Method

Agonist-induced functional analysis and cell sorting associated with single-cell transcriptomics characterizes cell subtypes in normal and pathological brain

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To gain better insight into the dynamic interaction between cells and their environment, we developed the agonist-induced functional analysis and cell sorting (aiFACS) technique, which allows the simultaneous recording and sorting of cells in real-time according to their immediate and individual response to a stimulus. By modulating the aiFACS selection parameters, testing different developmental times, using various stimuli, and multiplying the analysis of readouts, it is possible to analyze cell populations of any normal or pathological tissue. The association of aiFACS with single-cell transcriptomics allows the construction of functional tissue cartography based on specific pharmacological responses of cells. As a proof of concept, we used aiFACS on the dissociated mouse brain, a highly heterogeneous tissue, enriching it in interneurons by stimulation with KCl or with AMPA, an agonist of the glutamate receptors, followed by sorting based on calcium levels. After AMPA stimulus, single-cell transcriptomics of these aiFACS-selected interneurons resulted in a nine-cluster classification. Furthermore, we used aiFACS on interneurons derived from the brain of the Fmr1-KO mouse, a rodent model of fragile X syndrome. We showed that these interneurons manifest a generalized defective response to AMPA compared with wild-type cells, affecting all the analyzed cell clusters at one specific postnatal developmental time.

[Supplemental material is available for this article.]

The selection, cloning, and morphological/functional characterization of individual cells in a suspension or from a tissue is a fastidious and lengthy procedure, despite several techniques, including limiting dilutions, laser microdissection (Datta et al. 2015), cytoplasm aspiration (Cadwell et al. 2016; Fuzik et al. 2016), microfluidics (Pollen et al. 2014; Zeisel et al. 2015; Tasic et al. 2016), and flow cytometry (Fulwyler 1965), having been set up to reach this goal. The latter is a robust and powerful technique that allows fast analysis and sorting of cell subsets from an initial heterogeneous sample. Cells can be sorted according to various parameters and are amenable to further characterization by single-cell RNA-sequencing (scRNA-seq) or mass spectrometry. Single-cell transcriptomics is a unique tool that provides precise and simultaneous analysis of the expression levels of thousands of genes in a complex heterogeneous population at the single-cell level (Poulin et al. 2016). Applied to the study of a pharmacological stimulation, this technique can discriminate homeostatic gene regulation at the tissue level from the modulation of the abundance of a given cell population, a phenomenon that is widely observed during development (Poulin et al. 2016; Ofengeim et al. 2017).

Coupling flow cytometry analysis and cell sorting with single-cell transcriptomics makes it possible to link a cell phenotype to its genotype, providing direct access to the molecular cues of a given phenotype. In this context, the key point is to submit the cells under analysis to the same stimulus. Functional fluorescence-activated cell sorting (FACS) analysis of intracellular pH or calcium variations using fluorescent probes (Chow et al. 2001; Vines et al. 2010) commonly consists of adding a stimulation (e.g., drugs or osmotic variations) into the sample tube containing the stained cell suspension before analysis or sorting. Although it takes up to 30 sec for the sample to reach the flow cell (the chamber aligning the cells in order to pass one-by-one through the laser beam for sensing), it can be considered that the fluorescence signal is measured “at equilibrium,” as its detection and the physiological stimulus are temporally far from each other. This lag time prevents a comparable and accurate analysis of cell-to-cell calcium variations in commercial flow cytometers compared with the efficient sampling of calcium imaging (from a few milliseconds to seconds) obtained via fluorescence microscopy by combining the quick local delivery of drugs with high recording rates. As the cell suspension in the sample tube is currently injected under pressure into the cytometer fluids in many commercial flow cytometers, the sample tube has to be removed to study cellular responses to drug stimulations. This manual step increases the time lag contributing to the observation of “stationary” cellular responses.

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Moreover, this methodology presumes that the first and last cells analyzed in the tube behave in an equivalent manner. With the appearance of sample lines using peristaltic pumping in commercial FACS (i.e., sample tube at atmospheric pressure), technical approaches have been proposed to add drugs to the cells in a continuous manner (Vines et al. 2010; Arnoldini et al. 2013). Although the time lag corresponding to the tube removal was suppressed, all cells remained simultaneously exposed to the drug but got analyzed at different times. Encouraging approaches to fast calcium response measurements by flow cytometry have been proposed in the past based on a fluidic modification in a FACS analyzer (Tárnok 1996; Zwartz et al. 2011). A method to monitor fast kinetics and the possibility of sorting the cells by modifying the tubing configuration of a FACStar Plus cytomter was also described (Dunne 1991). However, no sorting evidence was shown. These approaches remained in the form of prototypes and were only used for analysis.

