评述

神经元活动依赖工具开发应用的研究进展与展望

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摘要 大脑存在数量众多而功能异质的神经元, 在响应刺激输入时, 特定的神经元类群集结成印迹, 构成细胞和环路水平的功能编码单元. 为了解析这些功能编码单元, 即早基因(IEG)激活、环腺苷酸反应元件结合蛋白(CREB)表达增高及 Ca^{2+} 内流等细胞活化的特性被相继开发成活动依赖工具, 并被大量应用于记忆印迹和事件编码神经集群的解析研究. 本文对LacZ-Daun02失活系统、CREB过表达系统、四环素标签工具(TetTag)、活性类群靶向重组工具(TRAP)、活化神经集群捕获工具(CANE)、人工改造的活性启动子工具(E-SARE和RAM)以及光控钙离子依赖工具(Ca-Light和FLARE)等活动依赖工具的原理及应用进行了综述, 并展望了活动依赖工具的开发前景.

关键词 活动依赖工具, 记忆印迹, 神经集群, 神经编码, 即早基因

当代神经科学细胞及环路水平的研究认为, 大脑的功能编码存在于特定的神经元类群激活/抑制的时间空间图式之中. 解析行为与神经元类群活动之间的关联是神经科学研究的重要方向. 大量研究表明, 编码特定功能的神经元类群通常具有相似的反应特征, 可被功能相关的刺激同步激活而集结成印迹(engram)^[1,2]/神经集群(cell assembly/neural ensemble)^[3].

为了检测脑区/神经元的活动, 一系列方案被开发应用, 有基于代谢的2-脱氧葡萄糖摄取放射自显影^[4]、近红外光谱技术(near infrared spectrum instrument, NIRS)^[5]、功能核磁共振(functional magnetic resonance imaging, fMRI)^[6]以及正电子发射断层成像(positron emission tomography/single-photon emission

computed tomography, PET/SPECT)^[7,8]; 基于电/磁信号的脑电图(electroencephalogram, EEG)记录^[9]、脑磁图(magnetoencephalography, MEG)记录^[10,11]以及电生理记录^[12-14]; 基于钙离子、神经递质及神经调质荧光指示剂荧光信号^[15-19]的光纤记录(fiber photometry)^[20,21]、双光子钙成像(two-photon Ca imaging)^[22,23]以及显微内窥镜(GRIN lens/microendoscope)^[24]; 检测神经化学变化的微透析(microdialysis)和快速扫描循环伏安法(fast-scan cyclic voltammetry, FSCV)^[25,26]; 检测即早基因(IEG)c-fos^[27], Arc/Arg3.1^[28,29], zif268/egr1^[30], homer1a^[31,32], Npas4^[33]等的表达以及检测环腺苷酸反应元件结合蛋白(CREB)^[34,35]和核糖体蛋白S6^[36]的磷酸化等.

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IEG是细胞活化经典的分子标记,在细胞受到外界刺激时迅速启动转录,不依赖于新的蛋白合成,其表达产物涵盖转录因子、突触后蛋白、信号分子、分泌因子以及膜蛋白^[37]。Ca²⁺内流、钙依赖激酶介导的级联反应及相关转录因子磷酸化活化是IEG表达的基本要素^[38]。以应用最为广泛的c-fos的表达为例,细胞活化引发NMDAR及VSCC介导的Ca²⁺内流,当胞内Ca²⁺达到一定的水平会进一步通过ras-raf-MEKK级联信号通路激活ERK/MAPK,最终导致转录因子Elk-1/SRF及CREB磷酸化激活,从而结合在c-fos基因的5'非编码调控区启动c-fos表达^[39],生成的转录因子Fos与同家族的Jun形成异二聚体,通过结合在AP-1位点上进一步调控晚期基因的转录^[40]。环腺苷酸反应元件(cAMP response element, CRE)几乎存在于所有IEG的启动子区域,CREB是调控IEG表达的核心因子。由于转录调控及钙调动力学的差异,不同IEG在不同脑区及细胞类型中表达存在差异^[38]。

活性检测为功能解析提供了基本的相关性证据,神经元类群更深层的功能编码则需要相应的功能获得及缺失实验加以验证。传统的电刺激、损毁、药理兴奋/抑制以及近些年广泛应用的光遗传学(optogenetics)、药理遗传学(DREADDs)、细胞凋亡、突触抑制、基因过表达、基因敲减及沉默技术为解析脑区、细胞、环路及分子的功能编码提供了干预的方法,然而不同脑区及神经元具有很大的功能异质性,通过特定脑区的核团亚区分布、神经投射模式或是细胞类型通常无法分选出功能类群,直接捕获而后操纵被刺激活化的神经集群是细胞水平研究功能编码最为直接的手段。自20世纪90年代以来,一系列活动依赖的遗传改造实验大鼠、小鼠及病毒工具被相继开发应用(表1)^[38]。

