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SorLA Controls Neurotrophic Activity by Sorting of GDNF and Its Receptors GFRα1 and RET

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SUMMARY

Glial cell-line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor that has reached clinical trials for Parkinson's disease. GDNF binds to its coreceptor GFRα1 and signals through the transmembrane receptor tyrosine kinase RET, or RET independently through NCAM or syndecan-3. Whereas the GDNF signaling cascades are well described, cellular turnover and trafficking of GDNF and its receptors remain poorly characterized. Here, we find that SorLA acts as sorting receptor for the GDNF/GFRα1 complex, directing it from the cell surface to endosomes. Through this mechanism, GDNF is targeted to lysosomes and degraded while GFRα1 recycles, creating an efficient GDNF clearance pathway. The SorLA/GFRα1 complex further targets RET for endocytosis but not for degradation, affecting GDNF-induced neurotrophic activities. SorLA-deficient mice display elevated GDNF levels, altered dopaminergic function, marked hyperactivity, and reduced anxiety, all of which are phenotypes related to abnormal GDNF activity. Taken together, these findings establish SorLA as a critical regulator of GDNF activity in the CNS.

INTRODUCTION

Neurotrophic factors are key molecules in sculpting the developing nervous system as well as regulating adult neuronal maintenance and plasticity. Glial cell line-derived neurotrophic factor (GDNF) is essential for the development of several neuronal populations (Airaksinen and Saarma, 2002), and a critical survival factor for a number of neuronal subtypes, most notably midbrain dopaminergic (DA) neurons (Lin et al., 1993). More specifically, GDNF protects and repairs nigral DA neurons in neurotoxin-lesioned animal models of Parkinson's disease (Kirik et al., 2004), and phase I clinical trials with parkinsonian patients, GDNF gave highly promising results that were unfortunately hampered by conflicting outcome from phase II studies (Gill et al., 2003; Lang et al., 2006).

GDNF is a homodimeric neurotrophic factor belonging to the GDNF family ligands (GFLs) together with neurturin, persephin, and artemin (Airaksinen and Saarma, 2002). GDNF signaling is conventionally mediated via two receptors. First, the GDNF dimer binds GDNF family receptor α1 (GFRα1), which is linked to the plasma membrane through a glycosylphosphatidylinositol anchor. The resulting tetrameric (2:2) complex interacts with Rearranged During Transfection (RET) receptor tyrosine kinase and activates Erk, Akt, Src, and PLCγ pathways (Airaksinen and Saarma, 2002). Although RET is the established GDNF signaling receptor, many cells responding to GDNF and expressing GFRα1 do not express RET (Trupp et al., 1997; Yu et al., 1998). To date, two alternative receptors have been discovered: neural cell adhesion molecule N-CAM and syndecan-3 (Bespalov et al., 2011; Paratcha et al., 2003). In both cases, the ligand-receptor interaction leads to the activation of Src family kinases, modulating cell migration, neurite outgrowth, and synapse formation (Bespalov et al., 2011; Ibáñez, 2010).

Despite the progress in characterizing GDNF receptors and their signaling pathways, still very little is known about their trafficking and how GDNF activity is regulated. Due to alternative splicing, GDNF is synthesized in two precursor forms (α)- and (β)proGDNF that are sorted differentially through the secretory pathway prior to propeptide cleavage by a proprotein convertase (Lonka-Nevalaita et al., 2010), a process proposed to involve the sorting receptor SorLA (Geng et al., 2011; Westergaard et al., 2004). SorLA is one of five members of the sortilin-related receptor family (sortilins), unified by the vacuolar protein sorting protein 10p (Vps10p) domain (Jacobsen et al., 1996; Willnow et al., 2008). The cytoplasmic tail of SorLA contains several consensus binding sites for adaptor proteins that mediate internalization from the cell surface, Golgi-endosome transport, and retrograde sorting to the TGN (Nielsen et al., 2007). In the present study, we find that SorLA conveys

internalization of GFRα1 alone and in complex with GDNF and mediates their sorting to endosomal compartments. GFRα1 bypasses lysosomal degradation, whereas GDNF does not. The SorLA/GFRα1 complex further mediates subcellular sorting of RET, influencing GDNF-induced neurotrophic activity including the survival of primary DA neurons. SorLA-deficient mice have increased GDNF levels in the striatum and midbrain and exhibit a behavioral phenotype characterized by reduced anxiety, hyperactivity, and insensitivity to amphetamine, symptoms reminiscent of attention deficit hyperactivity disorder (ADHD) in humans.

RESULTS

SorLA Selectively Binds GDNF

GDNF bound to the extracellular domain of SorLA in a concentration-dependent manner as assessed using Biacore (Figure 1A). The interaction was of high affinity with a K_d of 3 nM, and selective as no other GFL showed binding to SorLA (Figure 1B). GDNF is unique among the GFLs by containing an extended N terminus of 38 amino acids (Baloh et al., 2000). As this region might contribute to SorLA binding, we generated a glutathione S-transferase (GST) fusion peptide encompassing the GDNF propeptide followed by the N-terminal extension (GDNF aa 20–115). This peptide bound SorLA with a K_d of 76 nM, whereas neither GST-GDNF propeptide (GDNF aa 20–77) nor GST-neurturin propeptide displayed any binding, clearly indicating that the GDNF N-terminal region is involved in binding of SorLA (Figure 1C and data not shown). The ~25-fold difference in the affinity of GDNF to SorLA compared to the N-terminal peptide could be due to additional degrees of freedom of the peptide fragment when not covalently associated with the folded structure of GDNF. However, at present, the involvement of other GDNF regions in SorLA binding cannot be excluded either. SorLA is a large mosaic receptor containing several potential ligand binding domains, one of which is the Vps10p domain (Figure 1D). Prior to its removal in late Golgi compartments, the SorLA propeptide inhibits access to this domain (Jacobsen et al., 2001). We found that excess SorLA propeptide prevented the binding of GDNF to SorLA (Figure S1A). In analogy, 200-fold molar excess of the small neuropeptide neurotensin, predicted to bind inside the tunnel-like cavity of the SorLA Vps10p domain (Quistgaard et al., 2009), also abolished GDNF binding to SorLA in Biacore experiments (Figure S1B). The SorLA-GDNF interaction was further confirmed in HEK293 cells transfected with an endocytosis-deficient SorLA mutant where the binding of ^{125}I-GDNF (100 pM) was displaced by increasing concentrations of unlabelled GDNF (Figure S1C). The combined data demonstrate that the SorLA Vps10p domain specifically and selectively binds to the N-terminal region of GDNF.

SorLA and GFRα1 Direct GDNF to Lysosomes

SorLA rapidly traffics from the cell surface to endosomes (Nielsen et al., 2007). Thus, to study SorLA-mediated GDNF endocytosis and how this was affected by the presence of the GDNF signaling receptors, we used HEK293 cells transfected with combinations of SorLA, GFRα1, and RET. Cells were incubated with GDNF (3 nM) for 2 hr on ice to allow cell surface binding before changing to 37°C medium without GDNF to commence internalization for 30 min. Analysis using confocal microscopy revealed GDNF in vesicular structures of SorLA cells, whereas faint spots of GDNF lined the surface of cells transfected with RET or empty vector (Figure 1E). In contrast, GFRα1 or GFRα1/RET-expressing cells showed intense GDNF staining lining the plasma membrane, but no endocytosis was apparent (Figure 1E). GDNF endocytosis by RET/SorLA cells was comparable to SorLA alone (Figure 1E). Strikingly, coexpression of SorLA and GFRα1 resulted in a dramatic increase in GDNF endocytosis independent of RET (Figures 1E and S1D). In fact, SorLA and GFRα1 together resulted in detectable internalization at a 100-fold lower GDNF concentration compared to SorLA alone (Figures S1E and S1F), demonstrating a cooperative effect. Furthermore, GDNF surface staining was reduced by 75% during 45 min in SorLA/GFRα1 cells compared to only ~30% in GFRα1 cells (Figures S2A and S2B).

Interestingly, SorLA cells metabolized GDNF from the culture medium to a much greater extent than mock-transfected cells (Figure S2C), and ^{125}I-GDNF bound to the surface of cells expressing SorLA, decreased from 3,214 ± 450 to 1,046 ± 72 cpm during 60 min, and was accompanied by an equivalent accumulation of nonprecipitable radioactivity released into the culture medium, possibly derived from degraded GDNF (Figure S2D). Importantly, GDNF did not remain associated with SorLA and GFRα1 but colocalized with the endosomal marker EEA1 (Figure 1F), and the intensity of the GDNF staining decreased over time (from 30 to 60 min) (Figures 1Fand 1G). Lysosome inhibition by leupeptin and pepstatin (leu/pep) clearly augmented GDNF immunoreactivity in structures of SorLA/GFRα1 cells also positive for the lysosomal marker Lamp-1 (Figure 1G). In contrast, no change in GDNF staining upon leu/pep treatment was evident in GFRα1 cells, where abundant GDNF was associated with the plasma membrane (Figure 1H). Taken together, the data show that SorLA alone and in cooperation with GFRα1 of the GFRα1-RET complex binds extracellular GDNF and targets it for lysosomal degradation.

