ANESTHESIOLOGY

Genetic Analysis of Patients Who Experienced Awareness with Recall while under General Anesthesia

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The incidence of explicit recall of intraoperative events, or awareness with recall, is less than 0.2%
- Anesthetic dosing is apparently adequate in 10 to 25% of awareness with recall patients
- The awareness with recall phenotype only reveals itself when patients are exposed to anesthesia

What This Article Tells Us That Is New

- A preliminary study sought to determine whether there is evidence that awareness with recall is caused by a few rare variants with high penetrance in 12 patients who had experienced awareness with recall in the presence of apparently adequate anesthesia
- Whole exome sequencing was conducted and identified variants were filtered and prioritized to identify a candidate list that might be suitable for further investigation of causes of awareness with recall
- No candidate gene(s) suggestive of a monogenic etiology were identified, possibly because of the application of a filtering strategy, the small sample size, or use of exome sequencing, which does not interrogate potentially important regulatory noncoding sequences

ABSTRACT

Background: Intraoperative awareness with recall while under apparently adequate general anesthesia is a rare, unexplained, and often very distressing phenomenon. It is possible that a relatively small number of genetic variants might underlie the failure of general anesthetic drugs to adequately suppress explicit memory formation and recall in the presence of apparently adequate anesthesia concentrations.

Methods: The authors recruited 12 adult patients who had experienced an episode of intraoperative awareness with recall (compared with 12 controls), performed whole exome sequencing, and applied filtering to obtain a set of genetic variants that might be associated with intraoperative awareness with recall. The criteria were that the variant (1) had a minor allele frequency less than 0.1% in population databases, (2) was within exonic or splicing regions, (3) caused a nonsynonymous change, (4) was predicted to be functionally damaging, (5) was expressed in the top 50% of genes expressed in the brain, and (6) was within genes in Kyoto Encyclopedia of Genes and Genomes pathways associated with general anesthesia, drug metabolism, arousal, and memory.

Results: The authors identified 29 rare genetic variants in 27 genes that were absent in controls and could plausibly be associated with this disorder. One variant in *CACNA1A* was identified in two patients and two different variants were identified in both *CACNA1A* and *CACNA1S*. Of interest was the relative overrepresentation of variants in genes encoding calcium channels and purinergic receptors.

Conclusions: Within the constraints of the filtering process used, the authors did not find any single gene variant or gene that was strongly associated with intraoperative awareness with recall. The authors report 27 candidate genes and associated pathways identified in this pilot project as targets of interest for future larger biologic and epidemiologic studies.

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Explicit recall of intraoperative events is a rare, but often distressing, phenomenon. We will use the nomenclature *awareness with recall* for these episodes. For 90% of the population, the amnesic effects of general anesthetic drugs occur at concentrations well below those required for unconsciousness (typically 0.1 to 0.3 minimum alveolar concentration [MAC]).[1] However, in approximately 10 to 25% of awareness with recall patients anesthesia dosing is apparently adequate (greater than 0.5 MAC),[2,3] indicating that the anesthetic drug has failed to

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disrupt conscious perception and the memory consolidation process. A genetic predisposition for awareness with recall may exist and could explain resistance to levels of anesthesia that are normally considered adequate for the majority of patients. However, we are not aware of any study that has previously investigated this hypothesis.

Many neurobiologic events must be fulfilled to lay down a properly consolidated memory.[4–6] It is noteworthy that the awareness with recall phenotype only reveals itself when patients are exposed to anesthesia; typically, awareness with recall patients display no other identified phenotypic disturbance in day-to-day life, and awareness with recall has no clear demographic or disease associations. Also, the incidence of awareness with recall is low (less than 0.2%).[7] If there is a genetic component to awareness with recall, these observations would suggest one of two parsimonious hypotheses for potential genetic causes of awareness with recall: (1) awareness with recall could be a polygenic trait with common variants in many weakly penetrant genes—and having interactions with other genes or the environment—each contributing a small amount to the risk; or that (2) awareness with recall could be caused by a few rare variants, each with a high penetrance. Identifying common variants of low impact is difficult, and typically involves undertaking a genome-wide association study, usually requiring hundreds if not thousands of affected individuals. Without access to these resources, we undertook a preliminary study examining whether there is evidence for the rare-variant model. To do this we conducted whole exome sequencing of a set of carefully chosen patients who have experienced awareness with recall in the presence of apparently adequate anesthesia concentrations. The identified variants were filtered and prioritized to identify a candidate list of variants/genes that might form a reasonable basis for further investigation of the causes of awareness with recall.

