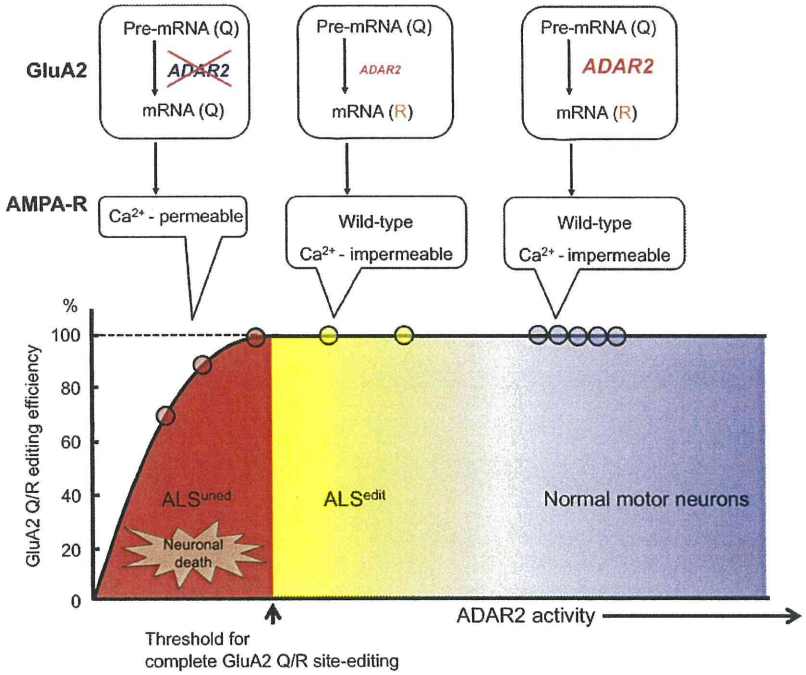

Fig. 5. Reduction of ADAR2 activity relative to expression of unedited GluA2 and death of motor neurons in sporadic ALS. ADAR2 activity was sufficiently high to edit the Q/R site of all the GluA2 mRNA expressed in the normal motor neurons (blue zone). In motor neurons of sporadic ALS, however, ADAR2 activity was lower than in normal motor neurons, even in those expressing only edited GluA2 mRNA (yellow zone). Motor neurons in which ADAR2 activity decreased below threshold (approximately 20×-10^{-3} relative to GluA2 mRNA) expressed Q/R site-unedited GluA2 and entered into the death cascade by expressing Ca^{2+}-permeable AMPA receptors containing GluA2 subunits unedited at the Q/R site (red zone).

pathology, including absence from the nucleus and mislocalization in the abnormal inclusion bodies, causes ADAR2 downregulation, or conversely ADAR2 downregulation causes TDP-43 pathology, remains to be elucidated. Recent microarray study demonstrated that ADAR2 mRNA was a target RNA of TDP-43 protein (Sephton et al., 2011) and ADAR2 gene expression was downregulated in the brain of TDP-43 knock-down animals (Polymenidou et al., 2011). Although these studies suggest that the reduction of ADAR2 expression resulted from the absence of TDP-43 in the nucleus of motor neurons with TDP-43 pathology, the quite modest extent of ADAR2 downregulation in the TDP-43 knock-down animal brain (Polymenidou et al., 2011) could not explain the loss of ADAR2-immunoreactivity in the motor neurons with TDP-43 pathology in patients with sporadic ALS (Aizawa et al., 2010). There remains a possibility that downregulation of ADAR2 causes TDP-43 pathology in ADAR2-lacking motor neurons. A close molecular link between ADAR2 downregulation and TDP-43 pathology in sporadic ALS is supported by the finding that neither inefficient GluA2 RNA editing nor mislocalization of TDP-43 occurs in SOD1 transgenic animals or SOD1-associated ALS patients (Kawahara et al., 2006; Mackenzie and Rademakers, 2007; Tan et al., 2007). These results suggest that death pathway involved in sporadic ALS including FTLD-MND is likely in common, while death pathway in SOD1-associated familial ALS may be one of other multiple different death pathways of motor neurons (Kwak and Weiss, 2006).

Because expression of ADAR2 was already reduced even in the motor neurons that express only edited GluA2 in ALS patients, downregulation of ADAR2 likely occurred before the onset of ALS symptoms and initiated the process of cell death when GluA2 RNA editing was incomplete. Therefore, investigation into the death cascade initiated by reduced ADAR2, including mislocalization of TDP-43, may provide insights into the pathogenesis of sporadic ALS. Unfortunately, little is known about the regulatory mechanism of ADAR2 expression in brains and spinal cords, and we do not know why ADAR2 is downregulated in ALS motor neurons. Because of the potentially critical role of ADAR2-mediated GluA2 RNA editing in sporadic ALS, elucidation of molecular mechanisms underlying ADAR2 downregulation is awaited. Demonstration of the disease-specific molecular abnormality that is involved in cell death provides a clue to the development of novel therapeutic strategies, and normalizing GluA2 Q/R site-editing in motor neurons is one potential method for treating sporadic ALS (Kwak et al., 2008).

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Disclosure statement

The authors declare that there are no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.nbd.2011.12.033.

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特集1

筋萎縮性側索硬化症（ALS）の基礎研究

ADAR2 発現低下と孤発性 ALS

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SUMMARY

筋萎縮性側索硬化症（ALS）は90%以上が孤発性である．我々は，少数例の孤発性ALS剖検組織の検討から，ALSの運動ニューロンでは，AMPA受容体のサブユニットであるGluA2が正常ではRNA編集されるべきQ/R部位が未編集のものが発現していることを発見した．このGluA2 Q/R部位のRNA編集はadenosine deaminase acting on RNA 2（ADAR2）により特異的に触媒され，運動ニューロン特異的ADAR2コンディショナルノックアウトマウスを作製した結果，ADAR2活性低下によるGluA2 Q/R部位RNA編集低下が原因で神経細胞死を引き起こされることがわかった．今回，約30例のさまざまな病型の孤発性ALS患者剖検組織を解析し，孤発性ALSの運動ニューロンには病型を問わず未編集型GluA2が発現していること，その原因がADAR2発現低下に依る活性低下であること，ALSの運動ニューロンにはpreclinicalな段階からADAR2発現が低下している分子異常が認められることを見い出したので紹介したい．

KEY WORDS
ALS
AMPA受容体
GluR2（GluA2）
RNA編集
ADAR2
ノックアウトマウス

I. AMPA受容体を介した神経細胞死

筋萎縮性側索硬化症（ALS）は90%以上が孤発性であり，有効な治療法の開発が切望されている神経難病である．発症の原因は不明であるが，現在最も有力な仮説がグルタミン酸による興奮性神経細胞死仮説である．特にAMPA受容体を介する神経細胞死がALSに関係する．AMPA受容体を介する神経細胞死は，チャネルからの過剰なCa^{2+}流入に引き続いて起こる．また，AMPA受容体はGluA1-4（GluR1-4）の4種のサブユニットからなる4量体で，Ca^{2+}透過性はGluA2サブユニットが含まれるかどうかにより決まる．GluA2を1個以上含むAMPA受容体はCa^{2+}非透過性で，1個も含まないものはCa^{2+}透過性である．ただし，GluA2は転写後にQ/R（グルタミン／アルギニン）部位のRNA編集を受けて初めてCa^{2+}非透過性を獲得するので未編集型GluA2を含むAMPA受容体もCa^{2+}透過性である．しかし，正常哺乳類では，脊髄運動ニューロンは全て編集型のGluA2を発現し，Ca^{2+}透過性は細胞の生命維持に関わるので，厳密に制御されている．

II. ADAR2活性とGluA2 Q/R部位のRNA編集

GluA2 Q/R部位のRNA編集はmRNAのアデノシ

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ン・イノシン置換（AI 編集）により CAG コドン（Q）が CIG=CGG コドン（R）に変わることによるアミノ酸置換を伴う．AI 編集は adenosine deaminase acting on RNA（ADAR）により触媒される．哺乳類では ADAR は 3 種（ADAR1, 2, 3）が知られているが，GluA2 Q/R 部位の RNA 編集は ADAR2 によって触媒されることがノックアウトマウスの検討から明らかにされている[1]．ADAR2 活性が低下すると AMPA 受容体サブユニットである GluA2 の Q/R 部位における AI 編集が行われなくなり，未編集型の GluA2 が発現する．未編集型 GluA2 をサブユニットに持つ AMPA 受容体はカルシウム透過性が高く，生理的にはニューロンには発現していない．そのため，未編集型 GluA2 の発現は神経細胞死を引き起こす分子変化であることが動物実験から示されている[1-3]．

孤発性 ALS 患者の剖検脊髄の検討では，半数以上の運動ニューロンにおける ADAR2 免疫染色性の低下[4]，前角組織では ADAR2 活性を規定する因子の一つである ADAR2 mRNA 発現量の減少が認められていた[5]．ラットの一過性脳虚血実験では海馬 CA1 錐体細胞で ADAR2 mRNA 発現が低下し，GluA2 Q/R 部位 RNA 編集低下が起こり，遅発性神経細胞死が生じ，同部位の RNA 編集を回復することで細胞死が回避されることが報告されている[6]．また全身的 ADAR2 ノックアウトマウスは，脳の GluA2 Q/R 部位の RNA 編集率が著減し，痙攣重積で死亡するが，GluA2 遺伝子を編集型 GluA2 をコードする遺伝子 GluR-B(R) に置換することにより正常化する[1]．ただし，この全身ノックアウトマウスは痙攣死であり，脊髄運動ニューロンの神経細胞死を確認出来なかった．

以上より，孤発性 ALS は，ADAR2 活性低下により GluA2 Q/R 部位の RNA 編集が低下することで神経細胞死が生じる，という仮説を立て，私たちはコンディショナルノックアウトマウスを作製した．

ADAR2 遺伝子の活性基をコードするエクソンを LoxP で挟んだ変異マウス（$ADAR2^{flox/flox}$）を作製し（**図 1A**），小胞性アセチルコリントランスポーター／アセチルコリン転換酵素のプロモータにより Cre recombinase をおよそ半数の運動ニューロンに発現する変異マウス（VAChT-Cre.Fast）[7] との交配により，$ADAR2^{flox/flox}$/VAChT-Cre.Fast（以下 AR2 マウス）を Cre-LoxP 系を用いて作製した[3]．この AR2 マウスは，運動機能が緩徐進行性に低下し（**図 1B, C**），有意な生存期間の短縮がみられた（**図 1D**）．また，運動ニューロンの変性像およびニューロン数の減少，脊髄前根においては有髄神経の変性と脱落が Cre の発現時期および発現頻度に一致して認められた（**図 1E**）．さらに，運動性脳神経核では，いずれにおいても GluA2 Q/R 部位の編集率低下を認め，顔面神経核，舌下神経核のニューロンは神経細胞の脱落を脊髄運動ニューロン同様に認めたが，動眼神経核においては，編集率は低下しているにも関わらず，神経細胞死は認めず，孤発性 ALS の臨床像に類似した（**図 1F**）[3]．さらに，この神経細胞死が未編集型 GluA2 のみによるかどうかを検討するために，ゲノムレベルで GluA2 Q/R 部位に R を発現するように操作した人工的な遺伝子を導入した $GluR\text{-}B^{R/R}$ マウス[8] と AR2 マウスを交配し，ADAR2 活性のないニューロンにおいても編集型 GluA2 を発現する変異マウス（AR2res）を作製した．その結果，AR2res マウスの運動機能の低下はみられず，ADAR2 を発現しない運動ニューロンにも変性が生じなかった（**図 1E**）．このことは，ADAR2 活性低下による運動ニューロン死は，未編集型 GluA2 の発現による AMPA 受容体の Ca^{2+} 透過性亢進を通じてのものであり，GluA2 Q/R 部位以外の ADAR2 編集部位は細胞死に関与しないことを示している（**図 1G**）[3]．