GFRα1 and SorLA Forms GDNF Sorting Complex

To study how GFRα1 delivers GDNF to SorLA, we probed for a receptor-receptor complex by coimmunoprecipitation (co-IP). Indeed, SorLA and GFRα1 co-IPed both in the presence and absence of GDNF (Figure 2A), whereas we were unable to co-IP SorLA and RET (data not shown). The SorLA/GFRα1 interaction was direct as assessed using Biacore (K_d = 6 nM) (Figures 2B and S3A), whereas we observed no binding between the SorLA and RET (Figure 2B). GFRα1 binding was inhibited by excess SorLA propeptide (Figure S3B), but, unlike for GDNF, neurotensin did not affect the SorLA-GFRα1 interaction (Figure S3C). We speculated whether SorLA-GFRα1-GDNF might interact simultaneously and tested this in a Biacore experiment where immobilized SorLA was first saturated with GDNF and subsequently tested for its ability to bind GFRα1. No reduction in the affinity for GFRα1 was observed for the SorLA-GDNF complex compared to SorLA alone (Figure S3D). In fact, picomolar GDNF concentrations increased SorLA/GFRα1 co-IP, indicating the potential formation of a ternary complex (Figure S3E). Finally, by crosslinking followed by sequential co-IP of GDNF and

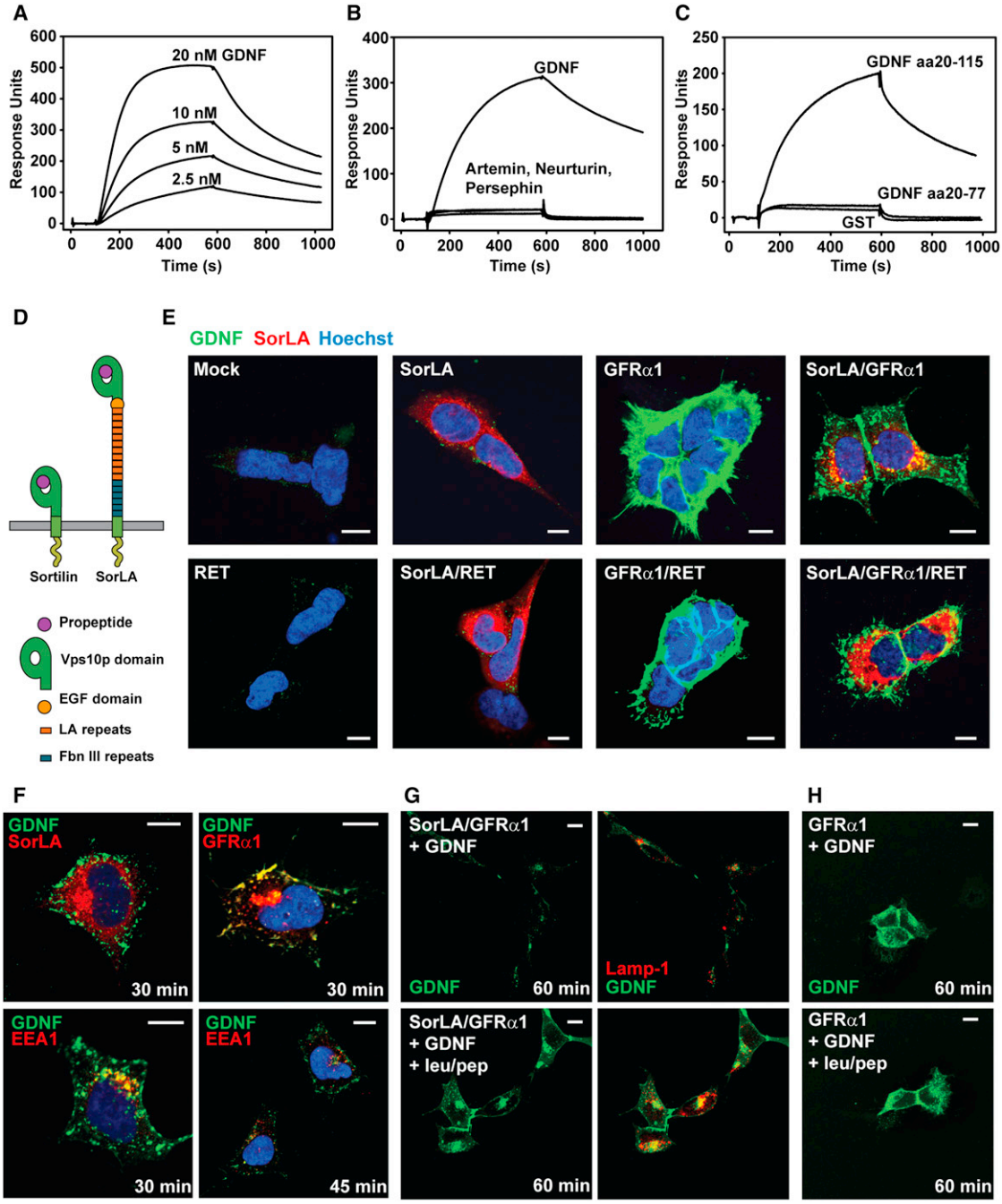

Figure 1. SorLA and GFRα1 Direct GDNF to Lysosomes

(A) GDNF binds SorLA in a concentration-dependent manner as shown using Biacore. K_d = 3 nM.

(B) Binding to SorLA is selective for GDNF and not observed for artemin, neurturin, or persephin (20 nM).

(C) Binding of proGDNF N-terminal fragments containing the proregion and first 38 amino acids (aa 20–115) from the mature GDNF fused to GST (200 nM) to immobilized SorLA ECD. The binding site in GDNF is encompassed within the N-terminal 38 aa of mature GDNF. Numbering starts from the human GDNF signal peptide. The aa 20–77 construct contains only the proregion of GDNF.

(D) The domain structure of SorLA and the related receptor sortilin is depicted. Vps10p, vacuolar protein sorting 10 protein; EGF, epidermal growth factor class B-like domain; LA, LDL class A repeats; Fbn III, fibronectin type III repeats.

(E) Mock-transfected HEK293 cells or HEK293 cells transfected with combinations of SorLA, GFRα1, and RET as indicated were incubated with GDNF (3 nM) at 0°C for 2 hr on ice to allow surface binding but not internalization. Cells were subsequently changed to 37°C culture medium for 30 min. GDNF (green) and SorLA (red) are visualized by IF. All images were obtained using the same laser power and microscope settings. Nuclei are stained using Hoechst. Scale bar, 10 μm. n = 10 independent experiments with over 300 cells evaluated for each condition.

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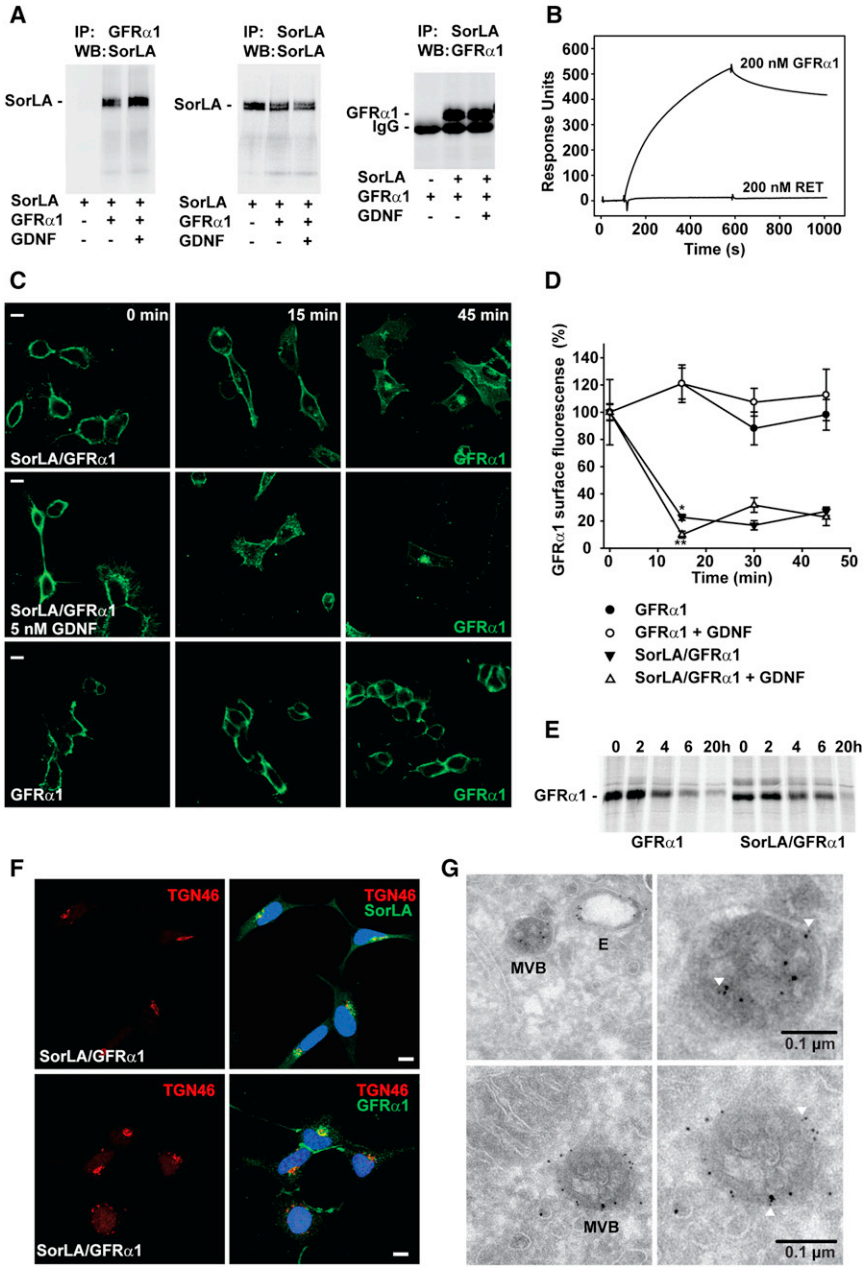