Materials and Methods

This study was conducted according to the Strengthening the Reporting of Observational Studies in Epidemiology guideline and Strengthening the Reporting of Genetic Association Studies extension.[8] Prospective approval was obtained from the ethics committees of Royal Melbourne Hospital, Australia (June 9, 2011; approval number 2011.008) and Waikato Hospital, New Zealand (June 29, 2011, Northern Y Regional Ethics Committee approval reference, NTY/11/03/027), as investigators at these sites recruited and interviewed the patients. The study was not registered because it was not a clinical trial.

Patient Cohort

Patients were eligible for this study if they were fluent in English, were at least 18 yr of age at the time of enrollment and at least 13 yr of age at the time of the awareness episode, experienced an awareness episode during the last 10 yr (on December 14, 2013 amended to the last 30 yr, *i.e.*, since the widespread use of end tidal volatile anesthetic agent monitoring), and reported an awareness episode that included the following features:

1. General anesthesia was intended for the case
2. Somatic sensations, pain, sounds, conversations, or emotions were experienced while the patient was supposed to be unconscious
3. These feelings were experienced during the procedure (*i.e.*, conversations confirmed to have occurred during surgery, sounds that could only have been heard during surgery)

Patients were recruited *via* advertisements in local newspapers, in the investigators' hospitals, on hospital websites and the website of the Australian and New Zealand College of Anaesthetists, and through free media opportunities (such as features in popular magazines and television programs). Patients who answered the advertisements were screened over the phone. Those who met the eligibility criteria were mailed the patient information and consent form. Patients were given an opportunity to ask questions of the investigators or obtain independent advice. After receipt of written informed consent, trained research nurses at each institution conducted a phone or in-person interview with the patient. The nurses or investigators provided general advice to patients, or referral to their own physician or hospital, if requested or required. The investigators did not seek access to medical records related to the reported awareness episodes.

The following information was obtained at interview:

1. Date of birth
2. Sex
3. Date of index surgery
4. Name of index surgery
5. Family history of awareness
6. Description of awareness episode
7. Presence of somatic sensations, pain, paralysis or weakness, sounds or conversations audible, visual perceptions, tried or able to move, emotions experienced, feelings of helplessness, anxiety, panic, or impending death, other (free text)
8. Description of any consequences of awareness episode
9. Presence of sleep disturbance, nightmares, daytime anxiety, depression, fear of future anesthetics, late psychologic problems, posttraumatic stress disorder
10. Description of treatment
11. Presence of consultation with health professional, counseling, medication, or other treatment for awareness episode
12. Details of the explanation provided to the patient by their anesthetist (especially in relation to the adequacy of anesthesia during the episode)
13. Any written material regarding incident in possession of patient and which patient is willing to share

Interview reports were adjudicated by three of the investigators (J.W.S., K.L., A.J.D.). The adjudicators independently classified the cases as "awareness," "possible awareness," and "no awareness," as per our previous studies.[9,10] Only cases that were universally coded as "awareness" were included. Furthermore, the adjudication panel decided by consensus whether a patient was aware despite apparently adequate general anesthesia.

DNA Sample Collection, Storage, and Analysis

A saliva sample was obtained from all included patients and genomic DNA was isolated using the Oragene kit as per the manufacturer's instructions (DNA Genotek, Canada). DNA quality and concentration was determined using a NanoDrop 2000c (ThermoFisher Scientific, USA) and samples were stored at −20°C until analyzed.

