lineage miR-150, miR-155, miR-221, and miR-222 are progressively down-regulated, with up-regulation of miR-451 and miR-16 occurring during the late phase of human erythropoiesis in *in vitro* study [89].

REPROGRAMMING FOR PLURIPOTENCY

Cell differentiation has been depicted as a ball rolling down an epigenetic landscape [90], starting from totipotency, moving through pluripotency, and finally reaching lineage-committed states. In the last three years, multiple studies have reported that the "ball" can actually be pushed back up the hill (Fig. 19.4): several types of differentiated cells have been shown to be reprogrammable back to the pluripotent state under the influence of a few factors such as Oct4, Sox2, Klf4, Myc, Nanog, and Lin-28 [91–101]. Such reprogrammed cells, which have been called induced pluripotent stem (iPS) cells [91], are similar to ESCs in terms of their morphology, expression of major ESC marker genes, and capacity to self-renew and to differentiate into various cell types of the three germ layers.

Induced pluripotency in iPS cells was shown to be caused by changes in epigenetic modification of the treated cells. The promoter regions of various pluripotency-associated genes are hypermethylated in differentiated cells, but in iPS cells these genes are hypomethylated, resembling their state in ESCs [102]. How the above-mentioned inducing factors can trigger the demethylation of pluripotency genes remains elusive, because it is unclear whether they possess direct or indirect DNA demethylation activity. Interestingly, nevertheless, generation of iPS cells can be promoted by demethylating agents such as 5-azacytidine [103], underlining the importance of the DNA demethylation process in mediating induced pluripotency.

Bivalent methylation marks on histone H3 are also re-established at the promoter regions in iPS cells [103,104]. Both ESCs and iPS cells have H3K4me3 in the promoter regions of pluripotency-associated genes, while both active H3K4me3 and repressive H3K27me3 are present at their differentiation-associated genes [62] (Fig. 19.3). Because differentiated cells such as mouse fibroblasts have the opposite histone methylation pattern [104], it is very likely that histone methylation also plays a role in reprogramming.

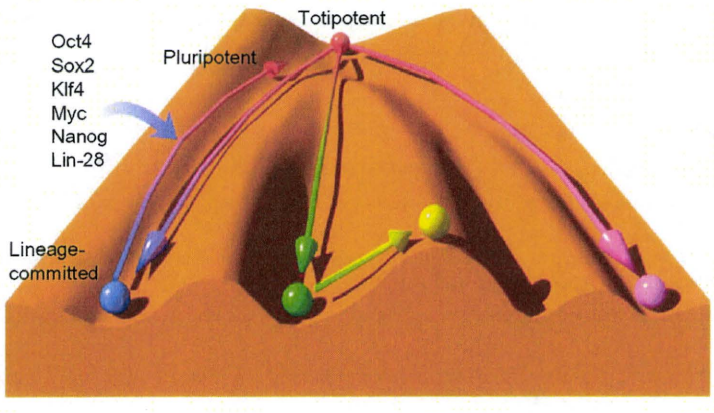

FIGURE 19.4

Epigenetic landscape. The totipotent fertilized egg can be depicted as a red ball that can roll down one of several possible valleys, passing through the pluripotent state and then differentiating into a particular tissue-lineage cell (blue, green, and pink balls). Reprogramming factors can push the ball back up the hill, enabling it to re-acquire pluripotency features. Lineage-committed cells can also trans-differentiate into cells of another lineage (yellow ball) by other epigenetic reprogrammings. The diagram is modified from Waddington [90].

In both ESCs and iPS cells, histones H3 and H4 at the promoter regions of pluripotency-associated genes are hyperacetylated, whereas these promoters in differentiated cells display hypoacetylated H3 and H4. Addition of the HDAC inhibitor VPA can lead to a hyperacetylated histone status, and has proved to be efficient in inducing pluripotency of human fibroblasts, with only Oct4 and Sox2 required as necessary supplemental factors [105]. Other factors may be dispensable under these conditions if any role they play in increasing acetylation by recruiting HATs, a known function in the case of c-Myc [106], can be substituted by VPA.

CONCLUDING REMARKS

Revealing the epigenetic mechanisms that contribute to stem cell potency and differentiation has been an exciting journey. Still, many avenues remain to be explored. For example, several HDAC inhibitors and DNA demethylating agents such as VPA and 5-azacytidine are now in clinical trials for therapeutic application to several disorders and diseases [107,108]. While HDAC inhibitors or DNA demethylating agents might be expected to affect a broad range of genes or their activator/repressor complexes, they actually do not. This differential effect must be attributable to characteristics that the affected genes alone possess. Therefore, the knowledge of how these compounds relieve or cure disorder and disease by changing epigenetic marks is highly important. It will be also interesting to explore the effect of disruption of certain miRNAs in order to generate iPS cells, because recent findings show that RNA binding protein Lin28 can promote reprogramming by selective inhibition of miRNA maturation machinery [109,110]. We also have to evaluate carefully the various origins of cells and induction methods available for stem cell differentiation in order to generate specific cell phenotypes that can be used for clinical applications. To develop an optimal method to generate specific cell types, we must urgently learn more about the precise mechanisms of stem cell fate specification.

ACKNOWLEDGEMENTS

We apologize to colleagues whose work, although relevant, we may not have included in this review due to space constraints. We thank our laboratory members for useful discussions on this topic, and Drs. Ian Smith and Siripong Thitamadee for critical reading of the manuscript. We have been supported by a Grant-in-Aid for Scientific Research in priority areas, the NAIST Global COE Program (Frontier Biosciences: Strategies for Survival and Adaptation in a Changing Global Environment), the Nakajima Foundation, and the Uehara Memorial Foundation.