The agonist-induced functional analysis and cell sorting (aiFACS) prototype we developed was built on a FACS Aria III as an add-on. It allows the analysis of all the aforementioned parameters and also sorts cells according to their immediate and individual responses to a stimulus. We used it to analyze neurons derived from mouse brains, and obtained enriched preparations of interneurons. Further application of aiFACS to Fmr1-KO mice—a rodent model of fragile X syndrome (FXS)—brains, associated with single-cell RNA-seq, highlighted the functional and molecular impairment of interneurons in this disease. Besides the brain, aiFACS can be used to analyze cell populations of normal and pathological tissues, including tumors.

Results

Setup of aiFACS

We designed the aiFACS prototype for sorting cells according to their responses to a pharmacological stimulation monitored in real time with a fluorescent calcium indicator (Fig. 1A). The sorter fluidics system (BD FACStar III) was modified to allow drug injection into the sample line. Tubing (Fig. 1A, in red) with a diameter of 0.19 mm, equivalent to that of the sample line (Fig. 1A, in blue), was connected to two syringes upstream of the solenoid valve and the flow cell via a “Y” connector. Two valves alternatively opened

Figure 1. The aiFACS technique. (A) Schema of the instrumental apparatus: BD FACStar III implemented with the aiFACS device. The sorter fluidics is modified to allow the injection of a pharmacological agonist. Two syringes, one containing D-PBS (in gray) and the other one containing the agonist (in green), are connected to their respective tubing: the D-PBS tubing (in gray) and the agonist tubing (in green). These are further connected to a downstream Y-shaped connector that enters the flow cell (the chamber in which the cells are aligned to pass one-by-one through the light beam for sensing). The sample is connected to the sorter through a tubing (in blue) having the same diameter as the agonist tubing (in green). A peristaltic micropump (P) allows control of the speed of solution injection and synchronization to the speed of sample flow in the sorter. The incubation time between each cell of the sample and the agonist is also controlled (red tubing). (B) Time versus Fluo-4 AM biparametric graph showing the response of the cells to different stimuli in real time. At time t0, the opening of the D-PBS valve starts the perfusion, and the baseline levels of fluorescence (in the red rectangle) are obtained with continuous perfusion. At time t1, the D-PBS valve is closed, and the one of the agonist is opened. The magnitude of the cellular calcium response to the KCl agonist (65 mM final) is shown in the light-blue rectangle. At time t2, ionomycin is added (6.5 μM final) to KCl as a positive control of stimulation. The maximal response of the cells is displayed in the green rectangle. (C) Addition of beads to the agonist solution allows real-time detection of the agonist presence. Bead fluorescence is shown in purple. (D) aiFACS allows viable recovery of stimulated cells. (Upper panels) Discrimination of cells based on scatter parameters (FSC) forward scatter; (SSC) side scatter) before sorting (55.3% of the total population; left panel). The pretreat viability is determined by labeling the cells with DAPI (95.5% of the cells in the red region; right panel). (Lower panels) Discrimination of cells based on scatter parameters (FSC and SSC) after sorting (90.9% of the total population; left panel). The viability of the cells after KCl stimulation and aiFACS sorting is determined by reanalyzing the DAPI staining (99.7% of the cells in the red region; right panel). (E) Sorted neurons are viable and can grow neurites when plated on L-ornithine–coated glass coverslips, cultivated for up to 6 d in vitro (DIV 6) in complete neurobasal medium, and analyzed by immunocytochemistry (MAP2 staining). 63× magnification; scale bars, 10 μm.
or closed to control the injection syringes containing PBS and agonist and allowed the injection buffer to be selected. The speed of injection of the solutions was controlled using a peristaltic micro-pump to synchronize it with the FACS flow rate. The incubation time between each cell and the drug was thus, strictly controlled (Fig. 1A, red tubing). At basal conditions, a sample resuspension buffer (D-PBS) was infused. To induce stimulation, the experimental drug was injected at 1.6× concentration. The cells were sorted according to their response to the drug. The precise dilution of the agonist was determined by the flow rates of the instrument and the pump. Its calibration was possible by using bead solutions of known concentrations (PE and APCDest) respectively instead of sample and agonist and by counting them at different flow rates, as shown in Supplemental Figure S1.

Using the Miltenyi neuron isolation kit (Holt and Olsen 2016; Berl et al. 2017), fresh postnatal day (PND) 18 adult mouse brains were dissociated to obtain RNA from the neuronal and nonneuronal populations, which were used to measure the expression of several neuronal (Gad2, Itp1, NeuroD1, and Ngr) markers by RT-qPCR. This fraction was depleted in microglia (Gm), immature neurons (Sox2), astrocytes (Gfap), oligodendrocytes (Mog2 and Mbp), and endothelial cells (Rmgep4) (Supplemental Fig. S2).