Whole Exome Sequencing and Variant Calling

Because the whole exome sequence of each individual can vary considerably, it is necessary to have a process to maximize the reliability of identification of the variants; which is done by comparing the sequence of participants with a reference genome databases. The technical description of the process we used is as follows. Whole exome sequencing was performed at the Australian Genome Research Facility, Melbourne, Australia, using the Agilent SureSelect Human All Exon V5+UTR capture platform.[11] The raw whole exome sequencing data from the 12 awareness with recall cases were analyzed using an in-house bioinformatics pipeline. In summary, the raw sequencing reads were aligned to the hg19 version of the reference human genome assembly with Novoalign (www.novocraft.com; accessed January 11, 2016) and polymerase chain reaction duplicates were removed using Picard MarkDuplicates.[12] It is necessary to determine the existence (likelihood) of the gene variants from the raw nucleotide sequence. To identify sequence variants in cases compared with the reference, the open source program (GATK HaplotypeCaller[13]) was used. The consequence and potential significance of identified variants was then determined by comparison with previously identified and reported variants using the bioinformatics tool ANNOVAR.[14,15] Standard quality control checks were performed during all stages of the analysis pipeline. We used our in-house database to select 12 other whole exome sequencing datasets as controls for filtering, in addition to the Genome Aggregation Database Exome Aggregation Consortium[16] (gnomAD/ExAC) and 1,000 Genomes variant frequency databases.[17] These are databases that catalogue the genetic variation in tens of thousands of unrelated individuals that can be used to establish the accuracy and incidence of any variants, and are critical in eliminating false positives, which can arise as a result of differences in the data analysis processing steps involved in variant calling. The controls were selected to match the samples with respect to sex, age, and ethnicity; they also underwent the same sequencing process at the same facility.

Filtering Strategies in Candidate Genes

Approximately 5.5 million variants were identified in the whole exome sequencing data generated for the 12 participants (fig. 1); therefore, a filtering strategy needed to be used. Filtering of variants was performed based on the assumption that the causal variants are rare and likely to affect either expression of the transcript or amino acid sequence of the protein. Application of these criteria reduced the candidate list to 8,706 variants. Initially, we searched this candidate list for the presence of variants in the Mendeliome, a subset of ~4000 genes with reported disease associations in the Online Mendelian Inheritance in Man.[18,19] We did not detect any variants fulfilling the criteria of either (1) previously reported as pathogenic or likely pathogenic or (2) novel/very rare high impact variants, that could plausibly explain either a dominant or recessive genetic model.

To further reduce the candidate variant list, we next focused on a subset of genes that had a higher *a priori* chance of being involved in awareness with recall. The R statistical software package KEGGgraph (http://bioconductor.org/packages/KEGGgraph/; accessed January 11, 2016) was used to extract the genes involved in Kyoto Encyclopedia of Genes and Genomes pathways to assemble the anesthetic awareness candidate gene list. Kyoto Encyclopedia of Genes and Genomes is a database of gene clusters associated with various known cellular functions. General literature searches involving likely targets of general anesthesia, drug metabolism, arousal, and memory were used to assist in finding the likely Kyoto Encyclopedia of Genes and Genomes pathways. The pathways and their member genes are listed in the Supplemental Digital Content (http://links.lww.com/ALN/C5).

To be included in the final dataset, all variants needed to pass the following filtration criteria: have a minor allele frequency less than 0.1% in the ExAC database; be within exonic or splicing regions; cause a nonsynonymous change; predicted to be functionally damaging by one of SIFT[20] and PolyPhen-2[21] (these databases predict the potential for a given variant to be detrimental to protein function based on sequence homology and physical properties of the amino acids involved); be expressed in the top 50% of genes expressed in the brain based on the genotype tissue expression (GTEx) dataset; be within genes in Kyoto Encyclopedia of Genes and Genomes pathways associated with general anesthesia, drug metabolism, arousal, and memory; and be detected in at least one of the 12 cases, but fewer than three controls. Genes were further annotated with residual variation intolerance scores,[22] genic intolerance scores[17] and Online Mendelian Inheritance in Man.

Validation of Variants

Prioritized variants were validated by standard polymerase chain reaction amplification and Sanger sequence analysis. This analysis was performed fee for service by Genewiz Corporation (USA).

Sample Size and Statistics

Descriptive statistics were used to describe the included patients and the quality of the sequencing data. Continuous data were summarized using median (range), and categorical data were summarized using number (percent). We did not perform formal inferential statistical testing because there was insufficient statistical power to achieve significance after multiple testing because of the small number of rare variants identified and the small sample size.

Results

Patients were screened between September 2011 and January 2014. Of 102 screened patients, 52 met the eligibility criteria and were interviewed. Among these, seven were determined to have had the awareness episode too long ago and one did not return the DNA sample. Twelve of these patients, with most phenotypic consistency, were selected for sequence analysis (fig. 2).

