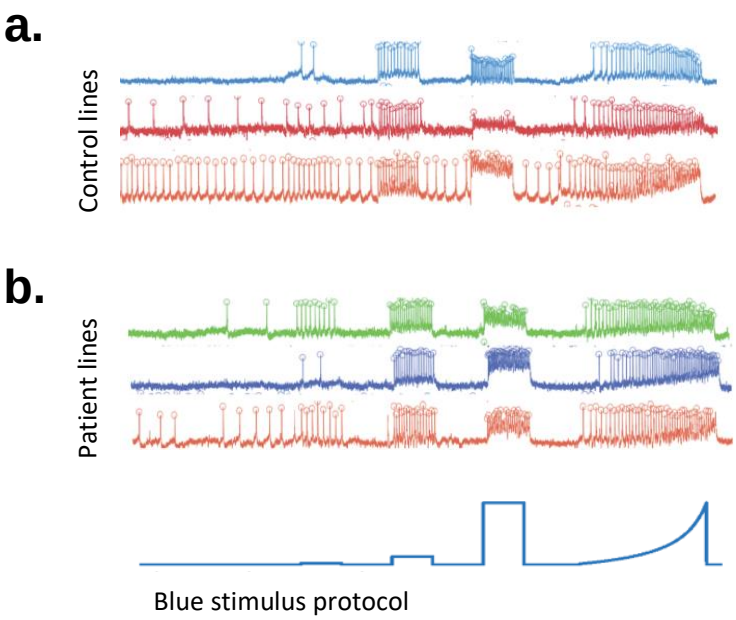
Cell lines	Patient	Control	Mycoplasma	Karyotype	Spiking Sources?	qPCR QC	FMRP qICC?	CGG Repeat Length
005-001-A		X	negative	normal	yes			30
005-001-B		X	negative	normal	yes			30
005-002-A	X		negative	Gain of ~2,500 kbp X Gain of ~25,00 kbp Y	yes			470, 578
005-002-D	X		negative	Gain of ~2,500 kbp X Gain of ~25,00 kbp Y	yes			554
005-003-C	X		negative	normal	yes			434
005-003-E	X		negative	normal	yes			484
005-004-A		X	negative	normal	yes			24
005-004-C		X	negative	normal	yes			22
005-005-A		X	negative	normal	yes			36
005-005-B		X	negative	normal	yes			25
005-006-A	X		negative	normal	yes			174, 912
005-006-B	X		negative	normal	yes			462
005-009-A	X		negative	normal	yes			294
005-009-B	X		negative	normal	yes			295
005-010-A		X	negative	normal	yes			29
005-010-B		X	negative	normal	yes			31
005-012-A		X	negative	normal	yes			28
005-012-B		X	negative	normal	yes			28
005-018-A	X		negative	normal	yes			364, 797
005-018-B	X		negative	normal	yes			366, 803