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トランスポートソームの世界

―膜輸送研究の源流から未来へ―

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編著

文部科学省科学研究費補助金　特定領域研究
「生体膜トランスポートソームの分子構造と生理機能」
（平成17年度～平成21年度）

京都廣川書店
KYOTO HIROKAWA

濃度の増加やCa^{2+}シグナルの亢進を引き起こすものと考えられた．興味深いことに，現在開発されているNCX阻害薬は，このCa^{2+}流入モードを選択的に阻害することが知られている[1]．そこで，NCX阻害薬は生理的なCa^{2+}汲み出し機能に影響を与えず，病態時のCa^{2+}流入を阻害する理想的な薬物になる可能性を秘めている．NCX阻害薬は，Ca^{2+}拮抗薬に続く新たなCa^{2+}調節薬として今後の臨床応用が期待される．

（4-2-5　岩本隆宏，喜多紗斗美）

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4-2-6 グルタミン酸性シナプス小胞のトランスポートソーム

(1) はじめに

神経間あるいは神経-効果器間のシグナル伝達は，シナプスと呼ばれる微小な細胞接着部位で行われる．神経終末には，シナプス小胞と呼ばれる直径約40 nmのオルガネラが多数存在し，内腔に神経伝達物質が濃縮されている．神経終末に活動電位が到達し，電位依存性カルシウムチャネルが開口すると，流入したカルシウムイオンによって小胞膜と形質膜の膜融合（＝エキソサイトーシス）が促され，神経伝達物質が細胞外に放出される．放出された神経伝達物質が隣接した細胞の受容体に結合することで，様々なシグナルが伝搬される．膜融合により，形質膜に挿入されたシナプス小胞は，エンドサイトーシスにより再び神経終末で合成され，神経伝達物質が再充填されることで，次の刺激に備える．従って，シナプス伝達が様々な強度の刺激に対応して維持されるためには，新たに合成されたシナプス小胞に神経伝達物質を再充填するシステムが必須であり，この過程を司るのが「小胞型神経伝達物質トランスポーター」である．本稿では，哺乳類中枢神経系で最も主要な興奮性伝達物質であるグルタミン酸を小胞内に輸送する小胞型グル

タミン酸トランスポーター（VGLUT：Vesicular Glutamate Transporter）に焦点を絞り，トランスポーターの特性と生理学的意義，VGLUT を取り囲む「トランスポートソーム」に関する最近の知見を概説する．

(2) グルタミン酸のシナプス小胞への取込活性

グルタミン酸は，生体内の全ての細胞内に比較的高濃度で存在する酸性アミノ酸である．それにも関わらず，哺乳類脳神経系においては，最も主要な興奮性神経伝達物質としてシグナル伝達を司り，知覚・認知・学習，記憶といった脳高次機能を支えている．特定の神経細胞のみがグルタミン酸をエキソサイトーシスによって放出する為には，シナプス小胞内腔にグルタミン酸を濃縮するための特別なシステムを備えていなければならない．脳から精製したシナプス小胞へのグルタミン酸取込活性は，1980 年代になってトリチウム標識されたグルタミン酸を用いた生化学

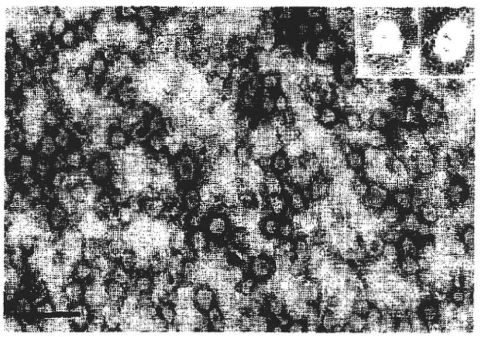

図 4-2-6a　ラット脳から精製したシナプス小胞の電子顕微鏡像（Scale bar, 100 nm）．

V-ATPase 依存的なグルタミン酸取込活性を保持している．

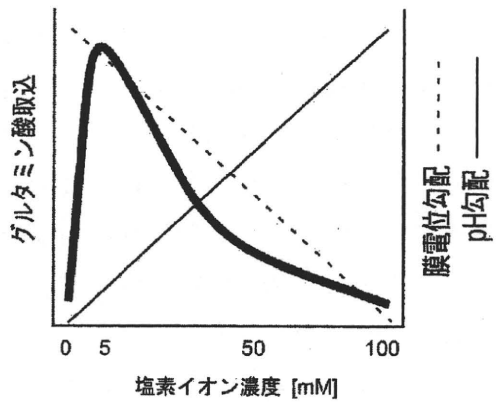

図 4-2-6b　グルタミン酸輸送の Cl-依存性

脳から精製されたシナプス小胞へのグルタミン酸輸送は，Cl-濃度に対して二層性の依存性を示す．また，V-ATPase によって形成されるプロトン電気化学勾配は，小胞膜の Cl-チャネルによって，電気的勾配（膜電位勾配，点線）と化学的勾配（pH 勾配，実線）の成分が変化する．

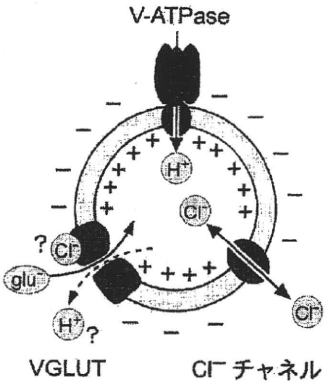

図 4-2-6c　グルタミン酸取込に関わる3つの成分

シナプス小胞へのグルタミン酸輸送は，プロトンポンプ（V-ATPase），トランスポーター（VGLUT），Cl-チャネルの3つの活性によって制御されている．

的取込アッセイによって初めて検出された[1]（図 4-2-6a）．シナプス小胞画分を用いた一連の生化学的な輸送活性測定から，グルタミン酸のシナプス小胞への取込は，液胞型プロトン ATPase（V-ATPase）が形成するプロトン電気化学勾配に依存した二次輸送であること，グルタミン酸に特異的な輸送であることに加えて，Cl-に対して特徴的な二層性の依存性を示すことが判明した[2]（図 4-2-6b）．また，シナプス小胞膜が Cl-を透過する性質が持っていることが，pH 勾配感受性蛍光色素を用いた実験から明らかになった．これらの実験を総合的に評価すると，シナプス小胞のグルタミン酸輸送系には，V-ATPase・小胞型グルタミン酸トランスポーター（VGLUT）・Cl-チャネルの3つのコンポーネントが必須であることが提唱された（図 4-2-6c）．

(3) 小胞型グルタミン酸トランスポーターの分子同定

長らく探し求められていた VGLUT の分子実体は，2000 年になって明らかになった．意外なことに，VGLUT の正体は，Na^+濃度勾配を駆動力として無機リン酸を細胞内に輸送する遺伝子（BNPI；Brain–specific Na^+-dependent inorganic Phosphate Transporter I）として既にクローニングされていた別の遺伝子産物であった[3]．当時の遺伝子データベースを用いたホモロジー検索から，BNPI と最も相同性の高い遺伝子はウサギ腎臓に発現する形質膜型リン酸トランスポーターであり，事実，BNPI の mRNA をアフリカツメガエル卵母細胞に発現させると Na^+依存的なリン酸輸送活性が顕著に上昇した[3]．ところが，BNPI タンパク質の脳内局在を詳しく調べると，形質膜よりもむしろシナプス小胞膜に局在しており，しかもグルタミン酸作動性ニューロンの神経終末に限定して発現していることが明らかになった[4,5]．また，線虫における BNPI 相同遺伝子 EAT-4 の機能解析から，EAT-4 変異体はグルタミン酸シナプス伝達の欠陥を呈するものの，グルタミン酸受容体の機能は損なわれていないことがわかった[6]．これらのヒントを元に，筆者らと UCSF の Robert Edwards のグループは，BNPI がシナプス小胞上でグルタミン酸の取込に関わる可能性を様々な観点から検討した結果，BNPI は小胞上でグルタミン酸トランスポーターとし

