HA/NA balance?

The evidence

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6th May 2010

DISCLAIMER

*I have not read **all** the papers I that I reference in detail. As a result, I cannot comment on the reliability of some of these experiments. I have tried to include the raw data here as much as possible so you can make up your own minds but to check the quality of the methods used, you need to look at the paper!*

Previously...

- We have talked a lot about HA/NA balance before (there is whiskey on the line!) – but never discussed the evidence in the literature for a balance existing.

- We all agree that there must be **some** degree of balance between the activity of HA and NA (think of extreme cases).

- What remains less clear is the **extent** of this balance – how much change can be tolerated in either protein before compensation is required by the other.

- Still not 100% convinced myself, but I accept that there are many theories that explain how it **could** work – the problem is finding evidence to support them!

A quick recap: HA, NA (and HEF?!)

- Influenza A and B viruses have two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), coded for by different gene segments.
- Both interact with sialic acid terminated glycan receptors
- HA binds to glycan receptors facilitating cell entry and membrane fusion.
- NA destroys glycan receptors to all progeny virion budding and disaggregation.

Note: Unlike A and B, Influenza C has a single glycoprotein (HEF) which performs all three functions on its own! There is one (3.2A) crystal structure for HEF but is there any genetic data? How much Influenza C surveillance is done?

Sources of evidence

1. Naturally occurring reassortments (1957 and 1968).
2. Lab generated reassortments.
3. NA inhibitor resistance studies (e.g. Zanamivir).
4. Crippling NA (no active site).
5. Varying NA ‘stalk’ length.

Naturally occurring reassortments

- 1957 H2N2 pandemic strain emerged after a reassortment between human (H) and avian (N) viruses.
- In 1968, there was a second reassortment: human HA2->HA3 (avian NA retained) giving H3N2.
- Baum and Paulson took 10 human viruses isolated between 1957 and 1987 and tested for NA and HA α(2,3)/α(2,6) linkage specificity.