这些工具基于CREB表达增高、IEG激活或Ca²⁺内流等细胞活化的特性,各具特色,以下分专题综述各类工具的原理及其代表性研究工作。

1 活动依赖工具原理及研究工作概要

1.1 即早基因报告品系、LacZ-Daun02失活系统及CREB过表达系统

早期的活动依赖工具利用IEG直接启动报告基因(β -半乳糖苷酶(β -gal)基因(*LacZ*)^[41,42,45,68,70]、荧光素酶

基因^[44,63,71]或荧光蛋白基因^[43,46,47,58-62,67,69])的表达标记活性神经集群。这些IEG报告品系被用于绘制特定刺激相关的全脑活性图谱^[67,83,84]、研究激活类群的电生理特性^[43,62]以及通过在体成像揭示集群的功能动态编码特征^[58,69],此外表达*LacZ*的工具还被用于活性神经集群的干预,Koya等人^[85]利用 β -gal能够催化前体药物Daun02转化成具有抑制神经元兴奋作用的柔毛霉素(daunorubicin)的特性,将Daun02给予到刺激后的c-fos-*LacZ*大鼠的感兴趣脑区,从而实现了刺激活化神经集群的失活(图1A)。应用*LacZ*-Daun02失活系统,一系列研究成瘾药物引发行为敏化^[85]、渴求^[86-89]、复吸^[39,90,91]等作用机制的工作发表。

CREB是调控IEG表达的核心因子,在记忆配置(memory allocation)与选择性募集编码的过程中扮演着重要角色^[92]。Han等人^[76]利用病毒过表达CREB证明声音偶联条件性恐惧记忆偏好激活外侧杏仁核(lateral amygdala, LA)过表达CREB的神经元,这些CREB水平更高的神经元在记忆配置过程中占据竞争优势从而被选择性募集编码记忆印迹(图1B),毒杀^[77]或可逆性失活^[78]偶联学习后过表达CREB的神经元抑制了相应的记忆,激活这一类群可引发相应记忆的提取及后续的记忆巩固^[82]。Zhou等人^[78]证明过表达CREB的神经元具有更高的兴奋性,相对活跃的神经元会被募集进行记忆编码。除LA之外,这一现象还被发现存在于海马齿状回亚区(dentate gyrus, DG)^[81]及岛叶皮层(insular cortex, IC)^[93]。CREB过表达系统在记忆配置研究领域发挥了重要作用,但这一方法可能并不适用于其他脑区功能编码的研究。

在IEG报告品系及CREB过表达系统开发探索的基础上,后续的活动依赖工具通过模块化系统调控目的基因条件性表达实现了特定时间区间内活性神经集群的持久标记,极大地提升了活动依赖工具的特异性(specificity, 标记与内源激活模式一致的保真信号)、外显率(penetrance, 标记尽可能多的保真信号凸显工具效能)、信噪比(S/N ratio, 工具灵敏性及背景泄露的综合度量)及应用拓展性^[94]。

1.2 四环素标签工具

2007年,Reijmers等人^[48]开发了基于Tet-off条件性基因表达系统^[95]的四环素标签工具(TetTag),首次实现了特定时间区间内活性神经集群的长时间标记。Tet-

表 1 基于活动依赖启动子、Ca²⁺内流以及CREB过表达的活动依赖工具

Table 1 Activity-dependent tools based on IEG promoters, Ca²⁺ influx and CREB overexpression

1 基于活动依赖启动子的工具				
基因	启动子类型	目的蛋白	工具类型	参考文献
<i>c-fos</i>	Mouse 5' regulatory region	c-fos-LacZ fusion	转基因小鼠	[41]
		Tau-LacZ fusion		[42]
		c-fos-EGFP fusion		[43]
		firefly luciferase		[44]
		c-fos-LacZ fusion	转基因大鼠	[45]
		c-fos-EGFP fusion		[46]
		PSD95-Venus fusion		[47]
		tTA/d2EGFP (TetTag*)	双转基因小鼠	[48]
		GFP	慢病毒	[49]
		ChR2-EYFP-2A-mCherry		
	Knock-in	NpHR-EYFP		[50]
		tTA		
		ChR2-EYFP-PEST	腺相关病毒	[52]
		ER ^{T2} -CreER ^{T2} -PEST		
		rtTA		[53]
		CreER ^{T2} (TRAP*)	基因敲入小鼠	[54]
		2A-dsTVA (CANE*)		[55]
		2A-iCreER ^{T2} (TRAP2*)		[56]
	RAM	d2tTA-pA::TRE-TdTomato	腺相关病毒	[57]
		d2tTA-pA::TRE-ChR2:EYFP		
		d2tTA-pA::TRE-ArchT:EGFP		
		d2tTA-pA::TRE-NLS-mKate2		
		d2tTA::TRE-FLEX-luc2p		
<i>Arc</i>	Knock-in	d2EGFP	基因敲入小鼠	[58]
		CreER ^{T2} (TRAP*)		[54]
	Mouse 5' regulatory region	d2Venus	转基因小鼠	[59]
		EGFP-Arc fusion		[60]
		d4EGFP		[61]
	Mouse BAC clone	firefly luciferase		[62]
		CreER ^{T2}		[63]
	SARE	d2EGFP	慢病毒	[64]
	E-SARE	d2EGFP	腺相关病毒	[65]
		ER ^{T2} -CreER ^{T2} -PEST		
<i>Egr1</i>	Mouse 5' regulatory region	d2EGFP	转基因大鼠	[66]
		LacZ	转基因小鼠	[67]
	Mouse BAC clone	EGFP		[68]
CREB结合位点	6xCRE-RSV	LacZ	转基因小鼠	[69]
	4xCRE-TK	firefly luciferase		[70]

