Ligand signature in the membrane dynamics of single TrkA receptor molecules

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Summary

The neurotrophin receptor TrkA is critically involved in several physio-pathological processes. Still, a clear description of the early steps of ligand-induced TrkA responses at the cell plasma membrane is missing. Here we exploit single particle tracking (SPT) and TIRF microscopy to study TrkA membrane lateral mobility and changes of oligomerization state upon binding of diverse TrkA agonists (NGF, NGF R100E HSANV mutant, proNGF and NT-3). We show that, in the absence of ligands, most of TrkA receptors are fast moving monomers characterized by an average diffusion coefficient of 0.47 $\mu$m$^2$/s; about 20% TrkA molecules are moving at least an order of magnitude slower and around 4% are almost immobile within regions of about 0.6 $\mu$m diameter. Ligand binding results in increased slow and/or immobile populations over the fast one, slowing down of non-immobile trajectories and reduction of confinement areas, observations which are consistent with the formation of receptor dimeric and oligomeric states. We demonstrate that the extent of TrkA lateral mobility modification is strictly ligand-dependent and that each ligand promotes distinct trajectory patterns of TrkA receptors at the cell membrane (ligand “fingerprinting” effect). This ligand-signature of receptor dynamics results from a differential combination of receptor-binding affinity, intracellular effectors recruited in the signalling platforms and formation of signalling/recycling endosome precursors. Thus, our data uncover a close correlation between the initial receptor membrane dynamics triggered upon binding and the specific biological outcomes induced by different ligands for the same receptor.

Introduction

A major open question in cell biology is how different growth-factor receptors achieve molecular specificity even if they activate apparently identical signalling events. Symmetrically, different ligands can activate the same receptor and result in distinct biological outcomes, although bearing similar receptor-binding affinities. Indeed, the mechanisms by which receptors transduce ligand-specific signals are not satisfactorily understood. Here we address this question by studying, at the single molecule level, the dynamics of the neurotrophin receptor TrkA (tropomyosin receptor kinase A) in the cell membrane of living cells.
The TrkA receptor plays a crucial role in the development and survival of sympathetic and sensory neurons, neuronal plasticity, and differentiation of central nervous system cholinergic neurons (Klein et al., 1991; Levi-Montalcini, 1987; Reichardt, 2006). Although Nerve Growth Factor (NGF) is the main ligand for TrkA, the latter can also be activated by other ligands such as proNGF, the unprocessed form of NGF, and by the neurotrophin NT-3. Each of these ligands exerts widely different cellular outcomes, which can only partly be accounted for by a varying contribution from TrkA coreceptor p75NTR (Fahnestock et al., 2001; Harrington et al., 2011; Kuruvilla et al., 2004; Lee et al., 2001). ProNGF can cause actions that are quite opposite to those driven by NGF (Lee et al., 2001) but it may also determine, albeit less effectively than mature NGF, TrkA-dependent neuronal survival (Fahnestock et al., 2001). The recent observation that NGF and proNGF promote different short-term gene expression profiles suggests that they activate different signalling pathways (D’Onofrio et al., 2011); to date, however, the description of the distinct molecular interactions between TrkA and proNGF/mature NGF leading to the observed different biological outcomes is still under debate.

On the other hand, NT-3 promotes, similarly to NGF, TrkA phosphorylation, and ERK, PLC-γ and Akt signalling activation (Harrington et al., 2011; Kuruvilla et al., 2004); differently from NGF, however, NT-3 is unable to elicit retrograde-transport-dependent neuronal survival. Moreover, a mutant form of NGF (NGF R100E or “painless NGF”) related to the human genetic disease HSAN V (Hereditary Sensory Autonomic Neuropathy type V) (Einarsdottir et al., 2004) was shown to bind TrkA with affinity close to that of wild-type NGF, but to activate a specific change in the downstream pathways: that is, a significant, selective reduction of PLC-γ dependent signalling activation, whereas ERK and Akt pathways are relatively unchanged (Capsoni et al., 2011). This was functionally related to the relative inability of this mutant form of NGF to activate nociceptive pathways.

The dynamics of TrkA in response to NGF was recently studied in living cells by different approaches. GFP-fused TrkA receptors were imaged in the presence or absence of NGF (Jullien et al., 2003). Membrane movements of bona fide NGF-TrkA complexes were investigated at the single-molecule level by NGF conjugated to Cy3 fluorophore (Shibata et al., 2006; Tani et al., 2005) or to quantum dots (Qdots) (Cui et al., 2007; Echarte et al., 2007; Rajan et al., 2008; Sundara Rajan and Vu, 2006). More recently, the simultaneous imaging of a GFP-TrkA fusion and Cy3.5-NGF allowed to discriminate the retrograde transport of vesicles
containing the receptor alone or the ligand-receptor complex (Nomura et al., 2011). However, none of these experimental approaches has yet allowed investigating how the lateral mobility of TrkA receptors at the cell membrane and the very early steps of receptor internalization are influenced by the binding of different ligands. Indeed, fusion of GFP to TrkA receptors labels both intracellular and membrane pools, and does not allow to track single receptors for a sufficiently long time, due to the poor photophysical properties of GFP at the single-molecule level. On the other hand, the alternative use of labelled NGF allows the study of TrkA receptor only in its activated form, so that the net effect of ligand binding on the receptor membrane mobility cannot be investigated. For these reasons, there are still relevant questions that must be explored: are TrkA membrane movements related to ligand-specific receptor activation processes and to its subsequent signalling cascade? How do different ligands affect TrkA dynamics, in comparison to NGF? Are NGF and its precursor proNGF different signalling molecules as far as TrkA binding is concerned?

In this article, by directly imaging single TrkA molecules on the membrane of living cells, we investigate whether and how receptor-membrane dynamics is linked to the specific mode of binding and activation exerted by NGF, “painless NGF”, proNGF and NT-3 ligands. To do so, we exploited a novel strategy to label TrkA (Callegari et al., 2012), which allows imaging and tracking of single receptor molecules both in absence and in presence of ligands. We provide a quantitative comparison of the diffusive properties and of the oligomeric state of free and NGF-bound TrkA receptors. Moreover, we describe the different receptor-motion patterns induced by the different ligands. TrkA dynamics changes were investigated, also in response to ligands, after inducing a set of biochemical modifications of the cell environment, affecting receptor kinase activity, clathrin-coated pits (CCPs) formation, or actin-meshwork integrity. By using a novel SPT analysis approach, we demonstrate that the pattern of receptor dynamics in the membrane carries a signature of the ligand by which it was activated.