We injected neurons labeled with Flu-4 AM to monitor the calcium response, and set up the machine using the parameters indicated in red in Supplemental Figure S3A. In the first step, based on our previous experience with ratiometric calcium imaging (Castagnola et al. 2018), we used KCl (65 mM final) as the agonist to elicit calcium entry into cells. We added fluorescent beads to monitor agonist perfusion and the increase in K⁺ ion concentration in the sample line. D-PBS perfusion started at time t0 and stopped at time t1, when the KCl perfusion was initiated (Fig. 1B). Each cell was in contact with KCl for 3.2 sec (Supplemental Fig. S3A). The appearance of the beads corresponded to the arrival of cells stimulated with KCl in the flow cell (Fig. 1C). The increased levels of calcium is shown in Supplemental Figure S3B. To verify the amplitude of the neuronal response and to have a positive control of stimulation, ionomycin was added to KCl at time t2 (Fig. 1B). Prepot viability was determined by labeling dissociated neurons with DAPI staining (Fig. 1D). The viability of the cells that responded to KCl and after sorting by aFACS was monitored using the previous labeling and was determined through reanalysis of the cells

![Figure 2](image-url)

**Figure 2.** Gating-dependent cell populations. (A) Discrimination of cells based on scatter parameters: (FSC) forward scatter; (SSC) side scatter. Cells were gated according to their size/structure. (GD1) Gating-dependent cell population 1; (GD2) gating-dependent cell population 2. (B) RNA was purified from WT GD1 and GD2 cells. Neuronal and nonneuronal marker levels were analyzed in both populations by RT-qPCR. The graph (on the left) shows mRNA expression relative to the total WT neuron suspension (WT input). GD1 and GD2 cells were live-imaged 2 h after aFACS sorting (on the right). 63× magnification; scale bars, 15 μm.

(a)FACS to study heterogeneous cell populations

To obtain a proof of concept that this new method allows the selection and study of neuronal populations using pharmacological stimulation, we decided to study the activation of ionotropic receptors by its agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). After brain dissociation, cells stained with Flu-4 AM were gated based on their fluorescence. We injected the cells in the FACS, supplying the flow cell with neutral D-PBS (baseline condition). After 3 min of recording, we stimulated GD1 cells with an AMPA solution (130 μM final). In this case, each cell was in contact with the drug for 3.2 sec. Selected interneuron GD1 cells were used to carry out single-cell transcriptomics using the 10x Genomics Chromium and Illumina sequencing platforms. After quality control, we analyzed 2170 cells (1287 AMPA and 883 baseline), with a median unique molecular identifier (UMI) per cell of 6652. The canonical correspondence analysis (CCA) of the two aggregated samples led to the identification of nine cell clusters (Fig. 3A,B). The AMPA response of each cluster is illustrated in Figure 3C. Selection of the various markers was performed on the basis of previous publications (Zeisel et al. 2015, 2018; La Manno et al. 2016; Tasic et al. 2016; Paul et al. 2017; Mi et al. 2018; Rosenberg et al. 2018).

Selected GD1 cells were broadly split into inhibitory (Dcx, Gad1, Gad2, Dlx6, Dlx1, Dlx5, and Dlx2) and excitatory neurons (S6a, Slc17a6, Nrn1, NeuroD1, and Neum2) (Fig. 3A,B). We identified two clusters of cycling progenitors (Top2a, Ube2c, Mki67, Hmyb2, and Capnp) (Fig. 3A; Supplemental Fig. S8) already engaged in inhibitory (Dlx1, Dlx5, and Dlx2) or excitatory lineages (Serh, Slc17a7, Slc17a6, Nrn1, NeuroD1, and Neum2) (Fig. 3A,B).
Meis1, Pax6, E2f1, Cog7, and Cad63). Inhibitory neurons were split into three main populations that express Meis2 in combination with other markers: Tiam2/Nrxn3, Pbx1/Sox4, and Synpr/Cabl2/Pbx3. We also identified two inhibitory neuron clusters further advanced in their maturation process: “Sema3c” (Cabl2, Sema3c, Id2, and Oprl) composed of VIP neuron precursors, and inhibitory mature neurons composed of “Rora” cells (Cdh3, Akap7, Chtipb2, Gad1, and Gad2) that may give rise to CCK neurons (Fig. 3A,B,G). Next, we identified a continuum of three excitatory cell clusters, separated by their differentiation status: ventricular zone or dentate gyrus granule cell intermediate progenitors (Hes6 and Cnd2), and two clusters of further differentiated cells (Neurod1, Neurod2, Apc, Nrxn1, Rhfox3, and Map1b), which could be split according to their intermediate or final maturation, as indicated by the expression of pre- and postsynaptic (Snap25, Grin2b, and Pclo), cytoskeletal (Mapt), and potassium channel (Kcnb2 or Kcna1 and Kcnk2) markers (Fig. 3A,B). The distribution of some specific markers (Meis2, Neurod1, Neurod2, and Gad2) is shown in the context of various clusters (Fig. 3D–G). In Figure 3H, the comparison of cell clusters before and after aiFACS selection is shown. In particular, the post-aiFACS selection clearly shows that AMPA stimulation promoted the positive selection of Meis2 interneurons (12%–38% of sorted cells) (Fig. 3B,D,H).