(续表1)

2 基于Ca²⁺内流的工具

工具名称	工作基础	工具模块	工具类型	参考文献
CAMPARI	GEC1 (Ca ²⁺ indicator) mEos2 (Photoconvertible chromophore)	NES-CaM-L1-mEos2-C-cpL-mEos2-N-L2-M13	腺相关病毒+ 紫外光	[72]
TRIC	GEC1 (Ca ²⁺ indicator) AD-DBD (TF activation) Gal4-UAS (Effector transcription)	p65AD::CaM MKII::GAL4DBDo UAS-X	转基因果蝇+ Q系统	[73]
Cal-Light	GEC1 (Ca ²⁺ indicator) LOV (Blue light Switch) Tango (Modularization)	TM-CaM-NES-TEV-N-AsLOV2-TEVseq-tTA M13-TEV-C-P2A-TdTomato TetO-X	腺相关病毒+ 蓝光	[74]
FLARE	GEC1 (Ca ²⁺ indicator) LOV (Blue light Switch) Tango (Modularization)	NRX-TM-Nav1.6 -CaMbp(M2)-eLOV-TEVcs(EN- LYFQ▲M)-tTA-VP16 CaM-V5-TEVp(Δ220-242) TRE-X	腺相关病毒+ 蓝光	[75]

3 基于CREB过表达的工具

工具元件	工具用途	工具类型	参考文献
GFP	标记分析		[76]
Cre+LSL-iDTR			[77]
AlstR			[78]
hM4Di	功能缺失	单纯性疱疹病毒	[79]
Kir 2.1			[80]
iC++			[81]
hM3Dq	功能获得		[79]
TRPV1			[82]

Tag包含c-fos-tTA和tetO-tau-LacZ两个基因表达级联模块,刺激活化的神经元首先通过c-fos启动四环素反式激活因子(tetracycline transactivator, tTA)表达,表达生成的tTA与四环素反应元件(tetracycline response element, TRE/tetO)结合进而调控LacZ基因的表达。tTA与tetO的结合可被外源给予的多西环素(doxycycline, Dox)所阻断,通过控制TetTag小鼠体内Dox的水平可以开放系统标记的时间窗,从而实现特定时间区间内的活性标记(图2)。

Reijmers等人^[48]利用TetTag的标记功能结合即早基因zif染色证明基底外侧杏仁核(basolateral amygdala, BLA)在恐惧习得时所激活的神经集群(LacZ⁺)在记忆提取时会被再度激活(LacZ⁺Zif⁺),且重激活的神经元数量与恐惧记忆的强度呈正相关,相关结果为记忆印迹理论提供了强有力的支持。

tTA的引入不仅提高了活性标记的时间精度也拓展了活动依赖工具的应用性,TRE/tetO随后被用于启

动其他目的基因如光遗传学^[96]和药理遗传学^[97]元件的表达,进一步实现了活性神经集群的操纵。Liu等人^[96]通过光遗传激活DG的恐惧记忆印迹成功引发了小鼠恐惧记忆的提取;更为有趣的是,在条件性恐惧学习时通过光遗传激活另一无关空白环境的记忆印迹将恐惧植入该空白环境的记忆中,制造出了虚假的恐惧记忆。应用同样的策略,Redondo等人^[98]激活DG的记忆印迹实现了正面与负面记忆的转换,而激活BLA的印迹则不会发生类似的情绪效价转换,经过人工激活转换之后DG的记忆印迹会支配不同的BLA神经集群从而介导不同的情绪效应,这表明BLA神经集群的功能编码是固定的,而DG的神经集群则具有很强的灵活性,可被募集编码写入不同的信息,这样的特性同样存在于可以编码社交记忆的腹侧海马vCA1亚区^[99]、编码情景记忆的海马CA1亚区^[100]以及压后皮质(retrosplenial cortex, RSC)^[101]。海马等区域编码的灵活性为记忆的更新和减退提供了基础,Khalaf等人^[102]的研究

图1 LacZ-Daun02失活系统(A)及CREB过表达系统(B)的作用机制图解

Figure 1 Diagram of LacZ-Daun02 inactivation system (A) and CREB overexpression system (B)

表明, DG恐惧记忆印迹的重激活有助于远期恐惧记忆的减退, 为创伤后应激障碍(posttraumatic stress disorder, PTSD)的暴露疗法提供了神经生理依据。

TetTag还被用于其他神经精神疾病研究, Ramirez等人^[103]发现激活DG正面记忆印迹可以逆转慢性压力引发的抑郁表型; 而Ryan等人^[100]则通过激活印迹找回了因逆行性遗忘丢失的记忆, Roy等人^[51]结合阿尔兹海默症(Alzheimer's disease, AD)模型小鼠进一步揭示了AD病理性早期遗忘的本质在于记忆提取而非记忆存储的破坏。

