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Impact of C57BL/6 substrain on sex-dependent differences in mouse stroke models

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ABSTRACT

We have recently found significant variation in stroke vulnerability among substrains of C57BL/6 mice, observing that commonly used N-lineage substrains exhibit larger infarcts than C57BL/6J and related substrains. Parallel variation was also seen with respect to sex differences in stroke vulnerability, in that C57BL/6 mice of the N-lineage exhibited comparable infarct sizes in males and females, whereas infarcts tended to be smaller in females than in males of J-lineage substrains. This adds to the growing list of recognized phenotypic and genetic differences among C57BL/6 substrains. Although no previous studies have explicitly compared substrains with respect to sex differences in stroke vulnerability, unrecognized background mismatch has occurred in some studies involving control and genetically modified mice. The aims of this review are to: present the evidence for associated substrain- and sex-dependent differences in a mouse permanent occlusion stroke model; examine the extent to which the published literature in other models compares with these recent results; and consider the potential impact of unrecognized heterogeneity in substrain background on the interpretation of studies investigating the impact of genetic modifications on sex differences in stroke outcome. Substrain emerges as a critical variable to be documented in any experimental stroke study in mice.

1. Introduction

Some of the earliest experimental stroke studies in mice recognized the importance of considering strain background as a variable that could impact ischemic injury (Barone et al., 1993; Huang et al., 1994; Fujii et al., 1997; Maeda et al., 1999; Majid et al., 2000; Pham et al., 2010). C57BL/6 is the most widely used inbred strain in biomedical research, and the majority of engineered lines are either backcrossed to C57BL/6 or directly generated on this genetic background. However, although all are descended from a colony established at The Jackson Laboratory (JAX), propagation of the original inbred strain at different locations has resulted in numerous substrains, generally due to fixation of spontaneous mutations within independent colonies (genetic drift). Such mutations are estimated to occur at a rate approaching 30 per generation in the C57BL/6 strain (Uchimura et al., 2015). The genetic and phenotypic divergence of the resulting substrains is increasingly appreciated (Mekada et al., 2009; Zurita et al., 2011; Simon et al., 2013; Kraev, 2014; Fontaine and Davis, 2016).

A primary distinction is usually made between J-lineage substrains that can trace their origins directly to JAX, most commonly C57BL/6J, versus the many commercially available N-lineage substrains that are descended from an NIH colony established with a transfer of mice in 1951 (Fig. 1). (Substrain nomenclature indicates first the strain and substrain lineage, e.g., C57BL/6J versus C57BL/6N, followed by specific information about the source of the mice. As examples, a J-lineage substrain from the colony of E. Eicher now maintained by JAX is identified as C57BL6/JEiJ, mice of the N lineage from the colony of Charles River Laboratories are designated C57BL/6NCrl, and the N-lineage substrain currently maintained at JAX is C57BL/6NJ. A substrain now maintained at JAX but originally descended from the colony of D. Bailey, obtained from the NIH colony soon after it was established, is named C57BL6/ByJ. These substrain names are used frequently below and will be abbreviated as simply J, JEiJ, ByJ, NJ and NCrl.) Emphasis on an initial discrimination between J and N is appropriate, since there is greater genetic divergence between than within these lineages (Mekada et al., 2009; Zurita et al., 2011). Recent genotyping efforts have revealed over 10,000 single nucleotide polymorphisms (SNPs) segregating between the J and NJ substrains (Keane et al., 2011), with far fewer variants segregating within each lineage. The issue has become of increasing practical importance with the selection of N-lineage mice as the embryonic stem cell (ESC) source for large scale knockout mouse projects (Pettitt et al., 2009). A well recognized

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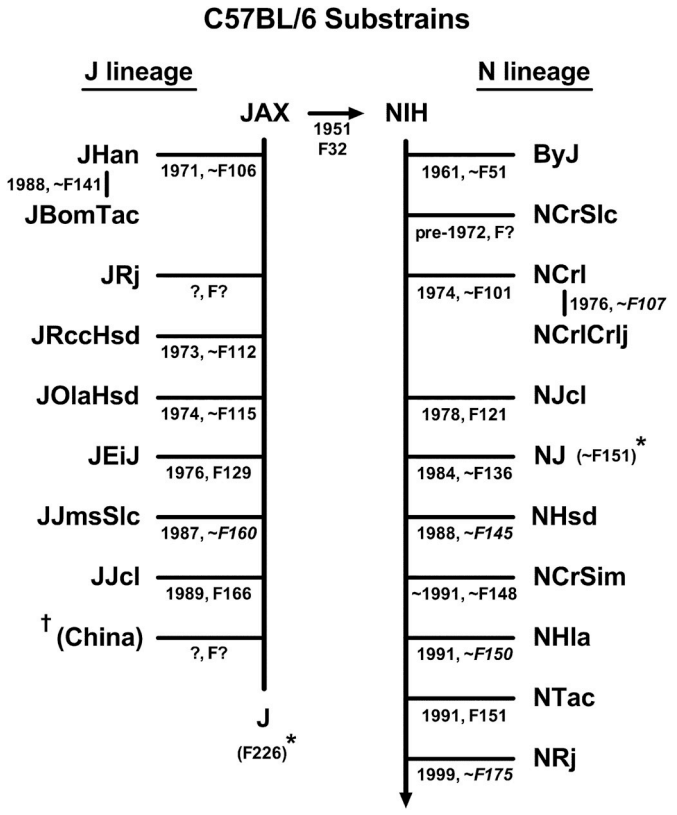