て働き，神経細胞がグルタミン酸を放出する為に必要不可欠なタンパク質であることを見いだし，VGLUT1（Vesicular glutamate transporter 1）と改名した[5,7]．更に，Edwards のグループは，VGLUT1 タンパク質が Cl-透過性を示す可能性を示した[7]．VGLUT が属する Solute carrier protein（SLC）17 ファミリーのメンバーである SLC17A1 や SLC17A3 に関しても，Cl-の透過性を強く示唆する報告があり[8,9]，Cl-による VGLUT の活性調節機構に対する関心が高まっている（後述）．

VGLUT1 の分子同定当初から，哺乳類脳では幾つかの VGLUT アイソフォームの存在が考えられた．VGLUT1 は大脳皮質・海馬で強く発現しているが，VGLUT1 の発現が認められない視床や間脳部にもグルタミン酸作動性シナプスが存在することは自明だったからである．実際，VGLUT1 に非常に高いアミノ酸相同性を示す遺伝子が次々と同定され，それぞれ VGLUT2, VGLUT3 と命名された．興味深いことに，VGLUT のアイソフォームは，（一部の例外を除いて）脳内で異なるニューロンに発現していることが明らかになっており，今日では部位特異的ノックアウトマウスの開発に基づいた神経回路の生理機能の解明が進み脳神経科学分野の発展に寄与している[10]．

(4) 小胞型グルタミン酸トランスポーターに関わるトランスポートソーム　その存在と分子実体

(4-1) 軸索輸送に関わるトランスポートソーム

VGLUT に限らず，シナプス小胞に局在するタンパク質が選択的に軸索を移動し，最終的に神経終末に到達するメカニズムは，殆ど分かっていないのが現状である．シナプス小胞の膜タンパク質は，細胞体で合成され，ER・ゴルジ装置の経路を経てから軸索を移行する．電子顕微鏡で軸索内をくまなく観察してもシナプス小胞と同等な大きさの小胞構造が見られないことから，シナプス小胞の膜タンパク質は何らかの Precursor 小胞に搭載されて軸索側に運搬されるとする説が有力である．興味深いことに，同じく軸索を通って神経終末に運ばれる Piccolo, Bassoon 等のアクティブゾーンを形成する足場タンパク質群は，直径 80 nm 程の有芯顆粒に搭載されて運ばれているが，その顆粒にはシナプス小胞膜タンパク質は存在しないことが報告された[11]．つまり，シナプス小胞の膜タンパク質はアクティブゾーンタンパク質とは異なる Precursor 小胞によって神経終末に運ばれると考えられる．また，シナプス小胞膜タンパク質の中でも，異なる軸索輸送のモータータンパク質を用いる Precursor 小胞が存在することが示唆されている．このように，個々のシナプス小胞タンパク質がそれぞれの Precursor 小胞によって運搬される過程で，何らかのタンパク質間相互作用やタンパク質-脂質相互作用が関与していることが考えられるが，相互認識を司るシグナル配列は明らかになっておらず，どのような「トランスポートソーム」が関与しているかは，今後の研究課題である．哺乳類の 3 つの VGLUT アイソフォームの内，VGLUT3 のみが神経終末のみならず，細胞体や樹状突起にも存在することは，VGLUT アイソフォームの選択的輸送のメカニズムを解明する上で興味深い[12]．

(4-2) 軸索終末におけるトランスポートソーム

Precursor小胞は神経終末に到達すると形質膜に融合し，運搬されたVGLUTをはじめとした小胞膜タンパク質は，一旦形質膜に挿入される．ここから，エンドサイトーシスによって合成されるシナプス小胞に選択的に挿入されることになる．VGLUTの3つのアイソフォームは，シナプス小胞タンパク質であるSynaptotagmin 1や他の小胞型神経伝達物質トランスポーター(VMAT2, VAChT）と同様，細胞質側に露出したC末端領域にdi-leucine様のモチーフを有している[13]（図4-2-6d）．この配列は，AP2に代表されるアダプタータンパク質に特異的に認識されることで，形質膜から作られるクラスリン被覆小胞への膜タンパク質の挿入を促すといわれている．実際，VGLUT1タンパク質のC末端にあるFVをアミノ酸置換した変異体は，エキソサイトーシスの後，エンドサイトーシスされる小胞への挿入効率が低く，形質膜に留まる[13]．一方，VGLUT1のシナプス小胞膜における発現量は，概日リズムと相関して6時間おきに変動することが報告された[14]．この変動は，時計遺伝子であるPer2欠損マウスでは見られず，また他のシナプス小胞タンパク質では見られないことから，VGLUT1特異的な形質膜—シナプス小胞間の分配機構の存在が想起されるが，その制御機構は不明である．

VGLUT1特有の輸送機構として知られているのが，エンドサイトーシス関連タンパク質であるエンドフィリンとの直接相互作用である[13]．VGLUT1のC末端にはdi-leucine様モチーフの他に，Proline-rich domain（PRD）が2つ存在する．この部分をベイトにしたYeast Two Hybridス

```
rVGLUT1     EKQPWAEPEEMSEEKCGFVGHDQLAGSDESEMEDEVE    529
rVGLUT2     EKQPWADPEETSEEKCGFIHEDEL--DEETGDITQNY    535
rVGLUT3     EKQDWAKPENLSEEKCGIIDQDELA--EETELNHEAF    539
rVMAT2      PLCFFLRSPPAKEEKMAILMDHNCPIKTKMYTQNNVQ    602
rVAChT      LRNVGLLTRSRRSERDVLLDEPPQGLYDAVRLREVQG    605
rVAMP2      SNRRLQQTQAQVDEVVDIMRVNVDKVLERDKLSELDD     65
mSYT1       RPIAQWHTLQVEEEVDAMLAVKK                  421
```