この結果の重要な点は，ADAR2 遺伝子のノックアウトにより，運動ニューロン死が起こること，その細胞死が内因性 GluA2 遺伝子を編集型 GluA2 を発現する変異遺伝子と置換することにより阻止されることから，細胞障害性の本質は，未編集型 GluA2 の発現が運動ニューロン死の直接原因である，ということが証明されたことである．さらに，AR2 マウスの検討から，未編集型 GluA2 が発現するにも関わらず眼球運動を司る運動ニューロンが保たれるといった（ALS の眼球運動が終末期まで保たれることに相同），同じ運動ニューロンでも部位により，細胞死の選択性があることが判明した．この結果は，孤発性 ALS では ADAR2 活性低下による GluA2 Q/R 部位の編集率低下が運動ニューロン死の直接原因であるという仮説[9] を支持する．

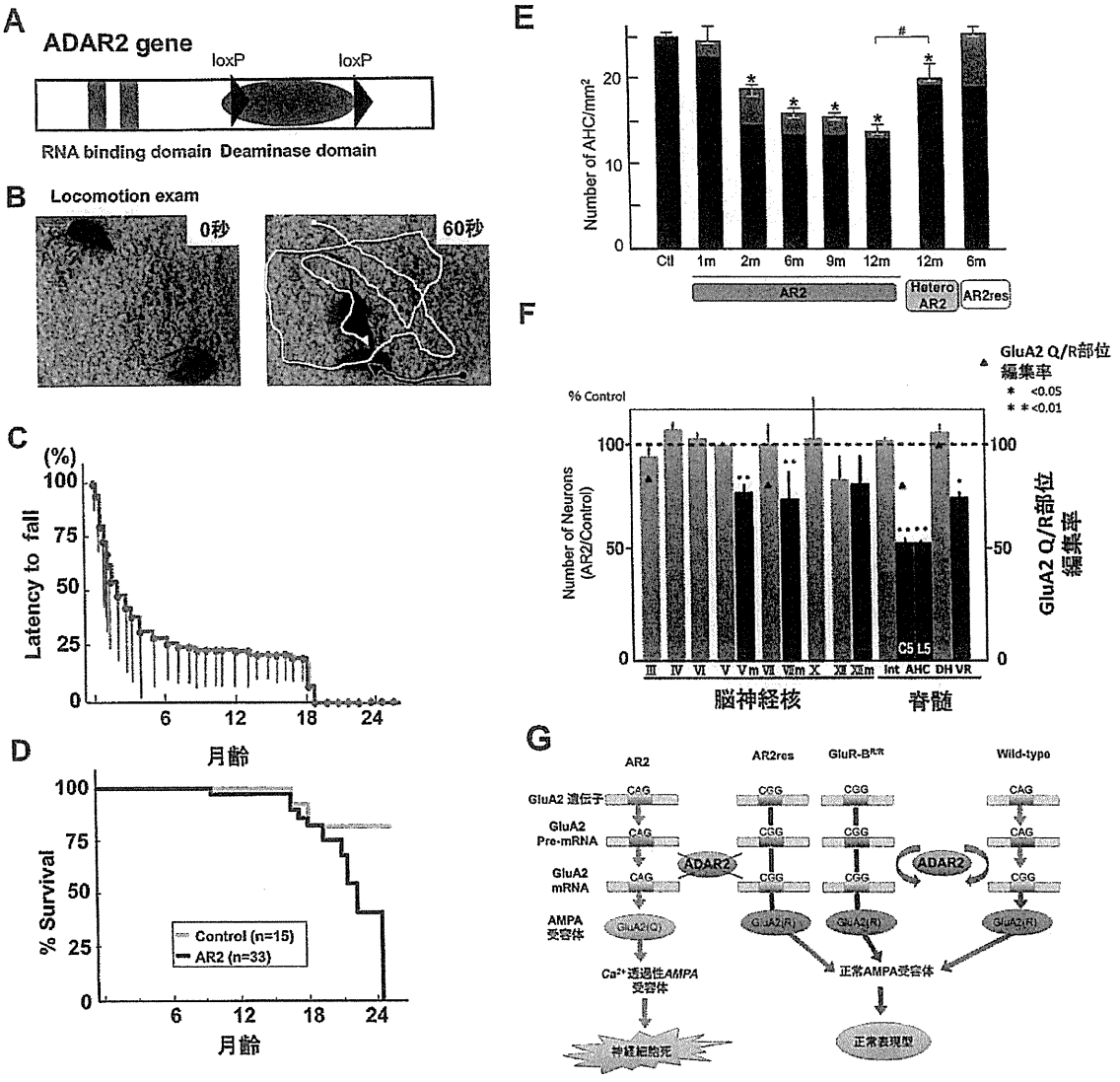

図1　ADAR2コンディショナルノックアウトマウス（p.5 カラー図参照）

A：ADAR2は，2個の2本鎖RNA結合部位（double-stranded RNA binding motifs: ds RBMs）と1ヵ所の編集触媒部位（catalytic domain）からなる．ADAR2遺伝子のcatalytic domainをコードするエクソンをLoxPで挟んだ変異マウス（ADAR2$^{flox/flox}$）を作製し，VAChT-Cre.Fastマウスとの交配により，ADAR2$^{flox/flox}$/VAChT-Cre. Fast（AR2マウス）をCre-LoxP系を用いて作製した．

B：12ヵ月齢では，同胞のADAR2$^{flox/flox}$マウスの表現型は活動性が正常（白ライン，カラー図：青ライン）であるのに対して，AR2マウスでは，後肢および尾，背筋の筋萎縮といった姿勢の異常が認められ，運動量が著減している（黒ライン，カラー図：赤ライン）ことが観察された．

C. ロータロッドは正常対照15匹，AR2マウス33匹に対して，毎週，10 rpmで180秒を最大値とし，3回施行した最大値で比較した．赤のプロットは，正常対照を100%とした際のAR2マウスの落下までの時間である．5週目からAR2マウスのロータロッドスコアは著減した．

D：生存期間は，正常対照（n=15匹）（灰色ライン，カラー図：青ライン），AR2マウス（n=33匹）（黒ライン，カラー図：赤ライン）が，105.1 ± 13.5週（mean ± SEM）に対して，AR2マウスは，81.5 ± 16.4週と短縮していた）$p = 0.0262$, Log-rank analysis）．

E. 脊髄前角大径運動ニューロンの特異的マーカーである，SMI32とADAR2の二重免疫染色を行い，正常対照（Ctl），1, 2, 6, 9, 12ヵ月齢（1 m, 2 m, 6 m, 9 m, 12 m）のAR2マウスについてSMI32陽性，ADAR2陽性の運動ニューロン数（黒カラム），SMI32陽性，ADAR2陰性の運動ニューロン数（灰色カラム）をカウントした．正常対照では全ての脊髄前角大径運動ニューロンは，SMI32陽性，ADAR2陽性であったが，AR2マウスでは1ヵ月齢から，Creの発現に一致し，ADAR2陰性の運動ニューロンが観察され，経時的にこれらのCreの発現する約半数のADAR2陰性の運動ニューロンが変性脱落した（$^*p < 0.01$, repeated ANOVA）．また，HeteroAR2（heterozygous ADAR2$^{flox/+}$/VAChT-Cre）マウスにおいてわずかな未編集型GluA2の発現による神経細胞死が生じた（$p < 0.01$, Mann-Whitney U検定），さらに，GluR-B$^{R/R}$マウスとAR2マウスを交配し（本文参照），

ADAR2 活性のないニューロンにおいても編集型 GluA2 を発現する変異マウス（AR2res）を作製した結果，ADAR2 を発現しない運動ニューロンにも変性が生じなかった．
F：縦軸の左側は，AR2 マウスのニューロン数を Ctl 正常対照マウスのニューロン数を 100% として表した数値である．縦軸の右側は，GluA2 Q/R 部位編集率である．運動性脳神経核（III, VII）では，いずれにおいても GluA2 Q/R 部位の編集率低下（黒三角，カラー図：橙三角）を認め，顔面神経核（VIIm），舌下神経核（XII）のニューロンは神経細胞の脱落を脊髄運動ニューロン（C5：第 5 頸髄，L5：第 5 腰髄）同様に認めた（黒カラム，カラー図：赤カラム）が，動眼神経核においては，編集率は低下しているにも関わらず，神経細胞死は認めなかった．脳神経系核：III（動眼神経核），IV（滑車神経核），V（三叉神経核），Vm（三叉神経運動性核），VII（顔面神経核），VIIm（顔面神経運動性核），X（迷走神経核），XII（舌下神経核），XIIm（舌下神経運動性核），Int（脊髄介在ニューロン），AHC（脊髄前角組織），C5（第 5 頸髄），L5（第 5 腰髄），DH（脊髄後角組織），VR（脊髄前根 L5）．$^{*} < 0.05$，$^{**} < 0.01$，いずれも Mann-Whitney U 検定．
G：野生型マウスでは，ADAR2 により，GluA2 Q/R 部位の RNA 編集が正常に行われ，編集型の GluA2 (R) が発現し，野生型の Ca^{2+} を透過しない AMPA 受容体が運動ニューロンに発現し，正常の表現型を示す．AR2 マウスは，ADAR2 ノックアウトにより，GluA2 Q/R 部位の RNA 編集が行われず，未編集型の GluA2 (Q) が発現し，Ca^{2+} 透過性の AMPA 受容体が運動ニューロンに発現し，緩徐進行性の神経細胞死を来す．AR2res マウスでは，ADAR2 活性はないが，編集型 GluA2 (R) が発現し，Ca^{2+} 非透過性の AMPA 受容体が運動ニューロンに発現し，正常の表現型を示した．以上から，ADAR2 活性低下による運動ニューロン死は，GluA2 Q/R 部位以外の ADAR2 編集部位は細胞死に関与しない，未編集型 GluA2 の発現による AMPA 受容体の Ca^{2+} 透過性亢進により生じたと考えられた．

III. 孤発性 ALS にユニバーサルな分子異常である GluA2 編集異常

さらに，私たちのグループは少数例の検討から，laser microdissector を用いて凍結剖検脊髄組織から単一運動ニューロンを切り出し，孤発性 ALS 脊髄運動ニューロンの単一神経細胞レベルおよび脊髄前角組織レベル[10]の検討において，部位選択的・疾患特異的に GluA2 Q/R 部位の編集率が低下していること，しかし GluA2 mRNA 発現量には有意な変化がないこと[11]，を確認した[12]．この分子変化の疾患特異性，部位選択性を検討し，孤発性 ALS で変性しない小脳プルキンエ細胞の他，さまざまな神経変性疾患における変性部位，すなわち，小脳を侵す神経変性疾患である脊髄小脳変性症のプルキンエ細胞[12]，ハンチントン病の線条体，アルツハイマー病の大脳皮質などにおいて，この部位の RNA 編集が保たれていることを明らかにした[13]．さらに運動ニューロンに変性を生じるトリプレット・リピート病である球脊髄性筋萎縮症の運動ニューロンと家族性 ALS で最も頻度が高い SOD1 関連性家族性 ALS（ALS1）の運動ニューロンでも GluA2 Q/R 部位の RNA 編集には変化がないことを明らかにし[14]，この分子変化が運動ニューロン死に伴う非特異的な変化である可能性を否定した．

今回，この分子異常が孤発性 ALS の病因に果たす意義を明らかにするために，多発例の孤発性 ALS において，運動ニューロンにおける未編集型 GluA2 の発現，ADAR2 の発現レベルおよび活性の変化を検討した．孤発性 ALS 29 例（古典型，進行性球麻痺，ALS 痴呆，Basophilic inclusion body のみられる ALS を含む），正常対照 6 例，疾患対照（多系統萎縮症）5 例の凍結脊髄剖検組織を用いた．脊髄前角および運動ニューロンをレーザーダイセクターで切り出し，回収した前角組織および単一脊髄運動ニューロンより総 RNA を抽出し，RT-PCR により cDNA を得た．得られた cDNA を用いて RNA 編集酵素 ADAR1, ADAR2, ADAR3 の mRNA を定量し，各 ADARs により特異的に触媒される RNA 編集部位の編集率を算定し，孤発性 ALS 群，正常群，疾患対照群の 3 群間で比較した．