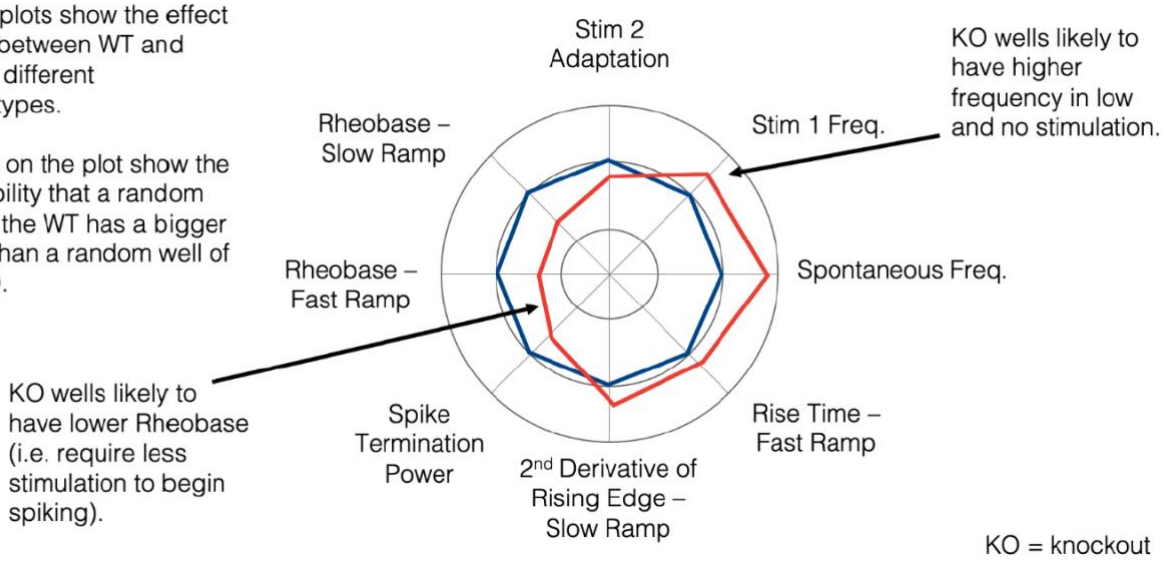
Supplemental Table 1: Patient cell line information and quality control. All patient and control cell lines were tested for mycoplasma, karyotype, the production of functional neurons (spiking sources), the correct FMR1 expression via qPCR, and the correct FMRP expression based on quantitative immunocytochemistry (qICC), and CGG repeat length. Green indicates successful passage of quality control criteria for that category. All cell lines were negative for mycoplasma and all produced functional neurons.