Results

Individual ACP-TrkA trajectories display different dynamical regimes at the membrane of SH-SY5Y cells
We recently demonstrated that the Acyl Carrier Protein (ACP) fused at the N-terminus of TrkA preserves the full functional responsiveness and signalling activation of the receptor, and allows its imaging at the single-particle level in the plasma membrane of living cells (Callegari et al., 2012). Here, the ACP-TrkA construct was transiently transfected in undifferentiated SH-SY5Y neuroblastoma cells. The receptor pool at the cell surface was selectively labelled by biotinylation of the ACP tag and subsequent addition of streptavidin-coated Qdots (S-Qdots) emitting at 655 nm. Bulk labelling was obtained using a concentration of 20 nM S-Qdots in the final labelling step. Fig. 1A shows our typical microimages: cells expressing the construct display S-Qdot red staining on the plasma membrane, while the cell body is green due to the simultaneous expression of GFP (the latter was inserted in the same expression plasmid). In order to achieve detection and tracking of single ACP-TrkA molecules in the cell membrane, we lowered the S-Qdots concentration down to 2 nM and selectively imaged the basal membrane of the cells facing the coverslip by total internal reflection fluorescence (TIRF) microscopy (Fig. 1B). We analyzed cells displaying 5-50 dots on the basal membrane (0.01-0.1 dots/µm²), as occurred with the same labelling reaction in the case of a SH-SY5Y stable cell line expressing low levels of the ACP-TrkA construct. The density of labelled receptors is an important issue when performing SPT studies (Jaqaman et al., 2011; Kasai et al., 2011) and in our case the selection of cells with low-expression profile of the construct is necessary also in order to avoid autoactivation of the receptor kinase activity in the absence of NGF (Fig. S1). The use of S-Qdots as a probe allowed long-term tracking of individual ACP-TrkA receptors and revealed a rather heterogeneous behaviour. A pool of receptors is almost immobile during the whole observation period (Fig. 1C); long trajectories also showed a multimodal dynamics, switching from periods of faster diffusion to phases of transient arrest of lateral diffusivity (Fig. 1D). TAD (Transient Arrest of Diffusion) analysis was performed to separate trajectory segments corresponding to confinement regions from those corresponding to diffusive or drifting traits. The resulting subtrajectories, if not assigned to TAD events, were pooled together with other receptor trajectories (usually shorter than the multimodal ones) that displayed a unique mode of diffusion: either slow or fast diffusing, sometimes within a confinement area and, more rarely, drifting (Fig. 1E, top, middle, bottom panels respectively). The quantitative parameters describing all classes of trajectories and subtrajectories and their respective frequency are summarized in Table 1 and further explained in the Methods section and in Fig. S7; Table S1
summarizes the results arising from multimodal trajectories only. The whole set of monomodal trajectories and subtrajectories analysed are hereafter referred to as (sub)trajectories, and their numbers are reported in the column “(sub)traj” of Table 1.

**NGF binding decreases TrkA diffusivity and confinement length**

The lateral mobility of ACP-TrkA was first investigated in absence and presence of saturating concentrations of NGF (125 ng/ml). The ligand was added to the cell medium and receptor mobility was monitored immediately afterwards, within a time window of 15 minutes. We found that NGF binding to TrkA results in macroscopic clustering of receptor molecules (Fig. 2A) and in decrease of its diffusivity (Figs 2B-C and Table 1). When analyzing the immobile receptor population (Fig. 1C), we observed that NGF addition causes an eightfold increase in the total time-length fraction of these trajectories from 3.8% to 29.7% (Table 1); furthermore, the average confinement length \( \langle L \rangle \) of immobile receptors is halved upon ligand binding, from 0.59±0.08 µm to 0.29±0.02 µm (uncertainty: s.e.; Fig. 2B). As for the TAD events and the slow and fast diffusing TrkA trajectories (Figs 1D-E), no significant variation of their confinement length is observed, while their total time length frequency is redistributed: both TAD events and slow-diffusing trajectories increase by a factor 1.7, while fast-diffusing receptor molecules are halved upon NGF addition (Table 1). Similarly, both the total time fraction and length of the drifted trajectories are shortened by NGF treatment (Table 1). We recorded a bimodal behaviour in the distribution of the diffusion coefficient \( D \) of non-immobile TrkA receptors (Fig. 2C), both in the absence and in the presence of NGF. Notably, NGF addition causes a 1.7-fold decrease of the integral of the peak corresponding to faster diffusion and a 2.4-fold increase of the integral of the lower-diffusivity peak together with a marked increase in the separation of the two peaks.

**NGF binding induces receptor di/oligo-merization**

In order to assess whether and how the receptor oligomeric status is related to the above-described changes of lateral diffusion, we changed labelling strategy of the receptor and covalently conjugated a CoA derivative of Atto633 fluorophore (CoA-Atto633) to the ACP-TrkA expressed at the membrane of SH-SY5Y cells. Indeed, organic fluorophores display more monodisperse photophysical features than Qdots, and thus are more suitable to directly link the fluorescence intensity of the tracked objects to the oligomeric forms of the receptor (Resch-
Genger et al., 2008). The average intensity for single fluorophores measured with our TIRF microscope set-up was around 3000 a.u. in all our experiments. As an example, left panel in Fig. 2D shows the intensity signal recorded in the case of a spot comprising two fluorophore molecules whose individual bleaching can be monitored.

In order to analyze correlations between oligomeric status and diffusion coefficient of Atto633-labelled TrkA receptors, we plot the 2D distribution of $D$ and spot intensity $\langle I \rangle$ averaged on each trajectory (Fig. 2D). In the case of unstimulated receptors, the peak of this 2D distribution lays at an $\langle I \rangle$ value corresponding to single molecules and at a $D$ value consistent with the fastest mobile population of TrkA (Fig. 2D, central panel). From these data we conclude that about 70% of TrkA receptors (as calculated from the integral of the distribution in the central panel of Fig. 2D within the black rectangle) is present as fast diffusing monomers at the cell membrane in the absence of any ligand. The remaining detected particles, displaying lower mobility, appear to have variable intensities spanning values between the ones expected for monomers and dimers or, albeit very infrequently, larger oligomers. When imaging in the presence of NGF was performed, the intensity of tracked Atto633-ACP-TrkA molecules displaying both fast and slow diffusion was significantly modified (Fig. 2D, right plot). On one hand, the fast population of tracked receptors displayed a more heterogeneous average intensity, with values intermediate between the intensity of monomeric and dimeric receptor forms (highlighted by the two white crosses on the graph). This is likely a result of the monomer-dimer equilibrium undergone by the TrkA receptors upon ligand stimulation (or possibly by the photobleaching of a single fluorophore in a dimer). Indeed, the shift towards the dimer fluorescence intensity corresponds to a slight decrease of $D$, possibly linked to the increase of the molecular mass of the tracked objects and/or to interactions with sub-membrane effectors. On the other hand, the receptor population with lower diffusivity was not only greatly increased (in agreement with data reported in Fig. 2B-C), but also displayed a wide range of average fluorescence intensities, spanning from monomers to oligomers containing up to about 8 individual receptor molecules.