Baum LG, Paulson JC, Virology 1991; **180**: 10-15

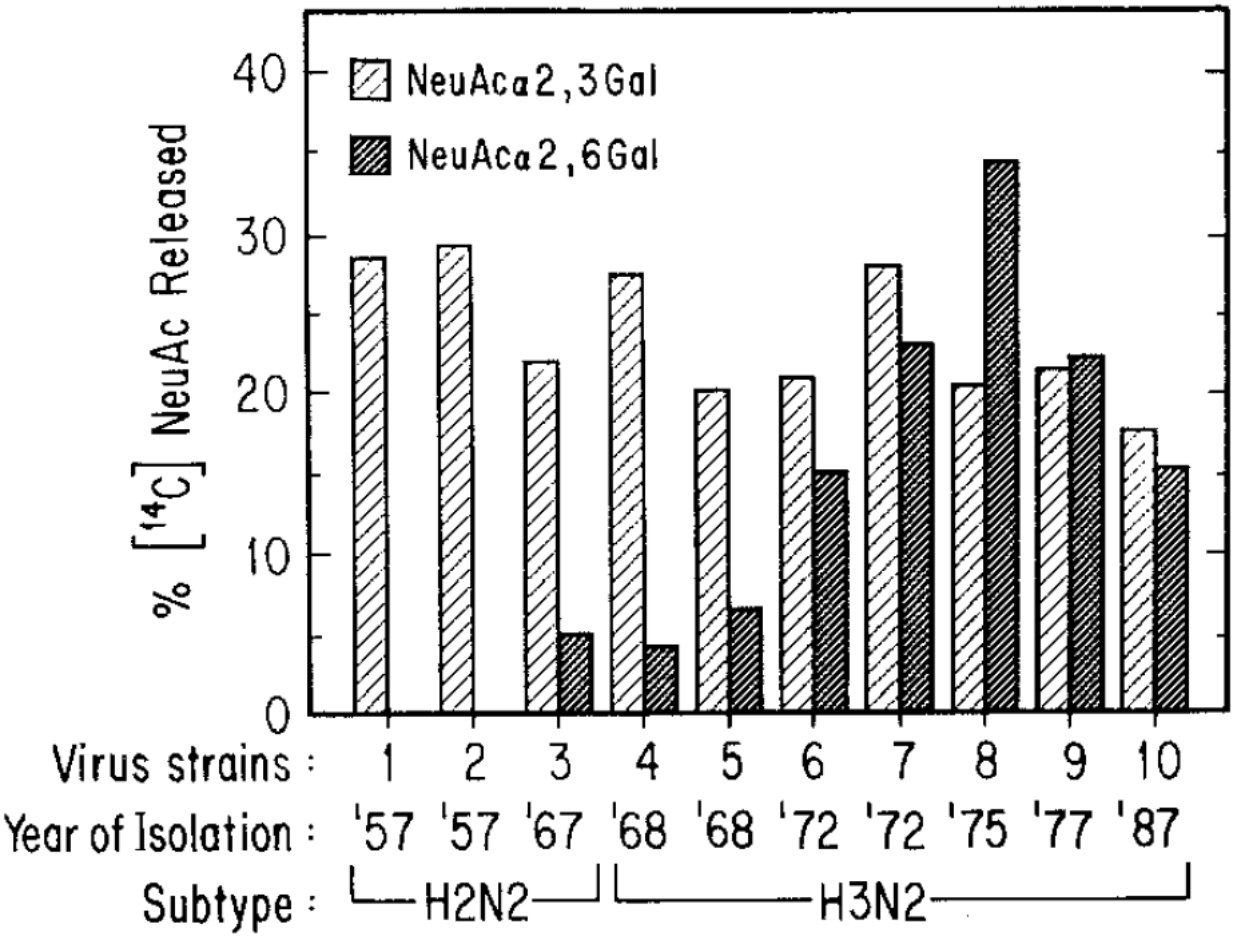
Naturally occurring reassortments

Viruses used:
A/RI/5+/57
A/RI/5-/57
A/Japan/305/57
A/Tokyo/3/67
A/Aichi/2/68
A/Hong Kong/8/68
A/Udorn/307/72
A/Memphis/102/72
A/Victoria/3/75
A/Texas/1/77
A/Los Angeles/2/87

- All were grown in eggs

- Avian NA gains activity for α(2,6) linked sialic acids

- NA retains dual specificity



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Naturally occurring reassortments

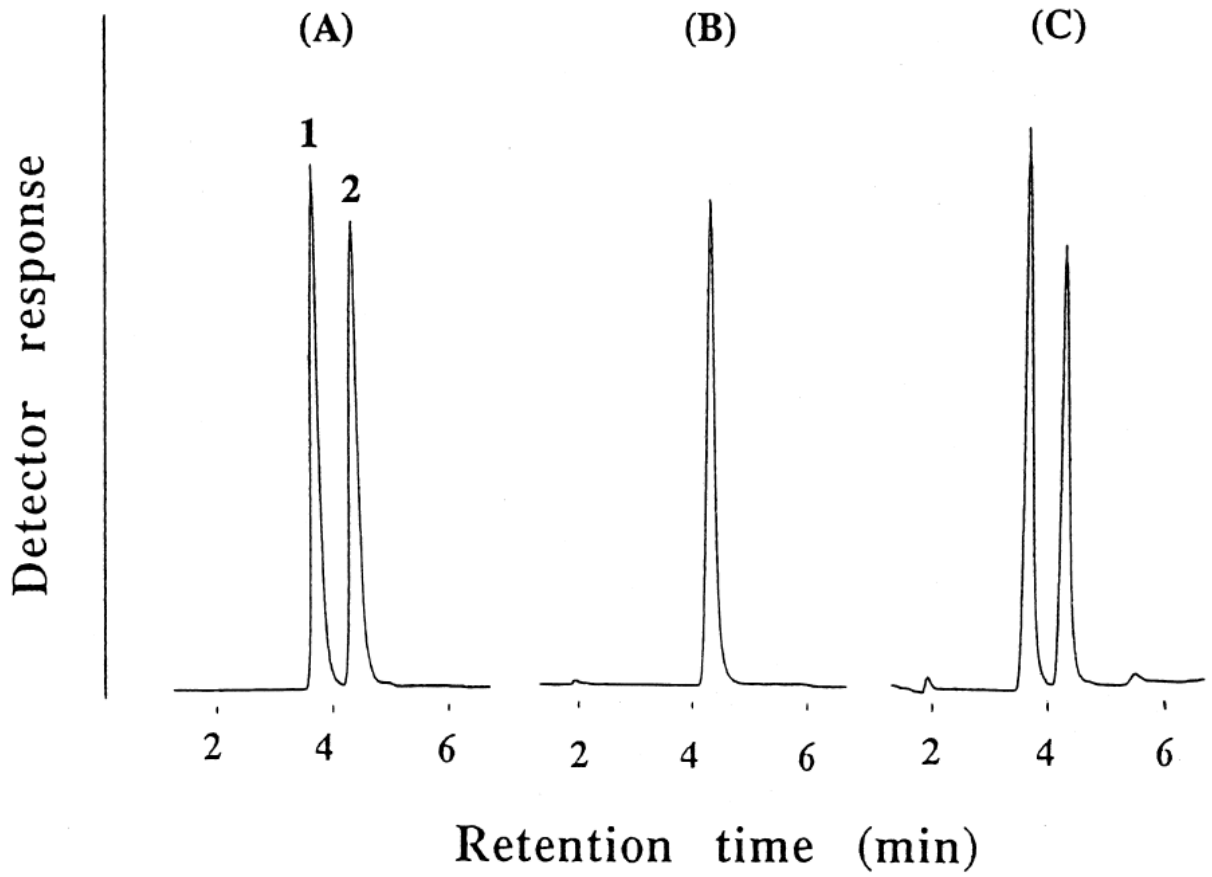
Suzuki *et al* compared the activity of swine and human NAs.