Supplemental Figure 1: All-optical electrophysiology. (A, B) Example fluorescence traces indicated action potential firing from 6 example neurons, one each from (A) 3 control cell lines and (B) 3 FXS patient cell lines. The blue light stimulus protocol to evoke activity is also depicted at the bottom. All cell lines were tested to confirm functional activity prior to phenotype exploration.

a.

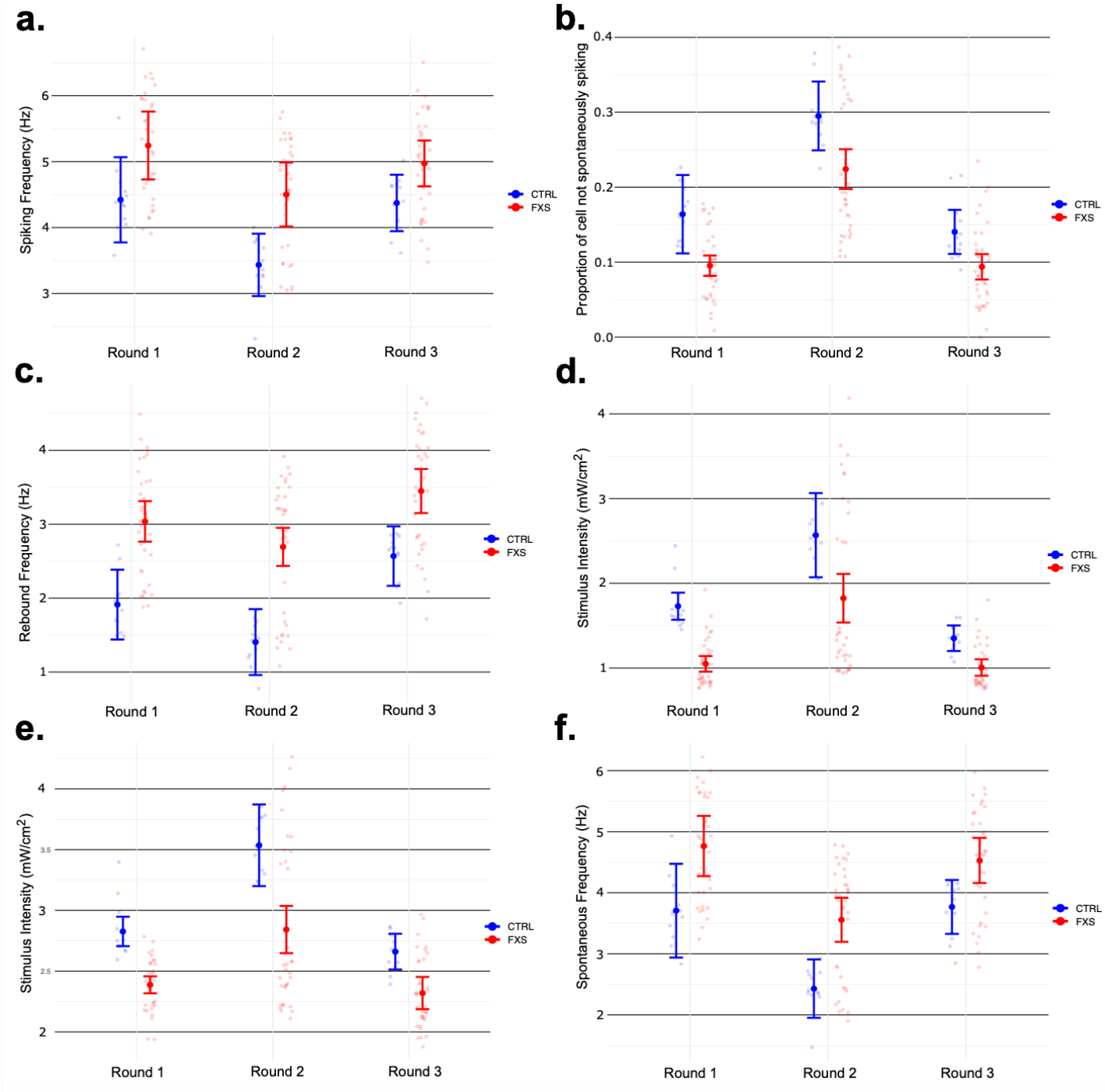
- Radar plots show the effect sizes (between WT and KO) of different phenotypes.
- Values on the plot show the probability that a random well of the WT has a bigger value than a random well of the KO.