**Effect of other TrkA ligands on TrkA lateral diffusion**

We next investigated how TrkA receptor dynamics is influenced by three other ligands (whose biological effect via TrkA binding is summarized in Fig. S2): a mutant form of NGF...
bearing the R100E point mutation (NGF R100E), the unprocessed pro-neurotrophin proNGF, and the neurotrophin NT-3. The distributions of confinement length and of $D$ values obtained from the analysis of tracked receptors in the three cases are reported in Fig. 3, panels A-C and D-F, respectively (data obtained for TrkA in the absence and in the presence of NGF are also reported in each panel as a reference). We found that all ligands induce increased immobilization (Figs 3A-C and Table 1) and a redistribution between the two peaks of TrkA diffusion in favour of the lower diffusivity one (Figs 3D-F and Table 1) when compared to the non-stimulated receptor. However, while NGF R100E induced an increase of the total time fraction of both TAD events and slow trajectories in a similar way as NGF, proNGF and NT-3 elicited the increase only of TAD occurrence (Table 1, columns Time% of TAD events and slow trajectories). Clearly, the extent of receptor mobility modification is very different for the various ligands, with NGF R100E displaying the highest similarity to the NGF-driven behaviour, proNGF and NT-3 being significantly different from NGF.

In order to better evaluate the differential effects exerted by the four analyzed ligands when compared with each other and with the non-stimulated receptor, we generated the 2D distribution of two parameters summarizing the diffusive behaviour of all (sub)trajectories obtained for each case (Fig. 4). These parameters are the $D$ coefficient, and the $\gamma$ coefficient calculated by the MSS algorithm (Ewers et al., 2005): the smaller the $\gamma$ value, the more sub-diffusive is the motion of the receptor (as it is for confined ones), with a theoretical $\gamma \sim 0.5$ for free-diffusing particles and $\gamma > 0.5$ for drifted ones. All graphs were divided into eight dynamics regions (delimited by the gray rectangles numbered in panel A) and the relative weight of trajectories within each region was quantified and reported as a stack-column graph in Fig. 4G (and as percentages in Table S2). It is evident that NGF and NGF R100E induce the most prominent shift towards the slow-diffusing receptor pool (regions 2-3) and the almost-immobile receptors fraction (regions 4-6); however, this analysis reveals a subtle difference, in that the slower trajectories are differently redistributed in the $D-\gamma$ distributions for these two ligands. ProNGF has a slowing effect on TrkA mobility similar to that of NGF, but weaker; finally NT-3-induced $D-\gamma$ distribution resembles that of the receptor in the absence of any ligand, except for a higher population in region 5 and a lower one in region 2. This analysis highlights the existence
of distinct, ligand-dependent modes of diffusion and confinement for ACP-TrkA upon ligand stimulation.

TrkA lateral mobility is modified by drug treatments affecting TrkA kinase activity, the early events of endocytosis and the actin meshwork

We next investigated how the different motion and diffusivity patterns described in the previous section depend on the TrkA receptor state and/or on the structural components of the plasma membrane. We first took into account the intrinsic receptor kinase activity. SH-SY5Y cells transfected with ACP-TrkA construct were treated overnight with the kinase inhibitor K252a, which resulted in the complete inhibition of ACP-TrkA phosphorylation (Fig. S3A). Cells were then labelled with S-Qdots and individual receptor molecules imaged either in the absence or in the presence of NGF. K252a treatment affects the mobility of the receptor, mainly by increasing the population in diffusivity region 3 (Figs 5A, G); more importantly, this treatment dramatically impairs the observed NGF-induced receptor slowing down and immobilization (Figs 5B, G, Table 1, Figs S4A-B and Table S2).

We then investigated how inhibition of CCPs formation affects TrkA membrane mobility and NGF-associated alterations in TrkA diffusion patterns. To this end, SH-SY5Y cells transfected with ACP-TrkA construct were treated with chlorpromazine (Chlprz), a selective inhibitor of CCPs formation and of clathrin-dependent early endocytosis, as assessed by the impairment of Alexa488-labelled transferrin internalization in SH-SY5Y cells (Fig. S3B). We found that also this treatment has only a slight effect onto TrkA lateral mobility in resting conditions (Figs 5C, G), mainly decreasing the slow population in regions 2 and 3 in favour of the fast population in region 1. On the contrary, inhibition of CCPs formation has a much stronger influence on the effect raised by NGF binding to the ACP-TrkA receptor (Figs 5D, G, Table 1, Figs S4C-D and Table S2), by inhibiting almost completely the population of regions 2 and 6, and changing the details of the distribution within regions 3 and 4.

Finally, SH-SY5Y cells expressing ACP-TrkA were treated with Cytochalasin D (Cyt-D) in order to disrupt the meshwork of actin filaments that are associated with the cytoplasmic face of the plasma membrane and contribute to its structure (Fig. S3C). We found that treatment with Cyt-D leads to a significant decrease of the fast-diffusing ACP-TrkA population in resting conditions (Figs 5E, G and Fig. S3E). Still, a number of ACP-TrkA receptors remains sensitive
to NGF stimulation (Figs 5F-G and Figs S4E-F), as demonstrated by the higher immobilization of receptors (Fig. S3E) and by the higher proportion of receptors residing in areas of low $\gamma$ and $D$ like regions 3, 4, 6 in presence of NGF.

Taken together, these results indicate that, while K252a and Chlprz treatments impair NGF-induced TrkA dynamic behaviour, Cyt-D administration affects mainly the fast diffusive component, while poorly influencing the NGF-dependent immobile and slow receptor populations. For this reason, the effect of Cyt-D can be appreciated significantly in the absence of any ligand addition.