Fig. 1. Substrain divergence of the C57BL/6 mouse. Common currently available substrains are listed according to their approximate order of derivation, in most cases without identifying intermediate colonies. The filial generation (F) at which a substrain originated, if available, is based on information from the supplier website or from estimates of Egan et al. (2007). Italicized approximations represent linear interpolations/extrapolations rounded to the nearest 5 generations, based on the years in which the separations occurred (Egan et al., 2007; Mekada et al., 2009, 2015; Zurita et al., 2011). Subsequent further separations within some of these substrains are not shown. † Several substrains available from suppliers in China are reported to have diverged from a common J lineage ancestry (Niu and Liang, 2009). * The JAX Genetic Stability Program is designed to limit further divergence of the J and NJ substrains to within 10 of the cryopreserved generation numbers indicated. This program also supplies J substrain mice available through a number of cooperating vendors. (Supplier designations: Crl, Charles River Laboratories; Crlj, Charles River Japan; Han, Central Institute for Laboratory Animal Breeding, Hannover; Hla, Hilltop Lab Animals; Hsd, Harlan Sprague Dawley (Envigo); J, JAX; Jcl, CLEA Japan; Slc, Japan SLC, Rj, Janvier Labs; Sim, Simonsen Laboratories; Tac, Taconic Farms.).

variant used to distinguish the J substrain from all others is a deletion in the gene encoding nicotinamide nucleotide transhydrogenase (*Nnt*), which became fixed in the J substrain in the late 1970s or early 1980s (Huang et al., 2006; Wong et al., 2010). This has been used to identify the potential for confounding mismatches of J and N substrains in studies comparing wild type and genetically modified mice (Bourdi et al., 2011).

However, absolute substrain identification remains important in many contexts, as lineage-specific genetic variation can have a profound impact on biology. More recently fixed variants can be unique to a single vendor colony (private mutations). For example, the above-mentioned *Nnt* deletion is associated with reduced tolerance to the oxidative stress imposed by a superoxide dismutase 2 deletion on the J substrain background (Huang et al., 2006), in addition to changes in insulin secretion and glucose tolerance (Freeman et al., 2006; Wong et al., 2010), but this mutation is absent from the closely related JEiJ substrain (Huang et al., 2006). Other such unique variants include an inactivating duplication in *Dock2*, resulting in loss of the encoded guanine nucleotide exchange factor and producing an altered immune phenotype specific to the N-lineage C57BL/6NHsd substrain (Mahajan et al., 2016), and a deletion in alpha-synuclein restricted to a particular J-lineage substrain, C57Bl/6JOlaHsd (Specht and Schoepfer, 2001). Some mutations are recognized to have occurred relatively soon after the founding of the NIH colony, and are therefore common to subsequently derived N-lineage substrains. For example, a mutation in the crumbs 1 gene that produces a retinal degeneration phenotype (*Crb1*^{rd8}) is present in all N-lineage mice, including ByJ (Mattapallil et al., 2012). In contrast, slightly later mutations are absent from ByJ, including cytoplasmic familial mental retardation 1-interacting protein 2 (*Cyfip2*) (Kumar et al., 2013) and inner mitochondrial membrane peptidase 2-like (*Immp2l*) (Egan et al., 2007). These will be considered below in more detail. SNP panels can be used to distinguish individual C57BL/6 substrains (Mekada et al., 2015; Morgan et al., 2016) and such screens are available through major vendors.

Unrecognized substrain variation has confounded studies in many fields, and cryopreservation strategies are now in place to minimize further genetic drift in commonly used mouse strains (Taft et al., 2006). On the other hand, the accumulation of a limited number of variants in closely related C57BL/6 substrains also provides opportunities to identify the genetic changes underlying the phenotypes that vary. Comprehensive genome sequence comparison of the J and NJ substrains has identified at least 50 genes in which mutations impact protein coding regions (Keane et al., 2011; Simon et al., 2013). The distribution of these variants among other J- and N-lineage substrains remains to be fully determined. Likewise, most studies comparing phenotypes have involved only select substrain sets, allowing limited genotype/phenotype correlations. With respect to cerebro- and cardiovascular phenotypes, there are recognized substrain differences in response to myocardial infarction (Gorog et al., 2003), cardiac pressure overload (Garcia-Menendez et al., 2013), neonatal hypoxia/ischemia (Wolf et al., 2016) and stroke (Zhao et al., in press). These indicate greater N-substrain vulnerability for those comparisons in which C57BL/6 lineage was specified (Garcia-Menendez et al., 2013; Wolf et al., 2016; Zhao et al., in press). This review will focus on recent results in experimental stroke that provide the most extensive substrain comparisons, and that also provide evidence for substrain variation with respect to sex-dependent differences in stroke outcome (Zhao et al., in press).