TetTag可快速高水平表达目的基因的特点使其在解析记忆编码方面具有独特优势, 应用TetTag, 一系列关于记忆巩固^[104,105]、遗忘^[51,100,102,106,107]及偶联^[108-112]等方面的研究报道刊载, 但是受Dox代谢时程影响, TetTag系统开放标记的时间窗比较大(1~2天), 致使TetTag不适用于背景活动高的脑区, 且TetTag目的基因表达的时程较短, 限制了它在神经集群长时程研究方面的应用。

1.3 活性类群靶向重组工具

2013年, Guenther等人^[54]通过活性类群靶向重组工具(targeted recombination in active populations,

TRAP)最早实现了活性神经集群的永久性标记。他们将CreER^{T2}敲入*c-fos*及*Arc*启动子的后面分别制备了FosTRAP和ArcTRAP小鼠, 通过结合Cre依赖的携带目的基因的小鼠或是病毒进行特定时间区间内活性神经集群的标记: 在自然情况下, 刺激活化的神经元生成的CreER^{T2}被锁定在细胞质, 当给予他莫昔芬(tamoxifen, TM)或其代谢产物4-OHT时, CreER^{T2}可解离出来进而转位入核介导重组, 使目的基因在活性神经集群里得以表达(图3)。

Guenther等人^[54]的测试表明TRAP小鼠具有很好的标记特异性, 且拥有比TetTag更小的标记时间窗(<12 h), 但是FosTRAP在部分皮层下区域的标记效率很低, ArcTRAP小鼠则在皮层-丘脑部分区域存在非活动依赖的背景泄露。Ye等人^[52]应用ArcTRAP小鼠标记了正面和负面刺激所激活的神经集群, 通过全脑对比分析, 筛选出了差异性响应负面刺激的脑区: 外侧缰核(lateral habenula, LHb)。

TRAP小鼠所采用的基因敲入策略存在对*c-fos*或*Arc*基因表达的干扰, Allen等人^[56]通过将*iCreER^{T2}*基因由2A连接敲入完整的*c-fos*基因的后面进一步开发了TRAP 2小鼠, 成功解决了TRAP小鼠存在的表达干扰问题, 显著提升了工具的信噪比。他们应用TRAP 2小

图 2 TetTag工具作用机制图解

Figure 2 Diagram of TetTag

图 3 TRAP工具作用机制图解

Figure 3 Diagram of TRAP

鼠解析了下丘脑正中视前核(median preoptic nucleus, MnPO)编码渴觉信息的神经集群并检测了这群神经元不同投射在渴觉信息编码中的作用。

此外Denny等人^[64]制备了同样基于CreER^{T2}重组标记的Arc-CreER^{T2} BAC转基因小鼠研究海马印迹对长期记忆的贡献。Root等人^[113]应用该转基因小鼠标记及激活了皮质杏仁核(cortical amygdala)响应不同类型气味分子的神经集群,复现了愉悦以及厌恶性气味所引发的吸引和回避的行为表现,为皮质杏仁核存在气味驱动的先天气行为神经编码提供了直接证据。

1.4 活化神经集群捕获工具

活化神经集群捕获工具(capturing activated neural ensembles, CANE)是Sakurai等人^[55]2016年报道的一项基于易降解受体dsTVA的活动依赖标记技术,由*c-fos-2A-dsTVA*基因敲入小鼠和鸟类肉瘤病毒包膜蛋白(EnvA)包裹的携带目的基因的慢病毒(lentivirus, LV)/狂犬病毒(rabies, RV)两部分组成。CANE通过EnvA与其改造后易降解的受体dsTVA的特异性结合来实现活性神经集群的标记:被刺激活化的细胞首先会表达出模拟内源c-fos代谢的dsTVA,而后dsTVA嵌入活化细胞的膜表面提供靶标,此时将EnvA包裹的LV/RV及时注入到目标脑区,EnvA-dsTVA结合使得刺激活化的细胞感染携带目的基因的RV/LV从而被捕获(图4)。

Sakurai等人^[55]应用CANE在腹内侧下丘脑腹外侧区(vMHvl)这一介导攻击行为的经典脑区捕获到一群社交恐惧激活的神经元,通过操纵这群神经元实现了社交恐惧的双向调控。vMHvl编码社交恐惧的神经元与攻击行为所激活的神经元在空间上交错分布,结合投射荧光标记和跨单突触逆向追踪技术,他们进一步解析出编码社交恐惧的vMHvl神经元存在高度离散但相互连结(recurrent)的环路结构。此外他们还对比分析了CANE分选标记的功能类群与经典的分子标记类群的共定位情况,发现雌激素受体1(Esr1)/类固醇生成因子-1(SF1)并不是理想的攻击/社交恐惧的分子标签。这一结果也表明大脑功能编码的复杂性,而利用活动依赖工具进行功能解析具有很大的应用前景。