The characteristics of the 12 included patients are reported in table 1. The median age was 44.5 (range 23 to 88) years at the time of surgery. Eleven of the 12 patients (92%) were female. The interval between the surgery and the interview was 6.5 (range 0 to 36) yr. The single patient who had an episode of awareness 36 yr ago was included because this patient had a very clearly defined episode and also reported a family history of awareness with recall. Patients had the following experiences during their awareness episode: somatic sensations (83%), pain (42%), weakness (33%), sounds (75%), voices (67%), visual perceptions (17%), attempts to move (33%), able to move (17%), emotions (75%), and feelings of helplessness (75%). Psychologic consequences were experienced by 42% of patients.

Whole Exome Sequencing and Variant Calling

The sequencing data generated were of high quality, the median depth of coverage ranged from 31 to 38 reads with a mean of 35. The percentage of targeted bases covered by at least 10 reads ranged from 92.4 to 94.9%, with a mean of 94.0%.

Filtering and Validation

The pathway search led to nine pathways containing a total of 658 genes that were chosen as the candidate gene set for variant analysis. Because we preselected pathways to examine, we were unable to perform pathway enrichment analyses.

The variant filtering strategy is summarized in figure 1. This reduced the number of variants from an initial 5,465,705 variants to a final list of 29 variants in 27 genes in the 12 individuals with awareness with recall (table 2). None of the final 29 variants was observed in any of the

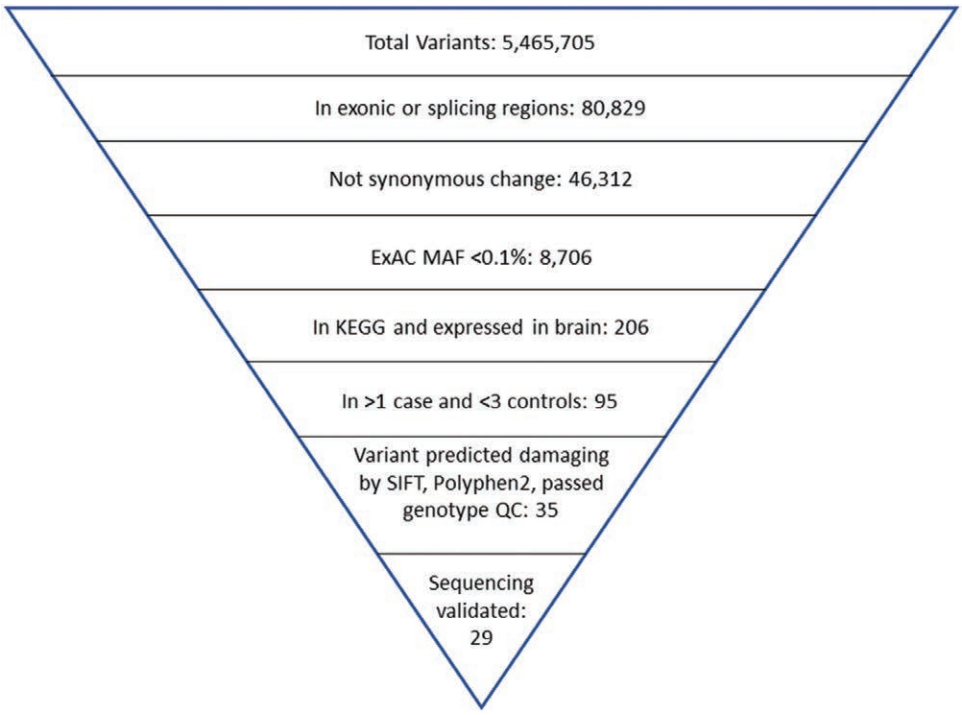

Fig. 1. Variant filtering strategy. ExAC, Exome Aggregation Consortium; KEGG, Kyoto Encyclopedia of Genes and Genomes; SIFT, sorting intolerant from tolerant database; polyphen2, polymorphism phenotyping v2.