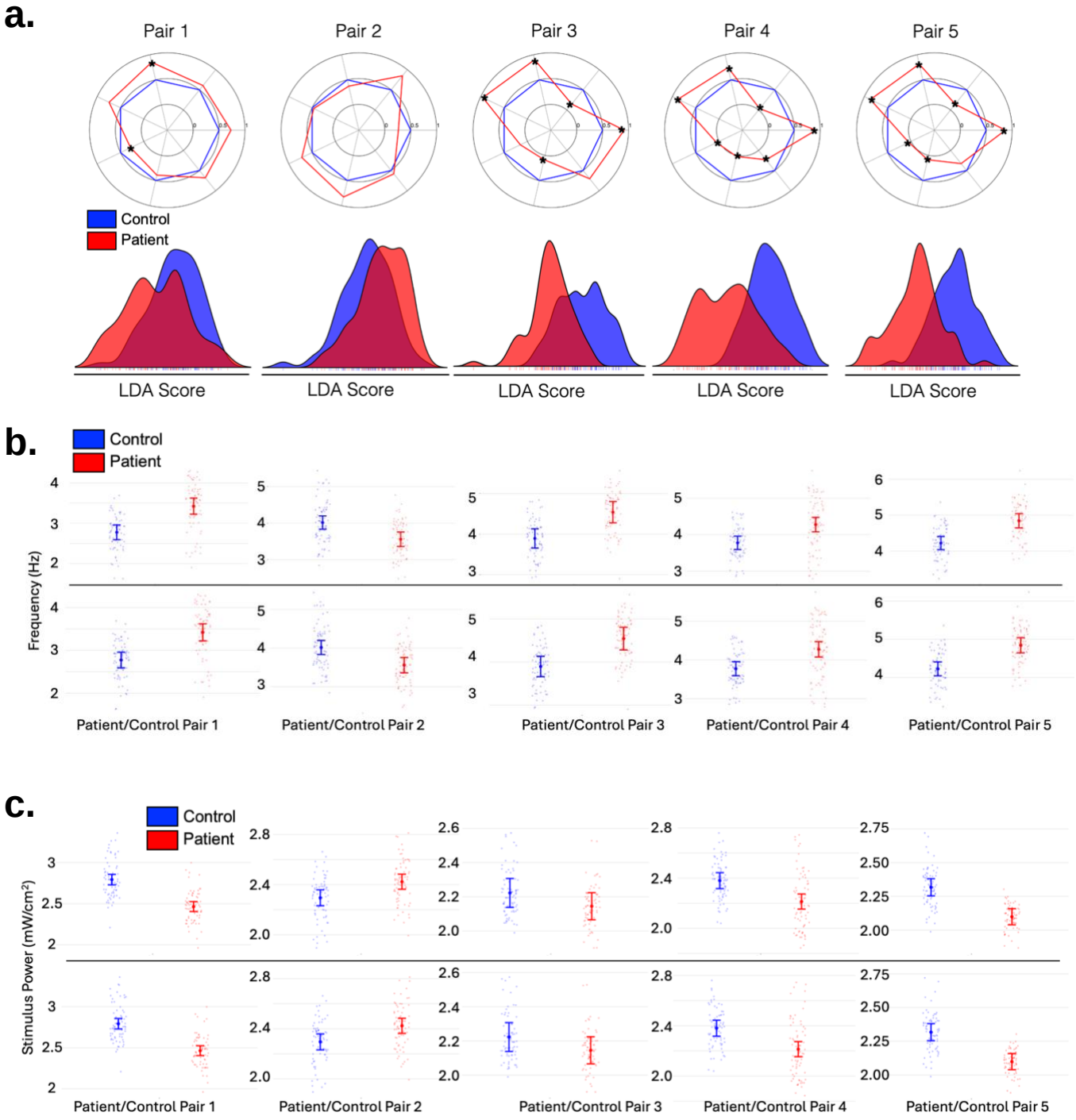
b.