Human NAs unable to cleave N-glycolyl (Neu5Gc) receptors

Swine NAs (except for Iowa!) could cleave both N-glycolyl and N-acetyl (Neu5Ac) receptors.

HPLC (on right) used to show that N-glycolyl receptors are absent in the human respiratory tract

Pigs have both in a roughly 1:1 ratio.



Chromatograms of Neu5Ac and Neu5Gc obtained from tracheal epithelia of humans (B) and pigs (C). A shows a standard mixture of both Neu5Ac (peak 2) and Neu5Gc (peak 1).

Suzuki T, Horiike G, Yamazaki Y, et al., FEBS Lett 1997;**404**:192-196

Lab generated reassortments

- Kaverin *et al* took NA from the human A/USSR/90/77/H1N1 strain and made reassortants with avian H3/4/10/13 viruses.
- Reassortant viruses replicated poorly in eggs (virus aggregates were seen) compared to the avian parent viruses.
- HPLC showed high levels of sialic acids on the reassortant virus particles compared to the avian parent

Is human NA able to cleave the α(2,3) linked receptors present in eggs?

Kaverin *et al.* Virology;**244**:315-321

Lab generated reassortments

- R2: HA from A/Duck/Ukraine/1/63/H3N8 (HA), rest from A/USSR/90/77/H1N1

TABLE 1

The Content of Sialic Acid in Purified Virus Protein

Virus	Subtype	Sialic acid content (pM per 1 μg of virus protein)[a]
A/Duck/Ukraine/1/63	H3N8	0.42 ± 0.12
A/USSR/90/77	H1N1	3.52 ± 0.28
R2	H3N1	3.55 ± 0.40

Passage in eggs?

[a] Mean of four determinations ± standard error. A separate virus preparation was used for each determination.

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R2-XXI	H3N1	2.65 ± 0.39
R2-XIIIa	H3N1	2.43 ± 0.25

[a] Mean of four determinations ± standard error. A separate virus preparation was used for each determination.

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Lab generated reassortments

- Both R2-XXI and R2-XIIIa have a mutation near the HA binding pocket - **N248D**
- HA binding affinity for high MW receptors was seen to decrease
- These variants grow well and do **not** aggregate!