2. Evidence for substrain differences in stroke vulnerability

In the course of other studies it was incidentally observed that infarct sizes differed between male C57BL/6 mice obtained from JAX and Charles River Laboratories, J being less vulnerable than NCrl. Parallel results were also reported for a comparison of J- and N-lineage mice in a neonatal hypoxia/ischemia model (Wolf et al., 2016). This led to a systematic investigation of mice of both sexes from these and other J- and N-lineage substrains, using a model of permanent tandem occlusion of the middle cerebral artery (MCA) and ipsilateral common carotid artery (CCA) (Zhao et al., in press). The main results are shown in Fig. 2. Mice of J and ByJ substrains exhibited smaller infarcts than NCrl and NJ mice. In addition, whereas infarct size was significantly lower in J and ByJ females relative to the corresponding males, no sex difference was seen for NCrl and NJ mice. These differences in stroke outcome were not associated with gross differences in blood flow or vascular anatomy, with respect to either the circle of Willis or the number and distribution of vascular collaterals (Zhao et al., in press), suggesting that they reflect an intrinsic difference in brain vulnerability. This mechanistically distinguishes C57BL/6 substrain variation from the genetic factors that underlie previously characterized differences in collateral perfusion among mouse strains (Keum and Marchuk, 2009; Zhang et al., 2010; Keum et al., 2013; Sealock et al., 2014).

These observations define the relationship between stroke vulnerability and the pattern of C57BL/6 substrain divergence (Fig. 3). The

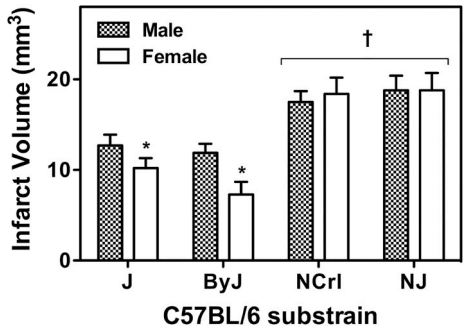

Fig. 2. Substrain- and sex-dependent differences in stroke vulnerability among C57BL/6 mouse substrains. Infarct volumes resulting from permanent focal ischemia are plotted for males and females as indicated (mean ± SEM). NJ and NCrl mice of both sexes exhibited larger infarcts than their J and ByJ counterparts (†). Females had significantly smaller infarcts than males of J and ByJ substrains (*), but no sex difference was observed for NJ and NCrl mice (Data of Zhao et al., in press.).

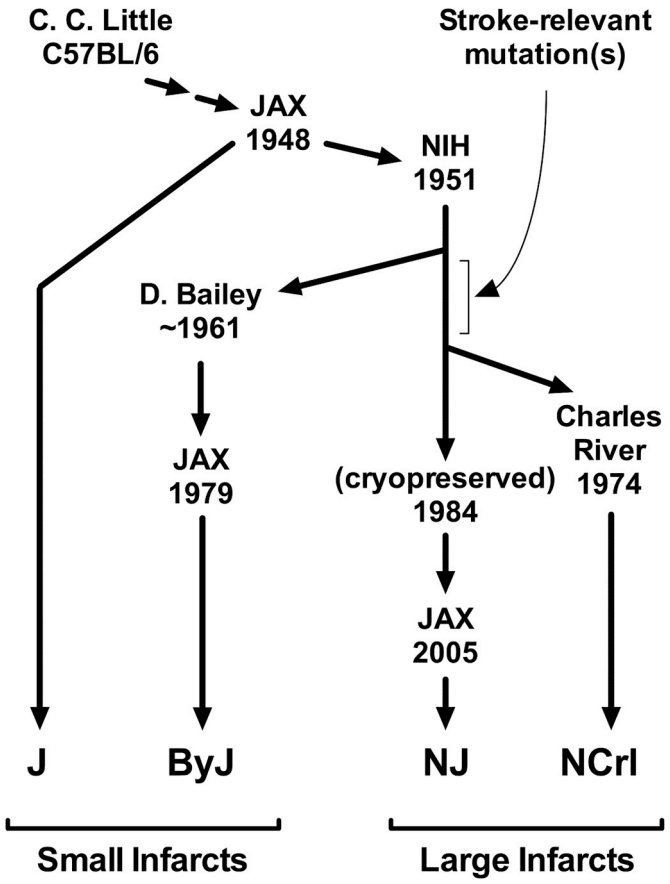

Fig. 3. Stroke phenotype and C57BL/6 substrain lineage. The increase in stroke vulnerability appears to have become fixed in the NIH lineage during the interval between separation of the ByJ and NCrl substrains.

increase in vulnerability and loss of sex difference both appear in the N lineage during an interval of approximately 50 generations spanning the 1960s and early 1970s, between the time points at which ByJ and NCrl substrains were established. Whether the two traits are linked consequences of the same mutation or reflect independent events will not likely be resolved until the variant gene(s) can be identified. To our knowledge there is only a single extant substrain that was derived during the interval separating ByJ and NCrl, C57BL/6NCrSlc (Mekada et al., 2009). Precise timing of its origin is uncertain, but sequence similarity indicates it is more closely related to ByJ than to NCrl (Mekada et al., 2015). The NCrSlc substrain has yet to be characterized with respect to stroke phenotype in this model.

Since larger infarcts and the absence of sex difference both appear as new traits in the C57BL/6 N-lineage, reduced female vulnerability (at least in hormonally intact young adults) may be viewed as the default phenotype. This would be consistent with evidence showing corresponding sex differences in mice of strains other than C57BL/6, e.g., SV129 (Yuan et al., 2009; Siegel and McCullough, 2013) and BALB/c (Xiong et al., 2015), as well as in rats (Alkayed et al., 1998; Selvamani et al., 2014). However, as considered in detail below, the more severe insult associated with a permanent occlusion model may have obscured detection of the sex difference that has been reported for N substrains in some transient occlusion studies (Alkayed et al., 2001; Liu et al., 2009a, 2009b). The present results can at least be taken as evidence for a relative blunting of sex difference in the N lineage.