Figure 2. SorLA and GFRα1 Form a GDNF Sorting Complex

(A) SorLA interacts directly with GFRα1 as shown by co-IP in transfected HEK293 cells ± GDNF (5 nM).

(B) Specific interaction of GFRα1 but not RET extracellular domain (200 nM of either) with SorLA as shown using Biacore.

(C) Surface-labeled GFRα1 (green) is endocytosed in HEK293 cells coexpressing SorLA ± GDNF. GFRα1 expressed alone is not endocytosed during the course of the experiment. Scale bar, 10 μm. n = 10 independent experiments with over 300 cells evaluated for each condition.

(D) Quantification of the decrease in GFRα1 surface IF over time in GFRα1- or GFRα1/SorLA-expressing HEK293 cells ± GDNF (*p = 0.005, **p = 3E-5). Error bars indicate SEM.

(E) Turnover of GFRα1 in transfected HEK293 ± SorLA was assessed by metabolic labeling followed by pulse-chase analysis and subsequent IP.

(F) SorLA and GFRα1 colocalize with the *trans*-Golgi marker TGN46 in HEK293/SorLA/GFRα1 cells.

(G) Immunoelectron microscopy showing colocalization of SorLA and GFRα1 in endosomes (E) and multivesicular bodies (MVB) visualized by staining of HEK293/SorLA/GFRα1 cells with first mouse anti-SorLA and goat anti-GFRα1, and subsequently with gold-particle-coupled donkey anti-mouse (10 nm particle diameter) and donkey anti-goat (6 nm particle diameter) secondary antibodies. MVBs are magnified in right images and white arrow heads indicate 6 nm particles close to 10 nm particles, representing colocalized SorLA and GFRα1. See also Figures S3 and S4.

GFRα1 as described in Figure S3F, SorLA was pulled down specifically as part of a ternary SorLA/GFRα1/GDNF complex, confirming its existence.

SorLA Sorts GFRα1 from the Cell Surface to the TGN

To monitor SorLA-mediated sorting of GFRα1, we labeled surface receptor with antibodies. In the presence of SorLA, the majority of GFRα1 accumulated in paranuclear compartments within 45 min, whereas no endocytosis was observed for GFRα1 alone (Figures 2C and 2D). To determine if SorLA destines GFRα1 for lysosomal degradation, we studied its turnover by metabolic labeling of cells expressing GFRα1 alone or coexpressing SorLA. Surprisingly, SorLA appeared to prolong GFRα1 half-life, ruling out lysosomal sorting (Figures 2E, S3G, and S3H). GFRα1 alone localized mainly to the plasma membrane, but coexpression with SorLA shifted a substantial

(F) GDNF (green) (3 nM) internalized by HEK293/SorLA/GFRα1 cells for the indicated time periods displays only modest colocalization with SorLA and GFRα1 (red) but instead colocalize with the endosomal marker EEA1 (red). All images were acquired at the same laser power and microscope settings and illustrate the decrease in GDNF vesicular staining over time.

(G and H) Inhibition of lysosomal proteinases using leupeptin and pepstatin (leu/pep) prevents degradation of GDNF (3 nM, 60 min) in HEK293 cells expressing SorLA/GFRα1 as visualized by the increase of GDNF IF (green) colocalizing with Lamp-1 (red). In contrast, intense GDNF staining associated with the plasma membrane was observed in HEK293/GFRα1 cells independent of treatment. Experiments in (F) and (G) were performed six times and at least 200 cells were evaluated for each condition. Representative images are shown. Scale bar, 10 μm. All images were obtained with the same laser power and microscope settings. See also Figures S1 and S2.

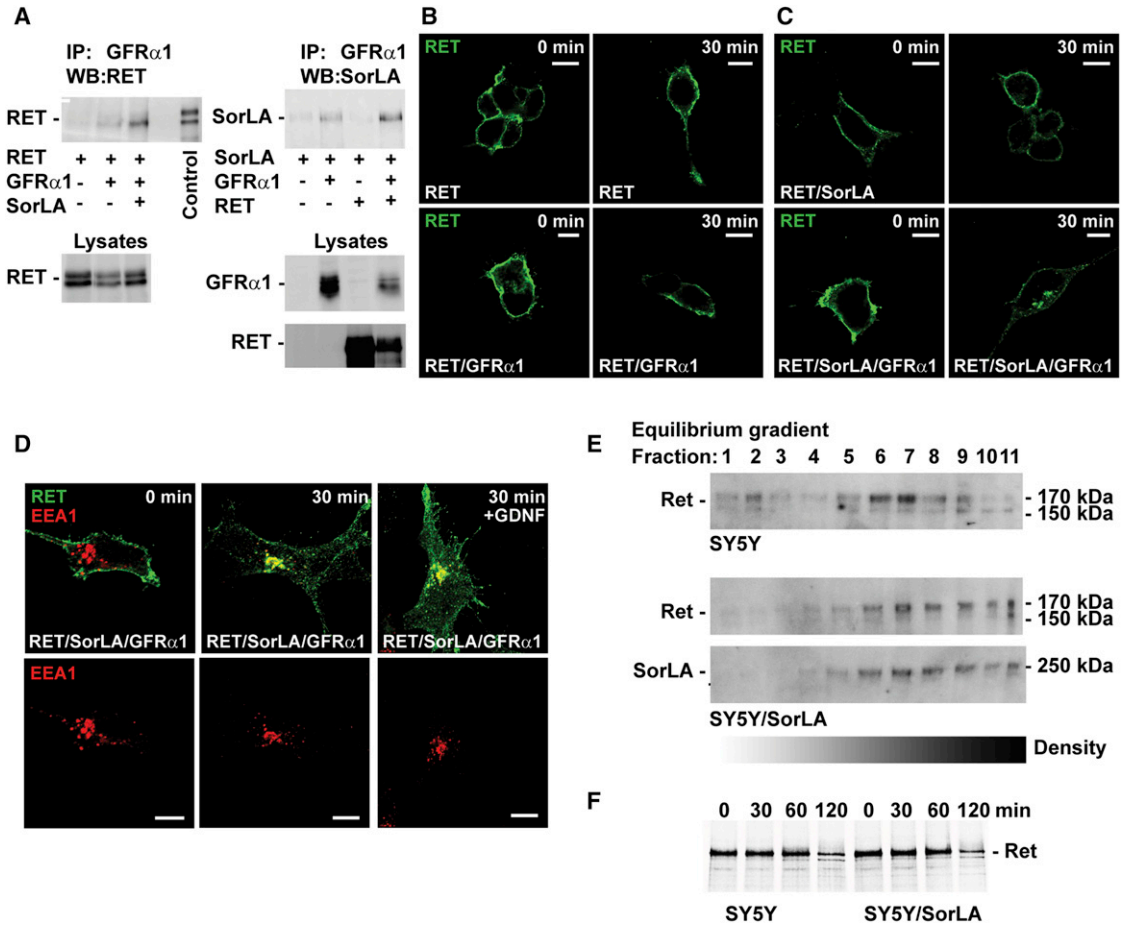

Figure 3. The SorLA/GFRα1 Complex Mediates RET Endocytosis

(A) Co-IP of RET together with GFRα1 ± SorLA from transfected HEK293 cells (left part of figure). Co-IP of SorLA together with GFRα1 ± RET (right part of figure). n = 4 independent experiments.

(B and C) HEK293 cells transfected with combinations of RET, SorLA, GFRα1, or empty vector were incubated on ice for 2 hr in medium containing antibodies against the RET extracellular domain (0.1 μg/ml) and subsequently 0 or 30 min at 37°C in normal culture medium. Scale bar, 10 μm. n = 4 independent experiments with over 100 cells evaluated for each condition.