- Spontaneous Frequency: the average firing rate (in Hz) during a 3s window prior to any stimulation. A phenotype here can be broken down into:
  - Spontaneous non-spiking: the proportion of neurons in a well that don't spontaneously spike during that 3s window (i.e. may require stimulation, or fire slower than 0.33 Hz).
  - Spontaneous frequency of spiking sources: the firing frequency (in Hz) of just the sources that spike spontaneously.
- Step 2 late rebound: the average firing rate (in Hz) 250-500ms after the 2nd stimulation (quite low but not the lowest we can give).
- Rheobase: the stimulation intensity required to elicit the first spike.
  - The fast ramp was designed to measure rheobase in these neurons with maximal signal.
  - The linear conductivity "LC" ramp attempts to linearly increase the proportion of open CheRiff channels over the span of 2.5s.
- Step 6 falling edge 2nd derivative: measures how quickly the downstroke of the action potential elicited by the highest stimulation shifts is transitioning towards the AHP plateau.

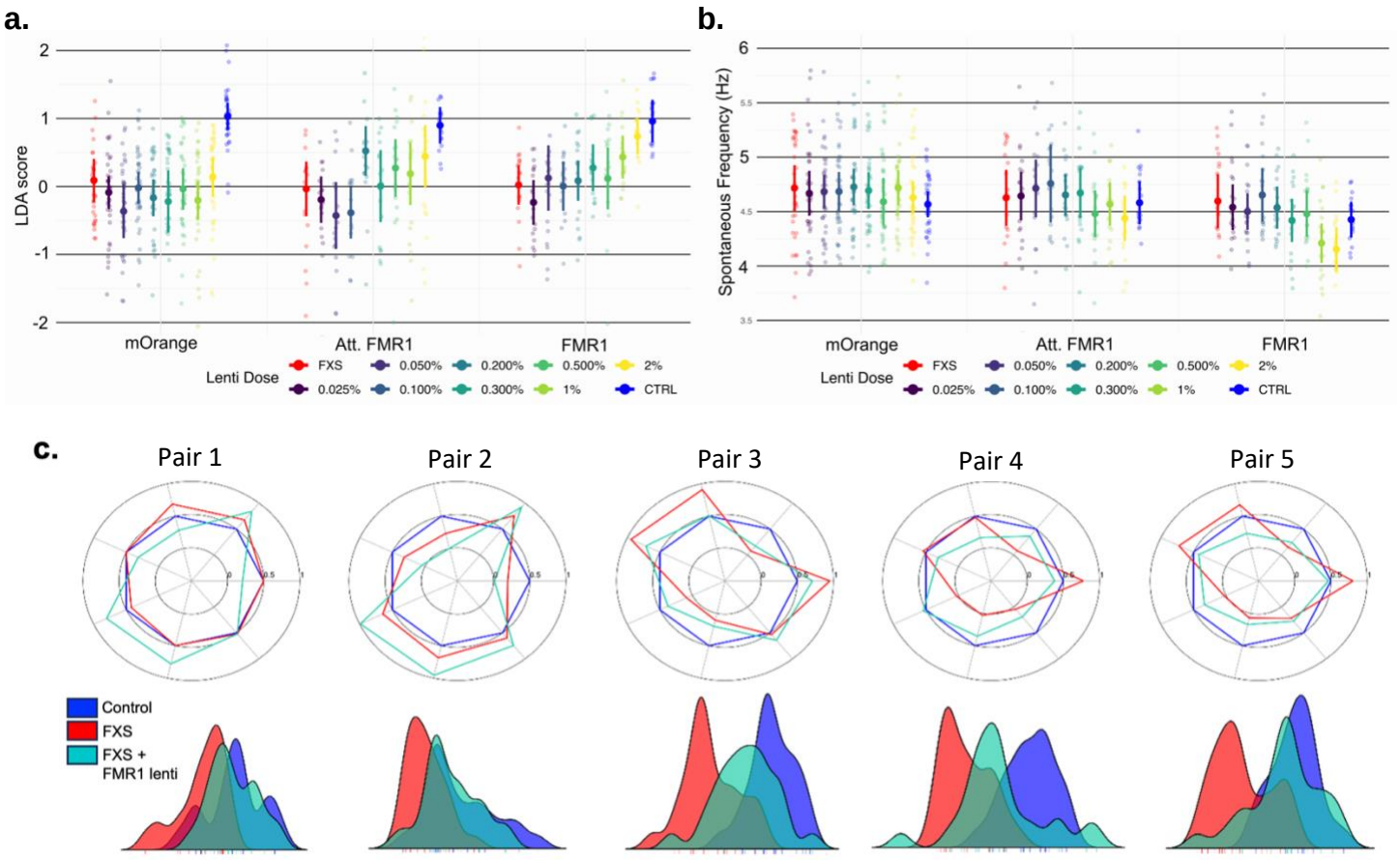
Supplementary Figure 2. Radar plot explanation and functional parameter definitions. (A) Example radar plot to illustrate interpretation of FXS phenotype fingerprint. Wild type (WT)/control condition is plotted as blue and is always normalized to sit at the middle ring of the radar plot. Disease/knock out (KO) condition is plotted in red against the blue control condition. Explanations are given for example features. (B) List of the aspects of the FXS-specific functional phenotype and the meaning of each functional parameter.