その結果，正常および疾患対照脊髄前角組織の GluA2 Q/R 部位の RNA 編集率はほぼ 100% であったが，孤発性 ALS では全例で 100% 未満に低下していた（Mann-Whitney U 検定，$p < 0.001$）（**図 2A**）．なお，病型，発症年齢，臨床経過での有意差はなかった．運動ニューロン組織の検討では，正常および疾患対照群，検索した全ての運動ニューロンにおいて GluA2 Q/R 部位の RNA 編集率は例外なく 100% であった（**図 2B**）．その一方で ALS 群の全例で，未編集型 GluA2 を発現する運動ニューロンが検出され，いくつかの運動ニューロンの編集率は 0% であった（図 2B）．

また，RNA 編集酵素 ADAR2 の GluA2 Q/R 部位以外の特異的な基質である cytoplasmic fragile X mental retardation protein interacting protein 2（CYFIP2）K/E 部位における編集率は，ALS 群の前角組織（$p < 0.03$）および運動ニューロン（$p < 0.005$）で

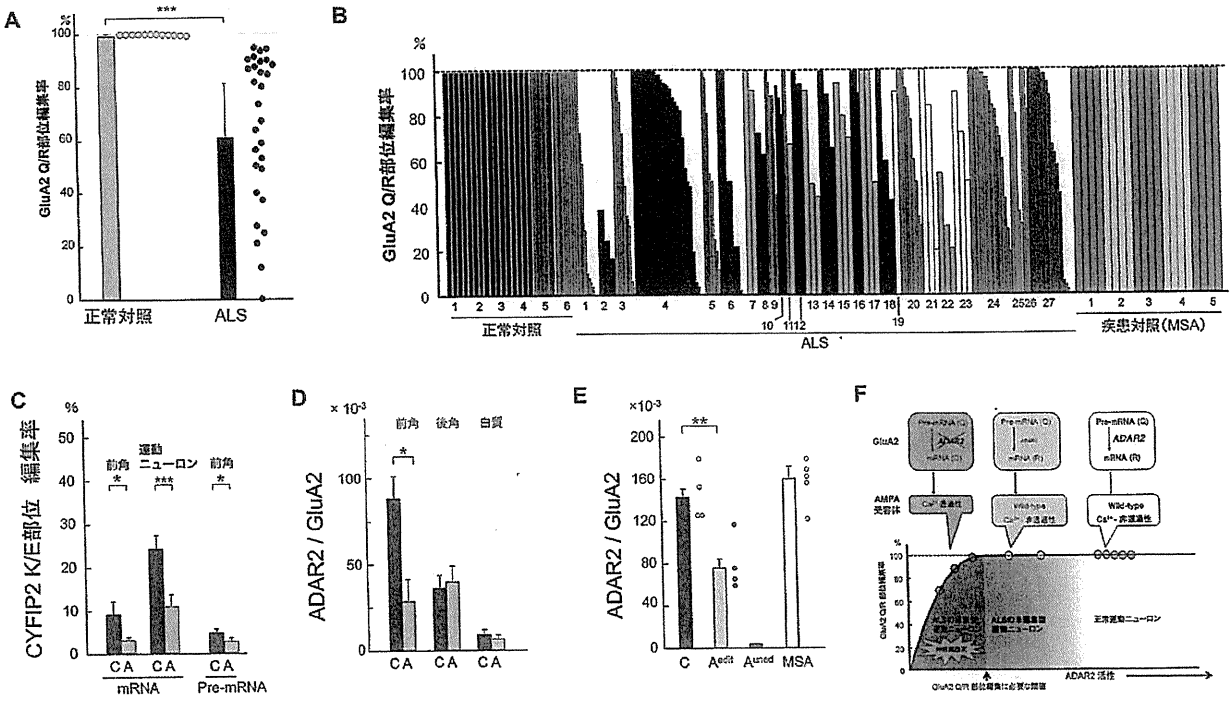

図2　孤発性ALSとADAR2活性低下(p.5 カラー図参照)

A：正常（n=12）および疾患対照脊髄前角組織（n=29）のGluA2 Q/R部位のRNA編集率はほぼ100%であったが，孤発性ALSでは全例で100%未満に低下していた（Mann-Whitney U検定，正常対照の編集率99.4% ± 0.7%に対して孤発性ALSは61.0% ± 22.7%．$^{***}p<0.001$）．

B：縦軸は，GluA2 Q/R部位のRNA編集率，横軸の数字は各症例を指す．運動ニューロン組織の検討では，正常（6例）および疾患対照群（MSA：多系統萎縮症，6例），検索した全ての運動ニューロンにおいてGluA2 Q/R部位のRNA編集率は例外なく100%であった．その一方でALS群の全例（青色系カラム：四肢型，黄色系カラム：球麻痺型，桃色系カラム：ALS痴呆，赤カラム：Basophilic inclusion body封入体を有する若年ALS例）で例外なく，未編集型GluA2を発現する運動ニューロンが検出された．細いカラムはシングルセル1個，太いカラムはシングルセル30個の結果である．

C：ADAR2のGluA2 Q/R部位以外の特異的な基質であるcytoplasmic fragile X mental retardation protein interacting protein 2（CYFIP2）K/E部位における編集率は，ALS群(A)の前角組織（Mann-Whitney U検定，$^{*}p < 0.03$）および運動ニューロン（Mann-Whitney U検定，$^{***}p < 0.005$）で正常対照(C)に比較し，有意に低下しており，GluA2 pre-mRNA Q/R部位の編集率もALS群の前角組織で有意に低下していた（Mann-Whitney U検定，$p < 0.01$）．

D：ADAR2 mRNAの発現レベルは，ALS群(A)の後角および白質では，正常対照(C)と同レベルであったが，前角組織では正常対照の1/3に低下していた（Mann-Whitney U検定，$^{*}p < 0.05$）．

E：運動ニューロンにおけるADAR2 mRNAの発現レベルは，正常対照群(C)と疾患対照群（MSA：多系統萎縮症）との間には有意差がなかったが，孤発性ALSの編集型GluA2のみを発現する運動ニューロン（A^{edit}）においても有意な活性低下がみられた（Mann-Whitney U検定，$^{**}p < 0.02$）．ALS群の未編集型を発現する運動ニューロン（A^{uned}）では，編集型（A^{edit}）のみを発現する運動ニューロンに比してもさらに著減していた．ALSの編集型運動ニューロンですでにADAR2活性が低下していることははじめての発見であり，編集が保たれていても既に疾患発症前状態になっていると考えられた．

F：縦軸は，GluA2 Q/R部位編集率で，横軸はADAR2活性を表す．正常（白の部分，カラー図：青の部分）は，ADAR2活性が十分あり，編集型GluA2を有するCa^{2+}非透過性の正常AMPA受容体を発現している．しかし，ALS発症準備状態に入るとADAR2活性が下がり始め（灰白の部分，カラー図：黄の部分），ある閾値を超えると確率的にdeath cascadeに陥り（黒の部分，カラー図：赤の部分），未編集型GluA2を有したCa^{2+}透過性のAMPA受容体を発現し，神経細胞死に陥る．

有意に低下しており，GluA2 pre-mRNA Q/R部位の編集率もALS群の前角組織で有意に低下しており（$p < 0.01$）（**図2C**），ADAR2活性低下を反映していた．

ADAR2活性を規定する因子の1つであるADAR2 mRNAの発現レベルは，ALSの後角および白質では，正常対照と同レベルであったが，前角組織では正常対照の1/3に低下していた（$p < 0.05$）（**図2D**）．さらに，運動ニューロンにおけるADAR2 mRNAの発現レベルは，正常対照群と疾患対照群との間には有意差がなかったが，孤発性ALSの編集型GluA2のみを発現する運動ニューロンにおいても有意な活性低下がみられた（$p < 0.02$）（**図2E**）．ALS群の未編集型を発現す

る運動ニューロンでは，編集型のみを発現する運動ニューロンに比してもさらに著減していた（図 2E）．ALS の編集型運動ニューロンですでに ADAR2 活性が低下していることは驚くべき結果で，編集が保たれていても既に疾患発症前状態になっていると考えられた（**図 2F**）[17]．

ADAR2 の isozyme である ADAR1 は活性レベル（GluA2 R/G 部位編集率，Bladder cancer associated protein（BLCAP）Y/C 部位編集率）および mRNA 発現とも，編集型 GluA2 のみを発現する運動ニューロンにおいても ALS 群での変化はなかった[17]．さらに，ADAR3 mRNA 発現量についても正常対照群と有意差はなかった[17]．ADAR2 同様 A-to-I 変換を触媒する ADAR1，ADAR3 は過剰発現系では，競合的 inhibitor になり得る報告があったが[15, 16]，今回の検討から，ADAR2 の活性低下が発現低下によるものであり，ADAR1 や ADAR3 との相互作用による影響は関与していないと考えられた．また，ADAR2 遺伝子発現の低下が，非特異的遺伝子発現低下を反映したものではなく，特異的な分子メカニズムにより引き起こされていることを示唆している。

これまで，孤発性 ALS 運動ニューロンに疾患特異的に GluA2 Q/R 部位の編集異常が生じていることを私たちは示してきたが[10, 12]，その検討は 10 例前後の少数例のものであった．今回の多数例での共通した結果から，この分子異常が，孤発性 ALS に共通する分子異常であることが判明した[17]．さらに，その原因が，運動ニューロンにおける GluA2 Q/R 部位を触媒する ADAR2 活性低下によることが ADAR2 の特異基質における RNA 編集率の低下と ADAR2 mRNA 発現量の低下から明らかになった．孤発性 ALS 運動ニューロンにおける ADAR2 発現レベルの低下は，免疫組織化学的に確かめられた ADAR2 タンパクの発現低下[4]とも合致する．今回の結果は，ADAR2 活性低下が ADAR2 発現の低下によりもたらされ，タンパク分解の亢進によるものではないことを示している．したがって，ADAR2 コンディショナルノックアウトマウスの結果と合わせると，孤発性 ALS に見出されている GluA2 の RNA 編集異常は神経細胞死の直接原因であり，ADAR2 活性低下が病因的意義を持っている可能性が高い．

さらに，未編集型 GluA2 の発現が neurotoxic である根拠として，私たちは，AR 2 マウスと同時に作製した，HeteroAR2（heterozygous ADAR2$^{flox/+}$/VAChT-Cre）マウスにおいてわずかな未編集型 GluA2 の発現による神経細胞死が生じていたこと（図 1E）[18]，AR2res による編集型 GluA2 の導入による細胞死抑制が（図 1E, G），それを支持する．とくに，Hetero AR2 マウスの検討からは，未編集型 GluA2 の発現が多寡を問わず neurotoxic であり[18]．AR2 マウスおよび Hetero AR2 マウスの検討[18]，孤発性 ALS 患者の運動ニューロンで GluA2 Q/R 部位が 0% のニューロンが検出されたことなどから[17]，仮説のように運動ニューロンおいて未編集型 GluA2 を発現する運動ニューロンが何時死ぬのかは，かなり確率的な現象であり，未編集型のみを発現していても 1 年以上生存するものもあるがあることもその考えを支持する（**図 2F**）．

IV. 今後の展望

以上から，編集型 GluA2 のみを発現している ALS 運動ニューロンでも ADAR2 活性が低下していたことは，孤発性 ALS では ADAR2 mRNA 発現レベルが進行性に低下していること，その低下が GluA2 の全てを編集型に維持するために必要なレベル以下にまで達すると未編集型 GluA2 を発現し，細胞死のカスケードに順次組み入れられることが想定される．すなわち，ALS 運動ニューロンは ADAR2 活性が低下していても，その活性が全ての GluA2 Q/R 部位の RNA 編集に必要な閾値に達するまでは，ほぼ正常な機能を保つことが出来るが，この閾値を超えて低下した時点で発症することを示唆している．このように孤発性 ALS の病因として ADAR2 活性低下が前臨床段階から生じていることが明らかになった．このことにより，孤発性 ALS の病因が機能分子の活性低下による可能性が高くなり，発症前診断，病因解明，特異的治療開発の展望が開けた．今後，さらに研究をすすめ，孤発性 ALS の病因，治療が開発できるように日々努力して行きたい．