TABLE 2

Changes in the Affinity of HA toward Sialic Acid-Containing Substrates Associated with the Loss of Virion Aggregation

	Substrate			
Virus	Fetuin–HRP conjugate	3′-Sialyllactose attached to a polyacrylic acid carrier	α-*N*-Acetyl neuraminic acid	3′-Sialyllactose
R2	0.16 ± 0.02[a]	0.97 ± 0.14	312 ± 43	463 ± 60
R2-XXI	1.17 ± 0.12	19.2 ± 2.2	420 ± 51	770 ± 98
R2-XIIIa	0.95 ± 0.08	14.6 ± 3.0	513 ± 33	460 ± 92

[a] K_{aff} (expressed in μM sialic acid) determined for fetuin conjugate by direct solid-phase enzyme-linked assay (Gambaryan and Matrosovich, 1992) and for SA-polyacrylate, Neu5Ac, and 3′-SL by competition assay (Gambaryan *et al.*, 1997). The data represent means ± standard error. For each substrate four preparations of each virus were assayed.

R2-XXI was passaged 21 times in eggs
R2XIIIa was passaged 13 times in eggs

Kaverin *et al.* Virology;**244**:315-321

NA inhibitor resistance studies

- NA inhibitor resistant mutants can be generated *in vitro* by passage in cell culture in the presence of an inhibitor such as Zanamivir (ZMR) or Oseltamivir (OTV).

- Two mechanisms for viruses to escape NA inhibitors have been observed:

(a) make changes to conserved amino acids in the NA binding pocket to lower binding affinity to the specific inhibitor used.

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- Two mechanisms for viruses to escape NA inhibitors have been observed:

(a) make changes to conserved amino acids in the NA binding pocket to lower binding affinity to the specific inhibitor used.

(b) make changes in and around the **HA** binding pocket to reduce receptor affinity. This makes the virus less reliant on high NA activity!

McKimm-Breschkin JL *et al*. Antimicrob Agents Chemother 1996;**40**:40-46

NA inhibitor resistance studies

TABLE 2. Sequence changes in the NA and HA proteins of resistant viruses[a]

Virus (drug used)	NA amino acid at position:		HA amino acid at position[b]:		
	90	346	155	223	229
NWS/G70C parent	G	N	T	V	R
Variant 1 (4-amino-Neu5Ac2en)	**Q**	N	T	**I**	**I**
Variant 5 (4-amino-Neu5Ac2en and then 4-guanidino-Neu5Ac2en)	G	N	T	V	**S**
Variant 7 (4-guanidino-Neu5Ac2en)	G	**S**	**A**	V	R

[a] Amino acid numbering is based on the N2 NA and the H3 HA. Sequence changes are shown in boldface.
[b] Changes were all in the HA1 chain.

- Mutations seen in NA were not anywhere near the receptor binding site. Mutations to HA however were!

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NA inhibitor resistance studies