Table 1. Characteristics of Included Patients

Patient	Sex	Type of Surgery	Age at Surgery (yr)	Years since Surgery (yr)	Family History	Somatic	Pain	Weakness	Sounds	Words	Visual	Move Attempt	Move	Emotions	Helpless	Consequences
1	f	gynae	36	28	–	–	–	–	–	+	–	–	+	+	–	+
2	f	gynae	36	0	–	+	+	–	–	–	–	+	+	–	–	+
3	f	spine	37	9	–	+	+	+	+	+	+	+	–	+	+	+
4	f	gynae	23	36	+	+	–	+	+	+	–	+	–	+	+	–
5	f	gynae	41	4	+	–	–	+	+	+	+	+	–	+	+	–
6	f	general	50	0	–	+	–	+	+	+	–	+	–	+	+	+
7	f	general	53	6	–	+	+	+	+	+	–	+	–	+	+	+
8	f	many	48	11	–	+	–	+	+	+	–	+	–	+	+	–
9	f	ortho	50	7	–	+	–	+	+	+	–	+	–	+	+	–
10	f	ortho	36	9	–	+	+	+	+	+	–	+	+	+	+	–
11	f	ortho	76	4	–	+	–	–	+	+	–	–	+	–	–	–
12	m	cardiac	88	0	–	+	+	–	–	–	–	+	–	+	+	–

Plus signs indicate present; *minus signs* indicate not present. f, female; general, general surgery; gynae, gynecologic surgery; m, male; and ortho, othropedic surgery.

12 control whole exome sequencing datasets, and all were observed in only a single patient, except for the variant in *CACNA1A* (NM_001127222.1:c.6658_6659insACC, p.[His2219dup]), which was observed in two patients. Two different variants in the gene encoding CACNA1S were identified (NM_000069.2:c.3322C>G; p.[Gln1108Glu] and NM_000069.2:c.1819G>A; p.([Val607Ile]).

Possible Associated Disorders

Awareness with recall has no known associations with other diseases. Twenty-five of the variants caused nonsynonymous coding sequence alterations, two resulted in frameshift insertion deletion (*P2RX1* and *CREB3L3 indels*), one resulted in a nonframeshift duplication of a histidine (*CACNA1A*), and one resulted in an intronic deletion of five base pairs 16 base pairs upstream of a splice site (*RPS6KA6*). Of the 27 genes of interest that we identified, a few had some reported association with various clinical disorders (such as malignant hyperthermia, long QT syndrome, epilepsy, spinocerebellar ataxia, myopathy, and leukemia [table 2]). However, the variants we identified in the awareness with recall patients are probably not clinically significant for these disorders, because they are described as either not present or of conflicting interpretation in ClinVar, which is the main publicly available database for linking gene variants with phenotypes.[23]

Likely Candidate Genes for Awareness with Recall

There was no single gene variant, or multiple different variants in a single gene, present in a high proportion of the awareness with recall patients that would indicate a monogenic or paucigenic mechanism in our cohort. We did identify a cluster of variants for nine CACN genes in 10 of the patients (table 2), and most of the other variants are linked to calcium signaling pathways in the Genecards database.[24] In addition, there were six variant identified in genes that encode proteins involved in purinergic receptor function and metabolic signaling. These observations are suggestive that a polygenic mechanism may be associated with awareness with recall in this cohort, although general conclusions regarding underlying genetic mechanisms will require analysis of larger cohorts.

Discussion

The purpose of this observational study is to report some possible genetic methodologies and explanations regarding the clinical issue of awareness with recall. We do not make any definitive causal claims, but present these as hypothesis-generating data. This study also highlights some of the methodologic difficulties in understanding the implications of genetic studies.

In the cohort analyzed we did not identify any candidate gene(s) suggestive of a monogenic cause. This may be attributable to one or more factors, including the