In summary, aiFACS is a tool to analyze a tissue response to a pharmacological stimulation, offering new information on ion homeostasis players and the cellular specificities that drive the heterogeneity of the cell response.

aiFACS selection through AMPA stimulation unveils impaired interneurons in Fmr1-KO mouse brain

To validate that aiFACS is helpful for studying brain disorders, we applied it to dissociated neurons from mouse Fmr1-KO brains. This disorder is owing to the lack of function of the FMRP translational regulator 1 (FMR1) protein. Indeed, recent studies have highlighted interneuron dysfunctions in FXS (Olmos-Serrano et al. 2010; Le Magueresse and Monyer 2013; Patel et al. 2013; Cea-Del Rio and Huntsman 2014; Goel et al. 2018; Yang et al. 2018), as well as alterations in the expression, trafficking, and functioning of AMPA receptors (Cheng et al. 2017). To carry out this part of the study, we implemented the technique by introducing a dynamic selection to simultaneously analyze wild-type (WT) and Fmr1-KO samples. We dissociated PND 18 WT and Fmr1-KO brains, and cells from both genotypes were stained with Fluo-4 AM. WT cells were further labeled with Alexa-Fluor 594-coupled wheat germ agglutinin (WGA 594), whereas Fmr1-KO cells were labeled with Alexa-Fluor 647 WGA. This labeling allowed the mixing of cells of both genotypes to perform a combined analysis (Fig. 4A), and did not influence the selection (Supplemental Fig. S9). Following the previous
levels were quantified by RT-qPCR and compared for WT and Fmr1-KO neurons subjected to aiFACS (input WT). Results are presented as the mean ± SEM, analyzed simultaneously. (A, left) PND 18 WT and Fmr1-KO brains were dissociated, and neuronal cells were selected. Neurons from both genotypes were multiplexed by fluorescent labeling with wheat germ agglutinin (WGA; WGA647 for WT, and WGA594 for Fmr1-KO) and were processed and analyzed simultaneously. (Upper right panel) The injection of fluorescently labeled beads simultaneously with AMPA (130 μM final) perfusion allows the monitoring of the agonist in the flow cell. (Central, lower panels) Real-time monitoring of neuronal responses to AMPA stimulation by Fluo-4 AM fluorescence quantification in Fmr1-KO (blue) and WT (green) cells. (B) mRNA was purified from 5000 GD1 cells, and inhibitory and excitatory marker expression levels were quantified by RT-qPCR and compared for WT and Fmr1-KO. Marker expression upon AMPA stimulation at PND 18 in both genotypes is presented as the fold change respective to the expression of WT neurons subjected to aiFACS (input WT). Results are presented as the mean ± SEM, Mann–Whitney U test; *(P < 0.05), (ns) not significant. For Meis2, Neurod1, and Gad2: WT, n = 4; Fmr1-KO, n = 5. For Neurod2: WT, n = 4; Fmr1-KO, n = 4. Each n corresponds to two (nonlittermate) mouse brains and is the mean of two independent replicates. (C) Percentage of cells belonging to the nine clusters after single-cell analysis of AMPA response in WT and Fmr1-KO GD1 cells. Ex-Neurod2, Ex-Sept4, Ex-Selm, and Ex-Top2a: excitatory clusters; Inh-Top2a, Inh-Sox4, and Inh-Synpr: inhibitory clusters; Sema3c: ependymal cells; Akap7: oligodendrocytes.