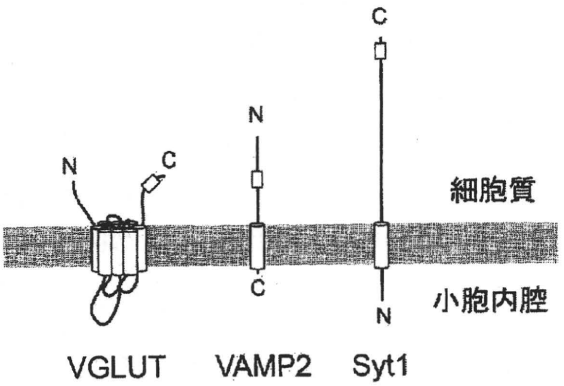

図4-2-6d　VGLUTの3つアイソフォームは，細胞質側のC末端部位にdi-leucine様モチーフを持つ（下線）

これらの配列は，他のシナプス小胞膜タンパク質であるVAMP2やシナプトタグミン1（Syt1）でも見られ，エンドサイトーシスを司るアダプタータンパク質（AP-2）と直接相互作用することによって，クラスリン被覆小胞への挿入を促す．

クリーニングの結果，Src homology 3 domain（SH3 domain）を持つエンドフィリンが見いだされた．VGLUT2/3 は PRD をもたず，エンドフィリンとの結合能を持たない．エンドフィリン結合能を欠損した VGLUT1 変異体を海馬培養細胞に発現させ，持続的な頻回刺激を与えると，エンドサイトーシスされた小胞に VGLUT1 変異体が取り込まれる速度が低下した．この実験結果は，異なる VGLUT アイソフォームを発現シナプスにおいて，神経活動依存的にグルタミン酸取込速度が異なる可能性を示唆している．

(4-3) 輸送活性に関わるトランスポートソーム

VGLUT のグルタミン酸輸送活性を調節するメカニズムの詳細は明らかになっていない．一方，前述の様に VGLUT は V-ATPase が形成するプロトン電気化学勾配に依存した二次輸送であり，シナプス小胞を用いた研究から，Cl-との何らかの機能的な相互作用が提唱されてきた．本項目では，VGLUT の輸送活性を制御する 2 つの異なるメカニズムについて紹介する．

(4-3-1) Cl-との機能的カップリング

グルタミン酸のシナプス小胞への取込は，小胞外の Cl-濃度に対して二層性の依存性を示すことが古くから知られていた（図 4-2-6b）．また，三量体 G タンパク質の構成成分である $G\alpha o_2$ を欠損したマウスから得られたシナプス小胞を用いた実験から，Cl-の二層性効果には $G\alpha o_2$ の活性化が関与していることが示唆された[15]．しかしながら，それらの分子メカニズムに関しては諸説あり，現在に至っても混沌としている．問題の本質は，Cl-の作用点はどこか？ にある．VGLUT の同定により，VGLUT と輸送の駆動力を供給するプロトンポンプをリポソームに再構

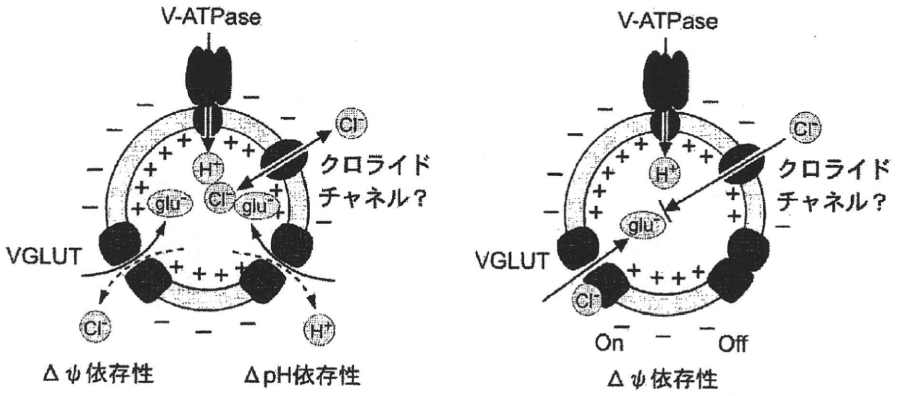

図 4-2-6e　グルタミン酸取込における Cl-依存性の 2 つのモデル

左）VGLUT が膜電位勾配と pH 勾配の双方を駆動力とするモデル．VGLUT が Cl-透過性を有している．小胞外の Cl-は V-ATPase と共役して pH 勾配を作ることにより pH 勾配依存性のグルタミン酸 /H^+ 交換輸送を行う．それに対して，小胞内に Cl-が存在する場合は，膜電位依存性のグルタミン酸 /Cl-交換体として働く．右）VGLUT が膜電位のみを駆動力とするモデル．この場合，小胞外の Cl-は VGLUT に直接結合し活性化する役割と，分子未同定の Cl-チャネルを通じて流入することで，駆動力である膜電位勾配を低下させる役割の 2 つを担う．

成することが可能になり，筆者らはVGLUT1タンパク質自身がCl-を透過することを見いだした[16]．これは，前述したEdwardsらの実験結果と一致する[7]．興味深いことに，内腔に高濃度のCl-を含むVGLUT1リポソームを作成すると，小胞外にCl-が無い条件においても高い‘膜電位に依存した’グルタミン酸輸送活性が検出された．この実験結果は，VGLUT1がグルタミン酸/Cl-交換体として働きうることを示している[16]．シナプス小胞がエンドサイトーシスにより形質膜から合成される時，小胞内には，高濃度のCl^-を含む細胞外液が流入すると考えられる．従って，筆者らの実験結果は，シナプス小胞内のグルタミン酸量を規定する因子として，細胞外液中のCl-濃度が重要な働きをする可能性を示唆している（図4-2-6e左）．一方で，人工的に膜電位のみ形成させる再構成実験系では，上記の実験結果とは異なり，小胞内のCl-の濃度はグルタミン酸輸送に無関係であることが報告された．この実験で採用された一過性の膜電位形成に依存したグルタミン酸取込測定では，シナプス小胞で見られる高濃度の小胞外Cl^-によるグルタミン酸輸送阻害が見られないことから，Cl-の作用はVGLUTタンパク質への直接結合によるアロステリック効果であると提唱された[17]（図4-2-6e右）．同じリポソームの再構成実験系ではあるが，検出されたグルタミン酸輸送活性は大きく異なっており，シナプス小胞で実際に起きている反応を理解するには，更なる実験系の改良が必要であろう．