As noted above, the N and J substrain lineages exhibit differences in numerous phenotypes, as well as considerable genetic heterogeneity. The finding that the ByJ substrain shares the J stroke phenotype permits a dissociation of stroke vulnerability from many of these previously recognized genetic variants. For example, ByJ does not harbor the deletion in *Nnt* that occurred relatively recently in the J lineage (Huang et al., 2006); conversely, ByJ shares the *Crb1*^{rd8} retinal degeneration mutation with other N-lineage substrains (Mattapallil et al., 2012) but differs in stroke vulnerability. The mutations in alpha-synuclein and *Dock2* are unique to specific vendor colonies, so these do not enter into consideration. However, two candidate genetic variants emerge with a substrain distribution that correlates with stroke outcome, both impacting N-lineage substrains subsequent to ByJ. These include the mutations in *Cyfip2* (Kumar et al., 2013) and *Immp2l* (Egan et al., 2007). An inactivating *Cyfip2* mutation is homozygous lethal (Han et al., 2015), whereas the N substrain variant exhibits altered cocaine responses (Kumar et al., 2013) and feeding behavior (Kirkpatrick et al., 2017). The encoded protein is proposed to have diverse roles in fundamental cellular processes (Schenck et al., 2001, 2003; Eden et al., 2002; Abekhoukh and Bardoni, 2014), including apoptosis (Saller et al., 1999). The *Immp2l* intronic deletion that distinguishes C57BL/6 substrains does not convey any previously recognized phenotype. However, another inactivating mutation in this gene, which encodes a peptidase required for processing of certain mitochondrial proteins, has been associated with increased oxidative stress (George et al., 2011; Liu et al., 2016), defects in nitric oxide mediated vasodilation (Lu et al., 2008; Soler et al., 2010) and increased vulnerability to stroke and hypoxia (Ma et al., 2011, 2017). This enzyme has recently been proposed to play a key role in the reprogramming of mitochondrial signaling pathways that determine cell fate, its inactivation being associated with the commitment to senescence (Yuan et al., 2018). How the substrain variants in either of these genes might influence acute stroke pathophysiology remain to be established, but they appear to be reasonable candidates for further study.

ByJ and NCrl differ at 64 of the 100 SNPs in one panel selected for use in C57BL/6 substrain discrimination (Mekada et al., 2015), with 45 of these in known or predicted genes, although only 5 introduce non-synonymous changes in protein coding sequence. One of these is the *Cyfip2* mutation noted above, and others include variants in an olfactory receptor (*Olfr577*), pro-melanin-concentrating hormone (*Pmch*), and a stop codon in spermatogenesis associated 31 (*Spata31*). A search of the CGA-MDA1 mouse diversity genotyping array in the JAX Mouse Phenome Database yielded several additional intronic SNPs but no other coding variants. However, each of the above panels represents only a sampling of the genome. Together, the recognized mutations that distinguish ByJ and NCrl comprise a subset of those identified in the high-density sequence comparison of J and NJ substrains (Simon et al., 2013), from which additional variants that differentiate ByJ and later-derived N-lineage substrains are likely to emerge.

3. Modeling considerations

Since the above results were obtained in a model of permanent tandem MCA/CCA occlusion, comparison with other studies requires a brief consideration of those features that distinguish the commonly used focal ischemia models that nominally target the MCA. The main factors to consider relate to the distribution of involved vascular territories and the effective occlusion duration, including some technical details that impact these variables. More comprehensive assessments of ischemia models are available in a number of recent reviews (Fluri et al., 2015; Yan et al., 2015; Sommer, 2017; McCabe et al., 2018).

3.1. Direct surgical approaches

C57BL/6 is among the least vulnerable mouse strains, often yielding negligible infarcts following MCA occlusion alone (Keum and Marchuk, 2009; Zhang et al., 2010). Limiting collateral perfusion in such models by also occluding the ipsilateral CCA (Brint et al., 1988; Barone et al., 1993; Zhao et al., in press), or temporarily even both CCAs (Chen et al., 1986; Hiramatsu et al., 1993), increases insult severity and infarct size. However, as might be expected for a strain that yields relatively small infarcts, there is considerable individual variability in outcome. The infarct size distributions that underlie substrain population differences are illustrated in Fig. 4, comparing pooled data for J-phenotype (J + ByJ) and N-phenotype (NJ + NCrl) mice. (For simplicity, ByJ is identified in this context as J lineage with respect to stroke phenotype, although it was derived following strain transfer to NIH.) A higher proportion of larger infarcts is clearly evident for males of N relative to J lineages. Some very small infarcts are seen for both sexes of all substrains, but these comprise a major fraction of the population of J-lineage females, whereas N-lineage females trend in the opposite direction. Reliable documentation of such differences required fairly large groups of animals, and was facilitated by a model in which there is negligible mortality, and in which only few animals are excluded based on other technical criteria. These results reinforce the concern that many studies in experimental stroke lack statistical power (Dirnagl, 2016). Indeed, the numbers of J-lineage mice required to detect an intervention effect using this model, particularly in females, would be prohibitively large, and such a study in N-lineage mice would still require approximately 20 mice per group (Zhao et al., in press).