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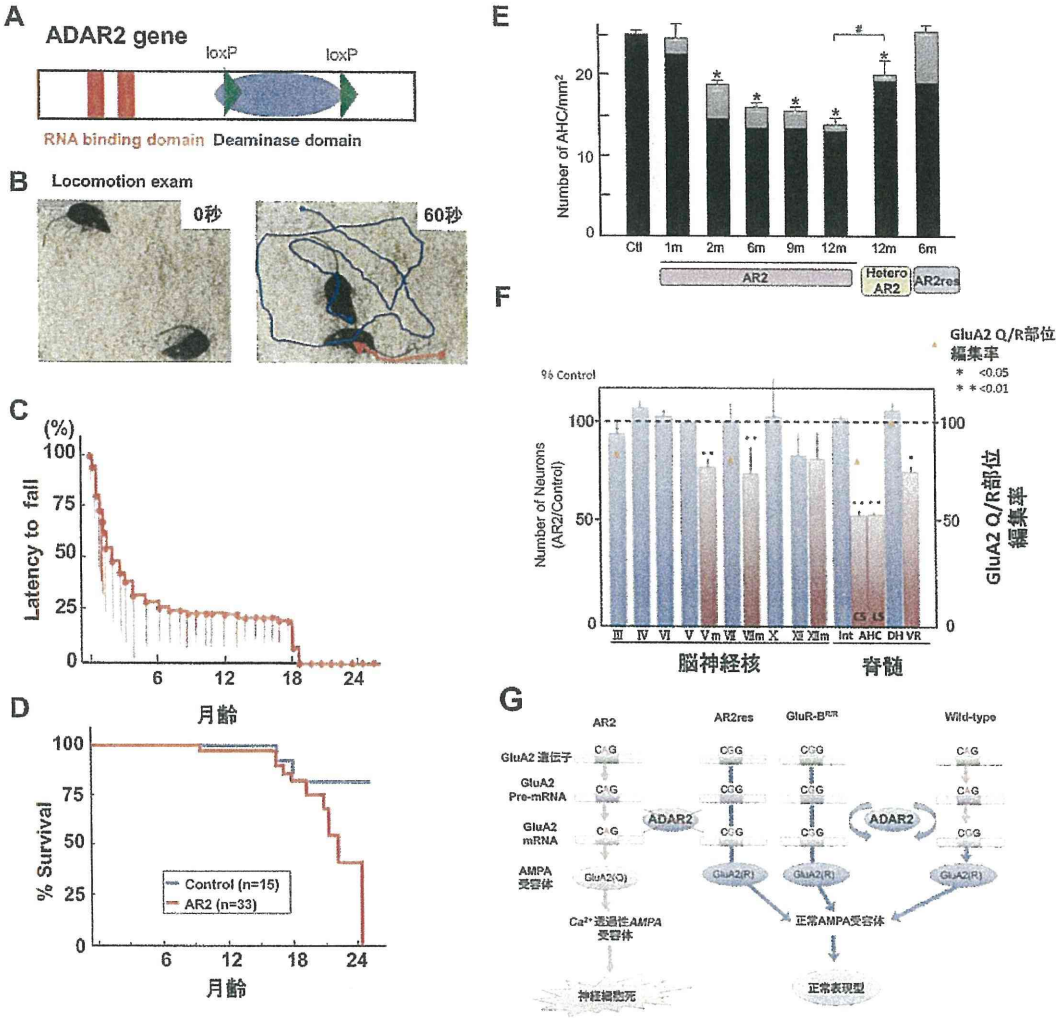
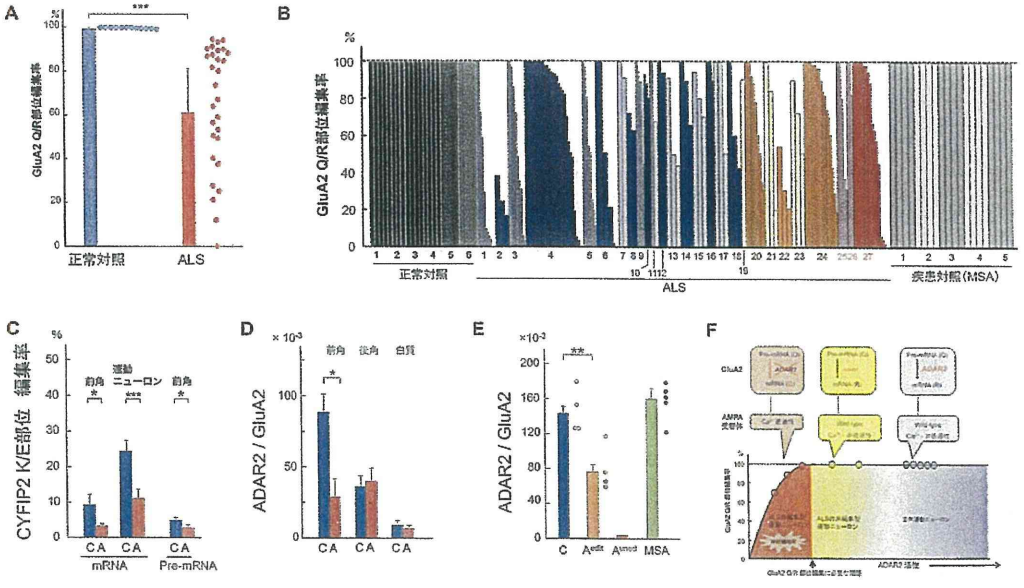


ADAR2 コンディショナルノックアウトマウス（p.36）



孤発性 ALS と ADAR2 活性低下（p.38）

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When does ALS start? ADAR2–GluA2 hypothesis for the etiology of sporadic ALS

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Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease. More than 90% of ALS cases are sporadic, and the majority of sporadic ALS patients do not carry mutations in genes causative of familial ALS; therefore, investigation specifically targeting sporadic ALS is needed to discover the pathogenesis. The motor neurons of sporadic ALS patients express unedited GluA2 mRNA at the Q/R site in a disease-specific and motor neuron-selective manner. GluA2 is a subunit of the AMPA receptor, and it has a regulatory role in the Ca^{2+}-permeability of the AMPA receptor after the genomic Q codon is replaced with the R codon in mRNA by adenosine–inosine conversion, which is mediated by adenosine deaminase acting on RNA 2 (ADAR2). Therefore, ADAR2 activity may not be sufficient to edit all GluA2 mRNA expressed in the motor neurons of ALS patients. To investigate whether deficient ADAR2 activity plays pathogenic roles in sporadic ALS, we generated genetically modified mice (AR2) in which the ADAR2 gene was conditionally knocked out in the motor neurons. AR2 mice showed an ALS-like phenotype with the death of ADAR2-lacking motor neurons. Notably, the motor neurons deficient in ADAR2 survived when they expressed only edited GluA2 in AR2/GluR-$B^{R/R}$ (AR2res) mice, in which the endogenous GluA2 alleles were replaced by the GluR-B^{R} allele that encoded edited GluA2. In heterozygous AR2 mice with only one ADAR2 allele, approximately 20% of the spinal motor neurons expressed unedited GluA2 and underwent degeneration, indicating that half-normal ADAR2 activity is not sufficient to edit all GluA2 expressed in motor neurons. It is likely therefore that the expression of unedited GluA2 causes the death of motor neurons in sporadic ALS. We hypothesize that a progressive downregulation of ADAR2 activity plays a critical role in the pathogenesis of sporadic ALS and that the pathological process commences when motor neurons express unedited GluA2.

Keywords: ADAR2, RNA editing, GluA2, Q/R site, ALS, neuronal death, AMPA

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease. ALS is characterized by progressive paralysis with muscle wasting due to a selective loss of upper and lower motor neurons. More than 90% of ALS cases are sporadic, whereas the remaining ALS cases have more than one other affected family member (familial ALS) (**Table 1**). The majority of sporadic ALS cases do not carry mutations in the genes that are known to cause familial ALS, including Cu/Zn superoxide dismutase (*SOD1*; Rosen et al., 1993; Jackson et al., 1997) *FUS/TLS* (Kwiatkowski et al., 2009; Vance et al., 2009) and *TARDBP* (TDP-43; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Yokoseki et al., 2008). In contrast, TDP-43 pathology in the spinal cord motor neurons is considered to be a neuropathological hallmark of sporadic ALS and is observed in most sporadic ALS cases (Arai et al., 2006; Neumann et al., 2006; Hasegawa et al., 2008) but not in the majority of familial ALS cases (Mackenzie et al., 2007; Tan et al., 2007; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Yokoseki et al., 2008; Kwiatkowski et al., 2009; Vance et al., 2009; Deng et al., 2010). These lines of evidence suggest that there is a common pathogenic mechanism of sporadic ALS, which is not among the mutations in the genes that cause ALS phenotype in familial ALS that have been identified to date. Therefore, an investigation of the molecular abnormalities that occur specifically in the pathological tissues of patients with sporadic ALS is required to elucidate the disease pathogenesis. Because molecular abnormalities found in the patients' pathological tissues include both the cause and the consequence of pathological changes, it is necessary to demonstrate that the molecular changes of interest induce the ALS phenotype in animals.

We have demonstrated that the RNA editing of GluA2, a subunit of the L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, at the glutamine/arginine (Q/R) site is inefficient in the motor neurons of sporadic ALS patients in a disease-specific and motor neuron-selective manner (Takuma et al., 1999; Kawahara et al., 2004). This is in marked contrast to the fact that all GluA2 mRNA was edited in the motor neurons of control subjects (Takuma et al., 1999; Kawahara et al., 2004), in patients with motor neuron diseases other than sporadic ALS (Kawahara et al., 2006), and in dying neurons in other neurodegenerative diseases,

Table 1 | Uniform sporadic ALS and multiple different familial ALS.

	Sporadic ALS	**Familial ALS**				
Incidence	**~90%**	**~10%**				
		ALS1	**ALS6**	**ALS10**	**Other fALS with known gene mutations**	**Other fALS with unknown mutations**
		20–40%	**4–5%**	**5–10%**	**~10%**	**30~60%**
Age at onset	64.6 ± 11.5	Average; 43–46 y.o.	Average; 44–45 y.o.	Range 30–75 y.o., average 55 y.o.	Juvenile; ALS2, ALS4, adult; ALS8, ALS9, OPTN	Juvenile; ALS5, adult; ALS3, 7
Causative genes	Unknown	Cu/Zn superoxide dismutase (*SOD1*)	Fused in sarcoma/translated in liposarcoma(*FUS/TLS*)	TAR DNA-binding protein (TDP)-43 (*TARDBP*)	ALS2; alsin, ALS4; senataxin, ALS8; VAPB, ALS9; ANG	ALS3; 18q21, ALS5; 15q15.1-q21.1, ALS7; 20ptel
Inheritance	Sporadic*	AD	AD/AR	AD	ALS2; AR, ALS4; AD, ALS8; AD, dynactin; PGRN; ANG; AD, OPTN; AD/AR	Variable
Clinical feature	Classic, PBP > dementia	Multisystem degeneration LMN dominant LMN > UMN, PBP, dementia	Classic, LMN dominant LMN > UMN > PBP > dementia	Classic, PBP > dementia	Variable OPTN: Classic	Variable
Pathology	UMN + LMN degeneration	Degeneration of Clarke's neurons, posterior horn, and spinocerebellar tract	LMN and spinal cord dominant degeneration	UMN + LMN degeneration	Variable	Variable
Bunina body	+	–	–	+	Unknown, OPTN(–)	Unknown
TDP-43 pathology	+	–	–	+	Unknown, OPTN(+)	Unknown
Basophilic inclusion	–	–	+	–	Unknown, OPTN(–)	Unknown
RNA editing	Under-edited	Normal	Unknown	Unknown	Unknown	Unknown
Model animal	AR2 mouse	SOD1 transgenic animal	FUS/TLS knockout/transgenic animal	TDP-43 knock-out/transgenic animal	ALS2 deficient mouse	Unknown

**A few% known gene mutations of FALS.*

FALS, familial ALS; AD, autosomal dominant; AR, autosomal recessive; UMN, upper motor neuron; LMN, lower motor neuron; VAPB, vesicle-associated membrane protein-associated protein B; OPTN, optineurin; classic, limb-onset classical ALS; PBP, progressive bulbar palsy; dementia, ALS with dementia; y.o., years old.

including the Purkinje cells of patients with spinocerebellar degeneration (Paschen et al., 1994; Akbarian et al., 1995; Suzuki et al., 2003; Kawahara et al., 2004). The high disease specificity warrants an investigation of how inefficient GluA2 RNA editing leads to neuronal death.