相比于TetTag和TRAP工具, CANE具有诸多优点^[55],但由于需要在刺激1~2 h后及时将EnvA包裹的病毒注入目标脑区,动物麻醉及手术损伤都可能会引发无关的背景标记,提前置入给药导管如无线光流控

系统(wireless optofluidic systems)^[114]或许是可行的解决方案。

1.5 人工活性启动子工具

相比于遗传改造的实验动物,病毒工具具有更大的灵活性和适用性,目前已有多个c-fos启动的病毒工具被开发用于情绪编码^[49,52,115]、记忆印迹^[51,104]及睡眠相关^[50]的研究。为了进一步提升IEG启动子标记的效率和性能,人工活性启动子E-SARE和RAM被相继开发。

2013年, Kawashima等人^[66]通过将5个串联重复的SARE(Arc转录调控的重要元件)与含有TATA盒子的Arc mini融合生成了人工活性启动子E-SARE。E-SARE的表达效率及标记性能显著优于c-fos及SARE-Arc mini^[65]启动子。在体测试表明,其标记的特异性大于60%且外显率高于50%,利用桶状皮层在体单细胞电生理记录及初级视觉皮层双光子钙成像进一步验证E-SARE启动子的表达与特异的刺激输入高度相关。此外他们通过制备E-SARE启动的CreER^{T2}病毒实现了特定时间区间活性神经集群的永久性标记, Kim等人^[116]应用该病毒证明了mPFC存在一群投往NAc的神经集群可抑制风险状态下小鼠寻求奖赏的行为。

2016年, Sørensen等人^[57]改造合成了基于Fos mini的RAM(robust activity marker)启动子。通过将AP-1位点及Npas4结合基序等构成的增强子模块4次串联重复再与Fos mini连接即生成了仅199 bp的人工活性启动子P_{RAM}。荧光素酶活性测试的结果表明, P_{RAM}具有比E-SARE更高的信噪比。随后他们采用Tet-off系统来控制活性标记的时间窗,通过将P_{RAM}启动d2fTA及TRE启动效应蛋白表达的双启动子系统置于同一个AAV载体合成了强活性标记的RAM系统。他们利用该系统在海马及杏仁核实现了记忆印迹的标记,并将RAM系统应用于大鼠及果蝇,此外还开发了Cre依赖的CRAM系统用于细胞类型或是投射特异的活性神经集群标记,极大地体现了病毒工具的多功能性。但需要注意的是RAM工具的在体性能还需要更多的特异性及功能操纵方面的验证。

1.6 光控钙离子依赖工具

IEG表达作为神经元激活的间接表征被广泛应用,但是从神经元激活到IEG表达需要经历一系列细胞电

图 4 CANE工具作用机制图解

Figure 4 Diagram of CANE

化学活动^[117],使其时间分辨率和活动表征灵敏性远不足即时标记高速变化的神经系统.随着基因编码钙指示剂(genetically encoded calcium indicators, GECIs)^[118-120]的开发应用,胞质钙离子浓度变化成为了实时检测神经元活性的一大主流方法, Fosque等人^[72]率先开发了基于GECIs的钙调光激活比率集成器(calcium-modulated photoactivatable ratiometric integrator, CAMPARI)永久活性快门技术,通过短促的紫外照射使与钙离子结合变构后呈绿色荧光的EOS荧光蛋白转变成红色,从而实现了高时间空间分辨率的活性标记.由于诱导转化所用的紫外照射存在损伤影响且无法将操纵元件等载入活性神经元进行功能的因果解析, CAMPARI的应用受到限制.2017年, Lee等人^[74]开发的Cal-Light和Wang等人^[75]开发的FLARE填补了这一空白.这两个新工具都是基于蛋白互作系统Tango^[121,122]的模块式基因表达结构、GECIs的钙检测以及向光色素光电压结构域(LOVs)^[123]的光反应特性,实现了高时间空间分辨率的光控钙离子依赖的活性神经集群标记及目的基因的表达.

他们将钙调蛋白(calmodulin, CaM)和CaM结合肽M13/M2两个GECIs的组分替换了Tango系统中的GPCR和 β -arrestin,并将转录因子tTA通过烟草蚀刻病

毒蛋白酶体(TEV Protease, TEVp)的酶切序列与其中的一个组分连接栓系在细胞膜上,而将另一组分与TEVp融合,当胞内 Ca^{2+} 浓度升高时, CaM与 Ca^{2+} 结合引发构象变化从而募集M13/M2,使得两个分离的结构模块靠近,此时给予蓝光照射引发LOVs构象变化使得掩藏的TEVp酶切位点暴露出来,随后TEVp的酶切反应将tTA从膜上释放出来,入核启动目的基因的表达从而实现光控 Ca^{2+} 依赖的活性标记(图5).