Discussion

In this work we explored the lateral mobility of TrkA receptors at the basal membrane of SH-SY5Y cells; in particular, we examined their response to a set of diverse biologically relevant TrkA ligands. We exploited TIRF microscopy and SPT together with the ACP-TrkA construct, which allows the selective labelling and imaging of single receptor molecules translocated at the plasma membrane (Callegari et al., 2012). We used two different receptor labelling strategies exploiting the versatility of the ACP tag (George et al., 2004; Johnsson et al., 2005). In one case, we biotinylated ACP-TrkAs using a CoA-biotin substrate and coupled them to S-Qdots; in the other, we covalently conjugated ACP-TrkAs to a CoA derivative of the Atto633 synthetic fluorophore. Tracking the receptor with these two probes allowed us to obtain different and complementary observations on TrkA dynamics. By long-term tracking of the receptor labelled with S-Qdots we observed that TrkA receptors display different dynamic behaviours in resting conditions (Figs 1C-F) and that the population of each dynamics category changes in response to ligand addition and drug treatment (Table 1 and Figs 2-5). On the other hand, by tracking Atto633-ACP-TrkA we could gain insights on the oligomeric status of TrkA, in the absence or presence of NGF (Fig. 2D).

We conclude that in the absence of any stimulus, the diffusion coefficient for TrkA receptors displays mostly a bimodal distribution, characterized by two components differing by one order of magnitude (Fig. 2C, top graph). The fast diffusion peak is the most populated: we found TrkA molecules moving with diffusivity higher than 0.1 $\mu$m$^2$/s for more than 70% of the
time, and this population corresponds mostly to TrkA monomers (Fig. 2D, black rectangle in the central panel). ACP-TrkA receptors follow (sub)trajectories contributing to the slow diffusion peak for ~1/5 of the time (Fig. 2C, top graph); these stem from receptors in both monomeric and di/oligomeric states (Fig. 2D, white rectangle in the central panel). These receptors are either moving slowly at the cell membrane or undergoing TAD events (Table 1, first row). The remaining trajectories are followed by TrkA molecules which are essentially immobile within a small area of ~0.6 µm diameter (Fig. 1C); these contribute to the histogram represented by the black curve in Fig. 2B and are detected for ~4% of the time (see Table 1).

NGF triggers a reduction of the fast diffusing population, greatly increasing the slow/immobile one (about eight-fold), displaying a more restricted confinement (Fig. 2B and C bottom panel, and Table 1). Furthermore, a striking shift of the monomer-dimer equilibrium towards the dimeric form can be observed in the fast diffusing receptor population, while receptor clusters containing several -up to eight- receptor molecules populate the slow and immobile populations (Fig. 2D, right panel). These considerations provide a direct demonstration, at the single particle level, of the NGF-induced TrkA dimerization and subsequent clustering at the cell membrane. Notably, our data, obtained from cells displaying low expression of ACP-TrkA, do not support the recent suggestion that NGF only induces dimer phosphorylation of preformed unstimulated TrkA dimers (Shen and Maruyama, 2011).

The observation that ligand binding leads to immobilization and slowing down of lateral mobility was reported for several membrane receptors other than TrkA (Chung et al., 2010; Kasai et al., 2011; Lill et al., 2005; Winter et al., 2011). To date, however, the specific influence of different ligands binding the same type of receptor was poorly investigated, and, to our knowledge, limited to a comparison between agonist and antagonist ligands (Jacquier et al., 2006). Here, we investigated TrkA lateral diffusion induced by the addition and consequent binding of four agonist ligands: NGF, “painless” mutant NGFR100E, proNGF and NT-3 (Figs 2-4). As schematically depicted in Fig. S2, these ligands are known to display different affinity for TrkA, as well as specific modes of receptor activation and/or recruitment of intracellular signalling effectors. Therefore they can be considered full (NGF), partial (NT-3 and proNGF) or biased (NGF R100E) TrkA agonists. Our experiments clearly show a ligand “fingerprinting” effect on the modifications in TrkA lateral mobility at the single molecule level. Each ligand
prompts peculiar changes in receptor diffusion, as well as in the fraction and type of immobile trajectories (Fig. 3). The observed heterogeneous responses suggest a complex set of TrkA-receptor dynamics modes upon ligand stimulation. These are only partially described by the diffusion-coefficient distributions and by histograms of the confinement length; therefore, we analyzed the 2D distribution of $\gamma$ and $D$ parameters for all TrkA (sub)trajectories (Fig. 4). Moreover, we investigated how TrkA lateral mobility is also influenced by drug treatments inhibiting its kinase activity or the early events of clathrin-dependent endocytosis; similarly, we studied the effects of actin meshwork disruption under the plasma membrane (Figs S3-S4 and Fig. 5). As such, we were able to quantitatively analyze how ACP-TrkA receptor molecules redistribute among all dynamic and immobile categories (plot-regions numbered from 1 to 8 in Figs 4A and 5A) as a result of specific ligand addition (Fig. 4) or drug treatment (Fig. 5). The results allowed us to hypothesize a biological significance for almost all of these dynamics regions and to propose a molecular mechanism explaining the heterogeneous responses of TrkA dynamics, as schematically depicted in Fig. 6 and explained in the following.

Region 1 comprises $D$ coefficients between 0.1 and 1 $\mu$m$^2$/s, and $\gamma$ values dispersed around 0.5, with a significant tail towards lower values down to 0.25. It is populated by fast almost free-diffusing trajectories, although relatively large regions of confinement (up to several microns) were occasionally detected for them (Table 1, $\langle L\rangle$ column for fast trajectories). Region 1 is significantly depleted by almost every ligand treatment (Fig. 4); crucially, the higher the ligand affinity for TrkA, the lower is the frequency of trajectories populating region 1 (compare ligand $K_d$ values in Fig. S2 to Fig. 4G and to Table 1, column Time% for fast trajectories). We suggest that this region constitutes the “monomer reservoir” of ACP-TrkA receptors available for ligand capture (it may also include freely diffusing dimers, with no or low interactions with other membrane or cytoplasmic components - see Fig. 6). Differently from region 1, the correlation of ligand-induced changes of the other dynamics regions (2-8) with ligand affinity is not obvious (Table 1). Thus, other factors besides ligand affinity have to be considered to understand the ligand “fingerprinting” effect.

Region 2 includes trajectories with a low degree of confinement ($\gamma$-0.5 or slightly lower) but displaying a significantly lower diffusion coefficient ($10^{-3}$-0.5x$10^{-4}$ $\mu$m$^2$/s). It becomes highly populated upon NGF addition (Figs 4B, G), but not when cells are previously treated with the
tyrosine kinase inhibitor K252a (Figs 5B, G; its population falls from ~13% to 3.5% as reported in Table S2), nor when the previous treatment with Chlprz causes the disassembly of CCPs (Figs 5C-D, G).