NWS/G70C WT

Variant 1

Variant 5

Variant 7

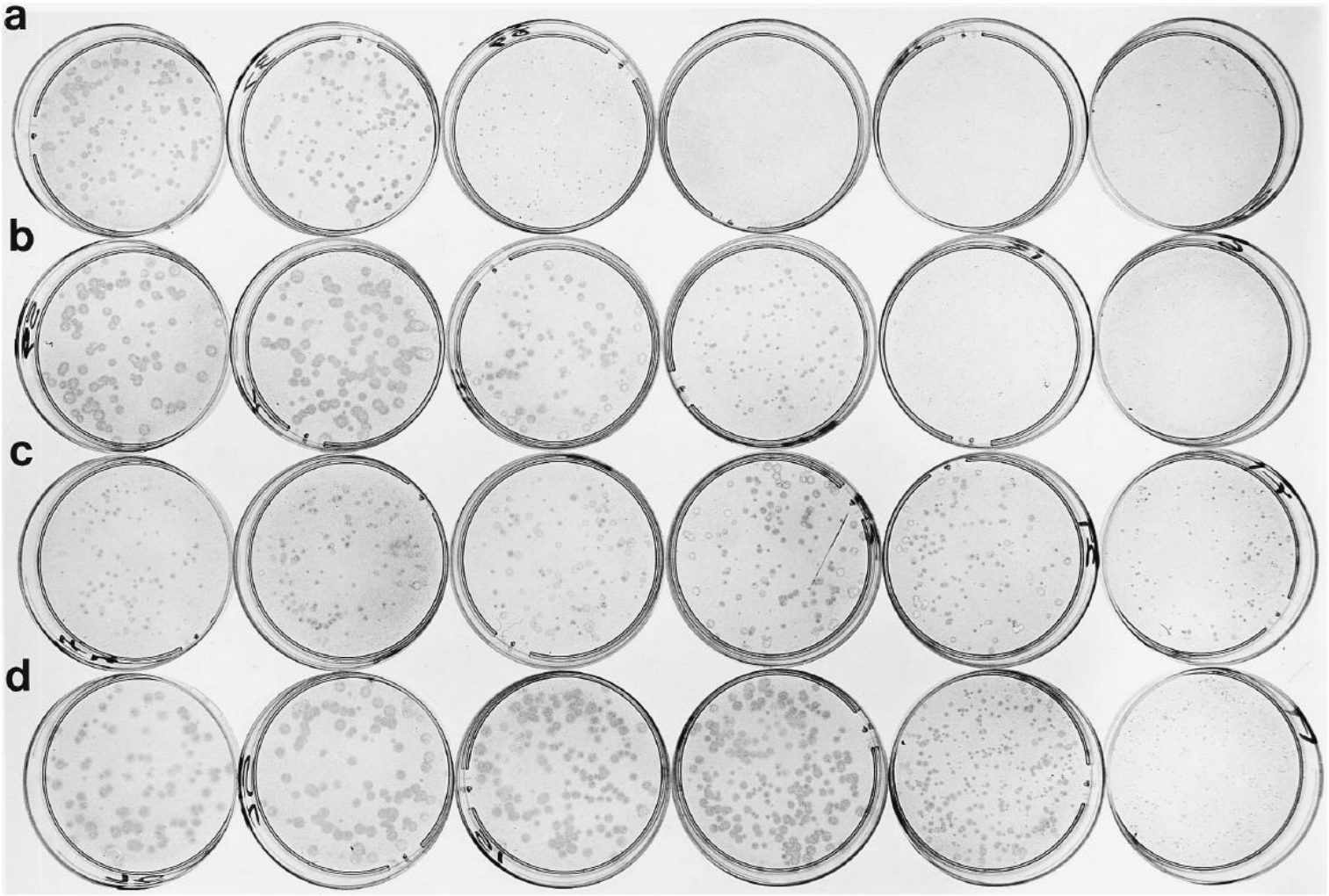

FIG. 1. Plaque inhibition assay with 4-guanidino-Neu5Ac2en in the overlay. Virus was plaqued, from left to right, without inhibitor and with 0.0003, 0.003, 0.03, 0.3, and 3 μg of 4-guanidino-Neu5Ac2en per ml. (a) NWS/G70C; (b) variant 1; (c) variant 5; (d) variant 7.

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Crippling NA (no active site)

- Mutants with an inactive NA can be created by serial passage in presence of anti-NA serum.
- The NA gene obtains a large deletion in its open reading frame plus a premature stop codon - only the stalk and transmembrane domain of the NA are expressed.
- Hughes *et al* reported that it is possible to get these mutants to replicate in eggs, mice or MDCK cells by repeated passage and selection for high growth variants:

Hughes *et al*. J. Virol 2000;**74**:5206-5212

Crippling NA (no active site)

- High growth variants have mutations to HA that reduce binding affinity

TABLE 2. Hemagglutination activity of parental virus and its NA deletion variants

Virus	ELISA titer	Hemagglutination titer at:		Specific hemagglutination activity (HA_4/ELISA titer)	Ratio of hemagglutination titer at 37°C to that at 4°C (HA_{37}/HA_4)
		4°C (HA_4)	37°C (HA_{37})		
NWS-G70c	6,000	1,600	1,200	0.3	0.75
23ΔNA	2,000	320	320	0.2	1.00
CK2-29	12,000	320[a]	40[a]	0.03	0.13
E17A	5,000	40[a]	<10	0.01	NA[b]
E17E	3,200	<10	<10	<0.003	NA
M18B	6,600	<10	<10	<0.003	NA

[a] Virus did not show complete agglutination.
[b] NA, not available.