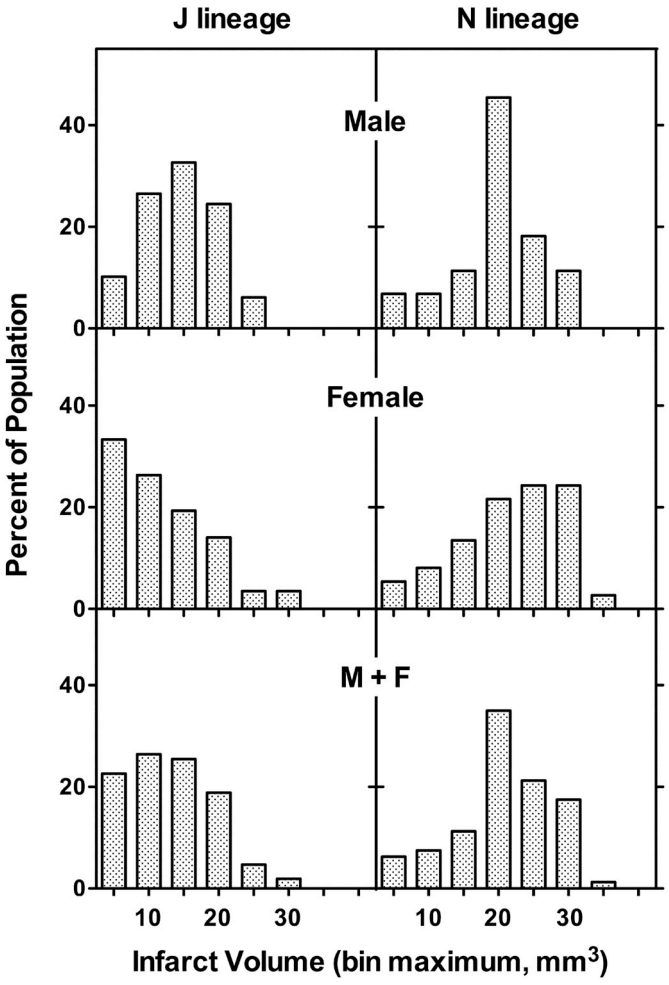

Fig. 4. Infarct volume distributions underlying sex- and substrain-dependent differences in mean stroke vulnerability. J and N lineage data represent pooled groups (n = 30–50) of mice from J plus ByJ substrains and NJ plus NCrl substrains, respectively. Histograms illustrate the proportion of animals exhibiting infarct volumes within 5 mm^3 intervals (Data of Zhao et al., in press.)..

3.2. Intraluminal filament occlusions

By far the majority of experimental stroke studies in rodents involve intraluminal filament occlusion. It is usually incorrect to refer to such an approach as “MCA occlusion” since the filament almost always occupies a considerable segment of the internal carotid artery (ICA) and impacts blood flow to a number of vascular territories in addition to that of the MCA (Özdemir et al., 1999; Kanemitsu et al., 2002; McColl et al., 2004). Interruption of hypothalamic blood flow is associated with profound hyperthermia in rats (He et al., 1999; Li et al., 1999), which can amplify ischemic injury (Zhao et al., 1994; Li et al., 1999; Ábrahám et al., 2002). Pathophysiological consequences of hypothalamic ischemia have been less studied in mouse models, in which unrecognized cooling is the more general concern (Barber et al., 2004; Wu et al., 2014), whereas delayed hyperthermia has been associated with sepsis (Meisel et al., 2004). In contrast, the impact of posterior cerebral artery (PCA) involvement has received considerable attention. If the origin of the PCA were to be obstructed by the filament then perfusion of its territory becomes dependent on the patency of the posterior communicating artery, which varies considerably among mouse strains as well as among individuals within a strain or substrain (Barone et al., 1993; Majid et al., 2000; Yuan et al., 2012; Zhao et al., in press). Use of a shorter silicone segment at the tip can effectively eliminate this source of variability in filament occlusion models, as demonstrated in both rats (Guan et al., 2012) and mice (Yuan et al., 2012), maintaining PCA territory perfusion and producing more consistent infarcts. Not often mentioned is that an obstruction lodged at the origin of the MCA necessarily also limits perfusion of the anterior cerebral artery territory to that which can be provided via the contralateral circle of Willis. Other refinements have been proposed to avoid the adverse consequences of sacrificing the external carotid artery and/or CCA during filament insertion (Dittmar et al., 2003; Trueman et al., 2011; Trotman-Lucas et al., 2017).

It is clear that typical filament approaches achieve more widespread reductions in hemispheric perfusion than occur following tandem MCA/CCA occlusion. This leads to large infarcts and high mortality if filaments are left in place, so transient occlusions are most often used. However, there is considerable risk of intravascular trauma in such models, which can generate microemboli even after very brief insertion intervals (Zhan et al., 2008), and overt thrombus formation would be expected to extend the effective duration of occlusion beyond the interval of filament removal. Recognition of such issues prompted the use of heparin in some early filament occlusion studies in rats (Memezawa et al., 1992; Kurokawa and Tranmer, 1995; Müller et al., 1995), and even in models involving temporary surgical occlusions in which vascular trauma can also occur (Hiramatsu et al., 1993). This agent was specifically demonstrated to reduce the risk of hypothalamic ischemia and hyperthermia due to ICA thrombus formation in a model using very fine filaments (Ma et al., 2006). Recent work in a mouse model also

Table 1
Stroke studies with apparent mismatches of C57BL/6 substrain backgrounds[a].