Region 5 includes receptor trajectories characterized by relatively high short-lag-time diffusion coefficients ($D$ between 0.013 and 0.3 $\mu$m$^2$/s) and restricted motion (very low $\gamma$ values). This region is virtually unpopulated when the TrkA receptor is not stimulated (Figs 4A, G). It becomes highly populated upon NGF addition (Figs 4B, G), but not if K252a is added to cell culture prior to NGF (Figs 5B, G) or if the actin cytoskeleton is disrupted (Figs 5C-D, G). Interestingly, this region does not appear to be affected dramatically by Chlprz addition prior to NGF treatment (Figs 5E-G and Table S2).

Thus, while TrkA trajectories populating region 5 are affected by ligand-dependent receptor activation by phosphorylation, region 2 is linked to both receptor activation and CCP formation. We may infer that region 5 includes trajectories involved in the formation of signalling platforms whose motion is hindered most likely by interactions with downstream signalling effectors, possibly linked to actin filaments (Fig. 6). Conversely, region 2 likely corresponds to trajectories of receptors assembling into precursors of signalling endosomes at the plasma membrane (Fig. 6). Interestingly, incubation with NGF R100E causes regions 2 and 5 to be populated at the same extent as in the NGF case (Figs 4B-C, G); this does not occur for proNGF and NT-3 (Figs 4D-E, G). This demonstrates that NGF R100E can induce TrkA activation and formation of signalling endosomes similarly to NGF. Thus, the SPT analysis of TrkA trajectories activated by NGF or by the HSAN V-related NGF R100E mutant confirms that the two proteins share a similar binding mechanism to TrkA; identical conclusions have been drawn from different studies (Capsoni et al., 2011). Instead, proNGF and NT-3 induce very different TrkA dynamics. In vitro proNGF shows a lower binding affinity to TrkA than NGF (Fig. S2 and (Paoletti et al., 2009; Covaceuszach et al., 2010)); consistently, in vivo proNGF induces lower receptor phosphorylation levels than NGF (Fahnestock et al., 2004). We find that proNGF induces a lower number of TrkA receptor trajectories populating regions 5 and 2 (Fig. 4D) that correspond, in our interpretation, to signalling platforms and signalling endosome precursors, respectively. This is consistent with its lower activity via TrkA in vivo. The actual contribution of p75NTR coreceptor in modulating proNGF-induced TrkA dynamics remains to be
elucidated, given the very low levels of p75NTR in SH-SY5Y cells (Fig. S5). It could be that the co-expression of p75NTR enhances proNGF-dependent responses, which could explain the neurotrophic activity displayed by proNGF in vivo (Fahrenstock et al., 2001). As for NT-3, in vitro data show that it binds to TrkA with a binding affinity that is two orders of magnitude lower than NGF (Fig. S2). However, increased doses of NT-3 have been shown to activate TrkA and downstream effectors in vivo (Harrington et al., 2011; Kuruvilla et al., 2004). Our data show that a 10-molar excess of NT-3 over NGF and proNGF is able to activate TrkA, more than proNGF, but less than NGF (region 5 in Figs 4E, G). However, NT-3 does not cause an enrichment of trajectories that we associate to the assembly of signalling endosomes; this can be deduced from the lack of trajectories in region 2 (but also 4 and 6) and from the lower $t_{\text{TAD}}$ of TAD events (see Table 1 and Table S1), comparable to the case where the formation of CCP (and hence of early endosomes) is inhibited by Chlprz. Failure of NT-3 to induce phosphorylated TrkA internalization (see Fig. S2 and (Harrington et al., 2011; Kuruvilla et al., 2004)) may represent the functional significance of this observed NT-3 signature at the level of receptor dynamics.

Region 3 includes trajectories with a higher degree of confinement than region 2 ($0.1<\gamma<0.35$), but with a slightly higher $D$ ($7\times10^{-3}$–$0.9\times10^{-1}$ $\mu$m$^2$/s). The addition of all TrkA ligands causes an enrichment of trajectories populating region 3, with a similar trend to that of region 2 (compare panels B-E with A in Fig. 4). The same region is highly populated when the non-stimulated ACP-TrkA receptor is inactivated with K252a (from ~6% in resting conditions to 15% upon K252a administration; Table S2). Moreover this region, similarly to region 2, is sensitive to Chlprz treatment, as is highly depopulated by this drug either in the presence or absence of NGF (Figs 5E-G). These data suggest that region 3 is populated mainly by receptors primed for constitutive recycling, internalization/degradation or a feedback loop regulating the functional response to ligands (Fig. 6). It was quite surprising to find out that NGF R100E populates region 3 slightly more than NGF (Figs 4B-C, G). NGF and NGF R100E indeed share a similar activation of TrkA receptor; however, these growth factors demonstrate a different extent of PLC$\gamma$ pathway activation (see Fig. S2 and (Capsoni et al., 2011)). Clearly, this functional difference corresponds to the subtle modification of the D-$\gamma$ distribution reported in Fig. 4C. The precise reasons as to why NGF R100E addition increases the number of recycling receptors
compared to NGF remains to be explored. Still, our SPT analysis method provides a very sensitive measure of TrkA receptor activation, and makes it possible to detect minimal differences between various ligands.

Regions 4 and 6 include the slowest-diffusing trajectories down to very low $\gamma$ values corresponding to the immobile ones; these are reported in Table 1 and their confinement length was analyzed in Figs 2B, 3A-C and Figs S3A, C, E. Given the susceptibility of these trajectories to Chlprz treatment (Figs 5C-D, G), they likely correspond to ACP-TrkA multimers inside more mature signalling endosomes ready to be actively internalized (Fig. 6). Indeed, these regions are mostly populated when incubating TrkA with NGF and NGF R100E ligands (Figs 4B-C), the two ligands leading to the highest TrkA internalization, but not with proNGF and NT-3 (Figs 4D-E).

Finally, regions 7 and 8 are populated by drifted trajectories which display $\gamma>$0.5 and can be fast (region 7) or slow (region 8). The fast drifted trajectories are reduced by ligand addition, like the trajectories in region 1 (Table S2), and can therefore be considered as the high-$\gamma$ tail of the fast freely diffusing population. As for the slow drifted population of region 8, it is very poorly populated in almost all considered conditions. These trajectories might stem from ACP-TrkA receptor molecules localized within internalized sub-membrane vesicles (Fig. 6), which are still detectable because of the high fluorescence quantum yield of Qdots and the higher number of Qdots present within such vesicles.