Figure 4. aiFACS multiplex analysis. (A, left) PND 18 WT and Fmr1-KO brains were dissociated, and neuronal cells were selected. Neurons from both genotypes were multiplexed by fluorescent labeling with wheat germ agglutinin (WGA; WGA647 for WT, and WGA594 for Fmr1-KO) and were processed and analyzed simultaneously. (Upper right panel) The injection of fluorescently labeled beads simultaneously with AMPA (130 μM final) perfusion allows the monitoring of the agonist in the flow cell. (Central, lower panels) Real-time monitoring of neuronal responses to AMPA stimulation by Fluo-4 AM fluorescence quantification in Fmr1-KO (blue) and WT (green) cells. (B) mRNA was purified from 5000 GD1 cells, and inhibitory and excitatory marker expression levels were quantified by RT-qPCR and compared for WT and Fmr1-KO. Marker expression upon AMPA stimulation at PND 18 in both genotypes is presented as the fold change respective to the expression of WT neurons subjected to aiFACS (input WT). Results are presented as the mean ± SEM, Mann–Whitney U test; *(P < 0.05), (ns) not significant. For Meis2, Neurod1, and Gad2: WT, n = 4; Fmr1-KO, n = 5. For Neurod2: WT, n = 4; Fmr1-KO, n = 4. Each n corresponds to two (nonlittermate) mouse brains and is the mean of two independent replicates. (C) Percentage of cells belonging to the nine clusters after single-cell analysis of AMPA response in WT and Fmr1-KO GD1 cells. Ex-Neurod2, Ex-Sept4, Ex-Selm, and Ex-Top2a: excitatory clusters; Inh-Top2a, Inh-Sox4, and Inh-Synpr: inhibitory clusters; Sema3c: ependymal cells; Akap7: oligodendrocytes.

Because upon AMPA stimulation GD1 cells were strongly enriched in Meis2-expressing cells, we measured the expression levels of Meis2 by RT-qPCR after AMPA stimulation of both WT and Fmr1-KO GD1 cells obtained from new sets of animals by evaluating the fold change respective to the expression of aiFACS-selected WT neurons (Fig. 4B). Our results showed that the AMPA-responding Fmr1-KO neurons expressed lower levels of Meis2, Neurod1, and Neurod2 mRNA compared with WT neurons, whereas no changes were observed for Gad2 (Fig. 4B). This suggests that AMPA-responding Fmr1-KO GD1 cells display abnormal expression levels of the analyzed genes compared with WT or that a different number of cells express these markers in WT and Fmr1-KO. To gain a deeper insight into this phenomenon, we performed single-cell sequencing on AMPA-stimulated WT GD1 cells, even if their proportions were altered (Fig. 4C). This suggests an overall impairment in AMPA response in the absence of FMR1, as cells from each cluster are affected. This variability could be explained by the different levels of maturity or developmental profile of the cells comprising each cluster in WT versus Fmr1-KO brains.

To evaluate this hypothesis, we measured the levels of the analyzed markers at baseline (Fig. 5A) by using new sets of nonlittermate PND 18 and PND 19 WT, and Fmr1-KO mouse neuronal populations subjected to aiFACS. RT-qPCR analysis revealed elevated expression levels of the two proneuronal genes Neurod1 and Neurod2 in Fmr1-KO baseline GD1 cells. To assess whether the phenotype we observed was associated with the developmental process, we repeated the analysis in mice older than PND 18. At PND 19, RT-qPCR analysis indicated that the expression levels of Meis2, Neurod1, Neurod2, and Gad2 did not show a significantly different abundance between the two genotypes (Fig. 5B). Consistently, the analysis of PND 19 GD1 Fmr1-KO neurons revealed that their response to AMPA was unchanged compared with WT neurons for all the analyzed markers (Supplemental Fig. S11). We can therefore suggest that AMPA-responding Fmr1-KO cells might be altered at this stage of development, at least for the GD1 population expressing these markers.

Discussion

A new approach to study pharmacological cell responses

We developed the aiFACS prototype with the aim of exploring the cell-specific responses to environmental and pharmacological stimuli, particularly for fast kinetic studies. Classical FACS analysis does not allow the sorting of living cells, which are all subjected to the same brief stimulus. Our technique showed feasibility of calcium monitoring in real time with a fluorescent indicator, as well as sorting of cells according to their calcium response upon
pharmacological treatment. The entry of calcium into neurons is a tightly controlled process. This is owing to the opening of $\text{Ca}^{2+}$-permeable channels, which are known to respond to a large array of stimuli, such as membrane depolarization or extracellular chemical messengers, that can directly activate the channel or can act indirectly via intracellular molecular signaling (Barratt 1999; Taylor 2002). In particular, fast and brief increases in intracellular calcium levels are known to be involved in various steps of neuronal development. These calcium transients play a pivotal role in the regulation of neurotransmitter phenotypes, dendritic morphology, axonal growth, and guidance (Rosenberg and Spitzer 2011). Modulation of all these parameters is critical for neuronal subtype specification. Hence, the duration and quality of the stimulus are the most critical aspects to manage while setting up the aiFACS method, because they represent the key modulators of the selectivity, activity, and expression of transcription factors, as well as the availability of high-throughput analysis tools that allow us to unravel cell identity, define the molecular determinants of the pharmacological response, and directly assess gene expression differences, including splice variants and edited transcripts. Furthermore, by introducing dynamic selection, WT and one or more mutants can be analyzed simultaneously in the same run, or multiple individuals having the same genotype can be multiplexed and analyzed separately but simultaneously.