Functional AMPA receptors are tetramers with various combinations of GluA1, GluA2, GluA3, and GluA4 that are produced in a non-stochastic fashion. All the GluA subunits are expressed in the human and rat spinal motor neurons (Tölle et al., 1993; Kawahara et al., 2003; Sun et al., 2005). In mammalian neurons, adenosine in the Q codon (CAG) is converted to inosine (A-to-I conversion) in the Q/R site of virtually all GluA2 mRNA (**Figure 1A**). This conversion results in the expression of the GluA2 protein with R in the Q/R site because the CIG codon

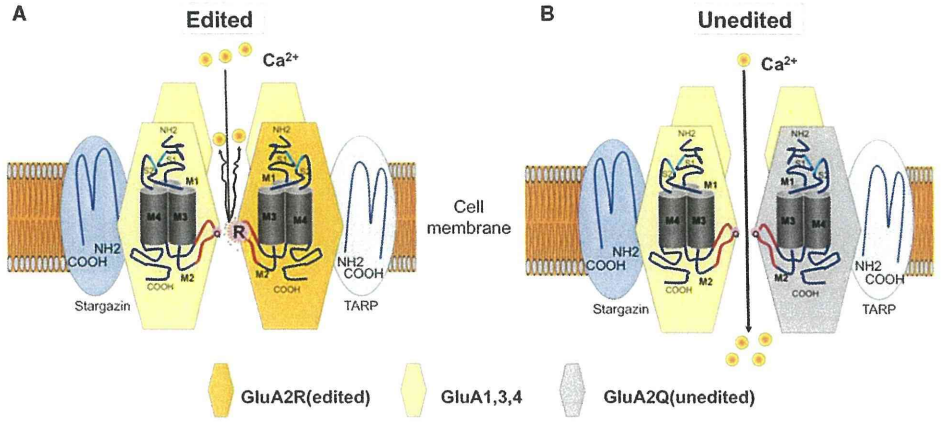


FIGURE 1 | The AMPA receptor and Ca^{2+}-permeability. The GluA2 subunit has four membrane domains (M1–M4). The glutamine/arginine (Q/R) site is located in the M2 domain, and this editing site is the major determinant of the Ca^{2+}-permeability of the AMPA receptor. **(A)** An AMPA receptor with the GluA2 subunit edited at the Q/R site (orange) in M2 (curved line in red). An arginine (R) residue at the Q/R site prevents Ca^{2+} (yellow circle) from passing through the channel pore. The AMPA receptors expressed on neurons contain Q/R site-edited GluA2 in their assembly. **(B)** Ca^{2+}-permeable AMPA receptors do not contain the Q/R site-edited GluA2 subunit. Naturally occurring Ca^{2+}-permeable AMPA receptors consist of GluA1, GluA3, and GluA4 (yellow). In sporadic ALS, Ca^{2+}-permeable AMPA receptors containing unedited GluA2 (gray) are expressed in motor neurons.

is read as the CGG codon (R) during translation. Because the A-to-I conversion at the Q/R site occurs only in the GluA2 subunit, and because the AMPA receptor subunit with R at the Q/R site critically regulates the Ca^{2+} permeability of AMPA receptors, AMPA receptors can be impermeable to Ca^{2+} only when they have Q/R site-edited GluA2 (Sommer et al., 1991; Nutt and Kamboj, 1994; Geiger et al., 1995). This evidence indicates that ALS motor neurons express abundant Ca^{2+}-permeable AMPA receptors with Q/R site-unedited GluA2 (Kwak and Kawahara, 2005) (**Figure 1B**). Because trafficking of unedited GluA2 is more efficient (Greger et al., 2002), a considerably high proportion of Ca^{2+}-permeable functional AMPA receptors will be expressed in the ALS motor neurons even with small amounts of unedited GluA2 expression.

RNA editing at the GluA2 Q/R site is catalyzed by adenosine deaminase acting on RNA 2 (ADAR2), a member of the ADAR family (**Figure 2**; Higuchi et al., 2000). Therefore, investigating the following questions would help to reveal a neuronal death-causing mechanism in sporadic ALS: whether ADAR2-lacking motor neurons die, whether deficient ADAR2 is a direct cause of neuronal death, and whether failure of the A-to-I conversion at the GluA2 Q/R site plays a critical role in the death of ADAR2-lacking motor neurons. To address these aims, we developed mutant mice in which the ADAR2 gene is targeted selectively to motor neurons using the Cre-loxP system.

ADAR2 CONDITIONAL KNOCKOUT MICE (AR2)

It is not clear whether neuronal death occurs in neurons lacking GluA2 Q/R site editing or in those lacking ADAR2 because both systemic ADAR2-null mice (Higuchi et al., 2000) and genetically engineered mice that cannot edit the GluA2 Q/R site (Brusa et al., 1995) die young from status epilepticus. We crossed $ADAR2^{flox/flox}$ mice with VAChT-Cre.Fast mice that displayed restricted Cre expression under the control of the vesicular acetylcholine transporter gene promoter in a subset of cholinergic neurons, including the spinal motor neurons (Misawa et al., 2003). By intercrossing the resulting $ADAR2^{+/flox}$/VAChT-Cre.Fast mice, we obtained $ADAR2^{flox/flox}$/VAChT-Cre.Fast (AR2) mice (**Figure 3A**; Hideyama et al., 2010). The AR2 mice displayed a slow, progressive motor dysfunction (**Figures 3B,C**) with a low rotarod performance (**Figure 4A**) and grip strength (**Figure 4B**). AR2 mice had slightly shorter life spans than the control mice (**Figure 4C**). We investigated the AR2 mice for morphological changes in the brain, spinal cord, motor nerves, and muscles, as well as for functional changes of the neuromuscular units at various postnatal periods. There were several degenerating large neurons in the anterior horn (AHCs) with cytoplasmic vacuoles (**Figure 5A**). To evaluate the progression of motor neuron death, we counted the number of motor neurons (SMI32-positive AHCs) with and without ADAR2 immunoreactivity. The number of SMI32-positive AHCs in AR2 mice markedly decreased between 1 and 2 months of age and slowly decreased beyond 1 year of age (**Figure 5B**). The motor neuron reduction is attributable to the loss of ADAR2-lacking AHCs. The number of ADAR2-positive AHCs remained unchanged after 2 months of age. There were darkly stained, degenerating axons with a decreased number of myelinated axons in the ventral roots (**Figure 5C**); these were the consequence of AHC degeneration. Skeletal muscles exhibited morphological characteristics of denervation, including muscle fiber atrophy, centrally placed nuclei, and pyknotic nuclear clumps. Electromyographic examination demonstrated fibrillation and fasciculation potentials, which are commonly observed in the denervated and reinnervated muscle fibers of ALS patients (Hideyama et al., 2010). Some neuromuscular junctions (NMJs) were abnormally innervated in AR2 mice; these NMJs were either lacking innervation (denervated NMJs), or they were innervated by ramified axons that innervated more than one NMJ (reinnervated NMJs; **Figure 5D**). The proportion of

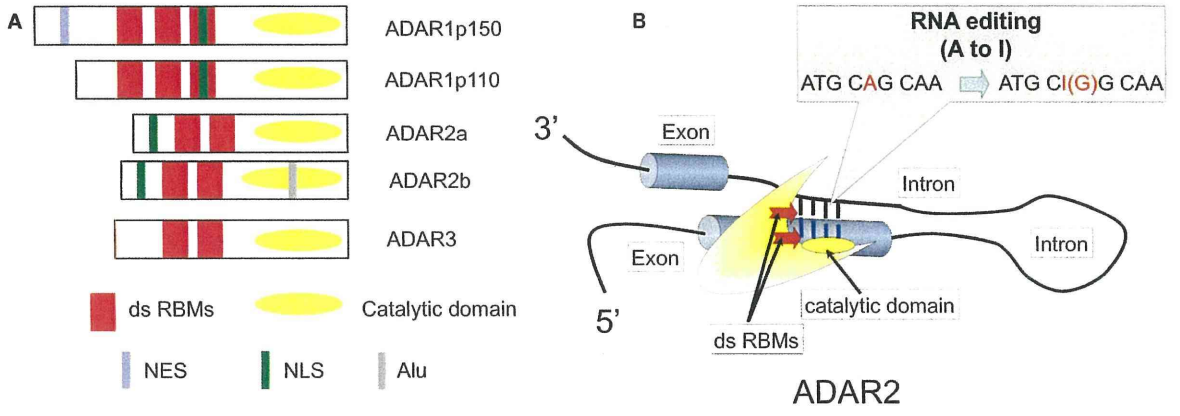


FIGURE 2 | ADAR structure and RNA editing. (A) Three members of the ADAR family. ADAR2 specifically catalyzes the A-to-I conversion at the GluA2 Q/R site, and two isoforms, ADAR2a and more abundantly ADAR2b, are expressed in human brains and spinal cords (Aizawa et al., 2010). RBM, RNA binding motif; NES, A nuclear export signal; NLS, A nuclear localization signal; Alu, Alu sequences; **(B)**. ADAR2 catalyzed adenosine to inosine conversion (A-to-I RNA editing) in the double-stranded RNA structure in both coding and non-coding regions of various transcripts expressed in mammalian brains. When this type of editing occurs in the reading frame, the resulting changes in the amino acid residues alter the biological function of the translated molecules, including 5-HT_{2c} receptors, GluAs, and GluKs. A-to-I RNA editing at the GluA2 Q/R site is specifically catalyzed by ADAR2, a member of the adenosine deaminase acting on RNA (ADAR) family. ADARs act on double-stranded RNA, which is formed with the distal end of exon 11 that includes the coding sequence for the Q/R site and the exon complementary sequence in intron 11 of GluA2 pre-mRNA (Higuchi et al., 1993).

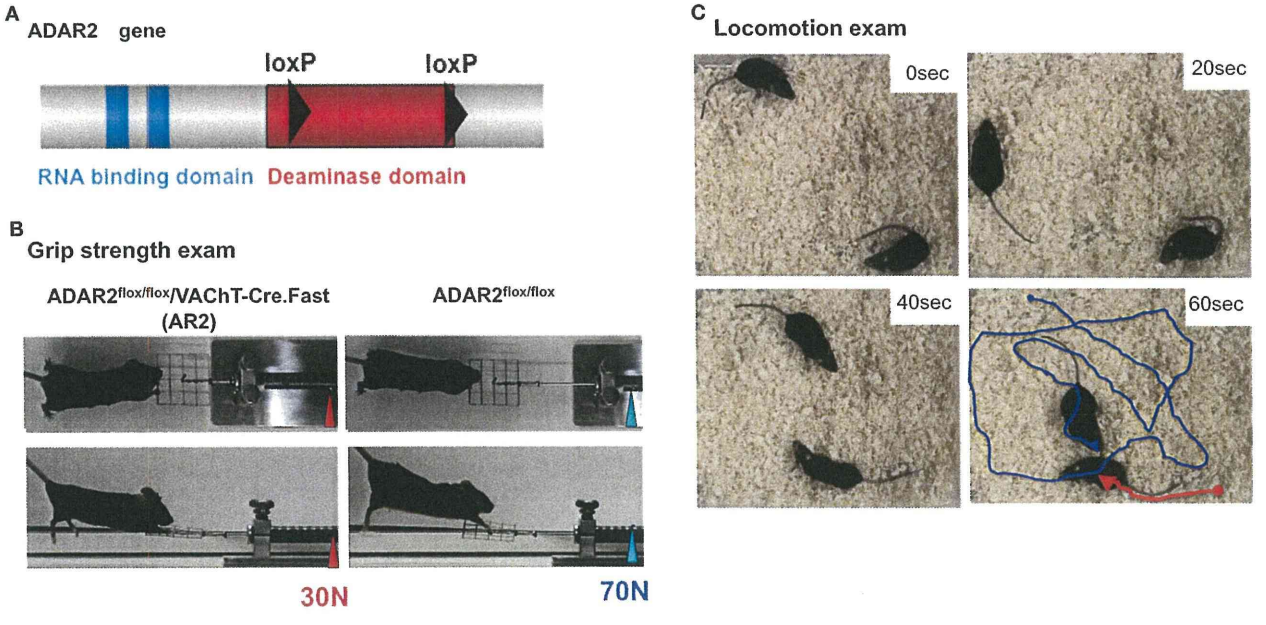


FIGURE 3 | Generation of conditional ADAR2 knockout mice (AR2). (A) One loxP sequence (filled triangle) was inserted into intron 6 and another into intron 9, which covers the majority of the catalytic domain encoded in the ADAR2 gene between the two loxP sites. **(B)** An AR2 mouse at 12 months of age (left panel) exhibits a low grip strength score with an abnormal hind limb and tail posture compared to a control littermate (right panel). **(C)** Locomotion and walking distances for an interval of 1 min were traced using a video capture system. An AR2 mouse at 12 months of age (red line) showed low locomotion levels compared to a control littermate (blue line).