JAX Stock	*Nnt* genotype[b]	Mutant	Common gene name	Stroke model	Reference
Infarcts larger in N-lineage mutant versus J control					
006659	+/+	*Cxcr5*$^{-/-}$	Chemokine (C-X-C motif) receptor 5	Filament (35 min + 2 w)	Chapman et al. (2015)
002251	+/+	*Il10*$^{-/-}$	Interleukin 10	Surgical (24 h)	Grilli et al. (2000)
002508	+/+	*Plat* $^{-/-}$	Tissue plasminogen activator	Microembolic (24 h)	Atochin et al. (2004)
004650	+/+	*Tlr2*$^{-/-}$	Toll-like receptor 2	Filament (60 min + 24 h)	Hua et al. (2009) [c]
				Filament (60 min + 7 d)	Bohacek et al. (2012)
Infarcts smaller than J control despite N-lineage background					
005582	+/−	*Cx3cr1*$^{-/-}$	Chemokine (C-X3-C motif) receptor 1	Filament (60 min + 3 d)	Jolivel et al. (2015)
–	–	*Serpinf2*$^{-/-}$	Alpha-2-antiplasmin	Thromboembolic (6 h)	Reed et al. (2014)
004650	+/+	*Tlr2*$^{-/-}$	Toll-like receptor 2	Filament (60 min + 3 d)	Tang et al. (2007)
				Filament (60 min + 3 d)	Bohacek et al. (2012)

[a] Cited studies involved use of C57BL/6J controls in comparisons with apparently N- or mixed-lineage knockout mice.
[b] Wild type (+/+) is consistent with N lineage, heterozygous alleles (+/−) with mixed N/J background, as identified in Bourdi et al. (2011). The N lineage is inferred for *Serpinf2*$^{-/-}$ mice based on Knockout Mouse Project sourcing.
[c] Used C57BL/10ScSn as control, stated to yield infarcts equivalent to C57BL/6J.

indicated that heparin treatment prior to filament withdrawal was essential to maintaining reliable reperfusion (Lin et al., 2013), consistent with earlier reports of infarct reductions by this and other anticoagulant interventions (Choudhri et al., 1999). Corresponding mechanisms may contribute to some of the beneficial effects of tissue plasminogen activator and other antithrombotic agents in mechanical occlusion models (Kilic et al., 1999; Nakano et al., 2015).

The combination of short filament tips with anticoagulant administration has the potential to achieve a more selective occlusion site and better control of insult duration by the intraluminal approach, and would result in filament models that more closely approximate the outcome of direct surgical MCA occlusions. A remaining distinction will always be striatal involvement. The most proximal surgical approach to the MCA that is compatible with routine modeling targets the artery at approximately the level of the rhinal fissure (Brint et al., 1988; Barone et al., 1993). This is distal to the branching of lenticulostriate arteries that supply the striatum, so the resulting infarcts are strictly cortical. In contrast, filament occlusions that obstruct the origin of the MCA necessarily include a component of striatal ischemia. The abundant medium spiny neurons of the lateral striatum constitute a comparatively vulnerable cell population (Pulsinelli et al., 1982), selective loss of which can sometimes masquerade as an infarct following short intervals of focal ischemia (Korematsu et al., 1993; Katchanov et al., 2003). The pathophysiology of infarction itself may differ between striatum and cerebral cortex in that, as in humans (Bozzao et al., 1989), striatum does not benefit from the collateral perfusion available to cortical MCA territory and may be more prone to hemorrhage following experimental ischemia (Yuan et al., 2013). Interventions that impact outcome in cortex can fail to affect striatum, including estradiol (Dubal et al., 1998; Alkayed et al., 2001). Cortical and striatal infarct volumes must therefore be considered as separate endpoints when evaluating outcome in filament occlusion models.

3.3. Thromboembolic models

Other models to be considered are those involving either injection of a preformed clot or local thrombus generation. These have the potential to better reproduce the spectrum of stroke pathophysiology, including the potential for spontaneous thrombolysis, which can impact effective insult duration in both of these modeling approaches. In practice, the methods that have been adopted are those that maintain effective occlusions for long enough to produce reliable infarcts.

The involved vascular territories vary with the experimental approach. The MCA receives a high proportion of the blood delivered via the ICA, and studies with synthetic emboli have shown that objects of appropriate size delivered by this route can often lodge within the MCA (Perez-Trepichio et al., 1993; Gerriets et al., 2003). Such localization has been described occasionally in models involving smaller clots (Zhang et al., 2005). However, in most implementations the injected material is expected to at least initially reside within the ICA (Overgaard et al., 1992; Busch et al., 1997; Zhang et al., 1997a; Ren et al., 2012), and distal positioning of the delivering catheter can provide reliable targeting to the MCA origin (Zhang et al., 1997a; Houng et al., 2014; Chen et al., 2015). Such models would be expected to have impacts generally comparable to those of intraluminal filament occlusions. This also holds for in situ thrombogenesis models that involve thrombin delivery by catheter within the ICA (Zhang et al., 1997b), or in which a thrombus is generated in the CCA and induced to migrate intracranially (Shibata et al., 2018). An equivalence to filament occlusion was explicitly demonstrated in an ICA collagen infusion model (Schunke et al., 2015).