(4-3-2) 異種トランスポーターとのシナジー効果

VGLUTの同定により，グルタミン酸作動性神経回路の解剖学的解析が容易になり，哺乳類中枢神経系におけるグルタミン酸放出部位が可視化できるようになった．これら一連の組織化学的解析から，それまで他の神経伝達物質を放出していると考えられていたシナプスの一部でもVGLUTの発現が認められ，グルタミン酸と他の神経伝達物質が共放出されている可能性が示唆された（ドーパミン作動性シナプスにおけるVGLUT2の発現，コリン作動性シナプスにおけるVGLUT3の発現等）．興味深いことに，VGLUT3欠損マウスやドーパミン作動性ニューロン特異

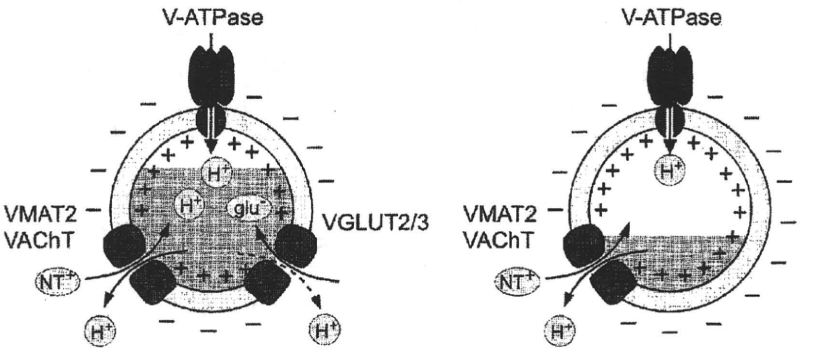

図4-2-6f　異種トランスポーターとのシナジー効果

VGLUTを共発現するモノアミン作動性シナプスやコリン作動性シナプスでは，グルタミン酸取込に伴う小胞内酸性化によってpH勾配が増大し，VGLUTが共発現していないシナプス小胞（右）に比べて，モノアミンやアセチルコリンの取込量が増大する（左）．

的 VGLUT2 欠損マウスの解析結果から，本来共局在している VGLUT が欠損すると，ドーパミンやアセチルコリンの小胞内含有量が低下することが分かった[18, 19]．ドーパミンやアセチルコリンは，溶液中で正の電荷を持つ神経伝達物質であり，それらを取り込むトランスポーターである VMAT2, VAChT は，基質と H^+ との交換輸送を司るため，小胞内外の pH 勾配を利用して小胞内に取り込まれる．一方，VGLUT を介したグルタミン酸輸送は，小胞内の pH を低下させる．従って，VMAT2 や VAChT を含む小胞上に VGLUT（2/3）が存在することでグルタミン酸が取り込まれると，ドーパミンやアセチルコリンの輸送の駆動力である pH 勾配が増大することで，ドーパミン・アセチルコリンの取込量が亢進すると考えられる（図 4-2-6f）．この新しい機構は，異種トランスポーター間の「シナジー効果」と呼ばれ，その生理学的意義や脳高次機能への寄与が注目されている．

(5) 展望

VGLUT の分子同定が達成されてから 10 年余りの間に，グルタミン酸神経回路の神経解剖学的理解が急速に進んだだけでなく，VGLUT タンパク質の輸送体としての構造-機能連関や VGLUT の局在や活性を調節する様々なトランスポートソームの存在が示唆されてきた．今後は，それらトランスポートソームの分子実体の解明や詳細な分子メカニズムの理解が深まっていくだろう．また，トランスポーター自体の作用機序に関してもまだまだ不明な点が多く，*in vitro* の実験結果から導かれたモデルを *in vivo* で再検証する流れの研究が重要になってくると思われる．

（4-2-6　高森茂雄）

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4-2-7 胃酸分泌細胞のトランスポートソーム

(1) 胃酸分泌に関与するイオン輸送タンパク質

胃酸は，胃粘膜の胃腺に存在する胃酸分泌細胞より分泌される．胃酸分泌細胞は，酸分泌時に劇的な形態変化をおこす．休止時に細胞内に多数存在する細管小胞が，アピカル膜と連結することで，大量の胃酸が分泌される（図 4-2-7a）．胃酸分泌細胞は，酸分泌に必要な ATP を大量に合成する必要があるため，細胞内に多数のミトコンドリアが存在している．その数はどの上皮細胞よりも多い．

胃プロトンポンプ（H^+, K^+-ATPase）は，サブユニット構造をとり，触媒サブユニット（α 鎖）と非触媒サブユニット（β 鎖）からなる．P 型 ATPase ファミリーに属し，ATP の加水分解の際に生成する無機リン酸を直接結合した中間体を一時的に形成する．H^+, K^+-ATPase は，細胞膜を隔てた 100 万倍以上ものプロトン濃度勾配に逆らって H^+ を胃管腔（pH は約 1）へ分泌する．我々は最近，H^+, K^+-ATPase のプロトン輸送に関与する電荷移動路を明らかにした[1]．

胃酸（HCl）の H^+ 輸送が H^+, K^+-ATPase により行われていることはよく知られている一方で，Cl^- 輸送を担う分子については種々議論されている．これまでに CFTR[2]，SLC26A9[3]，CLIC-6[4] などの Cl^- チャネルが候補として報告されている．また，H^+, K^+-ATPase が活性化され H^+ の分泌を維持するには，細胞内への K^+ の安定な供給が必要不可欠である．これまでに管腔側膜におけるこの K^+ 供給実体について，KCNQ1/KCNE2[5] や Kir4.1[6] などの K^+ チャネルが報告されている．

K^+-Cl^- 共輸送体（KCC）は，K^+ と Cl^- を細胞外に共輸送する二次性能動輸送体であり，種々の上皮細胞において細胞内 Cl^- 濃度調節や細胞容積調節などに関与している．KCC にはこれま