To appreciate the power of aiFACS, we analyzed the brain as a highly specialized and heterogeneous tissue. By applying this technique to PND 18 mouse brains, we showed the possibility of enriching our samples in interneurons responding rapidly to a pharmacological agonist. To date, the study of interneurons has been confined to restricted brain areas or circuits because of the limited availability of high-throughput analysis tools that allow a thorough and precise analysis of the complex molecular, spatial, anatomical, and connection heterogeneities of the brain (Le Magueresse and Monyer 2013). We are convinced that our approach will enable a novel function-based classification of interneuron cells rather than a classification based on (or in addition to) other parameters such as localization, morphology, and gene expression.

**A proof of concept in FXS**

We compared WT and $\text{Fmr1-KO}$ aiFACS-selected cell responses to AMPA. Our results show a global altered AMPA response in $\text{Fmr1-KO}$ cells, in agreement with other studies (Cheng et al. 2017). Based on RNA-seq profiles, the same clusters of cells were present, but their ratio was different between WT and $\text{Fmr1-KO}$ cells. Indeed, we repeated aiFACS selection on additional sets of brains, we showed the possibility of enriching our samples in interneurons responding rapidly to a pharmacological agonist. To date, the study of interneurons has been confined to restricted brain areas or circuits because of the limited availability of high-throughput analysis tools that allow a thorough and precise analysis of the complex molecular, spatial, anatomical, and connection heterogeneities of the brain (Le Magueresse and Monyer 2013). We are convinced that our approach will enable a novel function-based classification of interneuron cells rather than a classification based on (or in addition to) other parameters such as localization, morphology, and gene expression.

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Meis2 is a marker of lateral ganglionic eminence-derived interneurons that are medium spiny neurons of the striatum. Moreover, Meis2 interneurons are in the rostral migratory system, giving rise to dopaminergic periglomerular interneurons of the olfactory bulb (Allen et al. 2007; Agoston et al. 2014; Fujisawa and Cave 2016). Meis2 interneurons were never involved in the pathophysiology of FXS, and altered levels of Meis2 were never observed in the Fmr1-KO brain. Indeed, the previous analysis of cortex and hippocampus transcriptomics of the Fmr1-KO mice did not reveal substantial differences compared with WT samples (Maurin et al. 2018). Therefore, our data clearly show that aiFACS allows us to highlight unexpected impairments in cell subtypes by carrying out a sensitive functional cellular analysis of cortex and hippocampus transcriptomics of the Fmr1-KO mice (Tang et al. 2015; Maurin et al. 2019), we can consider that this difference in expression could be a consequence of an altered developmental profile of Fmr1-KO interneurons, dependent on the function of FMR1. This protein is known to modulate a network of pathways, including calcium signaling, which is supposed to be disorganized in its absence (Miashiro et al. 2003; Richter and Coller 2015; Ferron 2016; Castagnola et al. 2018; Maurin and Bardon 2018; Maurin et al. 2018).

aiFACS was developed on a simpler and similar basis to previous technical modifications on commercial flow cytometers designed to optimize real-time flow cytometry cellular assays in the past (Dunne 1991; Tarnok 1996; Vines et al. 2010; Zwart et al. 2011; Arnoldini et al. 2013). Flow cytometers fluids principally use two different technologies to inject the sample into the sheath fluid: by applying pressure on the sample tube or by peristaltic pumping. Some investigators (Dunne 1991; Tarnok 1996) used an approach similar to that described previously (Kelley 1989), with a derivation of the pressurization from the cytometer to create a stimulus line to mix drugs with the cells. Later, by using the Accuri C6 flow cytometer based on sample injection at atmospheric pressure, other investigators (Vines et al. 2010; Arnoldini et al. 2013) suggested simply adding the stimulation to the sample tube. Although simpler, the main drawback of the latter approach was in monitoring heterogeneous cellular stimulations, because the first and last cells monitored would be exposed to the drug for different lengths of time. If our aiFACS approach seems closer to the previous developments (Dunne 1991; Tarnok 1996), it offers the advantage of being more flexible in connecting a stimulus line to a commercial flow cytometer managing multiple drug injections and proving to be efficient with sorting strategies, whereas previous developments only showed cell analysis. This is a key aspect to perform further single-cell omics studies. The proof of concept on the FXS mouse model confirmed that aiFACS allows the collection of a wealth of new information concerning the molecular pathology of a brain disorder. This was possible by using both a sophisticated approach such as scRNA-seq and a simple and inexpensive technique such as RT-qPCR.