(D) RET (green) is internalized into EEA1- (red) positive endosomes by the SorLA/GFRα1 complex ± GDNF (3 nM). Scale bar, 10 μm.

(E) To separate the subcellular structures that contain endogenous RET, we performed a two-step sucrose gradient centrifugation of a postnuclear supernatant preparation of SY5Y or SY5Y/SorLA cells. We first fractioned cells by velocity gradient centrifugation and assessed the presence of SorLA and RET in collected fractions by western blotting as shown in Figure S5B. Fractions 7–9, enriched in both SorLA and RET, were pooled and further fractioned by an equilibrium gradient centrifugation, and collected fractions were analyzed again as indicated in the figure. n = 3 independent experiments.

(F) The effect of SorLA overexpression (SY5Y/SorLA) on turnover of endogenous RET in SY5Y neuroblastoma cells was assessed by metabolic labeling followed by pulse-chase analysis and subsequent IP of RET. n = 4 independent experiments.

See also Figure S5.

fraction of GFRα1 to vesicular structures (Figure S3I). SorLA and GFRα1 partially colocalized with the TGN marker TGN46 (Figure 2F) and were also found together in endosomes and multivesicular bodies as shown by immune electron microscopy (Figure 2G). Although SorLA does not bind the additional GFLs, it is tempting to speculate that it may sort neurturin coreceptor (GFRα2), artemin coreceptor (GFRα3) and persephin coreceptor (GFRα4) in a similar manner to GFRα1. Indeed, SorLA was specifically pulled down by co-IP with the individual GFRαs (Figure S4A). Furthermore, SorLA directed GFRα2 and -4 from the cell surface into vesicles (Figures S4B–S4D), suggesting that it may function as a general GFRα sorting receptor.

SorLA/GFRα1 Mediates RET Endocytosis

We next tested if SorLA affects RET/GFRα1 complex formation. Intriguingly, SorLA did not inhibit pulldown of RET and GFRα1; rather, it appeared to increase their interaction slightly (Figure 3A). Of note, the presence of SorLA mainly induced co-IP of GFRα1 with the lower molecular weight form of RET, likely representing immature intracellular receptor. Similarly, co-IP of SorLA by GFRα1 was also somewhat increased by RET despite the fact that we were unable to demonstrate an interaction between SorLA and RET (Figure 3A and data not shown). This was not altered by the presence of GDNF (3 nM) (data not shown). To study if RET trafficking is affected by SorLA, we

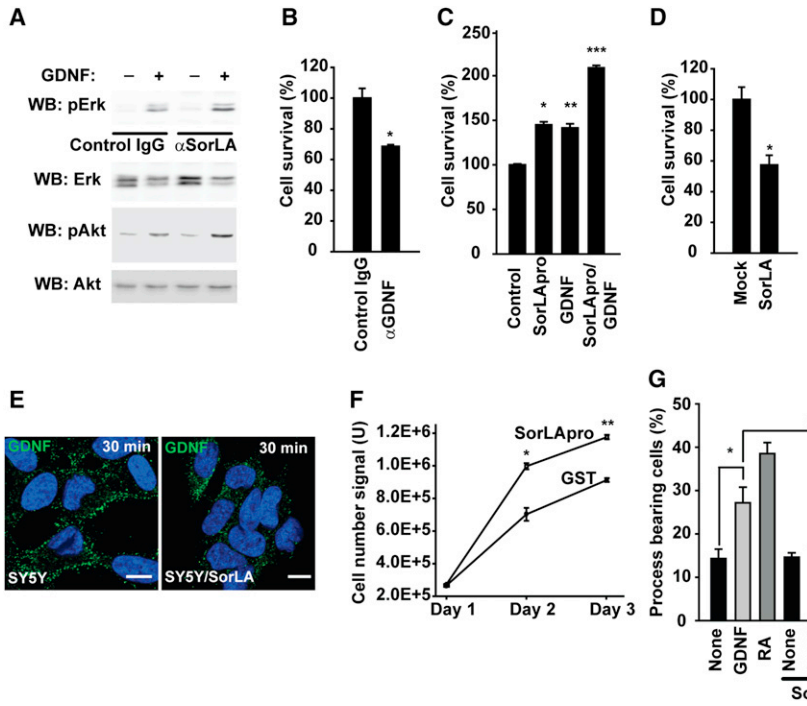

Figure 4. SorLA Inhibits GDNF-Induced Neurotrophic Activity

(A) SY5Y cells stimulated with GDNF (3 nM, 15 min) following 2 hr preincubation with the indicated antibodies (10 μg/ml). Representative immunoblots of phosphorylated Erk (pErk) and phosphorylated Akt (pAkt) and total levels of Erk and Akt proteins. n = 3 independent experiments.

(B) The presence of goat anti-GDNF (10 μg/ml) in the culture medium of serum-depleted SY5Y cells for 3 days reduces their survival compared to the presence of unspecific goat antibodies. *p = 0.04 (n = 4).

(C) Addition of exogenous GDNF (3 nM) and inhibition of endogenous SorLA by its propeptide (SorLApro, 1 μM) increases SY5Y cell survival upon serum depletion. *p = 6E-5 compared to the control. **p = 0.001 compared to the control. ***p = 1E-5 compared to SorLApro (n = 4).

(D) SorLA overexpression in SY5Y/SorLA cells reduces survival upon serum depletion compared to mock-transfected SY5Y cells. *p = 0.008 (n = 4).

(E) GDNF binding and uptake in SY5Y cells and SY5Y/SorLA cells for 30 min at 37°C following incubation in GDNF-containing medium (3 nM) for 2 hr on ice. n = 3 independent experiments with over 100 cells evaluated. Scale bar, 10 μm.

(F) Inhibition of SorLA by its propeptide (SorLApro, 1 μM) increases proliferation of SY5Y cells (*p = 9E-4, **p = 4E-6, n = 4). GST alone (1 μM) was added to SY5Y cells in the control experiment.

(G) Overexpression of SorLA inhibits GDNF-induced (3 nM) but not all *trans*-retinoic acid (RA)-induced (10 μM) neurite outgrowth in SY5Y cells. (*p = 0.03, **p = 0.04, n = 5).

Error bars indicate SEM. See also Figure S5.

labeled surface RET with antibodies and studied its internalization. At 30 min the vast majority of labeled RET remained at the surface of cells expressing RET alone, or RET together with GFRα1 or SorLA (Figures 3B and 3C). However, in SorLA/GFRα1/RET cells, RET was directed to EEA1-positive endosomes (Figures 3C and 3D), an observation that was independent of GDNF (Figure 3D, left panel). We speculated if the SorLA/GFRα1 complex sorts endogenous RET and tested this in SY5Y cells, which express all three receptors endogenously, and in SY5Y cells overexpressing SorLA (SY5Y/SorLA) (Figure S5A). To separate intracellular structures, we employed a two step gradient centrifugation protocol (Nielsen et al., 2007) and analyzed fractions by western blotting (Figures 3E and S5B). This experiment revealed a marked shift in RET localization in SY5Y/SorLA cells to fractions of higher density also enriched in SorLA (Figure 3E), strongly indicating that RET undergoes intracellular trafficking together with SorLA/GFRα1. Yet, such sorting does not affect RET degradation as newly synthesized receptor rapidly disappeared independent of SorLA overexpression (Figure 3F).

SorLA Inhibits GDNF-Induced Neurotrophic Activity

As SorLA targets all three components of the extracellular GDNF signaling machinery, we tested its impact on the activation of intracellular cascades. We treated SY5Y cells for 2 hr with SorLA antibodies that block GDNF/GFRα1 endocytosis (Figure S5C) and observed a marked increase in GDNF-induced Erk and Akt phosphorylation (3 nM, 15 min), suggesting an inhibitory role of endogenous SorLA (Figure 4A). Serum-depleted SY5Y cells depend on autocrine GDNF stimulation as anti-GDNF reduced survival by ~35% (Figure 4B). In contrast, survival was potentiated by the addition of exogenous GDNF (Figure 4C). The SorLA propeptide (SorLApro) can be used as a SorLA antagonist as illustrated in Figures S5D and S5E. Accordingly, SorLApro induced survival as efficiently as exogenously added GDNF, and their combination was even more effective (Figure 4C). In contrast, SorLA overexpression (SY5Y/SorLA) reduced survival to a similar extent as anti-GDNF (Figure 4D). Notably, SY5Y/SorLA cells also displayed markedly increased clearance of surface bound GDNF (3 nM) during 30 min compared to mock-transfected SY5Y cells (Figure 4E). GDNF-GFRα1-RET signaling is further known to promote proliferation of SY5Y cells (Hirata and Kiuchi, 2003), and in analogy the addition of SorLApro markedly increased the number of cells/well after 24 hr (Figure 4F). Proliferation could not be potentiated further by addition of both GDNF and SorLA propeptide, suggesting that it was already proceeding at its highest rate (data not shown). Cell differentiation, e.g., neurite outgrowth is a third downstream effect of GDNF. Addition of GDNF to the culture medium resulted in approximately 30% process-bearing SY5Y cells (Figure 4G). However, SorLA overexpression blocked GDNF-induced process outgrowth but did not significantly affect outgrowth induced by retinoic acid (RA) that initiates a different signaling cascade (Figure 4G). In summary, SorLA negatively

modulates downstream effects of GDNF signaling likely by controlling both GDNF levels and the subcellular distribution of GFRα1-RET.