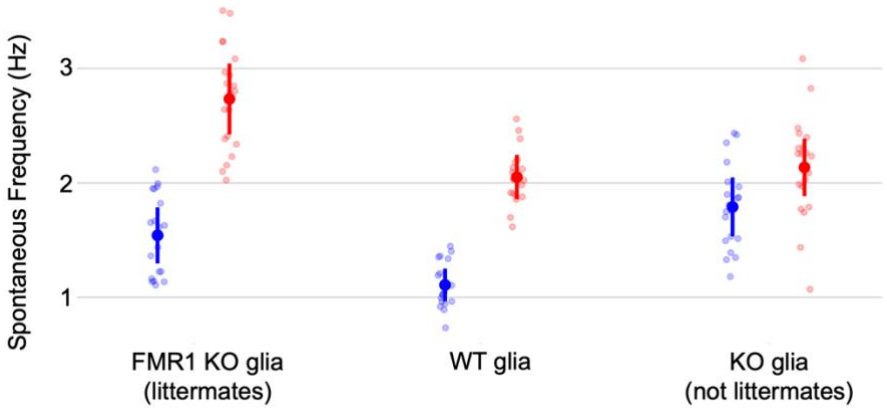
Supplementary Figure 3. Quantification of individual phenotype parameters. Similar to data presented in Figure 2D, A-F above depict raw values for each of the individual parameters of the FXS phenotype from the isogenic cell lines across all 3 rounds of measurements from the DIV30 timepoint. Error bars represent 95% confidence interval. Shown (A) frequency effect of non-spiking cells, (B) non-spiking cells, (C) rebound frequency, (D) linear conductivity (LC) ramp rheobase, (E) rheobase, and (F) spontaneous frequency, as defined in Supp. Fig. 2.



Supplemental Figure 4. DIV45 patient/control phenotyping replicates functional phenotype from DIV30. (A) top: FXS phenotype fingerprint represented as a radar plot for all 5 patient/control pairs replicates the DIV30 phenotype. The asterisk indicates features that were statistically significant. (B) Spontaneous frequency measured for control (blue) vs FXS neurons (red) across all 5 patient/control pairs comparing DIV30 (top row) vs. DIV45 (bottom row). (C) Fast ramp rheobase measured for control (blue) vs. FXS neurons (red) across all 5 patient/control pairs comparing DIV30 (top row) vs. DIV45 (bottom row). Phenotypes replicate across timepoints.



Supplemental Figure 5. *FMR1* lentiviral rescue with transduction 2 weeks prior to measurement. (A) LDA score and (B) Spontaneous frequency for FXS patient neurons treated with 8 increasing doses (represented as % volume of lentivirus) of either an mOrange fluorescent tag, an attenuated form of *FMR1*, or a full-strength *FMR1*. Cells were transduced on DIV16 and measured on DIV30. (C) Radar plots showing the FXS phenotype (red) vs CTRL wells (blue) alongside FXS+lentivirus at a single 1% by-volume dose (teal) for each of the five patient-control pairs (both clones averaged for each donor). Values reflect the common language effect size. Beneath is the LDA scores for each group, fit over the isogenic phenotype and applied to each patient-control pair.



Supplemental Figure 6. FXS phenotype in the presence of *Fmr1*$^{-/y}$ astrocytes/glia. Spontaneous frequency and for control vs. *FMR1*$^{-/y}$ isogenic lines co-cultured on either WT mouse glia or glia derived from *Fmr1*$^{-/y}$ littermates or those derived from non-littermate *Fmr1*$^{-/y}$ mice.

a.

Plate set 1

Genotype	01	02	04	04	05	06	07	08	09	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	*FMR1*$^{-/y}$	Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8	*FMR1*$^{+/y}$	PBS
C	PBS		Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8		PBS
D	PBS		Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8		PBS
E	PBS		Drug 9	Drug 10	Drug 11	Drug 12	Drug 13	Drug 14	Drug 15	Drug 16		PBS
F	PBS		Drug 9	Drug 10	Drug 11	Drug 12	Drug 13	Drug 14	Drug 15	Drug 16		PBS
G	PBS		Drug 9	Drug 10	Drug 11	Drug 12	Drug 13	Drug 14	Drug 15	Drug 16		PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

b.