Cal-Light和FLARE分别对不同的模块元件进行了改造优化,两个工具在离体培养神经元/脑片的测试中都表现出很好的光控钙离子(刺激)依赖的特性.在体测试方面, Wang等人^[75]使用FLARE标记了小鼠跑轮激活的运动皮层的神经集群, Lee等人^[74]则利用Cal-Light标记了小鼠压杆获取奖赏时所激活的初级运动皮层(M1)的神经集群,两个工具在不给光或是动物麻醉的情况下几乎都没有荧光标记,表明它们的在体标记同样具有很好的光及刺激共同依赖的特性.此外 Lee等人^[74]还通过在标记的M1神经元表达抑制性光敏感通道eNpHR进行了功能测试,当小鼠压杆时通过光遗传抑制标记的神经元显著降低了小鼠习得的压杆获取奖赏的行为,而在小鼠压杆之外的时间抑制这群神经元或是抑制小鼠走动所激活的神经元则不影响小鼠

图5 光控钙离子依赖新工具作用机制图解

Figure 5 Diagram of light- and Ca^{2+} -gated activity-dependent tools

的压杆行为, 表明Cal-Light的功能标记高度特异.

Cal-Light和FLARE的开发极大地拓展了活动依赖工具的时间精度和活动依赖特性, 但Wang等人^[75]表示FLARE的在体实验还存在一定的背景表达问题. 此外被标记的神经元需要同时感染三个不同结构元件的病毒致使诱导表达存在差异, 影响了光控钙离子依赖工具的稳定性, 有待进一步的改进和优化.

2 活动依赖工具应用概要

从1992年, Tom Curran和James I. Morgan开发第一个活动依赖工具小鼠c-fos-LacZ^[41]到最新的光控钙离子依赖新工具Cal-Light^[74]和FLARE^[75]的制备不过25年的时间, 但已有大量应用活动依赖工具解析记忆印迹和功能编码神经集群的工作发表. 活动依赖工具的应用涉及神经集群的标记、功能操纵、环路解析以及分子基础探究等, 以下进行简要概述.

2.1 多个活性神经集群标记

大脑的功能编码高度整合, 同一脑区通常可被多种不同的刺激所活化^[124-126], 同时标记多个活性神经集群是细胞水平鉴别不同刺激激活模式的基础. 自20世纪90年代末以来即有一系列基于IEG蛋白免疫组织化学染色(Immunohistochemistry, IHC)及mRNA荧光原位杂交(fluorescence *in situ* hybridization, FISH)的双刺

激标记方案报道: Chaudhuri等人^[127]利用Zif268的mRNA和蛋白质生成及代谢的时间差开发了I-FISH技术, 由此绘制了视觉皮层响应两次视觉刺激的活性图谱. Xiu等人^[128]将酪胺放大系统(tyramide signal amplification, TSA)引入I-FISH, 突破了I-FISH在部分脑区进行双刺激标记时信号难以分离的限制, 开发了TAI-FISH技术并成功绘制了边缘前脑的情绪效价图谱. 此外Guzowski等人^[129]利用mRNA随时间由细胞核转位到细胞质的定位变化特征开发了catFISH技术, 解析了两次空间探索经历激活的海马记忆印迹.

随着活动依赖工具的开发, 长时间标记活性神经集群逐渐被用于解析同一脑区不同的功能亚群. 通过活动依赖工具标记与IEG IHC结合的方法, Gore等人^[49]揭示了正面和负面刺激活化的BLA神经集群交错分布, Root等人^[113]则发现皮质杏仁核响应愉悦和厌恶性气味的神经集群相互分离. 这一双标方案的优势在于无需进行操作相对复杂的原位杂交实验且结果不受相继给予刺激可能存在干扰的影响, 但需要注意的是活动依赖工具本身的特异性及外显率都会影响结果的完整性和可信度.

除上述结合染色的方案之外, Okuyama等人^[99]将荧光计时蛋白(fluorescent timers) FT-Slow引入了活动依赖工具的标记系统, 利用FT-Slow的发色团随时间缓慢成熟由蓝变红的特点, 通过设置适当的刺激时间差直接实现了两次社交记忆印迹的双色荧光标记. 这种

基于同一系统的标记方案显著提升了活性标记的稳定性和可对比性. FT-Slow也可用于Cal-Light和FLARE系统进行双刺激标记以及检测工具的稳定性和特异性.

多神经集群标记应用最为出众的是CANE, Sakurai等人^[55]通过在同一只小鼠上依次注入两个不同的CANE病毒实现了社交恐惧和攻击行为激活的vMHvl神经集群的永久性双色标记, 除荧光标记之外, CANE还可将其他目的基因载入同一小鼠多个脑区不同的神经集群, 为系统解析和模拟多功能复杂编码提供了一种可能的方案.

2.2 活性神经集群环路解析

神经集群的功能依赖于其所在的神经环路和神经网络的活动来实现, 早期的研究通过将目标脑区IEG IHC或ISH与下游脑区注射逆行示踪剂(fluorogold, retrobeads, CTb)相结合的方法间接解析活性神经集群的投射^[130-132], 效率较低, 而活动依赖工具的应用则实现了活性神经集群环路结构的直接示踪. 除普通的荧光标记^[55]之外, 通过mGFP^[133,134]或EYFP-NRN^[52]可以更高效地显示出整个神经纤维, 而利用与小突触泡蛋白(synaptobrevin)^[135]或突触小泡蛋白(synaptophysin)^[136]融合的荧光蛋白则可更精确地标记投射末梢的突触形成位置, 此外基于组织透明技术及光切片荧光成像的BBAB, Scale, 3DISCO, ClearT, CLARITY, SeeDB, CUBIC, iDISCO, PACT/PARS, SWITCH等(详见综述^[137])以及基于断层成像技术的fMOST^[138,139], STP^[83,140]以及FAST^[141]等为重构神经集群全脑三维结构提供了可能. Ye等人^[52]利用CLARITY全脑成像揭示了mPFC响应正面和负面刺激的神经集群分别偏好投往NAc和LHb, 从环路水平解释了不同功能神经集群的差异所在, 为寻找功能相关的环路和靶点提供了参考.