SorLA Controls GFRα1 Localization and GDNF Uptake in Neurons

Using western blotting, we found SorLA expressed in all CNS tissues analyzed (Figure 5A), and immunohistochemistry of mouse brain sections revealed the presence of SorLA in distinct neuronal vesicular structures as exemplified in the cortex (Figure 5B) but also in GFAP-positive glia (Figures S6A and S6B). GFRα1 is highly expressed in the developing cortex and hippocampus where it acts independent of RET (Bespalov et al., 2011; Ledda et al., 2007; Pozas and Ibáñez, 2005). In primary hippocampal neurons, we detected high levels of endogenous GFRα1 but not RET, and all neurons also expressed variable levels of SorLA (Figure 5C and data not shown). Interestingly, GFRα1 was markedly enriched in the initial segment of filaments (Figure 5C). As SorLA-positive vesicles are abundant in the soma and protrude gradually through the initial segment of filaments (Figure 5C), we speculated if it might function here as sorting hub for GFRα1 targeted for axons and dendrites. Indeed, such GFRα1 enriched initial segments displayed much stronger surface immunoreaction in neurons derived from SorLA knockout (KO) mice (Figure 5D) albeit total GFRα1 levels were unaltered in KO neurons compared to wild-type (WT) (Figure 5F). We therefore performed GFRα1 immunostainings of nonpermeabilized neurons from both genotypes and quantified the relative intensity of surface staining in the initial segments as illustrated in Figure 5D. Notably, lack of SorLA resulted in a marked increase in surface GFRα1 in the soma and at the initial segment of filaments (Figure 5E), showing that SorLA regulates GFRα1 subcellular localization in neurons. We subsequently tested the ability of hippocampal neurons to bind and metabolize GDNF. Exogenous GDNF (3 nM) avidly bound to the surface of neurons derived from WT and KO mice upon incubation for 2 hr at 0°C (Figures 5G and 5H). Following 30 min incubation in 37°C culture medium, the GDNF signal in the soma markedly decreased and was now found in a few faint vesicles of WT neurons (Figure 5G). In contrast, GDNF remained associated with the surface of SorLA KO neurons, suggesting that endogenous SorLA/GFRα1 complex mediates its endocytosis (Figure 5H).

SorLA Regulates In Vivo GDNF Levels and GDNF-Induced Survival of DA Neurons

The survival of midbrain dopaminergic (DA) neurons in culture depends on exogenous GDNF in a RET-dependent manner, as well as on a supporting layer of glial cells (Burke et al., 1998; Lin et al., 1993). Interestingly, both glia and DA neurons were found to express high levels of SorLA (Figures 6A–6C, S6A, and S6B). Hence, SorLA might regulate DA survival by sequestering extracellular GDNF and altering the subcellular distribution of its receptors. Accordingly, we found that when GFAP-positive cortical glia were incubated with GDNF (3 nM), it accumulated in vesicular structures within 15 min (Figures 6D and 6E). In contrast, no GDNF internalization was observed in cells derived from SorLA KO mice (Figure 6F). In addition, GFRα1 appeared to be increased at the surface of DA neurons from SorLA KO compared to WT neurons where it was more pronounced in vesicular structures (Figure 6G). We therefore cultured rat DA neurons with anti-SorLA or unspecific immunoglobulin G (IgG) in the culture medium. In the absence of GDNF, anti-SorLA did not affect survival compared to the control (data not shown). However, in the presence of GDNF (0.3 nM), SorLA inhibition potentiated survival by nearly 50% (Figure 6H). Similarly, the GDNF-induced survival of SorLA knockout DA neurons was markedly increased compared to that of WT neurons (Figure 6I). To determine if SorLA regulates the amount of GDNF available to DA neurons in vivo, we first examined SorLA expression in the adult DA system by immunohistochemistry. A clear SorLA staining was observed in vesicle-like structures in the soma of tyrosine hydroxylase (TH)-positive midbrain neurons, and also in the surrounding cells (Figure 6J). In striatum, we observed abundance of SorLA-expressing cells (Figure S6E), some of which were also GFAP positive (Figure S6F). We next analyzed adult striatal and midbrain homogenates for their content of GDNF by ELISA and found it be highly elevated in KO mice compared to WT (Figure 6K).

SorLA Deficiency Affects the DA System and Anxiety-Related Behavior

We speculated that the absence of SorLA, and thus potentially altered GDNF activity, might lead to abnormal functionality of the DA system. The overall appearance of the midbrain DA system of SorLA knockouts was normal compared to WT mice as shown by TH staining (Figure S7A). Stereological counting showed that the number of midbrain TH^+ neurons in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) was unchanged in both young (5 weeks, n = 6 in each group) and older (45 weeks, n = 3 in each group, data not shown) *Sorl1*$^{-/-}$ mice compared to WT mice (Figures 7A and 7B). Also the length density of TH^+ nerve fiber protruding from the SNpc and VTA from sections of 10-week-old mice was similar between genotypes (Figures 7C and 7D). To assess DA connectivity in the nigrostriatal system, we performed unilateral intrastriatal injections of the retrograde tracer cholera toxin β subunit (CTb). CTb-labeled TH^+ neurons in the SNpc were then identified by double immunofluorescence (IF) and counted. Remarkably, the number of CTb-labeled DA neurons in knockouts was less than one-fourth of that of WT mice (Figures 7E, S7B, and S7C), showing that nigrostriatal connectivity was severely perturbed. The reduction in nigrostriatal connectivity was accompanied by approximately 50% reduction in TH protein levels in striatum of SorLA knockouts as assessed by western blotting (Figures 7F and 7G), and a slight reduction in dopamine levels as measured by high-performance liquid chromatography (HPLC) (Figures 7H and S7D–S7K).

The activity of midbrain DA neurons is instrumental in the behavioral response to phychostimulants. We therefore tested the role of SorLA in amphetamine-induced hyperlocomotion by injecting WT and SorLA knockouts with either saline or amphetamine and monitoring their behavior in an open field. The locomotor activity of WT mice was increased approximately three times upon amphetamine (10 mg/kg) administration and the average distance traveled during 40 min augmented from 58.28 ± 4.19 m to 172.51 ± 19.88 m (p = 2E-4). In contrast, saline-injected KO mice were already hyperactive compared to WT controls