Our parameters and working conditions resulted in the study of interneurons. However, by modulating the aiFACS selection parameters, testing different developmental times, using various stimuli, and multiplying the analysis of readouts, we think that it will be possible to extend the use of aiFACS to other brain cell types and also to a large panel of normal and pathological tissues, including tumors. In perspective, aiFACS can be applied to study second messenger modulations, kinase activations, ionic fluxes, and many other biochemical and pharmacological mechanisms at the level of individual cells.

Methods

Animal handling and care

Animal care was conducted in accordance with the European Community Directive 2010/63/EU. WT and Fmr1-KO mice on a C57BL/6j congenic background were obtained from Prof. R. Willemsen (Fmr1-KO line 2) (Mientjes et al. 2006). All animals were housed in groups of six under standard laboratory conditions (22°C, 55 ± 10% humidity, 12-h light/12-h dark diurnal cycles) with food and water provided ad libitum. Only the brains of male animals were analyzed. For timed pregnancies, noon on the day of the vaginal plug was counted as E0.5. The experiments were performed following the Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines (Kilkenny et al. 2010; https:// ARRIVEguidelines.org/publications). The experiments were approved by our local ethics committee and the Ministry of Education and Research (approval no. 00788.01).

Brain dissociation and neuron isolation

Full brains were dissected from PND 18 mice. A brief wash with complete D-PBS (supplemented with 0.5% bovine serum albumin, 1% pyruvate, and 15 mM glucose) was performed before cutting the brains in six equally thick sagittal sections (2 mm), using a mouse brain matrix slicer (CellPoint Scientific) and razor blades. Brain slices were dissociated using a gentleMACS octo dissociator and the adult brain dissociation kit (Miltenyi Biotec) following the manufacturer’s instructions. The neuron isolation kit (Miltenyi Biotec) was used for magnetic selection of neuronal cells.

Neuron labeling

WT and Fmr1-KO neuronal suspensions were labeled with a combination of the Fluor-4 AM calcium indicator (5 μg/mL; Invitrogen) and either Alexa-Fluor 594-coupled WGA or Alexa-Fluor 647-coupled WGA (5 μg/mL; Invitrogen) in D-PBS for 20 and 10 min, respectively, at 37°C. The dyes were switched in each experiment. Centrifugation at 300 g for 10 min at room temperature was performed subsequently, and the cells were resuspended in 300 μL of D-PBS. Before sorting, neurons were labeled with 0.05 μg/mL DAPI.

aiFACS and cell sorting

Cells were sorted using a 100-μm nozzle, on a FACS Aria III (BD Biosciences) equipped with four lasers. Fluor-4 AM, Alexa Fluor 594, DAPI, Alexa Fluor 647, and APC-Cy7 were excited at 488 nm, 561 nm, 405 nm, and 633 nm, respectively, and detected using BGP30/30, BP610/20, BP450/40, BP660/20, and BP780/60 filters. The sorter was implemented with a homemade injection system (Fig. 1A). The sample line was improved, upstream of the solenoid valve, with an injection system composed of two syringes controlled by valves and a peristaltic micropump. D-PBS was placed in the first syringe. A 1.6× concentrated agonist solution (100 mM KCl, 200 μM AMPA, or 10 μM ionomycin) was prepared, and APC-Cy7-labeled CompBead compensation particles (BD Biosciences) were added to the solution before putting it in the
second injection syringe. Both the MINIPULS 3 peristaltic pump (Gilson) and the cytometer flow rate were set to 39 μL/min. Baseline acquisition and sorting were performed with the valve of the buffer syringe opened. Once the valve of the agonist syringe was opened, the valve of the buffer syringe was closed. The agonist solution running in the flow cell was monitored by the appearance of the beads. At this point, the agonist-responding cells started to be sorted. Cells were collected in D-PBS. Data were analyzed using BD FACSDiva v6 and FlowJo softwares (BD Biosciences).

**Gating strategy**

Cells and beads were identified by their size/structure profiles. The first region, cells, was drawn around the GDI population. Next, living cells were identified as DAPI-negative. Doublets were excluded based on morphological parameters: side scattered (SSC) and forward scattered (FSC). Among the living cells, in singlets, a graph of the fluorescence of WGA coupled either to Alexa Fluor 594 (605/40 channel) or to Alexa Fluor 647 (APC channel) allowed us to identify the cells from each mouse phenotype. For each mouse, the basal level of Fluo-4 AM was represented as a function of cell size. A region above this baseline was defined and used for identifying the cells that responded to the agonist in order to sort them. The appearance of the beads gave the signal for the start of the stimulation and, thus, the sorting (Supplemental Fig. S4).