The actin meshwork disruption caused by Cyt-D treatment mostly induces a higher population in region 3, which we interpret as the group of inactive receptors that are constitutively recycled and possibly degraded. Moreover, we observed a small shift towards lower values of the characteristic $D$ value of the faster population. These data are apparently not in agreement with the fence-and-pickets model (Kusumi et al., 2005): according to that model, when inducing a partial actin depolymerization by short drug treatment, there are less barriers constituted by actin filaments so that an increase in receptor diffusion can be observed (Suzuki et al., 2007), eventually determining an enhanced availability of receptor molecules for signalling (Treanor et al., 2010). However, we should note that different receptors may have distinct sensitivities to disruption of the sub-membrane actin meshwork; moreover, the actin disruption performed in our experiments was not partial (see Methods and Fig. S3C). It is in principle
possible that after prolonged drug treatment the TrkA “monomers reservoir”, normally maintained by a functional actin meshwork, undergoes processes like constitutive internalization, while still being available for signalling events upon ligand treatments (Figs 5E-F). Indeed, we do see an increased fraction of either confined or immobile receptors upon NGF administration, in the presence of Cyt-D (Figs 5F, G and Figs S4E-F; Table 1), although the confinement and diffusivity features of signalling platforms (normally populating region 5) and signalling endosomes precursors (normally within regions 2, 4, 6) are here completely shifted by the drug treatment.

From a general perspective, the main conclusion from these experiments is that receptor dynamics in the plasma membrane carries a signature tracing back to its specific activation event. TrkA lateral mobility was shown to be a sensitive predictor of full, partial, or biased agonists that bind to the receptor. The ligand signature results from a different binding affinity of a given ligand for the receptor, from the specific intracellular effectors recruited in the signalling platforms and/or from the formation of endosome precursors. The second conclusion that we can draw from these experiments is that the specific biological outcomes induced by different ligands binding to the same receptor can be reliably traced back to the earliest events of receptor activation, namely the initial receptor membrane movements triggered upon binding. Indeed, different TrkA agonists, all binding TrkA specifically and activating, via TrkA, different cellular processes, trigger distinct trajectory patterns of TrkA molecules at the cell membrane.

How the ligand-signature of TrkA dynamics is influenced by its coreceptor p75NTR is an important question that remains to be addressed. Answering such a question requires the ability to perform SPT of the two receptors simultaneously. Another open issue is the generalization of the dynamic fingerprint concept to other receptors: hypothetically, there may be a causal correlation between dynamic fingerprints and signalling pathways.

Materials and Methods

Cell surface labelling of ACP-TrkA expressed in living SH-SY5Y cells

SH-SY5Y cells were transfected with ACP-TrkA construct (Callegari et al., 2012). After 3-5h from transfection, cells were trypsinized and transferred into Willco-dish® glass-bottom
dishes. Cells were allowed to attach overnight, then serum starved for 2 hours, and incubated for 30 minutes at 37°C in DMEM-F12 with 0.5% BSA, containing 1.0 μM of Sfp-synthase (New England Biolabs), 10 mM MgCl₂ and variable amounts of Coenzyme A derivative depending on the experiment. For the labelling with S-Qdots for single-molecule experiments, 5 μM biotin-CoA was used, then cells were washed twice in PBS before incubation for 2 minutes at room temperature with 2 nM S-Qdots (Qdot® 655 Streptavidin Conjugate, Invitrogen) in Borate buffer pH 8.3, 0.5% BSA and 215 mM sucrose (Bannai et al., 2006). These conditions ensured a minimal non-specific adhesion of S-Qdots to the glass surface. To achieve a “bulk” labelling of ACP-TrkA construct at the plasma membrane (Fig. 1A), 20 nM S-Qdots were incubated for 15 minutes, then cells were fixed, mounted in DAPI-containing VectaShield and imaged using an Olympus FV1000 confocal microscope with a 40X objective and the 488 nm Argon-laser line to image both the GFP and the S-Qdots655 channels and a 405nm laser for DAPI excitation. For the labelling with Atto633 fluorophore, 5 nM CoA-Atto633 conjugate was used as a substrate in the reaction mix to yield complete receptor labelling. Cells were imaged in Imaging Medium (20 mM HEPES, 6.6 mM D-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.5% B-27 supplement in MEM medium) after extensive washing (8-10 times with PBS). Notably, no significant differences in receptor immobile populations and in D distributions were detected when labelling the receptor with either monovalent Atto633-CoA fluorophore or multivalent S-Qdots. This observation supports the idea that potential S-Qdots mediated receptor crosslinking is minimized under our experimental conditions (low Qdot concentration, use of BSA in the labelling reaction and analysis of cells displaying low-expression levels of receptor).

**Synthesis of biotin-maleimide**

Biotin (10mg) dissolved in DMF (250 uL), NHS (1.1 eq), 2-aminoethylmaleimide (1.1 eq), and EDC (1.2 eq) were mixed, and the solution stirred 4h at 25 °C. The product was purified by semipreparative RP-HPLC.

**Synthesis of CoA-biotin and CoA-Atto633**

Coenzyme A (10 nmol), dissolved in DMF (2 uL), was mixed with the appropriate maleimido derivative (20 nmol) in DMF (10 mM). Solution was stirred at 35°C for 4 hours. The product was purified by RP-HPLC (Column: Phenomenex Fusion 150x4.6. Solvents: Ammonium formate 5mM/acetonitrile).
**Ligand treatments**

Transfected SH-SY5Y cells were treated with different ligands: native mouse NGF (Alomone Labs, 125 ng/ml), recombinant human pro-NGF (300 ng/ml), recombinant human NGF R100E (125 ng/ml) and recombinant human NT-3 (Alomone Labs, 1000 ng/ml). Equimolar doses of NGF, NGF R100E and proNGF were used, while NT-3 was administered in a 10-fold molar excess over NGF, as reported (Harrington et al., 2011; Kuruvilla et al., 2004). Recombinant proNGF and NGF R100E mutant were expressed in E. coli and purified as described (Covaceuszach et al., 2010). All ligands were added as 2-times concentrated, pre-warmed (37°C) solutions to an equal volume of imaging medium directly into WillCo glass-bottom chambers used for microscopy. Cells were always imaged for a maximum of 15 minutes upon ligand addition.

**Drug treatments**

For the inhibition of TrkA kinase activity, ACP-TrkA expressing SH-SY5Y cells were treated overnight with 200 nM K252a (Sigma-Aldrich) before the labelling reaction. To block the endocytosis machinery, transfected cells were exposed to 25 μM Chlprz (Sigma-Aldrich) during the ACP labelling reaction. In order to disrupt the actin meshwork at the plasma membrane, transfected cells were treated with 1μM Cyt-D (Sigma-Aldrich) for 2 hours, during the serum starvation preceding the cell surface labelling of ACP-TrkA. Each drug was added also in the imaging medium, keeping Cyt-D concentration unaltered, while K252a was lowered down to 100nM in the labelling reaction and imaging medium and Chlprz was increased to 50 μM in the imaging medium.