TABLE 3. Amino acid changes in NA deletion variants

Virus	Relative receptor-binding affinity[a]	Amino acid changes in the HA by H3 numbering (H1 numbering)[b]								
		96 (89)	132 (128)	135 (132)	140 (137)	141 (138)	145 (142)	193 (190)	205 (202)	220 (217)
NWS-G70c	+++++							S	V	
23ΔNA	+++++	A	T	V	S	H	S	R	M	R
CK2-29	++			A			N			K
E17A	+	T[c]		A			N			
E17E	−		K	A	P		N		V	K
M18B	−			A	P	Q	N			K

[a] Based on hemagglutination data in Table 2. +++++, highest affinity; specific hemagglutinating activity, >0.2; ++, moderate affinity; specific hemagglutinating activity, 0.03; +, weak affinity; specific hemagglutinating activity, 0.01; −, affinity below the sensitivity of the assay.
[b] Changes in the HA1 sequences represent differences between the 23ΔNA and the other viruses, including the parental strain. Blank spaces indicate that the amino acid is identical to that in 23ΔNA.
[c] Substitution A96T creates a potential glycosylation site at Asn-96.

Hughes *et al*. J. Virol 2000;**74**:5206-5212

Varying NA ‘stalk’ length

- NAs with a short 'stalk' have been shown to be inefficient in releasing and disaggregating progeny virions.

- This is likely to be a **geometric** effect i.e. the active site is not held far enough from the viral membrane and so cannot interact with its substrate efficiently.

Els MC, Air GM, Murti KG, *et al.* Virology 1985;**142**:241-247

Varying NA 'stalk' length

- Castrucci and Kawaoka created a set of mutants with stalk lengths between 0 and 52 AAs (the wild type A/WSN/33/H1N1 has a stalk length of 24).
- In MDCK cells, they were all able to replicate at least as well as the parent virus, but in eggs, the stalk length was well correlated with the efficiency of viral replication, the longer the better!

TABLE 1. Correlation between NA stalk length and virus replication in tissue culture and eggs

Virus	No. of amino acid residues in the NA stalk[a]	Titer in MDCK cells (log_{10} [PFU/ml])	Replication in eggs (log_{10} [EID_{50}[b]/ml])	PFU/EID_{50}
Wild type [WSN(NA15)]	24	7.3	5.5	63
SD0	0	7.3	—[c]	—
SD9	15	7.0	4.5	350
SA14	38	7.0	7.0	1
SA28	52	8.1	8.5	0.4

[a] Based on assignment of Harley et al. (18).
[b] EID_{50}, 50% egg infective dose.

Castrucci and Kawaoka . J Virol 1993;**67**:759-764

Varying NA ‘stalk’ length

• Mitnaul *et* repeatedly passaged the SD0 mutant (no stalk) in eggs, selecting high growth variants.
• As with drug resistance, there were two mechanisms seen that allowed the recovery of efficient replication in eggs:

(a) **insertions** acquired in the NA the gene derived from NP, PB1 and PB2 genes - **reconstituting the stalk**.

Mitnaul *et al*. J Virol 2000;**74**:6015-6020

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(a) **insertions** acquired in the NA the gene derived from NP, PB1 and PB2 genes - **reconstituting the stalk**.

(b) mutations acquired around the **HA** binding site, reducing receptor binding affinity. High NA activity is therefore no longer required to prevent aggregation and facilitate budding!

Mitnaul *et al*. J Virol 2000;**74**:6015-6020

Varying NA 'stalk' length

TABLE 1. Properties of egg-adapted SD0 strains[a]

Virus	Egg passage at which positive hemagglutination was detected	NA stalk insertion	
		No. of amino acids inserted	Origin of gene insertion
0EA	12	22	PB2
7EA	12	10	PB2
10EA	10	13	PB1
18EA	8	18	PB2
21EA	10	20	NP
2EA	12	0	NA[b]
5EA	8	0	NA
14EA	12	0	NA
19EA	12	0	NA
25EA	10	0	NA

TABLE 3. Mutations in the HA gene of egg-adapted viruses[a]