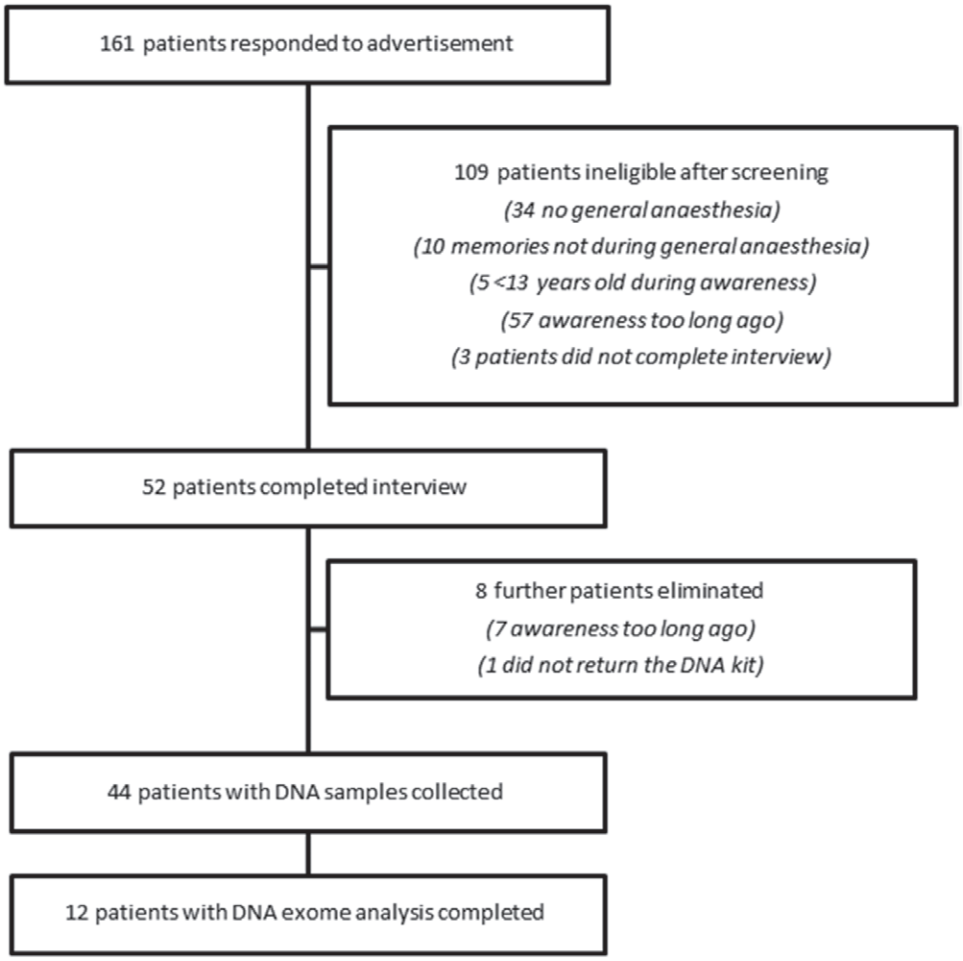

Fig. 2. Study flowchart.

application of our filtering strategy, the relatively small sample size, or the use of exome sequencing, which does not interrogate potentially important regulatory noncoding sequences. We did identify variants in multiple genes that function in the calcium signaling, purinergic receptor, and metabolic signaling pathways. These observations are consistent with the hypothesis that awareness with recall is a polygenic trait. Other large next-generation studies in neurogenetic disorders—which seek to identify rare causal variants—commonly identify multiple genes with different variants showing elevated risk.[25] The variants that we found merit further definitive investigation through a replication study in an independent cohort of awareness with recall patients to increase statistical power, and with experimental functional studies—such as mouse models with these variants inserted into the genome artificially by genome editing methodologies such as CRISPR/Cas9.

The role of calcium signaling pathways and their relationship to purinergic pathways is worth exploration. Clearly they are pivotal in memory formation[26] and arousal. In animal models mutations of these genes have been associated with hyperfunction diseases, such as epilepsy and migraine. As yet there is limited evidence to associate these mutations with anesthetic sensitivity, although Tatsuki *et al.*[27] suggested that impaired CACNA-1H /Cav2.3 decreases slow wave sleep and is a critical regulator of N-methyl-D-Aspartate receptor function—and hence memory. As regards general anesthesia, Takei *et al.* somewhat confusingly found that CACNA-1B mutants were resistant to propofol sleep time but more sensitive to halothane.[28]

Measures of likely harmfulness—such as residual variation intolerance score and the loss of function intolerant scores—do not appear to be a useful guide for determining potential pathogenicity in this setting as a broad range of often conflicting scores, including very high (tolerant to mutations) for the known malignant hyperthermia gene *CACNA1S,* and yet very low for *R*YR1, were observed. The interpretation of the possible role of the gene variations

Table 2. Final List after Filtering for 29 Variants in 27 Genes in the 12 Individuals with Awareness with Recall