除了解析投射输出, 通过结合RV逆向跨单突触追踪, 活性神经集群的输入表征也得以实现. Sakurai等人^[55]由此绘制了vMHvl编码社交恐惧的神经集群的单突触输入, Kitamura等人^[104]追踪了mPFC记忆印迹的信息输入源. 而减毒高效的RV病毒株CVS-N2cDG^[142]及自失活无毒性的RV-SiR^[143]的开发应用则进一步为操纵和监测跨单突触输入类群提供了可能, 极大丰富了功能神经网络及其信息流研究的工具库, 势必在未来功能神经集群的解析工作中大放异彩.

2.3 活性神经集群分子功能解析

功能的神经编码具有很强的时间和空间特异性, 以记忆编码为例, 海马CA1的光遗传急性抑制可以破坏远期记忆的提取, 然而通过早期损伤和药物阻断CA1的实验则没有观察到类似效应, 这可能是由于前扣带回(anterior cingulate, ACC)代偿了CA1的长时间失活^[144]; 另一项研究则表明, 选择性抑制DG记忆印迹会破坏相应记忆的提取^[64], 而无选择性泛抑制DG神经元并不会影响记忆的提取^[145].

记忆印迹和事件编码神经集群通常只占据功能核团很小的一群神经元(如DG记忆印迹仅占DG颗粒细胞的2%~4%^[96], 社交恐惧只激活不到3%的vMHvl神经元^[55]), 功能神经集群的稀疏分布表明活动依赖工具在解析神经编码方面具有重要意义.

如前所述, 利用活动依赖工具表达光遗传学、药理遗传学以及突触传递抑制元件为解析活性神经集群的功能编码提供了大量充分性和必要性证据. 此外活动依赖标记与在体电生理记录结合可用于揭示活性神经集群活动特性及其动态的功能编码. Tanaka等人^[146]利用ChR2标记DG记忆印迹, 通过Opto-Tagging证明DG记忆印迹是编码情景经历的细胞而非位置细胞, 故而人工激活DG记忆印迹可以实现相应情景记忆的提取^[96]. Trouche等人^[107]则通过ArchT标记海马CA1偶联可卡因的环境记忆印迹, 随后放回可卡因偶联的环境, 并在光遗传抑制印迹的情况下进行多通道记录, 他们发现CA1产生了编码这一环境的新的记忆印迹, 而原来与可卡因偶联的记忆也随之消除. 活动依赖工具还被用于突触可塑性方面的研究, Choi等人^[53]利用dual-eGRASP技术分别标记海马CA3印迹细胞的突触前和CA1印迹细胞的突触后, 通过结构和功能分析证实记忆形成过程中存在印迹细胞突触连接的增强.

2.4 活性神经集群分子基础检测

检测活性神经集群的分子基础对于寻找功能类群的分子标签、解析功能编码以及揭示功能异常引发的分子变化进而寻找药物干预靶点具有重要意义.

除Knight等人^[36]开发的PhosphoTRAP技术^[147,148]以及Wu等人^[149]开发的Act-seq技术之外, 活性神经集群分子基础的检测主要通过活动依赖工具与测序技术的联用实现. Ye等人^[52]通过活动依赖杂交小鼠使活性

神经集群的核糖体L10a亚基融合表达GFP, 随后应用翻译核糖体亲和纯化技术(translating ribosome affinity purification)检测了mPFC编码情绪的神经集群的分子特性; Kim等人^[115]则通过活动依赖病毒表达带Flag标签的RNA poly(A)结合蛋白Pabpc1与活性神经集群的mRNA结合, 随后利用Flag免疫沉淀反应(immunoprecipitation, IP)将活性神经集群的mRNA分离出来进行测序分析, 筛选出了BLA响应正面/负面刺激的神经集群存在的分子标记; 此外Allen等人^[56]通过流式细胞荧光分选术(fluorescence-activated cell sorting, FACS)将TRAP 2标记的MnPO编码渴觉信息的神经元(tdTomato⁺)分离进行单细胞测序, 解析了这群神经元的分子特性。