**Microscope Image Acquisition**

Cells prepared as described above were immediately imaged at 37°C, 5% CO₂ with a Leica DM6000 microscope equipped with Leica TIRF-AM module, incubator chamber, electron multiplying charge-coupled-device camera (ImagEM C9100-13, Hamamatsu), and 100X oil immersion objective (NA 1.47). TIRF time series were acquired on a ROI with constant size of 144x152 pixels (32.7x34.5 μm) within the basal membrane of each cell, using the 488 nm laser line with a Qdot655/10 emission filter, or the 632 nm laser line with a Cy5 Leica1152303 emission filter, and penetration depth set at 90 nm. The integration time per frame was 21 ms and
typical time series lasted 3000 frames. The lag time between two consecutive frames was minimized to the integration time (21 ms) by using the overlapping mode of the CCD camera with the reduced ROI. Cells with low density of Qdots where chosen to calculate trajectories.

**Data Analysis**

Detection and localization of spots corresponding to S-Qdots conjugated to biotinylated ACP-TrkA or to CoA-Atto633 labeled ACP-TrkA, linking and merging of the trajectories was done using Imaris software (Bitplane Scientific Software). We found the following software parameters to be optimal to overcome splitting caused by Qdot blinking: estimated spot diameter 0.8 \( \mu m \), Brownian motion algorithm, max spot distance 1 \( \mu m \), max gap size 30 frames. Visual inspection of the resulting trajectories and their manual editing was performed in case of linking mistakes by the algorithm. Spots corresponding to fluorophores non-specifically adhered to the glass out of the cell were discarded by considering the merge of the TIRF over the DIC image. Qdots non-specifically adhered to the glass under the basal membrane of investigated cells were found to be negligible or anyway not interfering with TrkA trajectories analysis (Fig. S6).

Further analyses on the trajectories were carried out almost automatically using codes written in MatLab, which performed the analyses described in Fig. S7 and summarized in the following. The trajectories acquired in the case of CoA-Atto633 labeled ACP-TrkA are relatively short and very numerous: they are analysed all together only considering their average short-lag-time diffusion coefficient \( D \pm \text{s.e.} \) as deduced from the initial angular coefficient of the mean square displacement (MSD) (Callegari et al., 2012), and considering the average intensity \( \pm \text{s.d.} \) on whole trajectories (Fig. 2D). In the case of Qdots-labelled receptors, we used first the moment scaling spectrum (MSS) analysis (Ewers et al., 2005) in order to separate self-similar from multimodal trajectories; the latter ones were analysed by an algorithm for the detection of non-random transient confinement zones (Simson, 1995), similar to the stimulation-induced transient arrest of lateral diffusion (STALL) method (Suzuki et al., 2007). Our algorithm is here referred to as TAD (transient arrest of diffusion) as such events were observed also for the TrkA receptor in the absence of any stimulation. For all trajectories and subtrajectories (determined by TAD analysis) we calculated the MSD, the MSS and the average spot intensity \( \langle I \rangle \pm \text{s.d.} \), also checking if the diffusing subtrajectories and the confinement events predicted by TAD were correct. The confinement length parameter \( L \) has been calculated using the MSD fit for “confined”
trajectories only if those were long enough to explore the confinement area substantially (Callegari et al., 2012); the sub/super-diffusive coefficient $\gamma$ with its uncertainty was determined by a fit of the MSS. A schematic view of this analysis and more details about it can be found in Fig. S7 and its caption.

All 1D or 2D distributions or histograms reported here are evaluated weighting each (sub)trajectory by the number of spots composing it and, except where stated differently, their integrals are normalized to 1. Except for $L$, the uncertainties of the parameters were considered by summing 1D or 2D Gaussians for computing the experimental distributions. A colour code in logarithmic scale, with the maximum normalized to 1, allows visualizing the 2D distributions.

Acknowledgments

The authors would like to thank Fulvio Bonsignore for investigating endogenous levels of p75$^{\text{NTR}}$ in PC12 and SH-SY5Y cells; Alessandro Duci for providing the starting material for coding the analysis algorithms; Francesca Paoletti, Doriano Lamba and Simona Capsoni for helpful discussions and critical revisions of the paper. This work was supported by a FIRB grant to FB (RBAP11X42L_003), a PRIN grant to SL (2009XPTWM2), and a FIRB grant to AC (RBAP10L8TY).

Author contributions

LM and AC performed experiments; SL set up and supervised microscopy experiments, and developed and coded the algorithms for data analysis; LM, AC, SL, FB and AC designed and analyzed experiments, and wrote the manuscript; GS synthesized CoA-conjugated substrates for ACP labelling; AV prepared and purified recombinant NGF R100E mutant and proNGF proteins.

References


Visualizing odorant receptor trafficking in living cells down to the single-molecule level. Proc Natl Acad Sci USA 103, 14325-30.


Figure legends

Fig. 1. Selective labelling of membrane-localized ACP-TrkA receptor for SPT experiments. (A) Confocal images of SH-SY5Y cells transiently transfected with ACP-TrkA-IRES-GFP construct. The green channel is GFP fluorescence; the red channel is ACP-TrkA receptor at the cell membrane after its biotinylation and subsequent conjugation with 20 nM S-Qdots655. Scale bar: 10 µm. (B) Typical S-Qdots distribution on the basal membrane of living SH-SY5Y cells maintained at 37°C upon labelling of biotinylated ACP-TrkA with 2nM S-Qdots (red channel, imaged by TIRF; DIC transmission microscopy image in gray and epifluorescence GFP channel in green are shown as references). Scale bar: 20µm in gray channel; 10µm in insets. (C-E) Representative trajectories of tracked receptors, with spot colour coding for the frame number (3000 frames being 63 seconds). Trajectories were classified into different diffusive categories, e.g. immobile (C) or multimodal (D); the latter ones may be composed by subdiffusive or slow (E, top panel), free diffusive or fast (E, middle panel) and superdiffusive or drifted (E, bottom panel) dynamics modes. (F) Colour-code map representing the frame number for spots in the trajectories.