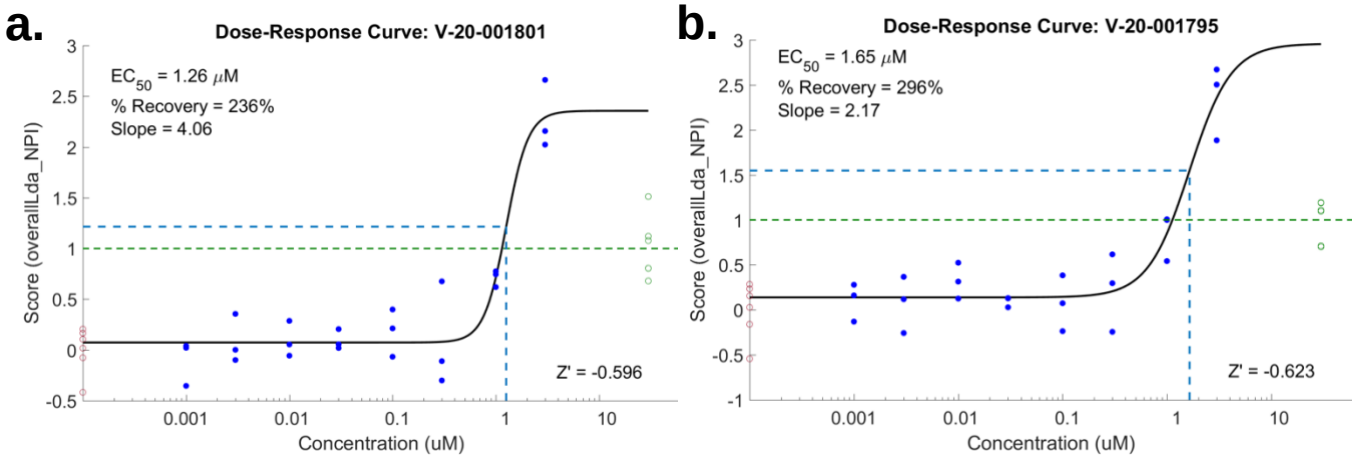
Plate set 2

Genotype	01	02	04	04	05	06	07	08	09	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	*FMR1*$^{-/y}$	Drug 9	Drug 10	Drug 11	Drug 12	Drug 13	Drug 14	Drug 15	Drug 16	*FMR1*$^{+/y}$	PBS
C	PBS		Drug 9	Drug 10	Drug 11	Drug 12	Drug 13	Drug 14	Drug 15	Drug 16		PBS
D	PBS		Drug 9	Drug 10	Drug 11	Drug 12	Drug 13	Drug 14	Drug 15	Drug 16		PBS
E	PBS		Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8		PBS
F	PBS		Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8		PBS
G	PBS		Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8		PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Supplemental Figure 7. Compound screen experiment design/plate layout. (A,B) Diagram of 96-well plates for compound screening in the isogenic cell line. Plate map shows the location of *FMR1*$^{-/y}$, *FMR1*$^{+/y}$, and the location of 16 compounds per plate (A: compound set 1, B: compound set 2).

Target	Pharmacology	Isogenic	Patient/Control
BK channel	blocker	Borderline	Hit
BK channel	opener	Anti	Anti
CaV channels (NS, 1.x, 2.x, 3.x)	blocker	Hit	Hit
CaV channels (NS, 1.x, 2.x, 3.x)	blocker	Hit	Hit
GABA reuptake	GAT1 inhibitor	Hit	Hit
GABA-B receptor	agonist	Borderline	Hit
Kappa opioid receptor	agonist	Hit	Hit
NaV channels (NS, 1.6, 1.8)	blocker	Hit	Hit
PKC-a	inhibitor	Borderline	Hit
Rac1	inhibitor	Borderline	Hit
Serotonin reuptake	inhibitor	Hit	Hit
SK channel	activator	Rec. for follow-up	Hit
SK channel	activator	Hit	Hit
SK channel	PAM	Hit	Hit
NaV Blocker	blocker	Hit	Hit
Slack channel	opener	Hit	Hit

Supplemental Table 2. Hits identified from tool compound screen.



Supplemental Figure 8. Additional dose-response data from hit confirmation studies. Dose-response graphs of LDA scores for V-20-001801 (left) and V-20-001795 (right) in the isogenic cell lines. On-plate controls shown in red (along y-axis; left; *FMR1*$^{-/y}$) and green (along y-axis; right; *FMR1*$^{+/+}$), with the dose and the response of the EC/IC50 marked with dotted blue line. Dotted green line is the mean of the controls.