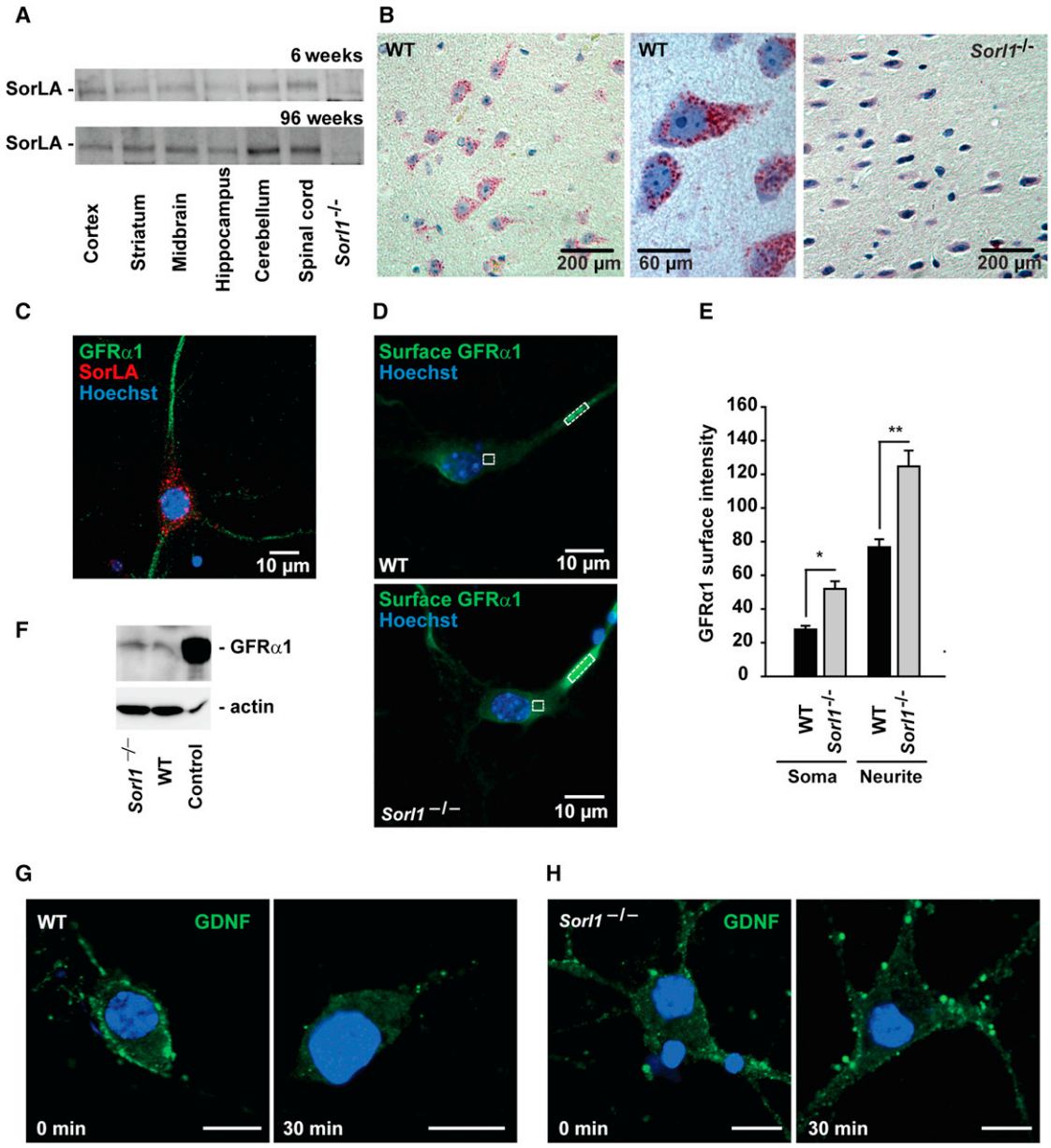

Figure 5. Neuronal Surface Level of GFRα1 Is Regulated by SorLA

(A) SorLA expression in the CNS of young (6 weeks) and old mice (96 weeks) shown in tissue homogenates analyzed by western blotting.

(B) Immunohistochemical staining for SorLA in cortical paraffin sections visualized using FastRed and counterstaining using hematoxylin. No immunoreaction was observed in sections from SorLA KO (*Sorl1*$^{-/-}$) mice. Eighteen-week-old mice were used.

(C) Confocal image of GFRα1 (green) and SorLA (red) in primary hippocampal neurons from WT mice (11 DIV). Nuclear staining was obtained using Hoechst (blue). Scale bar, 10 μm.

(D and E) Surface GFRα1 staining of nonpermeabilized hippocampal neurons (11 DIV) from WT and SorLA KO mice. White boxes indicate areas of soma and initial filaments where surface fluorescence was quantified from nonsaturated images using an optical slice of 7 μm of 94 WT and 109 KO neurons on six coverslips of each genotype from three independent cultures. *p = 3E-6, **p = 7E-6.

(F) Western blot showing GFRα1 in hippocampal neuron lysates derived from WT and SorLA KO (11DIV). Control is lysate of HEK293 cells stably expressing GFRα1.

(G and H) GDNF (3 nM) was allowed to bind to the surface of hippocampal neurons (11 DIV) of WT and *Sorl1*$^{-/-}$ mice for 2 hr on ice. Cells were subsequently washed to remove unbound GDNF and incubated for 30 min at 37°C to allow internalization. Scale bar, 10 μm. n = 3 independent experiments with over 200 cells evaluated.

See also Figure S6.

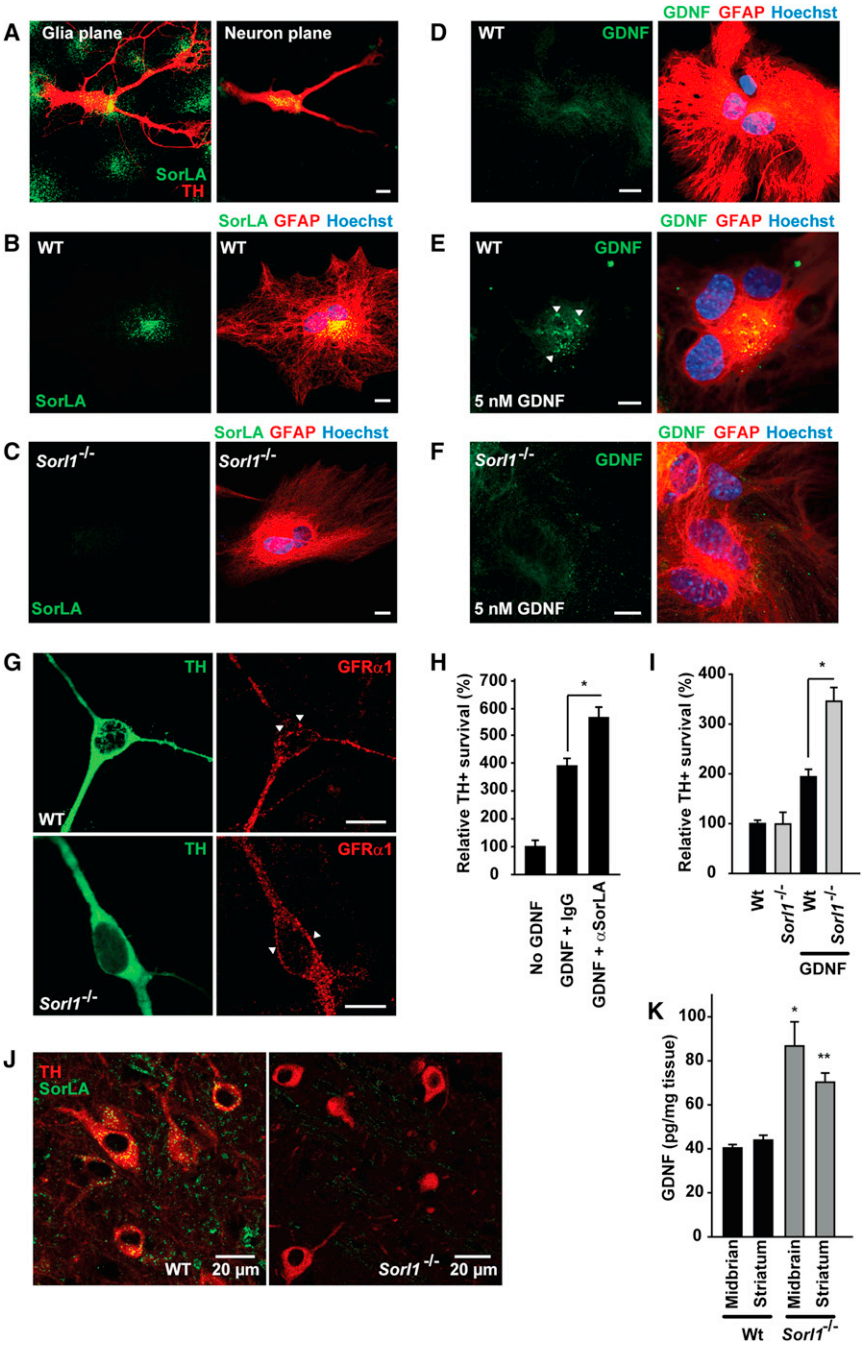

Figure 6. SorLA Reduces In Vivo GDNF Levels and the Survival of DA Neurons in Culture

(A) Confocal images of cultured midbrain TH-positive neurons (red) derived from P0 rats and grown on a layer of supporting cortical glial cells for 7 DIV. SorLA (green) is expressed in both glia and neurons. Scale bar, 10 μm.

(B–D) Primary cultures of mouse cortical glial cells stained with GFAP (red) display faint immunoreaction for endogenous GDNF (green) and GFRα1 (green, Figure S6C) but strong staining for SorLA (green). Nuclei were stained using Hoechst (blue). Cells from the *Sorl1*$^{-/-}$ mice are included as control for the specificity of the SorLA antibody.

(E and F) GDNF (green, 5 nM, 15 min incubation) is internalized by cortical glial cells from WT but not from *Sorl1*$^{-/-}$ mice as exemplified using GFAP-positive glia (red). These cells express some GFRα1 (Figure S6C). Representative examples of GDNF-containing vesicular structures are indicated by white arrow heads. n = 3 independent experiments with over 200 cells evaluated.

(G) Localization of GFRα1(red) in midbrain DA neurons (4 DIV) identified by TH staining (red). In the right images, white arrows indicate GFRα1 localized in vesicular structures in soma of a WT neuron, and surface localized GFRα1 in a KO neuron.

(H) Survival of primary rat DA neurons (7 DIV) requires GDNF (0.3 nM) and is enhanced by anti-SorLA but not by control IgG (10 μg/ml) (p = 0.02, n = 7).

(I) GDNF-induced survival of WT and KO DA neurons (4 DIV). TH^{+} neurons were counted on 14 coverslips of each genotype in three independent cultures. The survival of SorLA KO DA neurons stimulated with GDNF was 346% ± 28% of the unstimulated control, while GDNF-induced survival in WT neurons was only 194% ± 15% of the control (*p = 4E-4).