denervated NMJs was higher in AR2 mice at 4 months of age than at 12 months, whereas the proportion of reinnervated NMJs was higher in AR2 mice at 12 months of age than at 4 months of age (**Figure 5D**). These results indicate that degeneration of ADAR2-lacking AHCs led to the degeneration of their axons with a resultant denervation of NMJs, which were reinnervated by collaterally sprouted axons of the ADAR2-expressing normal AHCs. With the degeneration of AHCs, a marked proliferation of glial

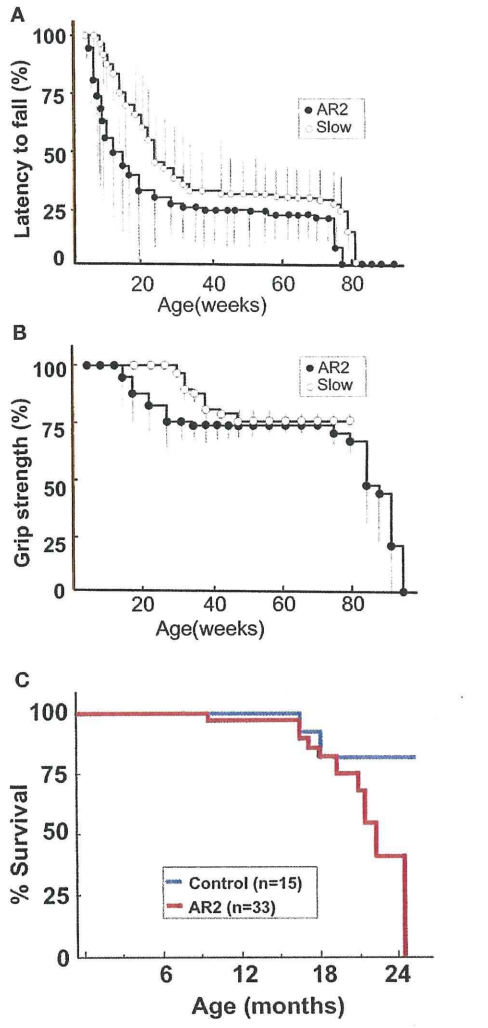
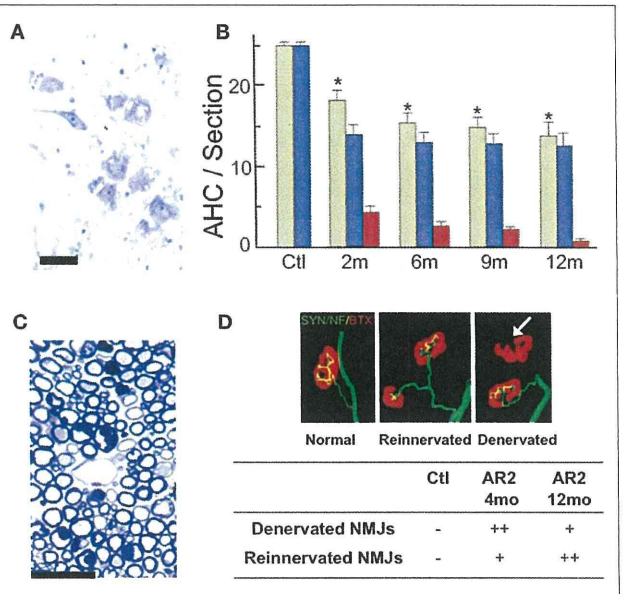


FIGURE 4 | The phenotype of conditional ADAR2 knockout mice (AR2) and slow mice. We generated two lines of conditional ADAR2 knockout mice with different Cre expression patterns: AR2 ($ADAR2^{flox/flox}$/VAChT-Cre.Fast) mice express Cre in progressively larger proportions of motor neurons, reaching ~50% of the maximum by postnatal week 5. Slow ($ADAR2^{flox/flox}$/VAChT-Cre.Slow) mice express Cre more slowly, reaching ~50% of motor neurons by 8 months of age. **(A)** Rotarod performance of AR2 mice (filled circle; $n = 28$) and Slow mice (open circle; $n = 33$). Using a mouse-specific rotarod (SN-445, Neuroscience Corp., Tokyo Japan), the maximal time before falling at 10 rpm during a 180-s period among three trials was used for the analysis. The scores are indicated as a percentage of the performance by control mice ($n = 15$), which exhibited full performance until approximately 12 months of age, followed by a slightly lower performance (greater than 164.5 ± 6.4 s) until 24 months. **(B)** Grip power measured with a dynamometer (NS-TRM-M, Neuroscience, Japan). The score is indicated as a percentage of the performance by control mice. **(C)** AR2 mice had long life spans, but the rate of death increased after month 18 compared to control mice.



	Ctl	AR2 4mo	AR2 12mo
Denervated NMJs	-	++	+
Reinnervated NMJs	-	+	++

FIGURE 5 | Loss of ADAR2-deficient motor neurons. (A) Degenerating large motor neurons in the anterior horn (AHC) of AR2 mice at 2 months of age (Nissl staining). Scale bar, 50 μm. **(B)** The numbers (mean ± SEM) of total AHCs (green columns), ADAR2-positive AHCs (blue columns), and ADAR2-negative AHCs (red columns) in AR2 mice at different ages (2, 6, 9, 12 months). The number of AHCs significantly decreased in AR2 mice after 2 months of age due to the reduction of ADAR2-lacking AHCs ($*p < 0.01$, repeated ANOVA). **(C)** The ventral root (L5) of AR2 mice at 4 months of age showing degenerating dark axons. Scale bar, 20 μm. **(D)** Neuromuscular junctions (NMJs) and distal motor nerve axons. The quadriceps muscles from an AR2 mouse (Reinnervated, Denervated) at 12 months of age and an age-matched, wild-type mouse (Ctl; normal) are stained with tetramethylrhodamine-bungarotoxin (BTX; red) and immunostained concomitantly with anti-synaptophysin and neurofilament antibodies (SYN/NF; green). The endplates (red) were counted as "innervated" if they were merged with axon terminals (yellow). A thick axon terminal in the Ctl mouse innervates each endplate, whereas in AR2 mice, some NMJs are innervated by axons that simultaneously innervate more than one NMJ (reinnervated NMJ), and other NMJs are devoid of axon terminals (denervated NMJs; white arrow).

fibrillary acidic protein (GFAP)-positive astrocytes and MAC2-positive microglial cells was detected in the anterior horns of AR2 mice (**Figures 6A,B**).

Other than the motor neurons in the spinal cord, large neurons in the facial (VII), and hypoglossal nerve nuclei (XII) in AR2 mice at 12 months of age were significantly decreased in number. In contrast, the number of neurons in the nuclei of the extraocular motor nerves (III, IV, VI) was not decreased (**Figure 6C**). Conversely, GluA2 Q/R site editing was significantly decreased in the oculomotor nerve nuclei (89.7% of control mice, $p = 0.0048$) and the facial nerve nuclei (83.3% of control mice, $p = 0.0017$) of AR2 mice at 12 months of age (**Figure 6C**). These results indicate that subsets of motor neurons, including those in the oculomotor nerve nucleus, are relatively resistant to cell death mediated by deficient ADAR2. Notably, the selective sparing of motor neurons that innervate the extraocular muscles as compared to those that innervate the bulbar and limb muscles is characteristically seen in ALS patients. Motor neurons in the nuclei of the oculomotor nerves are much less vulnerable in ALS patients. Notably, the expression of Ca^{2+}-binding proteins, particularly parvalbumin, is high in oculomotor neurons and low in the facial and spinal motor neurons (Ince et al.,

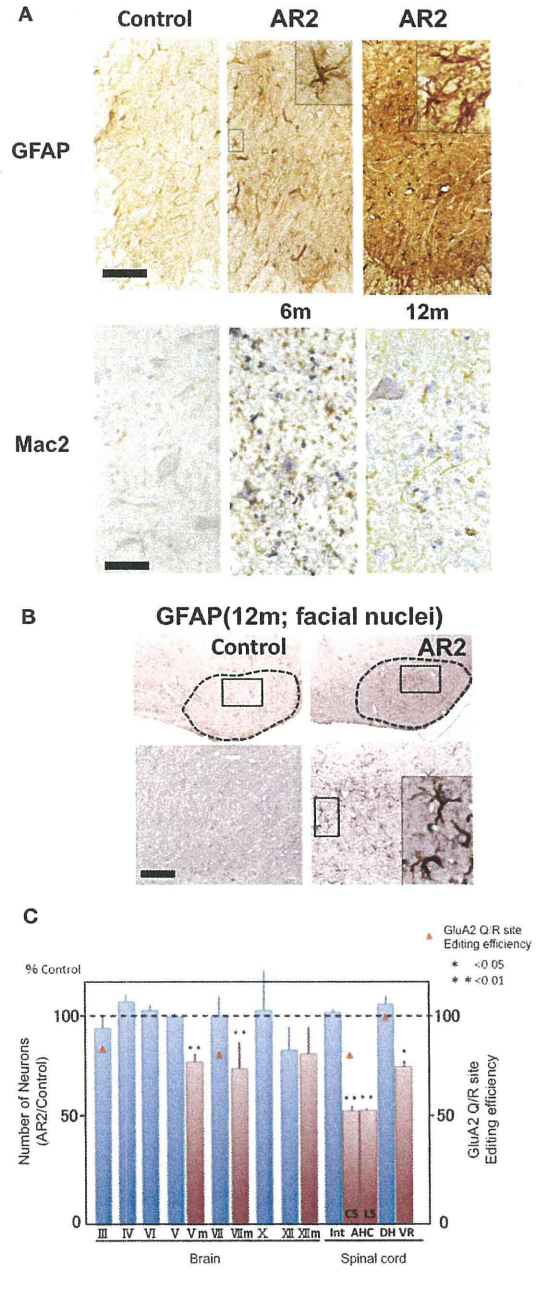


FIGURE 6 | Reactive glial cells in the spinal anterior horns and cranial motor nerve nuclei of AR2 mice (A,B). Immunohistochemistry in the anterior horn (C5) and motor trigeminal nuclei (Vm). There was an increase in GFAP- and MAC2-immunoreactivity in the spinal anterior horn of the AR2 mice, indicating the proliferation of activated astrocytes and microglia in association with motor neuron degeneration. m: months of age, inset; activated astroglia. Scale bars, 100 μm (GFAP) and 50 μm (insets and Mac2). **(C)** In AR2 mice at 12 months of age, the number of large neurons in the cranial nuclei of Vm, VII, and XII were lower than those in age-matched control mice, whereas those in the cranial nuclei III, IV, and VI were not. The GluA2 Q/R site editing efficiency was decreased in III, VII, and AH, indicating that the cranial motor neurons that innervate extraocular muscles are exceptionally resistant to deficient-ADAR2-mediated neuronal death.

1993). It has been shown that over-expression of parvalbumin attenuated kainate-induced Ca^{2+} transients and protected spinal motor neurons from the resultant neurotoxicity in parvalbumin transgenic mice (Van Den Bosch et al., 2002). In AR2 mice and in patients with sporadic ALS, it is likely that neurons with abundant parvalbumin, such as ocular motor neurons, are more resistant to Ca^{2+} overload from Ca^{2+}-permeable AMPA receptors than those with low parvalbumin levels, such as spinal motor neurons.