In contrast, local thrombogenesis often targets the distal MCA, whether achieved photochemically (Watson et al., 2002), by intravascular thrombin injection (Orset et al., 2007; Ansar et al., 2014), or by application to the vessel surface of agents such as ferric chloride (Karatas et al., 2011). These latter models are therefore comparable to surgical occlusions in that they produce infarcts limited to cortical MCA territory.

4. Evidence for substrain differences in other stroke studies

Although there do not appear to have been any previous attempts at direct substrain comparisons with respect to stroke vulnerability, some investigators have long appreciated substrain background as a potential issue. For example, wild type C57BL/6 mice from different vendors have been used occasionally in order to better match the backgrounds of genetically modified strains (Sampei et al., 2000; Zuloaga et al., 2015). This contrasts with the frequent absence of information about substrain background or even the source of animals, which presents a considerable limitation in assessing the literature. Nevertheless, it is possible to identify several examples of mismatches in substrain background that could explain or at least contribute to the experimental observations. Based on the presence of an intact *Nnt* gene, a number of knockout lines available through JAX were proposed to be on N or mixed N/J backgrounds (Bourdi et al., 2011). A few published stroke studies involved comparisons of one of these knockouts with J wild type mice, as summarized in Table 1. Seemingly straightforward examples include the finding of larger infarcts in mice having deletions in interleukin 10 (Grilli et al., 2000) or the chemokine receptor CXCR5 (Chapman et al., 2015), consistent with the greater intrinsic vulnerability of N-lineage. A tissue plasminogen activator (*Plat*) knockout strain was also reported to exhibit larger infarcts than the J substrain control in a microembolic stroke model (Atochin et al., 2004). Effects observed in these studies could potentially be attributable at least in

part to the difference in substrain background. One of the studies (Chapman et al., 2015) employed a very brief filament occlusion that produced only striatal damage. Together these results suggest that substrain differences seen after surgical occlusion can be generalized to other models, and that a difference in striatal vulnerability may parallel that in cerebral cortex. Conversely, there are examples of genetic modifications that confer benefit in a particular stroke model, despite their presence in a more vulnerable N-lineage background. These include knockout of α-2-antiplasmin in a thromboembolic stroke model (Reed et al., 2014) and of the chemokine receptor CXCR3 after filament occlusion (Jolivel et al., 2015). The former utilized N-background mice from the Knockout Mouse Project, and the latter were reported to be on a mixed N/J background (Bourdi et al., 2011). Other studies involving a toll-like receptor 2 knockout have yielded conflicting results, one showing exacerbation of injury in the knockout (Hua et al., 2009), one reporting reduced damage (Tang et al., 2007), and a third confirming early injury reduction yet finding larger infarcts at longer survival times (Bohacek et al., 2012). It should be noted that there is a substantial literature demonstrating infarct reductions in *Tlr2*$^{-/-}$ mice using control and knockout strains from sources other than JAX (Lehnardt et al., 2007).

The above survey is limited to a small number of studies in which use of divergent substrain backgrounds could be identified with reasonable certainty. An exhaustive review of the literature might well yield additional instances of substrain mismatch that would be informative. Caution is warranted in that genotype at a single locus does not fully define substrain background, although the presence of other segregating SNPs has been confirmed for the *Cxcr5*$^{-/-}$ and *Cx3cr1*$^{-/-}$ strains (JAX).

The above observations demonstrate the potential for substrain mismatch to impact experimental stroke. Interestingly, an analogous substrain difference in stroke vulnerability has been reported for mice of the C57BL/10 strain. In this case there is strong evidence that a deletion in toll-like receptor 4 (*Tlr4*) is the causal variant. This mutation was identified in the C57BL/10ScCr substrain (Poltorak et al., 1998), also known as 10ScNCr or 10ScNJ, whereas mice of the C57BL/10ScSn (or 10J) substrain retain the wild type allele. A deficient substrain exhibited smaller infarcts than the wild type in a filament model (Hua et al., 2009), as well as after surgical MCA occlusion (Caso et al., 2007; Moraga et al., 2014). Even more convincingly, comparison of F2 progeny of C57BL/10ScNJ and C57BL/10J substrains demonstrated smaller infarcts in *Tlr4*$^{-/-}$ relative to *Tlr4*$^{+/+}$ littermates, as well as markedly reduced hemorrhage following tPA administration, in a thromboembolic model (García-Culebras et al., 2017). If unrecognized, such a mutation would confound any stroke study comparing mice on mismatched C57BL/10 backgrounds, just as the yet unidentified mutation(s) impact C57BL/6. Conversely, this establishes the utility of substrain divergence as a source of phenotypic variation in stroke through which underlying pathophysiological mechanisms can be revealed.

5. Sex differences in stroke vulnerability and the impact of substrain

The above-mentioned studies involving mismatched strains used only male mice so do not address the substrain difference in sex-dependent vulnerability. A majority of published reports of smaller infarcts in hormonally intact young adult female C57BL/6 mice are based on studies utilizing filament occlusions in a J substrain. Examples include results from the McCullough (Liu et al., 2009a; Manwani et al., 2015), Offner (Banerjee et al., 2013; Dotson et al., 2015; Seifert et al., 2017) and Sobey laboratories (Broughton et al., 2014). Notably, the four core genotype (FCG) mouse lines, in which gonadal hormones determine sex differences in stroke vulnerability independent of sex chromosome complement (Manwani et al., 2015), are on the J background. Parallel results have also been obtained in a thromboembolic stroke model (Hoda et al., 2011). The presence of a sex difference favoring smaller infarcts in young J-lineage females is therefore well established.