(J) IF on midbrain cryosections showing the presence of SorLA (green) in TH-positive neurons (red) of substantia nigra and surrounding glial cells in 16-week-old WT mice but not in SorLA knockouts of the same age.

(K) GDNF levels determined by ELISA in tissue homogenates from WT and *Sorl1*$^{-/-}$ mice. GDNF is increased in midbrain and striatum of KO animals (p = 0.01, n = 3; each n comprising a pool of three 12- to 16-week-old animals).

Error bars indicate SEM. See also Figure S6.

(p = 0.006) and traveled a distance of approximately 108.62 ± 14.52 m, but no increase was observed following injection of amphetamine (104.33 ± 6.76 m) (Figures 7I–7J), strongly suggesting impaired DA functionality in SorLA KO mice. The pronounced hyperactivity of saline-treated knockouts led us to study their anxiety-related behavior. In the elevated plus maze, knockouts displayed markedly reduced anxiety levels evidenced by more entries and increased time spent in the open arms of the maze compared to controls (Figures 7K–7M). In striking contrast, *Gdnf*$^{+/-}$ mice displayed markedly increased anxiety characterized by reduced entries and time in the open arms (Figures 7N and 7O). The combined data show that SorLA is critical for DA function and anxiety-related behavior. Previous studies have shown that SorLA affects processing of amyloid precursor protein (APP) (Andersen et al., 2005) and sorting of lipoprotein lipase (LpL) to lysosomes (Klinger et al., 2011). However, neither APP nor LpL has been implicated in DA function so far. It is therefore tempting to speculate that the impaired DA function in SorLA KO is mainly caused by altered activity of GDNF and potentially of other GFLs.

DISCUSSION

We here identify SorLA as a sorting receptor for GDNF and GFRα1 that traffics a ternary SorLA/GFRα1/GDNF protein complex from the cell surface to endosomal compartments, thereby mediating uptake and clearance of extracellular GDNF as well as redistribution of the GFRα1 receptor pool. The SorLA/GFRα1 complex further targets RET for endocytosis independent of GDNF, thereby altering its subcellular distribution and signaling. Thus, SorLA represents an example of a transmembrane protein that actively mediates trafficking of complexes of a neurotrophic factor and its receptors. The SorLA/GFRα1 complex is most likely stable once formed, as the affinity is unusually high for a receptor-receptor interaction (K_d = 6 nM) considering the restricted diffusion in the cell membrane. For comparison, this affinity is approximately 5- to 30-fold higher than what has been reported for Eph-ephrin interactions (Himanen et al., 2004) and at least 70-fold higher than for neurexin-neuroligin binding in Biacore experiments (Koehnke et al., 2010). As GFRα1 itself has no cytosolic link to the intracellular sorting machinery, the docking of the SorLA tail to the adaptors AP-1 or the retromer complex (Nielsen et al., 2007) also results in retraction of GFRα1 from late endosomes, subsequent return to the *trans*-Golgi and then re-exit to the cell surface for another round of GDNF capture. The physiological role of SorLA/GFRα1-mediated trafficking of RET is unclear, but, in exerting its neurotrophic function, the RET/GFRα1/GDNF complex is well known to undergo internalization and retrograde transport from axon terminals to the soma. This is unlikely to involve SorLA, which is largely absent from nerve endings (Klinger et al., 2011), and because the SorLA/GFRα1-mediated RET endocytosis is independent of GDNF. However, it is evident, at least in overexpressing cells, that the SorLA/GFRα1-mediated endocytosis of GDNF and RET is quantitatively more important than internalization of the RET/GFRα1/GDNF complex.

Through this mechanism, SorLA inhibits downstream effects of GDNF-induced signaling such as cell survival, proliferation, and differentiation of neuronal cells, notably the survival of DA neurons, by lysosomal targeting of GDNF, and by altering GFRα1/RET subcellular distribution in both neurons and glia. Future studies will be carried out to dissect how SorLA/GFRα complexes affect sorting of neurturin, artemin, and persephin, and what role SorLA might play in other neuronal systems that depend on GFL activity. Finally, it would be important to address whether SorLA differentially affects GDNF-induced RET signaling versus RET-independent signaling.

GDNF has received massive attention as a potential treatment for Parkinson's disease due to its ability to protect and repair lesioned nigrostriatal pathway in vivo, but only very little is known about its role in the intact DA system. In both animal models of neurotoxicity and human patients, GDNF has been found to initially potentiate the DA system through increasing TH mRNA and protein levels, dopamine synthesis, and uptake, and by enhancing DA target innervation. However, long-lasting overexpression of GDNF in rats with lesioned nigrostriatal system led to downregulation of TH. Similarly, long-term GDNF overexpression in the striatum of rats with intact nigrostriatal pathway led to compensatory downregulation of TH with no effect on dopamine levels (Georgievska et al., 2002, 2004; Rosenblad et al., 2003) while another study reported reductions in both striatal TH and dopamine (Sajadi et al., 2005). These observations have been proposed to reflect compensatory effects of increased GDNF actions on the DA synapse and at the level of TH expression (Georgievska et al., 2004; Rosenblad et al., 2003). Contrary, dopamine levels are normal in the striatum and even increased in the nucleus accumbens of *Gdnf*$^{+/-}$ mice compared to their control littermates (Gerlai et al., 2001).

In the present study, we find that SorLA deficiency results in increased in vivo levels of GDNF that, as may be expected from the above, translates into reduced striatal TH albeit unaltered number of TH$^+$ neurons in the midbrain and normal nigral fiber length in SorLA KO mice. Increased GDNF activity in the VTA or nucleus accumbens has been reported to diminish the behavioral response to drugs of abuse (Carnicella and Ron, 2009). Conversely, infusion of GDNF inhibitory antibodies into the VTA or lack of one GDNF allele in *Gdnf*$^{+/-}$ mice increases the drug response (Messer et al., 2000). Intriguingly, SorLA knockouts display completely blunted response to amphetamine, which is most likely explained by a dramatic reduction in nigrostriatal connectivity as determined by retrograde tracing. Such reduced sensitivity to amphetamine is a characteristic of the dopamine transporter (DAT) knockout (Giros et al., 1996) and mice lacking α-synuclein (Abeliovich et al., 2000), both molecules involved in DA synaptic function. Blunted response to amphetamine has also been reported in mice transgenic for molecules involved in shaping DA connectivity such as the netrin-1-receptor-deficient (Grant et al., 2007) or EphA5-overexpressing mice (Sieber et al., 2004). The insensitivity toward amphetamine of SorLA knockouts stands somewhat in contrast to the previously reported increased response to amphetamine 4 days after a single dose of GDNF or neurturin into the striatum of mice (Horger et al., 1998). However, the SorLA KO phenotype may instead reflect an adaptive response to a long-term increase in GFL activity. SorLA KO mice were further characterized by marked hyperactivity and reduced anxiety levels, in striking contrast to mice lacking one GDNF allele, suggesting that the two could operate together to regulate anxiety-related behavior. This is interesting, as epigenetic regulation of GDNF expression was recently proposed to affect susceptibility to stressful events with its repression correlating with increased anxiety (Uchida et al., 2011). Thus, SorLA KO animals exhibit behavioral traits that may model symptoms of psychiatric disorders, in particular, ADHD, and further studies should validate the potential of SorLA-deficient mice as an animal model of ADHD. Interestingly, components of the GDNF system show no genetic association with the development of neurodegenerative diseases, but linkage has been suggested to ADHD, schizophrenia, and other neuropsychiatric disorders related to dysfunction of the DA system (Michelato et al., 2004; Souza et al., 2010; Syed et al., 2007).

We conclude that the interaction among SorLA, GFRα1, and GDNF is a key regulatory element in the GDNF signaling through GFRα1-RET. Our results also suggest that SorLA is a potential target for the treatment of pathological conditions related to the DA system such as drug abuse and Parkinson's disease.