目前的测序工作几乎都集中于寻找功能类群的分子标签, 尚未涉及所得标签分子的功能检验, 随着活性神经集群分子基础研究的深入, 新的功能编码相关基因可能被发现。

3 总结与展望

应用IEG活动依赖工具目前已有一系列关于记忆印迹、感觉信息、情绪效价、行为及其异常的研究工

表 2 活动依赖工具性能比较

Table 2 Comparison of different activity-dependent tools

工具	优点	缺点
c-fos-LacZ	可通过将Daun02转化成绒毛霉素用于活性神经集群的失活	表征活性神经集群需要通过组织固定和染色反应, 无法进行活体研究
		报告蛋白 β -gal比较稳定使得标记的背景较高, 影响工具的特异性
		在丘脑等区域的特异性较低
		标记的时间短, 无法用于长时程研究, 无法载入其他目的蛋白
TetTag	可快速高效表达各类目的基因, 适合短期效应如近期记忆的解析研究	需要1~2天时间清除小鼠体内的Dox, 系统开放标记的时间窗比较大, 背景较高
	能够通过控制小鼠体内Dox的水平可逆性标记活性神经集群	不能用于活性神经集群永久性标记
		转基因小鼠品系不能完全模拟基因内源表达模式
TRAP	可用于活性神经集群永久性标记	系统开放标记的时间窗依赖TM/4-OHT的代谢, 开放标记的时间窗较大
	可表达各类目的基因	CreER ^{T2} 的诱导及标记的效率不高
	可以通过报告小鼠品系进行全脑活性神经集群系统标记	ArcTRAP在丘脑等区域存在背景泄露
		Arc-CreER ^{T2} 是转基因小鼠品系不能完全模拟基因内源表达模式
CANE		TRAP破坏了内源c-fos/Arc的表达
	模拟了内源c-fos的代谢, 活性标记的时间窗较短	需要定点注射CANE病毒, 无法进行全脑活性神经集群系统性标记
	可通过使用不同的CANE病毒标记以及操纵同一脑区的多个活性神经集群	小鼠刺激后的麻醉和手术可能引入背景, 对于实验员技术有一定的要求
	可高效表达各类目的基因	需要足够的时间达到目的基因的高水平表达, 不适于短程效应的研究
Cal-light/FLARE	可用于活性神经集群永久性标记	
	具有很高的活性标记时间空间分辨率	需要感染三个不同结构的病毒致使标记存在差异
	可快速高效表达各类目的基因	无法进行啮齿类全脑活性神经集群系统性标记
	具有病毒的灵活性和不同物种的适用性	FLARE在体标记存在背景表达
		FLARE缺乏在体功能操纵验证

作被报道,极大地丰富了人们对大脑功能编码的认识. 这些工具无疑将在后续的功能解析中继续发挥重要作用,与此同时也需要认识到基于IEG的活动依赖工具存在一定的缺陷: IEG的表达需要神经元激活超过一定的阈值,且在不同类型的细胞中存在差异^[33,150,151],某些脑区(如黑质(substantia nigra))的神经元即使存在很强的电生理活动也不表达c-fos^[152~154],使得IEG表征的活性神经集群并不完整,限制了IEG活动依赖工具的应用. 此外TetTag系统中Dox的清除速率以及TRAP工具中TM/4-OHT的代谢速率都会影响工具的标记时间窗和效率,不同的活动依赖工具各有其优缺点(表2)^[38,55,150],开发更加高效而特异的神经元活动依赖标记方案依然是解析功能神经集群领域重要的方向.

tTA和CreER^{T2}以及dsTVA的引入使活动依赖工具条件性表达目的基因得以实现,而拥有比CreER^{T2}更低背景泄漏的Cre*PR^[155]以及融合了降解结构域通过药

物诱导稳定的DD-Cre^[156]为优化活动依赖工具标记时间窗提供了备选元件;光控钙离子依赖工具^[74,75]及电压敏感指示剂^[157]为高时间空间分辨率及活性特异的工具开发指示了新方向;而基于钙成像的闭环光遗传(closed-loop optogenetics)^[158,159]和双光子全息光遗传(holographic optogenetics)^[160,161]为系统模拟功能神经集群的生理活动提供了可能.

此外,目前开发的活动依赖工具都是基于神经元的激活,但这只是功能编码图式的一部分,抑制在功能编码中同样发挥着重要作用,开发抑制类群的标记工具无疑也是重要的方向.

单细胞水平在体成像监测神经集群活动^[162,163]与高分辨率活动依赖工具操纵神经集群活性的结合,为破译认知与意识、智力与情感、记忆与社会行为等复杂生物学功能编码开辟了道路,激发着一批又一批神经科学研究者探索人类对大脑的认知边缘.

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Research progress and prospect of neural activity-dependent tools

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Despite existing in large numbers with great heterogeneity, neurons form activity clusters in response to specific stimulus inputs. These activity clusters, also called engrams, are functional units that encode information at the cell and circuitry levels. Cell activation generally induces elevated CREB level, IEG expression and Ca^{2+} influx, from which a variety of activity-dependent tools have been developed. These tools are widely used in memory engram and neural ensemble research. In this article, we review the principles and applications of these activity-dependent tools, including LacZ-Daun02 inactivation system, CREB overexpression system, TetTag, TRAP, CANE, two synthetic activity-regulated promoters: E-SARE and RAM, and two calcium- and light- gated tools: Cal-light and FLARE. We also discuss the future development of activity-dependent tools.

activity-dependent tool, memory engram, neural ensemble, neural encoding, IEG

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