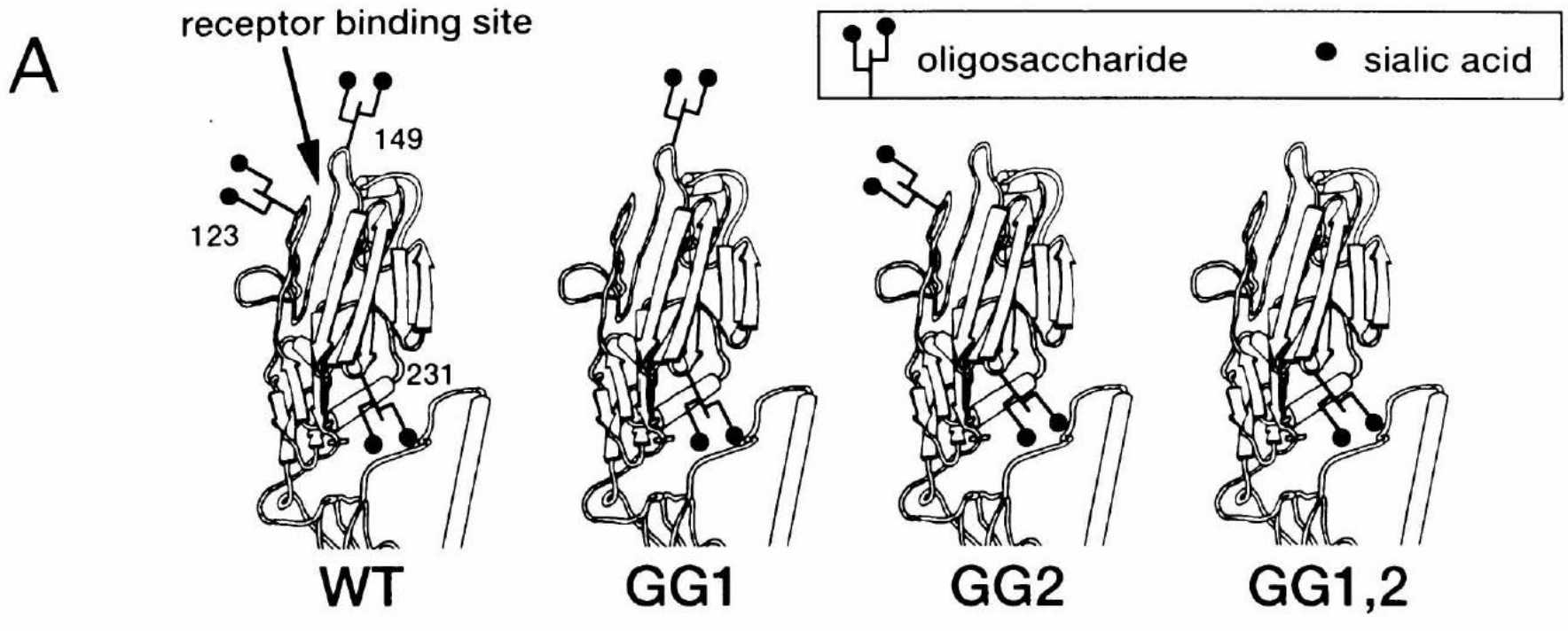
Virus	NA insertion	Mutation(s) in:	
		HA1	HA2
0EA	YES	95Asn→Asp	72Asn→Lys
21EA	YES	135Val→Ile	
2EA	NO	146Ser→Gly 262Arg→Lys	106Arg→Lys

[a] For each adapted strain, HA cDNA was synthesized by reverse transcriptase PCR followed by cDNA sequencing as described in Materials and Methods.

Mitnaul *et al*. J Virol 2000;**74**:6015-6020

Varying NA ‘stalk’ length

- HA binding affinity can also be modulated by **glycosylation**. It has been shown that removing N-glycans flanking the binding site gives improved HA binding.