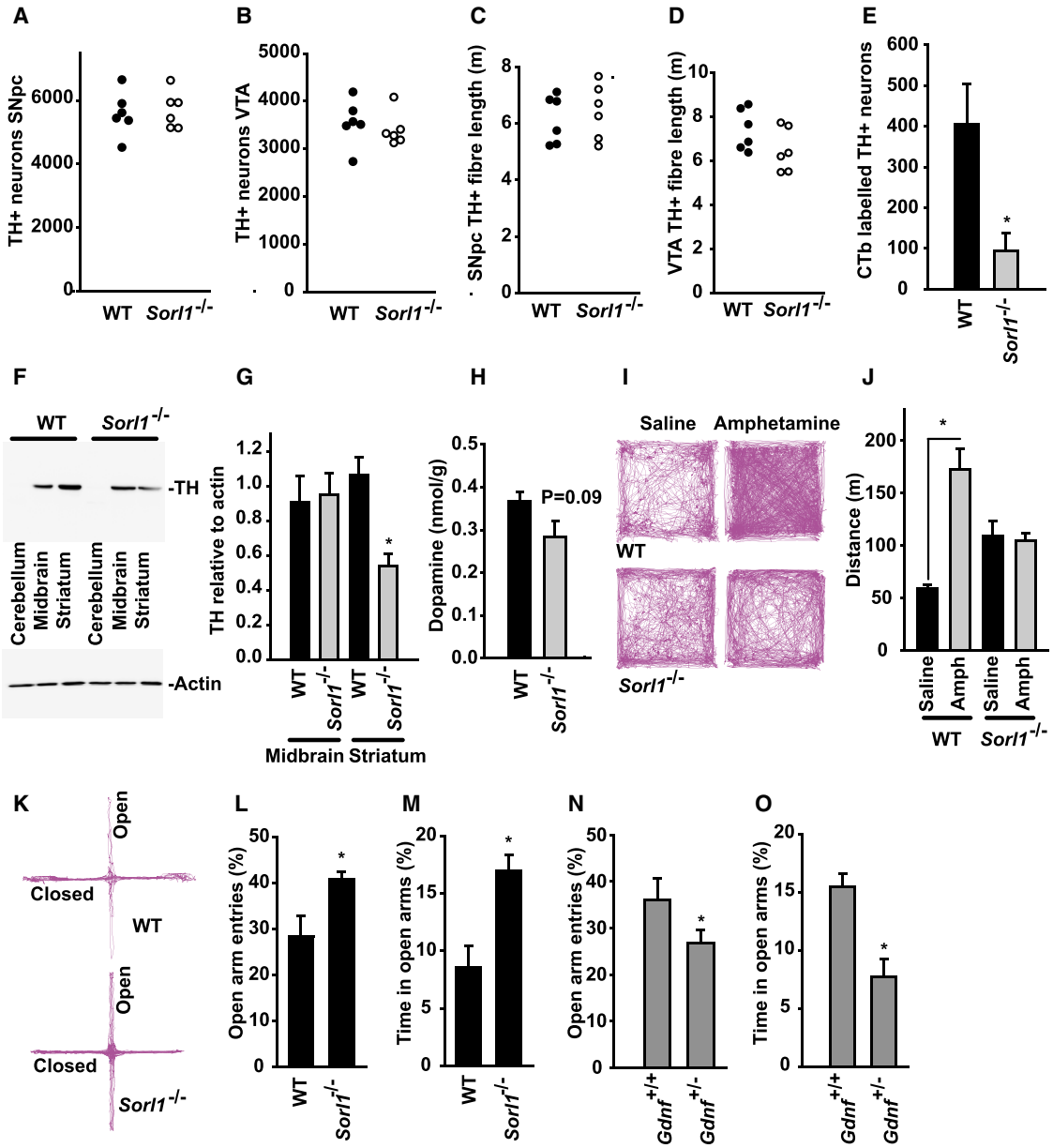

Figure 7. SorLA Affects DA Function and Behavior

(A and B) Number of TH$^+$ neurons is unaltered in the VTA and SNpc of WT and *Sorl1*$^{-/-}$ mice as determined by stereological counting (n = 6 of each group, 5-week-old mice).

(C and D) Normal fiber length density in VTA and SNpc as estimated using global spatial sampling of isotropic virtual planes in thick arbitrarily oriented sections stained with anti-TH and visualized using DAB (n = 6 of each group, 10-week-old mice).

(E) Retrograde labeling of SNpc TH$^+$ neurons by striatal injection of cholera toxin β subunit (CTb) and subsequently stereological counting of SNpc neurons positive for both TH and CTb as visualized by double IF. WT (n = 3) and *Sorl1*$^{-/-}$ (n = 4) mice were 10 weeks old. *p = 0.02.

(F and G) Reduced striatal TH levels in SorLA KO mice as assessed by western blotting and quantified relative to β-actin (n = 7, n = 5, respectively, each n comprising a pool of three 12- to 16-week-old animals). p = 0.01.

(H) Dopamine levels in the striatum of *Sorl1*$^{-/-}$ mice measured using HPLC. p = 0.09, n = 7 and n = 10 for wild-type and knockouts (12–16 weeks old), respectively.

(I) Representative track plots of WT (n = 12) and *Sorl1*$^{-/-}$ mice (n = 11) during 40 min in the open field following injection of saline or amphetamine (10 mg/kg).

(J) Quantification of the distance traveled following the injection of saline or amphetamine by WT (n = 6 and n = 6, respectively) and SorLA KO mice (n = 5 and n = 6, respectively). *p = 2E-4. The activity of saline treated knockouts was also significantly higher than WT (p = 0.006). Mice were 12–16 weeks old.

(K) Representative track plots of WT and *Sorl1*$^{-/-}$ mice during 10 min in the elevated plus maze. Note that while WT mice are most active in the bottom of the closed arms, knockouts display higher preference toward the open arms.

(legend continued on next page)

EXPERIMENTAL PROCEDURES

Biacore

Biacore was performed as described (Jacobsen et al., 2001). Binding was expressed in relative response units (RUs), the difference in response between the immobilized protein flow cell and the corresponding control flow cell. Kinetic parameters were determined using BIAevaluation 4.1.

Internalization Assay

Cells were cultured on poly-L-lysine coated coverslips and incubated with cold medium containing GDNF (3 nM), anti-GFRα1 (0.1 μg/ml), or anti-RET (0.1 μg/ml) for 2 hr on ice. The culture medium was then changed to 37°C normal medium and at specific time points, and the cells were fixed in 4% paraformaldehyde (PFA) (pH 7.4) and permeabilized with 0.1% Triton X-100 unless indicated otherwise. Internalized ligand and subcellular markers were subsequently visualized using IF on a LSM 710 (Carl Zeiss). In different experiments, cells were incubated with GDNF added to 37°C medium for defined time periods. Internalization was quantified from nonsaturated immunofluorescent images using ImageJ. Regions of interest were randomly selected along the plasma membrane of at least 120 cells for each time point. Inhibition of lysosomal proteinases was achieved by adding fresh medium containing leupeptin and pepstatin (50 μg/ml, Sigma-Aldrich) every 6 hr, starting 24 hr before the experiments.

Metabolic Labeling

Transfected HEK293 or SY5Y cells were labeled using ~200 μCi/ml L-[^{35}S]-cysteine and L-[^{35}S]-methionine (Pro-mix; GE Healthcare) in medium without methionine and cysteine in the presence of Brefeldin A (Pierce). After 4 hr, the cells were washed and changed to normal culture medium, and newly synthesized GFRα1 or RET were chased for specific time periods. GFRα1 or RET was subsequently immunoprecipitated from cell lysates, separated by SDS-PAGE, and visualized using phosphoimaging.

DA Cultures

Cultures of DA neurons were prepared from the midbrain of P0 rats or mice and grown on a layer of cortical glial cells as described (Burke et al., 1998). Culture medium was supplemented with 0.3 nM GDNF and changed every 3 days. The effect of endogenous SorLA in rat DA neurons was assessed by adding 10 μg/ml SorLA antibodies (rabbit anti-human SorLA) or control antibodies to the medium (rabbit IgG, R&D Systems). The number of surviving DA neurons was counted at 7 DIV (for rats) or at 4 DIV (for mice) after TH staining.

Behavior

Behavioral tests were performed during the light phase (9 a.m. to 4 p.m.) Mice were tested for anxiety levels in an elevated plus maze essentially as described (Chen et al., 2006). In the open-field test, wild-type and *Sorl1*$^{-/-}$ mice were administered amphetamine (10 mg/kg) or saline by intraperitoneal (i.p.) injection, and placed in the corner of a (40 × 40 × 35 cm) clear Plexiglas arena and their activity was recorded over a 40 min session and analyzed using the Any-maze tracking software.

Animals

The SorLA KO mouse was first described in Andersen et al. (2005) and has been backcrossed for ten generations into C57/BL6J. Behavioral studies were done with the backcrossed homozygous mice compared to the same C57/BL6J substrain that was used for backcrossing. The behavioral studies have subsequently been recapitulated using KO and WT offspring from heterozygous breedings. *Gdnf*$^{+/+}$ and *Gdnf*$^{+/-}$ littermates (Pichel et al., 1996) were obtained by heterozygous breeding using a GDNF KO line that has been backcrossed for five generations into C57/BL6J. Male mice were used for all experiments shown, but similar results were obtained using both genders (data not shown). Animal experiments were performed according to institutional and national regulations.

For further details, please see the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.12.011.

LICENSING INFORMATION

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(L and M) *Sorl1*$^{-/-}$ mice (n = 12) show more entries (p = 0.01) and spend increased time (p = 0.002) in the open arms of the elevated plus maze compared to WT control mice (n = 9). Mice were 12–16 weeks old.
(N and O) *Gdnf*$^{+/-}$ mice (n = 4) show fewer entries (p = 0.05) and reduced time (p = 0.006) in the open arms of the elevated plus maze compared to *Gdnf*$^{+/+}$ littermates (n = 7). Mice were 11 weeks old.
Error bars indicate SEM. See also Figure S7.

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