THE CRUCIAL ROLE OF GluA2 RNA EDITING IN THE DEATH OF ADAR2-LACKING MOTOR NEURONS IN AR2 MICE

ADAR2 predominantly catalyzes the RNA editing at the Q/R site of GluA2 both *in vivo* and *in vitro* (Melcher et al., 1996; Higuchi et al., 2000; Wang et al., 2000), but there are numerous A-to-I positions in mammalian brains, some of which are specifically catalyzed by ADAR2. Among the A-to-I positions specifically mediated by ADAR2 (Nishimoto et al., 2008), we found a significant reduction in the editing efficiency at the GluK2 (GluR6) Q/R site (AR2 mice vs. control mice, 15.3 vs. 31.8%, $p = 0.04416$) and at the K/E site of cytoplasmic fragile X mental retardation interacting protein 2 (CYFIP2) mRNA (**Figure 7**). These results indicate that RNA editing at the ADAR2-mediated A-to-I positions is universally defective in AR2 motor neurons.

To examine the possible role of defective RNA editing at A-to-I positions other than the GluA2 Q/R site in motor neuron death, we investigated the effects of edited GluA2 expression in ADAR2-lacking motor neurons (**Figure 8**). We exchanged the endogenous *GluA2* alleles that encoded Q at the Q/R site in AR2 mice with the GluR-B^R alleles (Kask et al., 1998), which encode R at the Q/R site of GluA2. This exchange circumvented the need for ADAR2-mediated RNA editing in the expression of edited GluA2. We intercrossed *ADAR2*$^{flox/+}$/VAChT-Cre.Fast/GluR-$B^{R/+}$ mice to generate AR2/GluR-$B^{R/R}$ mice. AR2/GluR-$B^{R/R}$ mice (AR2res) were phenotypically normal and had full motor function until

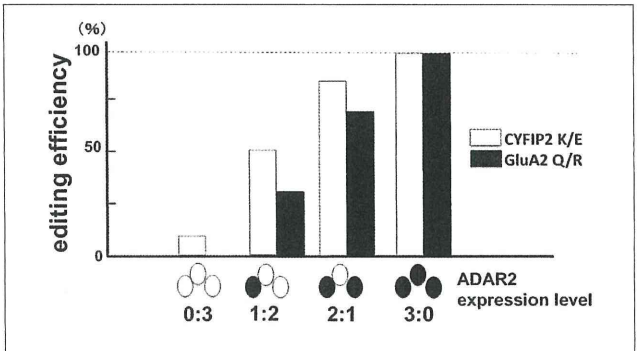


FIGURE 7 | The failure of CYFIP2 K/E site editing and GluA2 Q/R site editing in ADAR2-lacking motor neurons. When the extent of RNA editing was measured in the pooled lysates of three motor neurons obtained from AR2 mice, the proportions of CYFIP2 mRNA edited at the lysine/glutamic acid (K/E) site and those of Q/R site-edited GluA2 mRNA were lower in the lysates containing ADAR2-lacking motor neurons. This finding indicates that ADAR2 specifically mediates A-to-I conversion at the K/E site of CYFIP2 pre-mRNA. Ratios in abscissa indicate the number of ADAR2-expressing motor neurons (filled circle) and ADAR2-lacking motor neurons (open circle) in the three motor neuron lysates of AR2 mice. (see **Figure 10**).

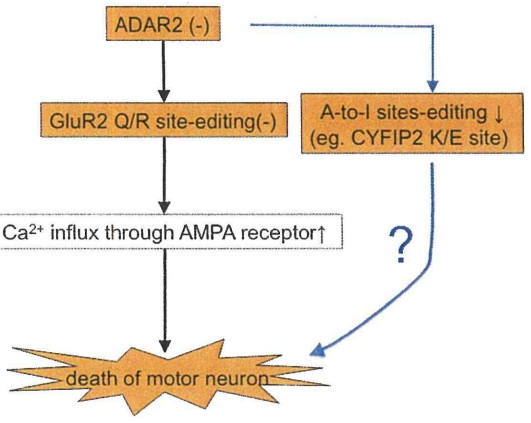


FIGURE 8 | Do other ADAR2 substrates play a role in the death of ADAR2-lacking motor neurons? There is a possibility that the death of motor neurons in AR2 mice is mediated by a failure of RNA editing at ADAR2-mediated A-to-I positions other than the GluA2 Q/R site.

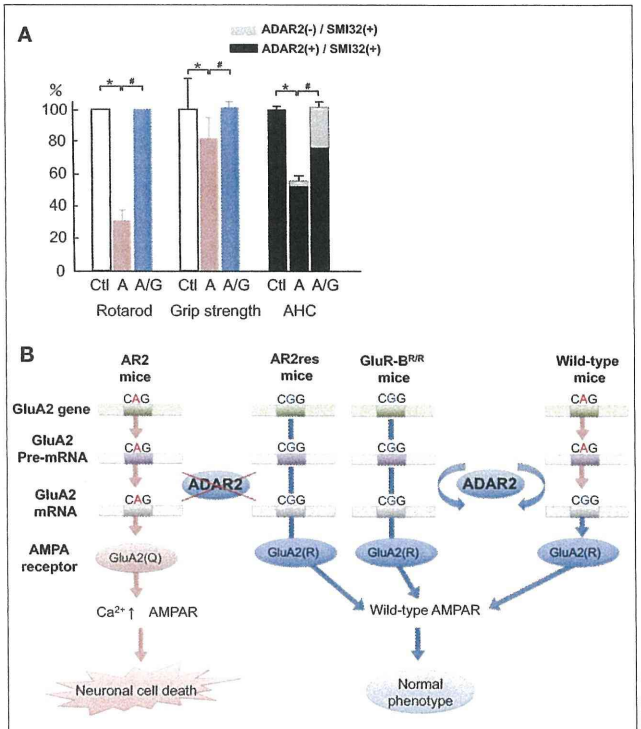


FIGURE 9 | The crucial role of GluA2 Q/R site editing in the death of ADAR2-deficient motor neurons. (A) AR2/GluR-$B^{R/R}$ mice (AR2res) are phenotypically normal with a preservation of ADAR2-lacking AHCs that account for 30% of the total AHCs at 6 months of age. **(B)** A scheme illustrating that the death of ADAR2-lacking motor neurons due to failure to edit the GluA2 Q/R site in the AR2 mice is rescued in the AR2res mice. The exonic Q codon (CAG) for the Q/R site of GluA2 is substituted by an R codon (CGG) in the endogenous *GluA2* alleles, and only edited GluA2 is expressed in the absence of ADAR2 activity.

6 months of age. The AHCs, including those lacking ADAR2 due to Cre-mediated recombination, were viable in AR2res mice at 6 months of age, and the total number of AHCs was the same as in age-matched control mice (**Figure 9A**). Consistent with a lack of AHC loss, there was no detectable increase in GFAP- or MAC2-immunoreactivity in the anterior horns (Hideyama et al., 2010). These results demonstrate that an ADAR2 deficiency induces the slow death of motor neurons specifically via the GluA2 Q/R site editing failure (**Figure 9B**).

DEATH OF MOTOR NEURONS IN HETEROZYGOUS AR2 (HETEROAR2) MICE

The results of our experiments with the AR2 mice indicate that deficient-ADAR2-mediated death of motor neurons in the spinal cord and the cranial motor nerve nuclei is specifically mediated by the failure to edit the GluA2 Q/R site. In the ALS spinal cord, some motor neurons express unedited GluA2, but others express only edited GluA2. The majority of motor neurons expressing unedited GluA2 also express edited GluA2 (Kawahara et al., 2004). Furthermore, a recent immunohistochemical study demonstrated that both ADAR2-positive and ADAR2-negative motor neurons coexist in patients with sporadic ALS, whereas all motor neurons are ADAR2-positive in control subjects (Aizawa et al., 2010). However, we do not know the expression level of ADAR2 that is required to edit all GluA2 mRNA or the proportion of unedited GluA2 that is not harmful to motor neurons.

To answer these questions, we investigated the extent of GluA2 Q/R site editing in motor neurons lacking one ADAR2 allele in the heterozygous $ADAR2^{flox/+}$/VAChT-Cre (HeteroAR2) mice. Additionally, we investigated whether motor neurons lacking one ADAR2 allele can survive in HeteroAR2 mice compared to AR2 and control mice. In HeteroAR2 mice, the proportion of motor neurons that express Cre is the same as in AR2 mice; the Cre-expressing motor neurons express only one ADAR2 allele. Therefore, if the expression level of ADAR2 in normal motor neurons is sufficiently above the requirement to edit the Q/R site of all GluA2 mRNAs expressed (i.e., threefold or more), all motor neurons would express only edited GluA2. However, if normal motor neurons express ADAR2 at a level that is only sufficient to edit GluA2 (i.e., less than twofold), motor neurons with one ADAR2 allele would express abundant unedited GluA2 and die. Furthermore, if motor neurons expressing one ADAR2 allele express both edited and unedited GluA2 and undergo degeneration in HeteroAR2 mice, we would expect to find the proportion of unedited GluA2 that is toxic to motor neurons.

When the extent of GluA2 Q/R site editing was examined in the lysates of three laser-captured motor neurons of 2-month-old HeteroAR2 mice, GluA2 Q/R site editing was incomplete in approximately 20% of the lysates (**Figure 10**). The proportion of edited GluA2 was above 70% in all the lysates; however, in the AR2 mice of the same age, unedited GluA2 was detected in more than 60% of the lysates of three motor neurons, and the editing efficiency was 0 in 7% of the lysates examined (Hideyama et al., 2010). Therefore, it is likely that motor neurons exhibit considerable editing activity in the expression of only one ADAR2 allele; however, this is not sufficient to edit the Q/R site of all GluA2 mRNA in HeteroAR2 mice.

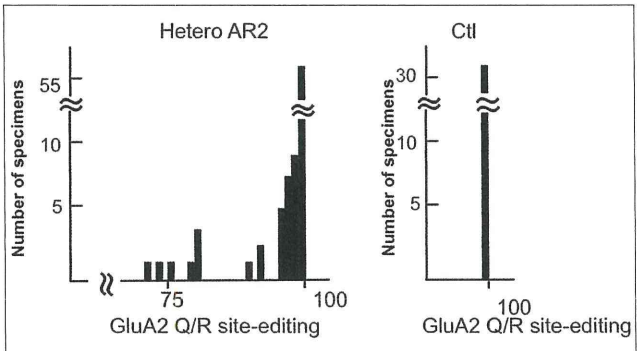


FIGURE 10 | Some of the motor neurons from HeteroAR2 mice express Q/R site-unedited GluA2 mRNA. A frequency histogram of editing efficiency at the GluA2 Q/R site in the lysates of three motor neurons obtained from heterozygous ADAR2$^{flox/+}$/VAChT-Cre.Fast (HeteroAR2) mice and control mice (Ctl). Motor neurons (252 neurons) were dissected from 14-μm-thick frozen sections of spinal cord obtained with a laser microdissector from HeteroAR2 mice at 2 months of age ($n = 4$). The lysates containing three motor neurons (84 specimens) were subjected to a measurement of the editing efficiency of GluA2 mRNA at the Q/R site. This histogram shows that approximately a quarter of the lysates, or 15–20% of AHCs, express unedited GluA2. Notably, the extent of GluA2 editing is above 70%, which is in contrast with the AR2 mice, in which approximately 8% of the lysates expressed only unedited GluA2. All 96 motor neurons dissected from control mice (Ctl; ADAR2$^{flox/flox}$ and VAChT-Cre mice) expressed only Q/R site-edited GluA2 mRNA.

We also examined whether there was a loss of AHCs in HeteroAR2 mice. The extent of AHC loss in HeteroAR2 mice was approximately half (26%) of that observed in AR2 mice (46%) at 12 months of age (**Figure 11A**). A moderate increase in GFAP- and MAC2-immunoreactivity was detected in the anterior horns of HeteroAR2 mice at 12 months of age (**Figure 11B**). HeteroAR2 mice did not exhibit significant behavioral changes until 12 months of age, indicating that mild loss of motor neurons would not affect motor function at least until 1 year of age.

