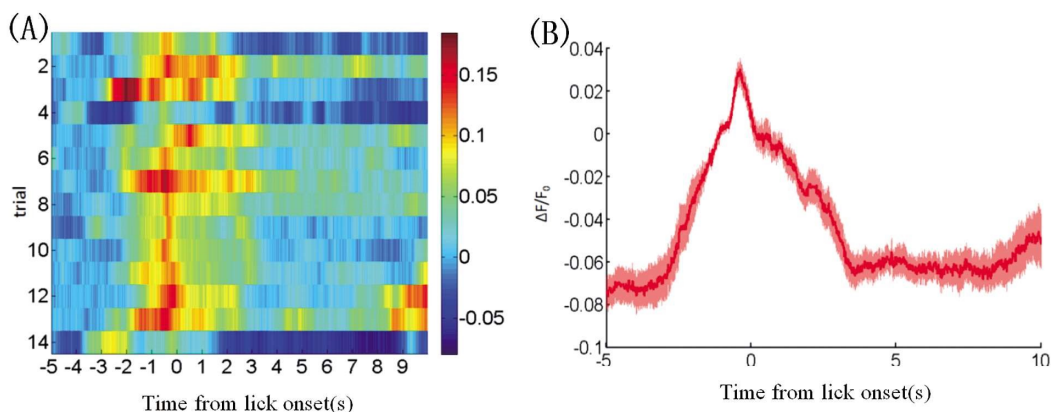


图2 热度图及事件相关的平均线图

Fig.2 Heatmap and event-related average plot

注:热度图和平均线图显示小鼠舔舐糖水这一行为引起钙信号的明显升高。

较粗的实线代表所有试次钙信号变化的平均值,周边的阴影区域代表均数的标准误。

Note: Heatmap of Ca^{2+} transients and average plot show that licking sucrose resulted in significant increase of Ca^{2+} signal across multiple behavioural bouts of a mouse. Thick lines indicate mean and shaded areas indicate s.e.m.

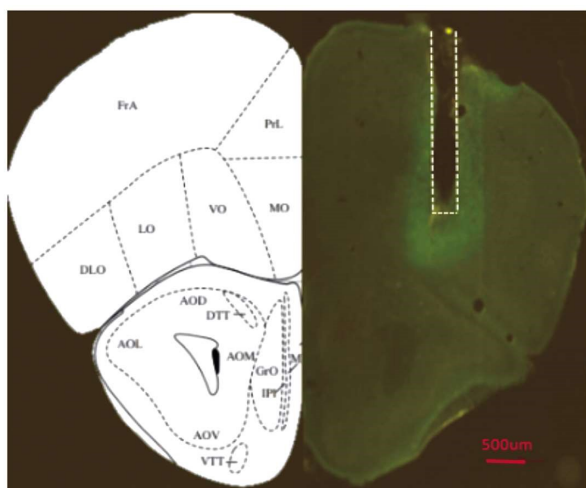


图3 组织学切片及成像

Fig.3 Histological section and imaging

注:小鼠脑组织冠状切片显微荧光成像结果显示 GCaMP6m 在眶额皮层中成功表达。图中虚线代表光纤植入位置。比例尺=500 μm 。相关的英文缩略词:DLO,背外侧眶额皮层;LO,外侧眶额皮层;VO,腹侧眶额皮层;MO,中间/内侧眶额皮层。

Note: Fluorescent photomicrograph of the coronal section showing the GCaMP6m was expressed in the OFC, and the dash line shows that the fiber implanted in the same area. scale bar=500 μm . Related abbreviations: DLO, dorsolateral orbital cortex; LO, lateral orbital cortex; VO, ventral orbital cortex; MO, medial orbital cortex.

荧光探针可以特异地标记某种类型的神经元,而且荧光信号的变化可间接反映神经元的活性变化。我们实验用的钙离子荧光探针 -GCaMP6^[4],是2013年 Douglas.Kim 等开发出的新一代超敏感的钙活动指示剂。较以往的钙活动指示剂相比,GCaMP6 对钙信号的敏感性提高了十倍之多。GCaMP6 又分为3种常见的亚型,分别为 GCaMP6s, GCaMP6m 和 GCaMP6f,它们的动力学特征分别是 slow, medium 和 fast。GCaMP6 动力学特征的快慢与其对钙信号的敏感性呈反比。由于 GCaMP6s 动力学特征较慢而 GCaMP6f 的动力学特征又较快,所以我们

实验选用的是动力学和敏感性均为中等的 GCaMP6m。组织学实验结果显示了 GCaMP6m 在小鼠眶额皮层中可以稳定表达,并且光纤成像记录到了较强的荧光信号。

实验之前,实验人员对小鼠的禁水处理、环境适应处理及糖水装置识别训练是至关重要的一个环节。忽略任何一个细节都可能导致实验的失败。在实验过程中,糖水装置在实验箱中放置的时间不宜过长以使小鼠不能长时间饮水从而使其保持相对饥渴的状态,以保证实验顺利进行。

基于光纤成像具有抗干扰,长时程记录等性能,我们成功记录到了自由活动的小鼠眶额皮层中与奖赏相关的神经元的活性,这是传统电生理等技术所不能实现的。该实验是对光纤成像技术及其性能的一次成功探索,之后我们将结合其他的行为学范式去记录一些疾病模型(如:癫痫,自闭症等)的病变脑区的神经元活性。这将有利于发现疾病的早期生物学靶点,从而为疾病的临床治疗提供更有思路或方法。

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