Prior studies do not generally suggest an absence of sex differences in N-lineage mice. Young adult mice obtained from Charles River Laboratories were found to yield smaller infarcts in females (Alkayed et al., 2001; Liu et al., 2009a, 2009b). Furthermore, although substrain comparison was not an intended focus, J- and N-lineage mice have been used as separate control groups in some studies, with no obvious differences emerging (Sampei et al., 2000; Zuloaga et al., 2015). On the other hand, in one comparison of wild type and genetically modified strains (Sakata et al., 2009), mutant mice showing a sex difference were stated to have been on a J background, whereas wild type mice with comparable infarct sizes in males and females were obtained from Clea Japan, a colony derived from the N lineage (Mekada et al., 2009). Although the result is consistent with divergent substrain effects, infarcts tended to be larger in the J background males in this study, which is the converse of the substrain difference documented in Fig. 2. This could reflect an impact of the knockout, but the study also involved permanent intraluminal filament occlusion with high mortality (40–60%) and overall exclusion rates of 70–80%, so preferential loss of mice with larger infarcts could have biased the outcome.

Model differences may provide a framework within which to resolve some of these apparently conflicting results. In at least one study, sex differences seen in wild type C57BL/6J mice after transient filament occlusions were no longer evident after permanent occlusions (Brait et al., 2010), indicating that differences in vulnerability can be masked with increasing insult severity. The endpoint in that study also included a prominent subcortical component, whereas sex differences may be more pronounced in cortex (Seifert et al., 2017). The residual sex effect seen in J-lineage mice in a permanent surgical occlusion model (Fig. 2) could reflect in part the strictly cortical distribution of the infarct that was produced, as well as the use of large enough groups to detect even a modest difference. Conversely, the sex differences reported after transient occlusions in other studies involving N-lineage mice may have been obscured by the increase in damage after permanent occlusions, in combination with the intrinsically greater vulnerability of N substrains.

6. Additional considerations

Increasingly, stroke studies involve comparisons of strains with multiple genetic modifications, including the study of sex-dependent differences (Sampei et al., 2000; Zhao et al., 2009; Zhu et al., 2017). The potential for obtaining mixed substrain backgrounds has been noted as a particular concern in the context of complex breeding strategies to generate conditional or tissue- or cell type-specific knockouts (Fontaine and Davis, 2016). Although many such strains are maintained by backcrossing to C57BL/6, substrain backgrounds are not always straightforward to identify. The scope of the problem is demonstrated in a recent study reporting that half of a sample of 68 strains maintained in investigator colonies were of mixed N/J autosome sequence, with an additional high proportion of mispaired Y chromosomes including some of non-C57BL/6 origin, resulting in the identification of over 70% of strains on a mixed genetic background (Dobrowolski et al., 2018). Since the pattern of inheritance of stroke vulnerability has not yet been established, it is risky to speculate regarding the intrinsic stroke phenotype to expect from inter-substrain crosses. A breeding program has been initiated to generate F1 mice from J and NJ parent substrains, in which stroke phenotype will then be evaluated, to begin to address this question.

There is also the general problem that a significant and variable extent of residual genome from the ESC strain of origin flanks the locus of any modified gene, even after extensive inbreeding to another background strain (Eisener-Dorman et al., 2010; Almodovar et al., 2013; Mahajan et al., 2016). Although we are not aware of any similar evaluations of mouse strains harboring multiple mutations the burden

of such persisting residual sequences must certainly increase with the complexity of the breeding program. In addition, the originating ESC has been identified as a frequent source of Y chromosome mismatch (Dobrowolski et al., 2018).

Finally, although most attention has focused on coding variants, mutations in non-coding genomic regions can affect transcription factor binding and the regulation of gene expression, thereby impacting disease phenotypes (Soccio et al., 2015). This considerably broadens the range of mutations to be considered in the search for those underlying the variation in stroke vulnerability among C57BL/6 substrains. However, the pool of candidate variants that distinguish these substrains is several orders of magnitude smaller than that distinguishing commonly used inbred strains (thousands versus millions). Forward genetic approaches combined with rapidly advancing next-generation sequencing strategies are thus expected to facilitate the identification of functional variants (Ashbrook et al., 2018).

7. Summary and conclusions

Recent evidence indicates that genetic variation introduced during the course of N lineage divergence of the C57BL/6 mouse has increased stroke vulnerability and blunted the preferential sparing seen in young adult females. The resulting confounds impact the interpretation of some prior studies that involved comparisons of stroke outcome between wild type and genetically modified strains. Given past inattention to substrain background, it seems likely that other examples of such mismatches could emerge. Going forward, identification of vendor and substrain must be considered essential information in any report, and investigators with established colonies should verify the substrain background of each line that is maintained. Further studies are needed to characterize the stroke phenotype of mice on mixed substrain backgrounds. The impact of substrain also remains to be more fully examined after transient occlusions, in other stroke models, and in the context of the changing interaction of sex with age in determining stroke outcome. The limited range of genetic variation among C57BL/6 substrains holds considerable promise for elucidating the specific variant(s) underlying differences in stroke vulnerability.

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