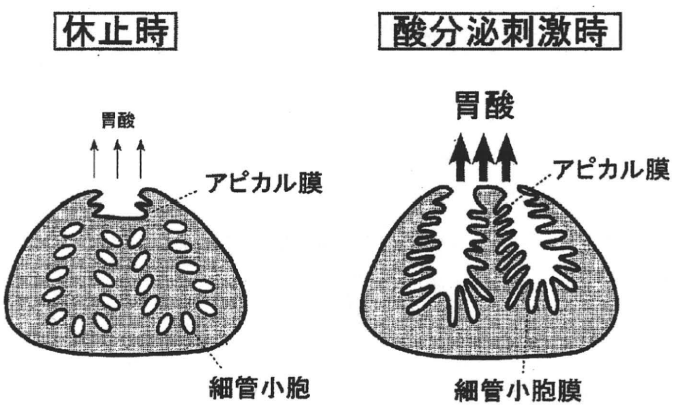

図 4-2-7a　休止時および酸分泌刺激時の胃酸分泌細胞の形態

Behavioural Brain Research 213 (2010) 126–129

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Short communication

Strain differences of selective attention in mice: Effect of Kamin blocking on classical fear conditioning

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ARTICLE INFO

Article history:
Received 6 January 2010
Received in revised form 20 April 2010
Accepted 23 April 2010
Available online 29 April 2010

Keywords:
Kamin blocking effect
Classical fear conditioning
Contextual conditioning
Selective attention
Strain difference
Schizophrenia

ABSTRACT

Selective attention was assessed in mice using a classical fear conditioning procedure. Mice were trained by three regimens involving contextual and/or cued conditioning (classical fear conditioning). Most notably, C57BL/6J mice exhibited salient contextual blocking, whereas DBA/2J and ICR mice did not. This study provides a new method to assess the selective attention of mice and describes an animal model for certain human mental disorders, such as schizophrenia.

In traditional learning theory, it has been proposed that after an initial conditioned stimulus (CS1) is learned to predict an outcome, a second conditioned stimulus (CS2) presented in combination with CS1 that leads to the same outcome cannot be learned to predict the outcome completely. This phenomenon is called blocking (Kamin blocking effect) [11]. The principle of blocking provides a model for investigating the selective attention process in animals and humans [6,11,12]. Selective attention is one of the principle mechanisms for information processing, especially with respect to how an organism obtains the information it needs (e.g., cocktail party effect) [3]. Disruption of attention leads to the breakdown of normal information processing, and causes some mental disorders such as schizophrenia in humans [1,13]. Some studies have reported schizophrenia patients that exhibited a disrupted blocking effect [2,7,9,10,15,16]. Therefore, blocking protocols provide not only useful tools for screening humans for schizophrenia, but also unique animal models for studying mental disorders.

One of the most basic procedures for studying the blocking effect uses the conditioned emotional response (CER) in rats [11,17,18,22]. In CER experiments, rats are first trained an instrumental behavior such as lever pressing (reinforced by a reward such as a food pellet) or licking (reinforced by a reward such as water). When they have acquired the desired behavior, the rats are trained by classical fear conditioning using a tone or light as a conditioned stimulus (CS) and an electric foot shock as an unconditioned stimulus (US). In the test trial, the rats are again exposed to the instrumental and/or appetitive behavior situation and eventually the CS. During CS presentation, rate of lever pressing or licking is suppressed by the "memory of fear experience". In the classical fear conditioning phase of a blocking study, the first CS (e.g., light) is conditioned to foot shocks, and the second CS (e.g., tone) is conditioned to foot shocks with light. During the subsequent training phase, the rats suppress their lever pressing or licking when a light CS is presented, but do not suppress their behavior when a tone CS is presented.

Although this procedure is simple and the results are reproducible, a long training period is required for instrumental conditioning. Furthermore, this procedure is quite difficult to adapt for mice, which do not press levers, poke holes or lick water as frequently as rats. This presents a serious limitation, because animal models for mental disorders have been shifting from rats to genetically modified mice, such as transgenic (TG) and knockout (KO) mice [5,24,25]. Moreover, as screening of genetically modified mice often relies on high-throughput procedures, more simple procedures for blocking experiments are needed.

Another procedure used to assess attention mechanisms in mice is latent inhibition, in which mice are exposed to a CS without a prior US conditioning trial [14,20]. This method is very simple and is frequently used for phenotyping genetically engineered mice. Although, the "memory" that inhibits the second conditioning in a

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doi:10.1016/j.bbr.2010.04.037

Table 1
Summary of conditioning schedule.

	Conditioning 1	Conditioning 2	Test
Group CN	No treatment	Context + noise	Noise
Group C-CN	Context	Context + noise	Noise
Group CC	Context	Context	Noise
Group LN	No treatment	Light + noise	Noise
Group L-LN	Light	Light + noise	Noise
Group LL	Light	Light	Noise

Note: Noise was 70 dB of broadband white noise. Light was supplied by white LED (150 lx).

blocking experiment is explicit, the "memory" that inhibits the later CS-US learning in latent inhibition is implicit. This fact suggests that blocking and latent inhibition may reflect different neuronal mechanisms. The aim of this study was to develop a simple procedure for examining the blocking effect in mice, and to evaluate the performance of this procedure in mouse strains commonly used in behavioral experiments in order to develop a new animal model of mental disorders.

Subjects included 48 male C57BL/6J mice, 21 male DBA/2J mice, and 20 male ICR mice. All mice were commercially obtained (JCL Inc., Tokyo, Japan) at 8 weeks of age and were housed individually for 1 week prior to the experiment. The breeding room and experimental room were air-conditioned (22 °C, 50–60% humidity), and a 12-h light–dark cycle was implemented (lights on at 08:00). Food and water were freely available except during experimentation. All experiments were conducted during the light cycle, between 13:00 and 17:00. All animal experiments in this study were performed in strict accordance with the guidelines of The Institute of Physical and Chemical Research (RIKEN), and were approved by the institute's Animal Investigation Committee.

Experimental apparatuses consisted of two types of sound-proof boxes (40 cm × 50 cm × 60 [H] cm each; inside wall white or black), a shock chamber (10 cm × 10 cm × 10 [H] cm; clear Plexiglas with 2-mm-diameter stainless steel shock grids), a test chamber (10 cm × 10 cm × 10 [H] cm; clear Plexiglas without shock grids), and a shock generator and scrambler (O'Hara, Tokyo, Japan). The ceiling of each sound-proof box was equipped with a fluorescent bulb (white box; 4 W; ~70 lx on the floor of the box) or light-emitting diodes (LED; black box; ~90 lx on the floor of the box). In addition, two LEDs (for CS; ~150 lx) were placed on the side walls of the black box. Experiments were controlled by a Macintosh computer and data were collected and analyzed using Image FZ software (O'Hara) modified from NIH Image (free software available at http://rsb.info.nih.gov/nih-image/).