Chr	Start	Ref	Alt	Gene	Gene Number	Protein	dbSNP	ExAC/ gnomAD	RVIS	R	Is Variant in ClinVar?	OMIM
1	181767498	G	A	CACNA1E	NM_001205293.1:c.6467G>A	p.(Arg2156His)	rs2480373	0.000465	3.34	6.61 (pLI=1)	No	NA
1	201029878	G	C	CACNA1S	NM_000069.2:c.3322C>G	p.(Gln1108Glu)	rs765685405	0.00002436	61.36	-0.97 (pLI=0)	Uncertain	MALIGNANT HYPERTHERMIA, SUSCEPTIBILITY TO, 5
1	201046056	C	T	CACNA1S	NM_000069.2:c.1819G>A	p.(Val607Ile)	rs377461013	0.0000528	61.36	-0.97 (pLI=0)	No	MALIGNANT HYPERTHERMIA, SUSCEPTIBILITY TO, 5
2	128083346	G	A	MAP3K2	NM_006609.4:c.635C>T	p.(Ser212Phe)	NA	0.0000041	19.17	0.93 (pLI=1)	No	NA
4	26483637	T	G	CCKAR	NM_000730.2:c.910A>C	p.(Asn304His)	rs201121639	0.000364	74.38	0.12 (pLI=0.02)	No	NA
4	104640318	C	G	TACR3	NM_001059.2:c.515G>C	p.(Ser172Thr)	rs201237591	0.00000416	31.78	1.06 (pLI=0)	No	HYPOGONADOTROPIC HYPOGONADISM
4	140624641	T	C	MGST2	NM_002413.4:c.262T>C	p.(Tyr88His)	NA	NA	63.43	-0.27 (pLI=0.06)	No	NA
5	159399049	C	CCGCCGCCGA	ADRA1B	NM_000679.3:c.1113_1114insCGCCGCCGA	p.(Arg378_ Arg380dup)	rs764801657	0.000948	31.87	3.79 (pLI=0.18)	No	NA
7	31864488	G	A	PDE1C	NM_005020.2:c.1399C>T	p.(Arg467Cys)	rs765961590	0.000008132	49.05	1.14 (pLI=0)	No	NA
10	18827163	C	T	CACNB2	NM_201596.2:c.1357C>T	p.(Leu453Phe)	rs145638628	0.000209	73.81	0.36 (pLI=0)	Uncertain	Not specified
11	1580181	C	T	DUSP8	NM_004420.2:c.475G>A	p.(Val159Met)	rs747581475	0.0000244	N	4.06 (pLI=0.68)	No	NA
11	67432827	C	T	ALDH3B2	NM_000695.3:c.635G>A	p.(Arg212Gln)	rs61736819	0.0005779	99.55	0.23 (pLI=0)	No	NA
11	113813713	C	T	HTR3B	NM_006028.4:c.706C>T	p.(Arg236Cys)	rs150117061	0.0000434	91.61	-0.51 (pLI=0)	No	NA
12	2797785	G	A	CACNA1C	NM_001129827.1:c.6101G>A	p.(Ser2034Asn)	rs755280013	0.0000325	3.51	6.41 (pLI=1)	Uncertain	Long QT syndrome
12	49221405	C	G	CACNB3	NM_000725.3:c.1178C>G	NM_000725.3:p. (Ser393Cys)	rs749648436	0.0000181	23.44	1.71 (pLI=0.05)	No	NA
12	121603187	C	G	P2RX7	NM_002562.5:c.561C>G	p.(Asn187Lys)	rs112631221	0.0000541	95.98	0.34 (pLI=0)	No	LEUKEMIA, CHRONIC LYMPHOCYTIC
14	70634202	C	T	SLC8A3	NM_033262.4:c.938G>A	p.(Arg313His)	rs149478505	0.0000397	7.02	0 (pLI=0.62)	No	NA
15	42442027	G	A	PLA2G4F	NM_213600.3:c.943C>G	p.(Arg315Gly)	rs144191503	0.0001	92.74	-1.71 (pLI=0)	No	NA
16	1252303	C	T	CACNA1H	NM_021098.2:c.1853C>T	p.(Pro618Leu)	rs60734921	0.000541	25.99	-2.59 (pLI=0.76)	Uncertain	EPILEPSY, CHILDHOOD ABSENCE, SUSCEPTIBILITY TO, 6
16	47703291	C	G	PHKB	NM_000293.2:c.2593C>G	p.(Pro865Ala)	rs142281844	0.000195	35.71	-0.55 (pLI=0)	No	NA
17	3593422	C	T	P2RX5	NM_002561.3:c.556G>A	p.(Glu186Lys)	rs147009070	0.00052	78.48	-0.22 (pLI=0)	No	NA
17	3806566	-	G	P2RX1	NM_002558.3:c.676_677insC	p.(Leu226Profs*23)	rs758752485	0.0000108	41.88	1.06 (pLI=0)	No	NA
17	3844399	G	A	ATP2A3	NM_005173.3:c.1966C>T	p.(Arg656Cys)	rs140404080	0.000663	5.77	3.13 (pLI=0.06)	No	NA
19	4171445	C	T	CREB3L3	NM_001271997.1:c.934C>G	p.(Arg312*)	rs143545033	0.000743	83.25	-0.55 (pLI=0)	No	NA
19	13318222	A	G	CACNA1A	NM_001127222.1:c.7426T>C	p.(Tyr2476His)	rs779631503	0.0000795	1.68	7.23 (pLI=1)	No	SPINOCEREBELLAR ATAXIA 6
19	13319691	-	GGT	CACNA1A	NM_001127222.1:c.6658_6659insACC	p.(His2219dup)	rs768950814	0.00828	1.68	7.23 (pLI=1)	Uncertain	SPINOCEREBELLAR ATAXIA 6
19	38995965	C	T	RYR1	NM_000540.2:c.8327C>T	p.(Ser2776Phe)	rs147707463	0.000712	0.03	4.44 (pLI=0)	Uncertain	MINICORE MYOPATHY WITH EXTERNAL OPHTHALMOPLEGIA
20	9401989	G	A	PLCB4	NM_000933.3:c.2164G>A	p.(Val722Ile)	rs772944610	0.00000406	25.88	2.78 (pLI=0.27)	No	NA
X	83372466	AAACA	-	RPS6KA6	NM_014496.4:c.790-16_790-12delTGTTT	NA	rs778822899	0.000181	16.12	1.7 (pLI=0.74)	No	NA