Ohuchi *et al.* J Virol 1997;**71**:8377-8384

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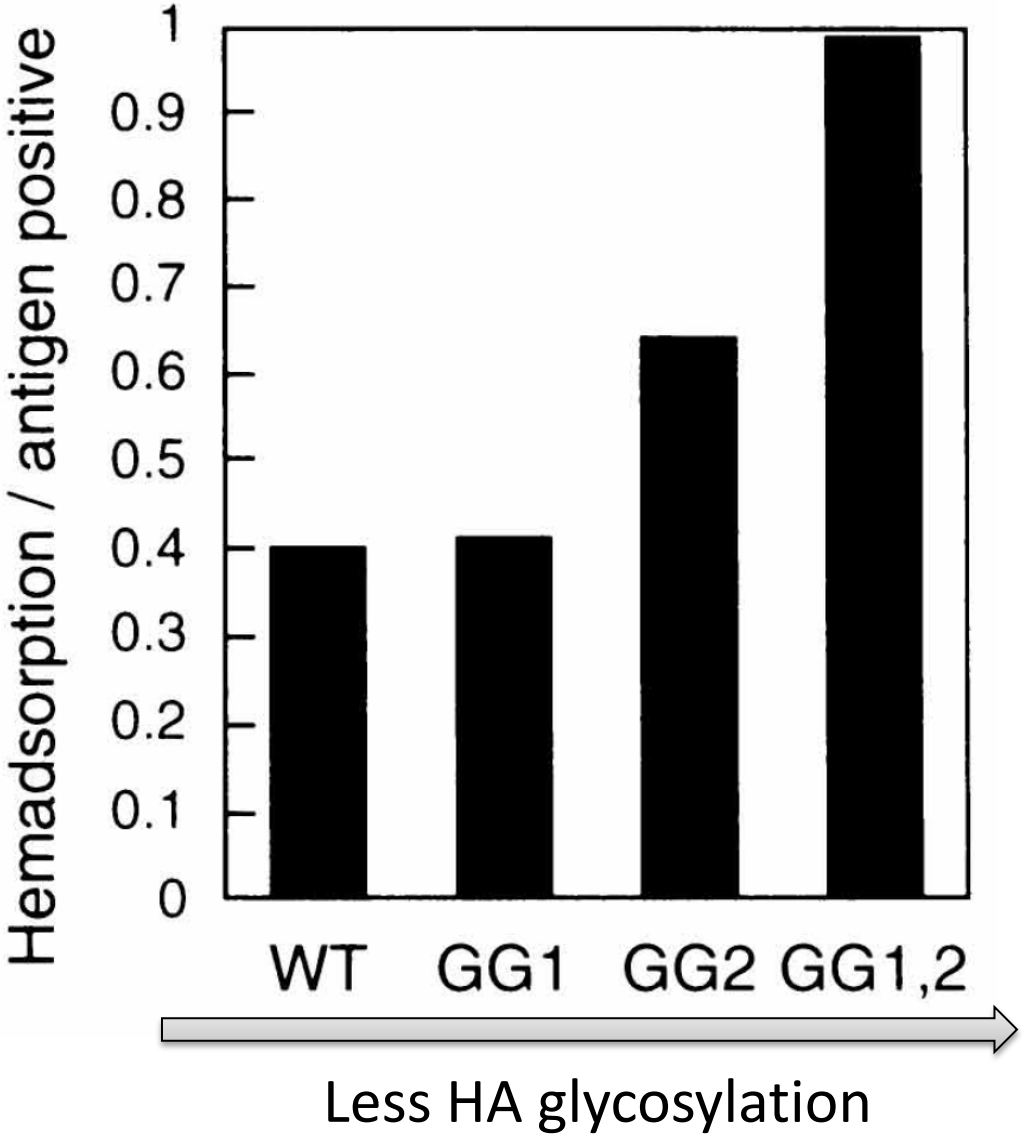

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Discussion time!

Here are some thoughts to get us started:

• Are **you** convinced that there is a functional balance between HA and NA?

• What experiment(s) could we do to convince ourselves further?

• Other mechanisms could compensate for variability in one protein or the other without requiring a functional balance e.g. Altering the distribution of HA/NA on the virus, or a change in sialic acid expression in infected cells.

• What does this mean for fitness exchange?

Ohuchi *et al.* J Virol 1997;**71**:8377-8384