Fig. 2. Effects of NGF on ACP-TrkA mobility at 37°C. (A) TIRF microimages showing the distribution of ACP-TrkA receptor labelled with 2nM S-Qdots655 before (red channel, left panel) and after NGF administration (red channel, right panel) at the SH-SY5Y plasma membrane. Areas outside cells are greyed. NGF-induced receptor clustering is highlighted by arrowheads in right panel; scale bar: 5 µm. (B) Distributions of the receptor confinement length $L$ for “immobile” trajectories of S-Qdots-labelled ACP-TrkA receptors in the presence (red curve, n=121) or absence (black curve, n=13) of NGF, with areas under the curve normalized to the fractions of spots assigned to immobile trajectories. (C) Distribution of short-lag-time diffusion coefficient ($D$) of S-Qdots-labelled ACP-TrkA receptors for non-immobile trajectories in the presence (red curve, n=788) or absence (black curve, n=664) of NGF. The areas under the reported distributions are normalized to the fraction of spots in non-immobile trajectories. The percentages are calculated considering the areas under the curve above and below $D=0.1 \mu m^2/s$. (D) Fluorescence intensity ($I$) analysis of single Atto633 molecules and Atto633-labelled ACP-TrkA observed by TIRF microscopy. Left panel: typical time-course of $I$ for a spot comprising two molecules of the organic dye CoA-Atto633 adhered to the Petri bottom glass. Right panels:
2D distribution of the average fluorescence intensity \( \langle I \rangle \) of Atto633-labelled ACP-TrkA trajectories and of \( D \) in the untreated (central panel, \( n=7367 \)) and NGF-treated (right panel, \( n=10288 \)) cases. NGF treatment induces receptor dimerization within higher-\( D \) trajectories (the white line connects the fluorescence intensity of the monomer to that of the dimer, which appears to have a halved \( D \)) and oligomerization (white rectangular area) corresponding to increased average fluorescence and decreased diffusivity of receptor aggregates.

**Fig. 3.** Effect of ligands on ACP-TrkA mobility. (A-C) Distributions of the confinement length \( L \) for “immobile” trajectories of S-Qdots labelled ACP-TrkA receptors (blue curves) upon treatment with NGF R100E mutant (NGF R100; panel A, \( n=121 \)), pro-NGF (panel B, \( n=59 \)) and neurotrophin-3 (NT-3; panel C, \( n=37 \)). Normalization as in Figure 2B, whose content is reported here as dashed curves for comparison. (D-F) Diffusion coefficient (\( D \)) distribution (blue curves) of non-immobile trajectories of S-Qdots labelled ACP-TrkA receptors upon treatment of cells with NGF R100E (panel D, \( n=1142 \)), pro-NGF (panel E, \( n=1175 \)) and NT-3 (panel F, \( n=1483 \)). Normalization as in Figure 2C, whose content is reported here as dashed curves for comparison.

**Fig. 4.** Effects of ligands on ACP-TrkA dynamics modes. (A-E) Total \( D-\gamma \) distributions according to MSS-TAD analysis of ACP-TrkA trajectories in non-stimulated cells (A, \( n=677 \)), and after addition of NGF (\( n=909 \)), NGF R100E (\( n=1263 \)), proNGF (\( n=1234 \)) and NT-3 (\( n=1520 \)) (B-E, respectively). Dynamics regions (numbered from 1 to 8 in panel A) are superimposed on each plot. The diffusion coefficient \( D \) reflects the short-term diffusivity and the \( \gamma \) factor reflects the long-term trajectory behaviour (\( \gamma \sim, >, < 0.5 \) for Brownian, drifted, and subdiffusive/confined dynamics, respectively). (F) Logarithmic-scale colour code for the frequency of the total \( D-\gamma \) distributions, normalized to 1 at the peak. (G) Frequency % of different dynamics modes for ACP-TrkA receptors, as calculated from the integral of the experimental \( D-\gamma \) distribution in each numbered region of panels A-E. These values are reported extensively in Table S2.

**Fig. 5.** Effects of drug-induced inhibition of tyrosine-kinase activity, clathrin-coated pits (CCPs) formation and actin meshwork on ACP-TrkA lateral dynamics. (A-F) 2D distribution of \( \gamma \) and \( D \) for S-Qdot labelled ACP-TrkA trajectories, as obtained by MSS-TAD analysis (as in Fig. 4A-E; same colour scale of Fig. 4F). The cells were either unstimulated (left panels) or stimulated (right panels) by NGF, and were treated with K252a (A, \( n=854 \); B, \( n=2077 \)), Chlprz (C, \( n=1152 \); D, ...
n=1546), or Cyt-D (E, n=1274; F, n=686). (G) Stack column graph representing the frequency % of different dynamics modes for ACP-TrkA receptors under different treatments, calculated as for Fig. 4G (data reported in Table S2). The first two stacked-bars, reported here for comparison, are the same of Fig. 4G.

**Fig. 6.** Model illustrating the fingerprinting effect. This schematic view of possible states of TrkA in its different dynamics modes (numbered as the regions in Figs 4A and 5A) summarizes our conclusions, better discussed in the main text. Insets show a typical trajectory for each dynamics mode, zoomed when necessary to appreciate its details. Population in regions 1 and 7 decreases upon each treatment; changes induced by ligands and/or drug treatments with respect to the unstimulated case are also indicated schematically. Big ellipses schematize TrkA extracellular domains (red if bound, gray if unbound), small ellipses the intracellular domains (red if phosphorylated, gray if inactive). Membrane bilayer is in blue, brown rectangles depicts clathrin, brown lines represent actin filaments, possible intracellular effectors are schematized in yellow and green.
A. Immobile trajectories

B. Non-immobile trajectories

C. Normalized Frequency

D. Normalized Frequency

E. Normalized Frequency

F. Normalized Frequency
### Table 1

Quantitative parameters describing ACP-TrkA single molecule dynamics in response to different stimuli. Cells: number of cells analyzed, #trajs: number of trajectories detected, #multi: number of multimodal trajectories detected, (sub)trajs: total number of trajectories and subtrajectories after TAD analysis; Trajs: total number of (sub)trajectories of each type; Time%: total time fraction for the (sub)trajectory type; \( \bar{t} \): average trajectory time length; \( \tau \): parameter in the exponential fit for the distribution of the time lengths of the trajectories (reported for TAD events in order to reduce the impact of long slow or immobile subtrajectories that were actually the most part of a trajectory; in the other cases it is not significantly different from \( \bar{t} \)); \( \bar{L} \) and \( \Delta L \): average and s.d. of the distributions for the confinement length L considering only the (sub)trajectories where \( L \) were in agreement with the calculated s.d. \( \sigma \) for the x-y positions in the corresponding trajectory (1.25 < \( L/\sigma \) < 4.75).

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