Mice were randomly divided into three groups, as summarized in Table 1. The first group received only context and cued conditioning (classical fear conditioning) on day 2 (group CN). The second group received a series of contextual conditioning on day 1 and classical fear conditioning on day 2 (group C-CN). The final group received a series of contextual conditioning on day 1 and another contextual conditioning on day 2 (group CC). In the contextual conditioning, there was no obvious stimulus except the inside of the sound-proof box and experimental chamber. In contrast, in the context and cued conditioning trial, three 15-s pulses of white noise (70 dB) were presented as CS, each followed by a foot shock (0.3 mA, 2 s). In the first contextual conditioning of group C-CN and group C-C, eight foot shocks (0.3 mA, 2 s each) were administered at 1-min intervals without noise presentation. In the second contextual conditioning of group C-C, mice were given three foot shocks (0.3 mA, 2 s each) at 1-min intervals. On day 3, a test trial was conducted for all groups. Mice were individually introduced to a test chamber that was a completely different environment from that of the conditioning chamber. The two-part test trial consisted of 2 min for assessing the non-specific fear response to the new environment, followed by 2 min for assessing the fear response to the white-noise CS. In C57BL/6J mice, a light CS experiment (150 lx, 15 s) was conducted in addition to the noise CS experiment. Fear response was assessed by freezing rate, which was calculated as freezing rate = (no movement time/duration of experiment) × 100.

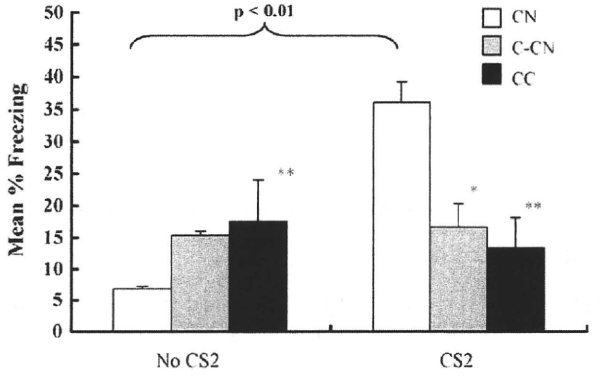

Fig. 1. Context-noise conditioning in C57BL/6J mice ($n = 9$ in each group). Data represent mean + SEM. Group names are described in the text. CS2 was white noise. Asterisks indicate statistical significance compared to CN (*$p < 0.05$; **$p < 0.01$).

Fig. 1 shows the results of the C57BL/6J white-noise CS experiment. In the first 2 min (left; no CS2), one-way ANOVA revealed a significant conditioning effect ($F(2,24) = 10.69$, $p < 0.001$), and post-hoc analysis (Turkey's test) showed that the freezing rate of group CC was statistically higher than that of group CN ($T = 6.45$, $p < 0.01$). This enhanced fear response in group CC was a non-specific fear response caused by repeated contextual conditioning over 2 consecutive days. In the second 2 min (right; CS2), one-way ANOVA again revealed a significant conditioning effect ($F(2,24) = 7.34$, $p < 0.01$), and post-hoc analysis (Turkey's test) showed that the freezing rates of groups C-CN and CC were both statistically lower than that of group CN ($T = 4.29$, $p < 0.05$ and $T = 5.03$, $p < 0.01$, respectively). Within-group comparisons revealed a significant statistical difference only in group CN ($T = 2$, $p < 0.01$, Wilcoxon's test). These results indicate that conditioning prior to the contextual CS may block subsequent conditioning to another CS (white noise).

Fig. 2 shows the results of the C57BL/6J light CS experiment. In the first 2 min (left; no CS2), one-way ANOVA revealed no significant conditioning effect ($F(2,21) = 1.57$, n.s.). These results indicate that there was no enhanced non-specific fear response in either group following CS conditioning with light. In the second 2 min

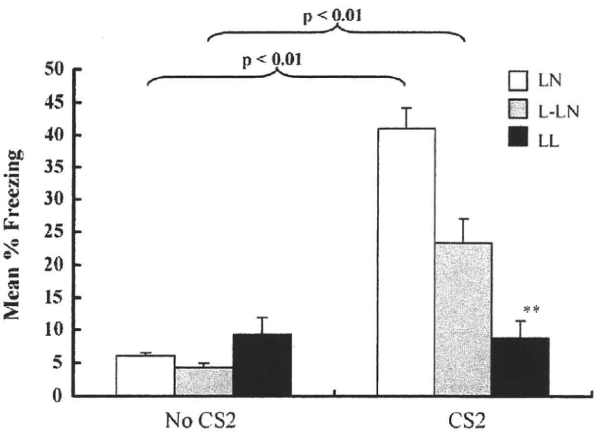

Fig. 2. Context-light conditioning in C57BL/6J mice ($n = 7$ in each group). Data represent mean + SEM. Group names are described in the text. CS2 was light stimulus. Asterisks indicate statistical significance compared to LN (**$p < 0.01$).

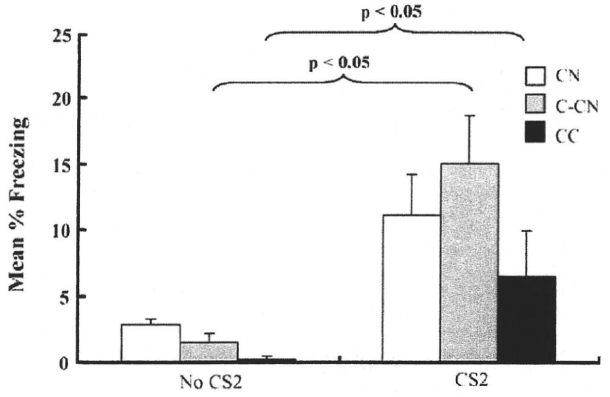

Fig. 3. Context-noise conditioning in DBA/2J mice ($n = 7$ in each group). Data represent mean + SEM. Group names are described in the text. CS2 was white noise.

(right; CS2), one-way ANOVA revealed a significant conditioning effect ($F(2,21) = 9.31$, $p < 0.005$), and post-hoc analysis (Turkey's test) showed that the freezing rate of group LL was statistically lower than that of group LN ($T = 6.09$, $p < 0.01$), but the freezing rate of group L-LN was not significantly lower than that of group LN. Within-group comparisons revealed statistical differences in groups LN and L-LN ($T = 0$, $p < 0.01$ and $T = 0$, $p < 0.01$, respectively; Wilcoxon's test). These results indicate that repeated conditioning to the light CS used in this study produced only an incomplete blocking effect.