These results show that one ADAR2 gene allele is sufficient to edit all GluA2 mRNA in half of the motor neurons but is insufficient in the other half of motor neurons. Because the editing efficiency was above 70% in the motor neuron lysates of HeteroAR2 mice, it is likely that the minimal expression level of ADAR2 required for complete GluA2 editing is slightly higher than half of the normal level in mouse motor neurons.

More than a quarter of the motor neurons in HeteroAR2 mice underwent degeneration by the age of 1 year. Furthermore, from the results of our experiments with AR2 and AR2res (Hideyama et al., 2010), it is likely that only the motor neurons expressing unedited GluA2 have undergone degeneration. The proportions of unedited GluA2 are 30% at the maximum and less than 10% in the majority of motor neurons in HeteroAR2 mice, indicating that expression of unedited GluA2, even in a small proportion, is not favorable for the survival of motor neurons in mice.

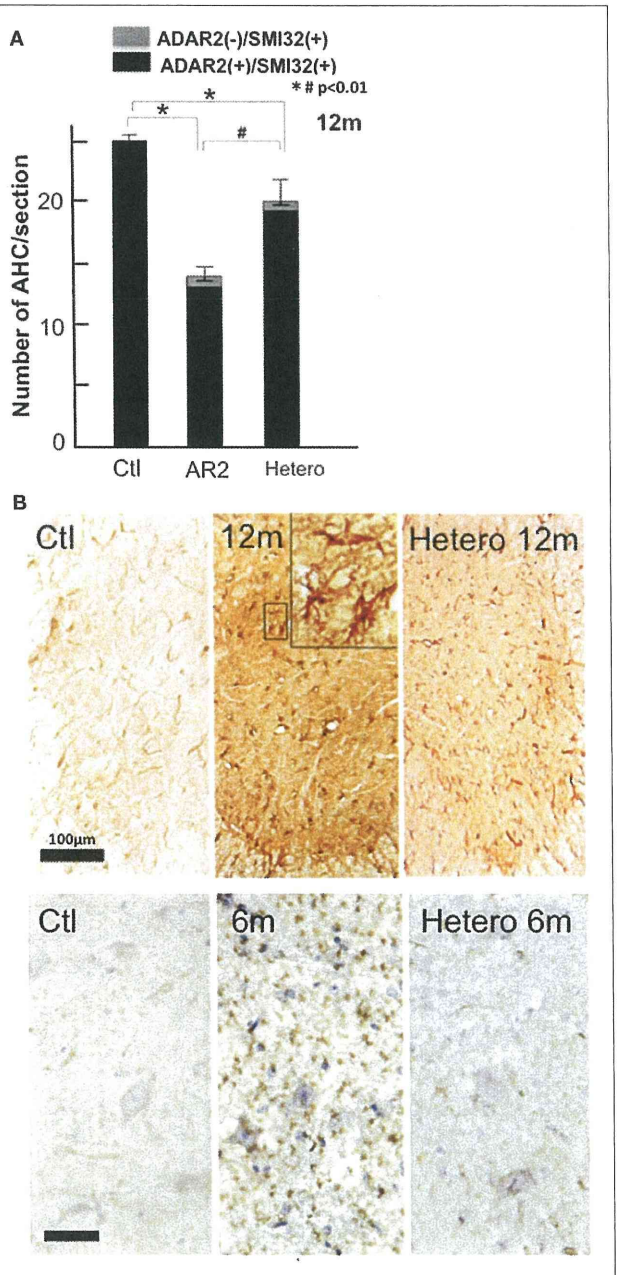


FIGURE 11 | HeteroAR2 mice display a loss of AHCs. (A) The numbers (mean ± SEM) of ADAR2-positive AHCs (black columns) and those of ADAR2-lacking AHCs (gray columns) in HeteroAR2 mice at 12 months of age compared to age-matched AR2 and control mice. There was a loss of AHCs in the HeteroAR2 mice (Hetero; $*p < 0.01$) but to a lesser extent than in the AR2 mice ($\#p < 0.01$). **(B)** Immunohistochemistry in the anterior horn of the spinal cord (C5). There was an increase in GFAP-immunoreactivity but to a lesser extent than in the AR2 mice (upper panel) and a slight increase of MAC2-immunoreactivity (lower panel) in the HeteroAR2 mice. Scale bars: 100 μm (upper panel) and 50 μm (insets and lower panel).

THE ADAR2 HYPOTHESIS FOR SPORADIC ALS

Progression of ALS is rather slow, taking several years to progress from the onset of the initial symptoms to death, which results from failure of the respiratory muscles. From our findings in the AR2 mice, we learned that some ADAR2-lacking motor neurons died within 1 month of ADAR2 knockout, whereas others could survive more than 1 year even with only unedited GluA2

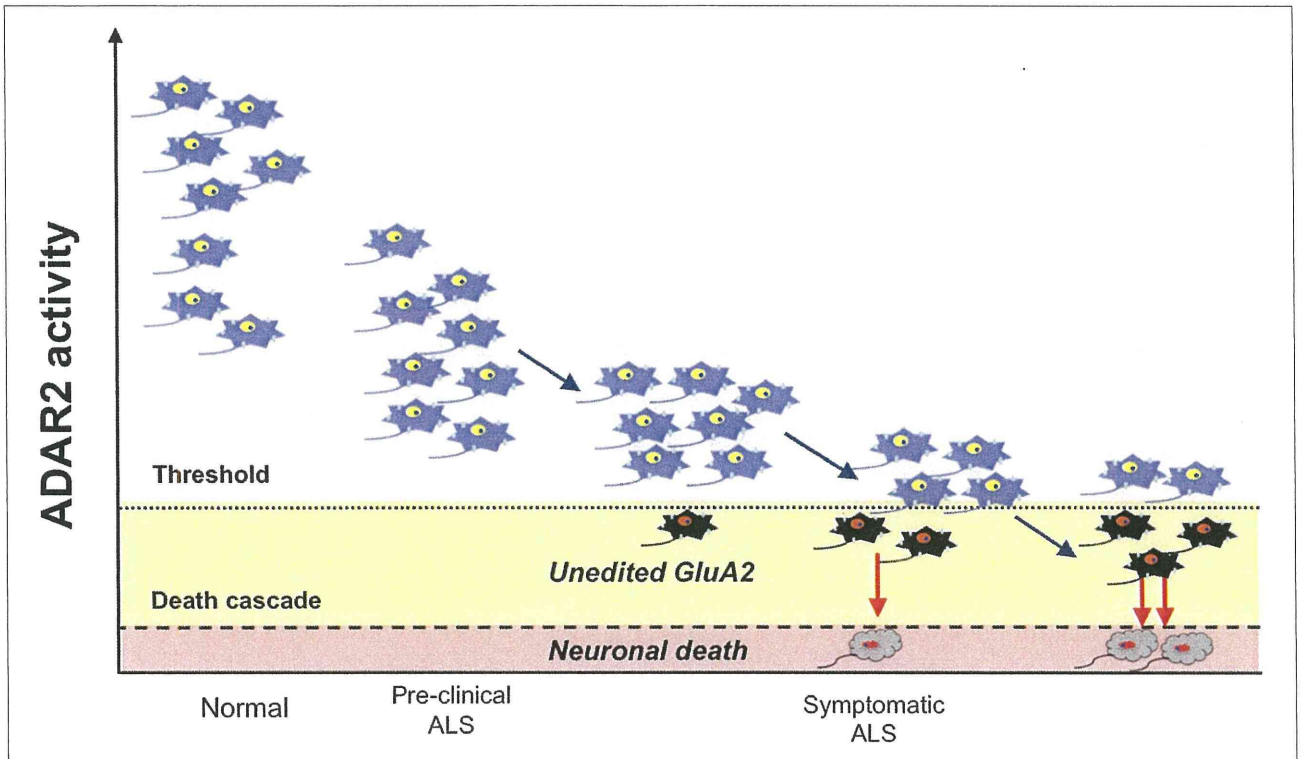


FIGURE 12 | The ADAR2-GluA2 hypothesis for ALS pathogenesis. ADAR2 activity is preserved well above the level that enables motor neurons to edit the Q/R site of all GluA2 mRNA expressed in healthy individuals (normal; neurons in blue). In the motor neurons of sporadic ALS patients, ADAR2 activity progressively decreases, and the ADAR2 activity in all motor neurons is initially low but is within a level that is sufficient to edit the Q/R site of all GluA2 mRNA; thus, motor neurons function normally (pre-clinical ALS). With a decline of ADAR2 activity, some motor neurons express ADAR2 below a threshold level for GluA2 RNA editing (dotted line) and express unedited GluA2 (neurons in black). These motor neurons can survive for some period of time, but they do not function normally and ultimately enter into the death cascade in a stochastic manner. With the progressive decline of ADAR2 activity, the number of motor neurons expressing unedited GluA2, and therefore those destined to die, increases. Patients and neurologists recognize ALS only after a considerable proportion of motor neurons has already malfunctioned (neurons in black) or been lost (neurons in gray).

expression (Hideyama et al., 2010). Therefore, it is likely that the timing of motor neuron death may be a stochastic phenomenon that depends on environmental factors and the level of the compensatory activity in the individual neurons, including the firing frequency of the motor neurons, the strength of the Ca^{2+} buffering system, and the density of functional Ca^{2+}-permeable AMPA receptors. Furthermore, the results from the HeteroAR2 mice indicate that motor neurons expressing unedited GluA2, regardless of the proportion, are destined to die in sporadic ALS patients.

The progressive downregulation of ADAR2 activity increases the number of motor neurons expressing unedited GluA2 in sporadic ALS. The mechanism underlying the reduction of ADAR2 activity in ALS motor neurons is not clear; however, considering that inefficient GluA2 RNA editing was found only in sporadic cases (Kawahara et al., 2006), undefined postnatal factors regulating the ADAR2 activity should not be neglected. Because ADAR2 SNPs are associated with longevity syndrome (Sebastiani et al., 2009) and the age-dependent downregulation of ADAR2 activity has been shown in human brains (Nicholas et al., 2011), the acceleration of age-related neuronal dysfunction may have a role in the progressive reduction of ADAR2 activity in ALS motor neurons.

We recently reported that TDP-43 pathology, which is a hallmark of ALS, appeared only in the motor neurons lacking ADAR2 immunoreactivity in patients with sporadic ALS (Aizawa et al., 2010). It is likely that motor neurons lacking ADAR2 immunoreactivity represent those expressing unedited GluA2, and motor neurons expressing ADAR2 immunoreactivity with normal TDP-43 immunoreactivity represent those expressing only edited GluA2. Because a reduction of ADAR2 likely begins before motor neurons express unedited GluA2 in ALS motor neurons, TDP-43 pathology may be induced by the expression of unedited GluA2 rather than TDP-43 pathology causes reduced ADAR2 activity. We do not know whether the reduction of ADAR2 immunoreactivity in the ALS motor neurons results from a reduced gene expression or accelerated ADAR2 protein degradation catalyzed by Ca^{2+}-activated proteinase as demonstrated in ischemic rat brains (Mahajan et al., 2011).

Based on this evidence, we propose a hypothesis for the pathogenesis of sporadic ALS. ALS motor neurons express progressively lower ADAR2 activity before manifesting an ALS phenotype, and the pathological process commences when motor neurons begin to express unedited GluA2. Motor neurons expressing unedited GluA2 do not function normally but do not immediately die.

The timing of the entry of these motor neurons into the death cascade may be regulated in a stochastic manner. With a sequential progression of these events, the pool of normally functioning motor neurons expressing only edited GluA2 decreases, which ultimately induces the ALS phenotype in patients (**Figure 12**). Because mutations in the coding molecules of the genes involved in RNA regulation, including TDP-43 and FUS/TLS, were recently found in patients with familial ALS (Arai et al., 2006; Neumann et al., 2006; Kwiatkowski et al., 2009; Vance et al., 2009), dysregulation of RNA metabolism in ALS pathogenesis is now attracting the interest of researchers (Lemmens et al., 2010). Because ADAR2 is an RNA regulatory molecule, future studies are needed to elucidate the molecular link between abnormalities of these ALS-linked RNA regulatory molecules with the ADAR2 downregulation in ALS motor neurons. Forcing motor neurons to express only edited GluA2 may be a future therapy for sporadic ALS.

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