**RNA preparation and RT-qPCR**

Total RNA was extracted from aiFACS-sorted cells using TRIzol reagent (Sigma-Aldrich) according to the manufacturer’s instructions. In each experimental sample, 1 μg of RNA (Supplemental Fig. S2) or 5000 cells (Figs. 2B, 4B, 5A,B; Supplemental Fig. S11) were used for each condition. RNA was purified using 500 μL of TRIzol reagent (Sigma-Aldrich) and precipitated from the aqueous phase with 500 μL of isopropanol (VWR Medicals) and 1 μL of glycogen (20 μg/μL, Invitrogen). RNA was resuspended in 20 μL (Supplemental Fig. S2) or 11 μL (Figs. 2B, 4B, 5A,B; Supplemental Fig. S11) of nuclease-free H2O. Either 1 μg (Supplemental Fig. S2) or 11 μL (Figs. 2B, 4B, 5A,B; Supplemental Fig. S11) of RNA was added to the RT reaction that was performed using the SuperScript IV synthesis kit (Invitrogen). Initial amplification was performed with a denaturation step for 5 min at 65°C, followed by oligo(dT) annealing for 10 min at 25°C, primer annealing for 10 min at 53°C, and primer extension for 10 min at 80°C. Upon completion of the cycling steps, the reactions were stored at −20°C. Quantitative PCR (RT-qPCR) was performed on a light cycler 480 (Roche) with MasterMix SYBR Green (Roche) following the manufacturer’s instructions and according to the MIQE guidelines (Bustin et al. 2009). Primer sequences are listed in Supplemental Table S1.

**Immunofluorescence**

aiFACS-sorted neurons were plated on ornithine-coated glass coverslips (35-mm diameter) and cultivated in complete medium: neurobasal (Invitrogen) supplemented with B-27 (Invitrogen) and GlutaMAX (Invitrogen) as previously described (Abekhoukh et al. 2017; Maurin et al. 2019). Neurons were fixed, and immunofluorescence was performed with microtubule-associated protein 2 (MAP2) antichicken polyclonal antibody (BioLegend 822501) detected with a secondary goat antichicken Alexa 594 (Invitrogen A32759) 6 d after the selection, as previously described (Drozd et al. 2019). Fluorescent images were taken using a wide-field upright fluorescence microscope (Axioplan2, Carl Zeiss), with an ORCA ER CCD camera (Hamamatsu), through a rhodamine filter set (BP565/30; LP585; BP620/60) and a PlanApoChromat 63×/1.4 DIC oil immersion objective (pixel size: 100 nm).

Microglia were labeled as previously described (Cazareth et al. 2014) using the following antibodies: BV510 anti-mouse CD45-clone 30-F11 (BD Biosciences 563891) and AlexaFluor700 anti-mouse CD11b-clone M1/70 (Sony Biotecnology 1106110).

**Droplet-based scRNA-seq**

Single-cell suspensions were converted to barcoded scRNA-seq libraries using the Chromium Single Cell 3’ Library, Gel Bead & Multiplex Kit, and Chip Kit (10x Genomics), aiming for an estimated 2000 cells per library, following the manufacturer’s instructions. Samples were processed using kits pertaining to the V2 barcoding chemistry of 10x Genomics. Libraries were sequenced using Illumina NextSeq 500 and mapped to the mouse genome (build mm10) using Cell Ranger (10x Genomics). Gene positions were annotated as per Ensembl build 84.

**Single-cell gene expression quantification and determination of major cell types**

Raw gene expression matrices, generated per sample using Cell Ranger (version 2.0.0), were loaded and processed in R (version 3.4.3) (R Core Team 2018). Samples were analyzed independently within the Seurat workflow using the Seurat R package (version 3.0.0) (Stuart et al. 2019). First, cells that had >95% dropouts were removed. Gene expression matrices from the remaining cells were normalized using SCTransform (Hafemeister and Satija 2019) from the Seurat package. To reduce the dimensionality of each data set, the resulting variably expressed genes were summarized by principal component analysis, and the first 30 principal components were further summarized using UMAP dimensionality reduction. The three samples from independent analyses were then integrated using CCA. The analysis workflow was then run on an integrated data set. Cell clusters in the resulting UMAP two-dimensional representation were annotated to known biological cell types using canonical marker genes described in the literature (Zeisel et al. 2015, 2018; La Manno et al. 2016; Tasic et al. 2016; Paul et al. 2017; Mi et al. 2018; Rosenberg et al. 2018).

**Statistics**

Statistical tests used in each experiment are indicated in the figure legends. Data are expressed as mean ± SEM, and P-values (or adjusted P-values) < 0.05 were considered statistically significant. Statistical analysis was performed using Prism Software version 7 (GraphPad Software).

**Data access**

The single-cell transcriptomic data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE142274. Data processing scripts (R code) and data used in this study are publicly available through the GitHub repository (https://github.com/ucagenomix/sc.castagnola.2020) and as Supplemental Code.

**Competing interest statement**

The authors declare no competing interests.
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