Fig. 3 shows the results of the tone CS experiment using an inbred mouse strain, DBA/2J. In the first 2 min (left; No CS2), because an initial Bartlett's test was significant, ANOVA could not be applied, so a non-parametric test (Steel Dwass's test) was conducted. This test showed that there were no statistical differences between groups. In the second 2 min (right; CS2), one-way ANOVA revealed no significant conditioning effect ($F(2,18) = 0.99$, n.s.). Within-group comparisons revealed statistical differences in groups C-CN and CC ($T = 0$, $p < 0.05$ and $T = 0$, $p < 0.05$, respectively; Wilcoxon's test). These results indicate that DBA/2J mice can easily learn certain types of classical fear conditioning but did not exhibit any blocking effect, at least in this protocol.

Finally, Fig. 4 shows the results of the tone CS experiment using ICR mice. In the first 2 min (left; no CS2), as for DBA/2J mice, the initial Bartlett's test was significant, but subsequent analysis by Steel Dwass's non-parametric test revealed no statistical differences. In the second 2 min (right; CS2), one-way ANOVA revealed no significant conditioning effect ($F(2,17) = 2.52$, n.s.). However, within-group comparisons revealed statistical differences in all groups (CN: $T = 0$, $p < 0.05$; C-CN: $T = 9$, $p < 0.05$; CC: $T = 0$, $p < 0.05$; Wilcoxon's test). These results indicate that ICR mice can learn classical fear conditioning, but the context CS did not block the response to the noise CS under these experimental conditions.

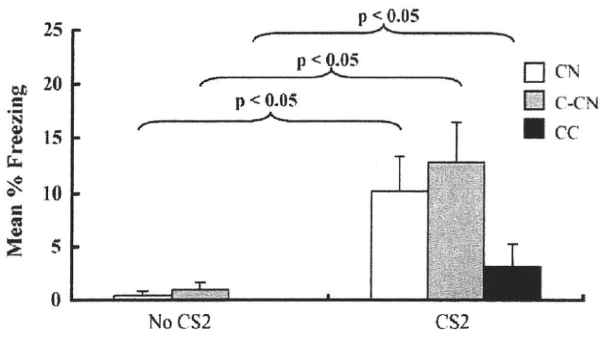

Fig. 4. Context-noise conditioning in ICR mice ($n = 7$ in group CN and C-CN, $n = 6$ in group CC). Data represent mean + SEM. Group names are described in the text. CS2 was white noise.

In this study, a simple blocking experiment for mice was developed and tested using several commonly used laboratory mouse strains. To simplify the methodology, a conventional classical fear conditioning protocol was used, and a successful blocking effect was obtained in the C57BL/6J strain, which has been widely used in learning and memory experiments and is the background strain for many genetically modified mice [5]. In contrast, DBA/2J and ICR mice only exhibited cued (auditory) fear conditioning, and did not exhibit any blocking effects. These results suggest that there are strain differences in the blocking effect using contextual conditioning. Therefore, studies of selective attention using a contextual blocking procedure should be conducted in the C57BL/6J strain and genetically modified mice of C57BL/6J background.

In the present protocol, multiple foot shocks were presented to mice in the first contextual conditioning phase (groups C-CN, CC, L-LN, and LL). Previous studies reported that inescapable foot shock could lead to learning deficits (e.g., learned "helplessness") [19]. Although multiple inescapable foot shocks were presented to the mice in this study, none of the mice in any strain displayed "helplessness" (being unable to learn second conditioning and/or immobile) and all strains were able to learn auditory-cued conditioning. Furthermore, all mice could explore freely in the cage, both with and without white-noise presentation (data not shown). These observations indicate that mice were able to learn in this procedure, and that the test can be used fairly to assess the blocking effect in mice.

Recently, latent inhibition has been used to assess functional properties of mutant mice [14,20]. Because mice are left only in the conditioning chamber, this procedure may be simpler than the blocking method. However, latent inhibition reflects the effect of implicit learning (CS evokes no events) on later conditioning, whereas blocking reflects the effect of explicit events (CS evokes foot shock) on later conditioning. This difference likely underlies different mechanisms for these two phenomena. Therefore, it may be better to apply both methods to the analysis of selective attention mechanisms in mice.

The three mouse strains used in this study (C57BL/6J, DBA/2J, and ICR) have been used in many behavioral studies and their behavioral properties have been well characterized [4]. Although C57BL/6J mice have often been reported to have hearing deficits, such hearing deficit progresses slowly over a year [23]. In this study, mice were 8 weeks of age at the beginning of the experiment, so their auditory abilities were intact. Hearing deficits in DBA/2J mice more rapidly progress than in C57BL/6J mice [23], but these mice still maintain their hearing ability until at least 6 months. Thus, no effect of hearing loss should confound the results of the current study. DBA/2J mice have also been reported to have learning deficits in trace fear conditioning [8]. However, a delayed conditioning procedure was used in this study, and these mice successfully learned auditory fear conditioning. Therefore, the hearing and/or learning deficiency in DBA/2J mice seem unlikely to have affected this results. DBA/2J mice learned the fear-conditioned response to the auditory CS but did not exhibit any blocking effect in the test phase. As the freezing rate of DBA/2J mice was almost identical to that of C57BL/6J mice, the absence of a blocking effect might be attributable to lower attention to the contextual stimuli in DBA/2J mice. A recent study reported that DBA/2J mice showed schizophrenia-like endphenotypes, including impaired latent inhibition [21]. In the present study, DBA/2J mice did not exhibit blocking. Taken together, these results suggest that the DBA/2J strain may be, a useful animal model of human mental disorders. Like DBA/2J mice, ICR mice displayed a normal but lower auditory fear-conditioned response and exhibited no contextual

blocking effects to the white-noise CS (group C-CN). Nevertheless, the absence of a blocking effect might be attributed not only to lower attention to the contextual stimuli but also to their higher activity in the test cages than other strains (data not shown).

The results of the present study indicate that mice, especially of the C57BL/6J strain, can be used to assess selective attention (blocking). In so doing, I have demonstrated that mice have the capacity for selective attention and can be used as an animal model of selective attention. Furthermore, the method described herein can be applied to the analysis of genetically engineered mice that lack the blocking effect, which can serve as an animal model of mental disorders such as schizophrenia. Application of the present protocol to the analysis of genetically engineered mice will further our understanding of the mechanisms underlying selective attention.

Acknowledgement

This study was supported in part by the Ministry of Health, Labour and Welfare (Tokyo, Japan; H20-Chemistry-General-009).

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