Alt, alternative nucleotide in the variant; chr, chromosome; dbSNP, reference number of the variant; ExAC.gnomAD, minor allele frequency in ExAC (Exome Aggregation Consortium) database; OMIM, Online Mendelian Inheritance in Man; ref, reference nucleotide; R, ExAC missense constraint; RVIS, Residual Variation Intolerance Score. 0, no missense constraint; –ve is more variation than expected; +ve is less variation than expected; pLI near 1, LoF constraint.

beyond those already implicated through clinical databases, such as ClinVar, is difficult.

Limitations

This study is of a preliminary nature, primarily to explore methodologic issues and generate hypotheses. There are two important limitations to acknowledge. The first is the question of whether our filtering processes were over restrictive. Because we filtered the genes that were analyzed according to the gene variant frequency and known association with likely anesthesia mechanisms, we have therefore precluded discovering unexpected genes, whose function lies outside our present understanding of brain function. Our approach was primarily driven by the fact that there is a growing recognition that unfiltered genome-wide analyses result in too many false positives—especially with such small sample sizes. We refer readers to an excellent review paper on the problems of rare variant association studies by Bomba *et al.*[29] They highlight the fact that false positives arise both because of incorrect statistical assumptions and bias, as well as heterogeneity in allelic estimation.

Successful implementation of genetic analysis is clearly a balance between overrestrictive filtering *versus* excessive false positive results. A minimum plausible filter would be that the gene variant: was expressed in the brain; caused a change in the protein (non-synonymous, or copy-number variants); was exonic; and was not very common (perhaps less than 1% if the variant had poor penetrance). The analysis of the resultant set of hundreds, or thousands, of genetic variants would probably produce a false positive result; even for a paucigenic Mendelian pattern with our small data set. If we had the resources for analyzing a large data set, this reduced filtering approach might accurately detect a paucigenic pattern. However, our study suggests that awareness with recall may represent a disorder with a substantial polygenic contribution, as observed in late onset diabetes. So there will be substantial difficulties in teasing out the complex web of gene-expression-protein interactions, even with a large dataset.

The other limitation of this study is the accuracy of determining the phenotype from a retrospective study design. awareness with recall may occur as a result of delivering low concentrations of anesthesia either intentionally if the patient cannot tolerate higher doses, or unintentionally as a result of error. In our selection we chose cases where it was thought highly likely that anesthesia should have been adequate, however without prospectively collecting detailed data this assumption can never be completely verified.

In conclusion, we hypothesize that a number of genetic variants found in our sample of awareness with recall patients—in particular those related to calcium signaling and purinergic pathways—could be associated with awareness with recall and should be considered as putative targets in future prospective studies into awareness with recall. To this end a collaborative international anesthesiology database would be a useful tool to start collecting rare variants of interest for anesthesia.

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Competing Interests

The authors declare no competing interests.

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